



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-116255-00000-0000

Fecha: 2010-09-20 16:43:47

Dep. 2020 DIR.NUEVASCR

Tra. 11 PATENTEIYII

Eve: 379 FASENACIONALII

Act. 411 PRESENTACION

Folios: 210

Industria y Comercio

SUPERINTENDENCIA

PCT

DELEGATURA PROPIEDAD INDUSTRIAL

SOLICITUD DE PATENTE DE INVENCION - ENTRADA EN FASE NACIONAL -

10-116255

21. EXPEDIENTE No.

54. TITULO COMPOSICIONES QUE COMPRENDEN
FITOESTROGENOS SELECTIVOS PARA RECEPTOR BETA DE
ESTROGENOS Y FIBRAS DIETETICAS

51. CLASIFICACION INTERNACIONAL A61K 31/357 (2006.01) – A61K
31/352 (2006.01) – A61K 31/05 (2006.01) – A61K 31/716 (2006.01) –
A61K 45/06 (2006.01) – A61P 1/00 (2006.01) – A61P 35/00
(2006.01)

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74. APODERADO DRA. MAGDA SALZBURG GARROS

22. BOGOTA D.C., SEPTIEMBRE 20 DE 2010

PETITORIO



Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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FORMULARIO ÚNICO DE SOLICITUD DE PATENTE PCT ENTRADA EN FASE NACIONAL

SOLICITUD DE:

1

☒ X

Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Capítulo I.

☒ X

Capítulo II.

2

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5

PCT (86)

Solicitud Internacional No. **PCT/EP2009/052038**
Publicación Internacional No. **WO 2009/103788 A1**

Fecha: **20 FEBRERO 2009**
Fecha: **27 AGOSTO 2009**

6

Título (54)

**COMPOSICIONES QUE COMPRENDEN FITOESTROGENOS
SELECTIVOS PARA RECEPTOR BETA DE ESTROGENOS Y FIBRAS DIETETICAS**

7

Clasificación Internacional (51):

**A61K 31/357 (2006.01) - A61K 31/352 (2006.01) - A61K 31/05 (2006.01)
A61K 31/716 (2006.01) - A61K 45/06 (2006.01) - A61P 1/00 (2006.01)
A61P 35/00 (2006.01)**

8

Prioridad

(33) País de Origen

(32) Fecha

(31) Número de Solicitud

SI

☒ X

NO

☐

ITALIA

22/02/2008

BA2008A000008

9

Comprobantes de pago No. 10 - 78,291 y No. 10 - 78,290

Fecha: **20 SEPTIEMBRE 2010**

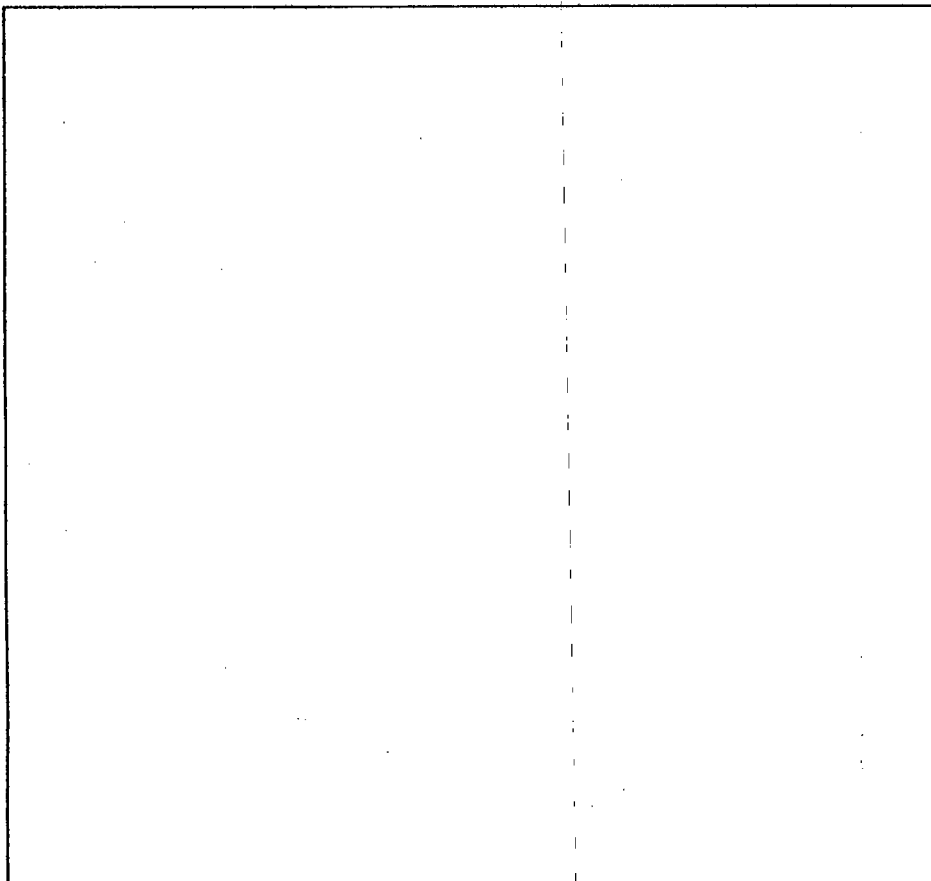
10

ANEXOS

- ☒ Comprobante de pago de la tasa de presentación de la solicitud **\$554.000.00**
- ☒ Comprobante de pago por reivindicación de prioridad **\$234.000.00**
- ☐ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica
- ☐ Poderes, si fuere el caso
- ☐ Documento de cesión del inventor al solicitante o a su causante
- ☐ Declaraciones
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA)
- ☐ Traducción del examen preliminar internacional efectuado por la IPEA
- ☐ De ser el caso, copia del contrato de acceso
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas
- ☐ De ser el caso, certificado de depósito del material biológico
- ☐ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud
- ☐ De ser el caso, modificaciones efectuadas a la solicitud internacional
- ☐ Otros

11

FIGURA CARACTERÍSTICA:



12

Solicito la concesión de la patente, NOMBRE: **MAGDA SALZBURG GARROS**

FIRMA:

C.C.: **35.458.610** Usaquén T.P.: **35.337** C.S.J.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 10 - 78,290
FECHA : SEPTIEMBRE 20 DE 2010

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-116255-00000-0000

Fecha: 2010-09-20 16:43:47 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 411 PRESENTACION Folios: 210

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
MAGDA SALZBURG	CONSIGNACION	BANCO DE BOGOTA	062754387	173393835	20/09/2010	234,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
9 50005-01-01 SOLICITUDES	*** REIVINDICACION UNITARIA ADICIONAL	234,000.00
	TOTAL :	\$ 234,000.00

SON: DOSCIENTOS TREINTA Y CUATRO MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 10 - 78,291
FECHA : SEPTIEMBRE 20 DE 2010

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-116255-00000-0000

Fecha: 2010-09-20 16:43:47 Dep. 2020 DIR.NUEVASCR
Tral 11 PATENTEIYII Eve: 379 FASENACIONALI
Act: 411 PRESENTACION Folios: 210

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
MAGDA SALZBURG	CONSIGNACION	BANCO DE BOGOTA	062754387	173393824	20/09/2010	554,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	554,000.00
	TOTAL :	\$ 554,000.00

SON: QUINIENTOS CINCUENTA Y CUATRO MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

**(12) SOLICITUD INTERNACIONAL PUBLICADA BAJO EL TRATADO DE
COOPERACIÓN EN MATERIA DE PATENTES (PCT)**

**(19) ORGANIZACIÓN MUNDIAL DE LA
PROPIEDAD INTELECTUAL
Oficina Internacional**

**OMP
PCT**

(43) Fecha de Publicación Internacional:

27 de Agosto de 2009 (27.08.2009)

(10) Número de Publicación Internacional:

WO 2009/103788 A1

(51) Clasificación Internacional de Patentes:

A61K 31/357 (2006.01)	A61K 45/06 (2006.01)
A61K 31/352 (2006.01)	A61P 1/00 (2006.01)
A61K 31/05 (2006.01)	A61P 35/00 (2006.01)
A61K 31/716 (2006.01)	

(21) Número de Solicitud Internacional: PCT/EP2009/052038

(22) Fecha de Presentación Internacional:

20 de Febrero de 2009 (20.02.2009)

(25) Idioma Presentado:

Inglés

(26) Idioma de Publicación:

Inglés

(30) Fecha de Prioridad:

BA2008A000008 22 de Febrero de 2008 (22.02.2008) IT

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(81) Estados Designados: (a menos que se indique de otro modo, para cada caso de protección nacional disponible): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Estados Designados: (a menos que se indique de otro modo, para cada caso de protección regional disponible): patente ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), patente Eurasiática (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), patente Europea (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), patente OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaración bajo la Regla 4.17:

- de la invención (Regla 4.17(iv))

Publicada:

- con el reporte de búsqueda internacional (Artículo 21(3))

(54) Título: COMPOSICIONES QUE COMPRENDEN FITOESTRÓGENOS SELECTIVOS PARA RECEPTOR BETA DE ESTROGENOS Y FIBRAS DIETÉTICAS

(57) Resumen:

Se describen composiciones orales que comprenden una asociación de uno o de una mezcla de fitoestrógenos, selectivos para el receptor-beta de estrógenos, con fibras dietéticas. Las composiciones de los mismos se pueden usar de manera ventajosa para el tratamiento profiláctico y terapéutico en mamíferos, incluyendo humanos, de condiciones de salud caracterizadas por un alto riesgo de comienzo y recurrencia de adenomas intestinales (adenopoliposis coli), y del progreso de los adenomas hacia carcinoma colorrectal. En la presencia de lesiones pre-cancerosas en el colon, caracterizadas por mutaciones del supresor de tumor de APC y definidas como pólipos o adenomas, las composiciones orales descritas se pueden usar en realidad de manera ventajosa para reducir el número y volumen de los pólipos, y para impedir su progreso a transformación neoplástica, reduciendo su grado de displasia.

WO 2009/103788 A1

COMPOSICIONES QUE COMPRENDEN FITOESTROGENOS SELECTIVOS PARA
RECEPTOR-BETA DE ESTROGENOS Y FIBRAS DIETETICAS

Campo de la Invención

5 La presente invención se refiere a composiciones que comprenden uno o más fitoestrógenos en asociación con fibras dietéticas, insolubles y resistentes a digestión enzimática, y al uso de estas composiciones para el tratamiento profiláctico y/o terapéutico de la mucosa intestinal y en particular de
10 adenopoliposis coli, y de su progreso a carcinoma colorrectal.

Antecedentes de la Invención

El adenocarcinoma, o carcinoma colorrectal (CRC), es la tercera causa de muerte en el mundo. La patogénesis de CRC incluye formación de pólipos en la mucosa intestinal,
15 consideradas lesiones pre-cancerosas. Cuando se diagnostica por colonoscopia, los pólipos intestinales usualmente se remueven de manera endoscópica. Después de la polipectomía, la frecuencia de recurrencia es de aproximadamente 10-15 % por año. Entre los pólipos recurrentes, 10 % se presenta
20 como adenoma avanzado, caracterizado por un diámetro ≥ 1 cm e histológicamente vellosos. La presencia de un adenoma avanzado, o la presencia de dos nuevas formaciones después de la polipectomía, independientemente de sus características histológicas, caracteriza la población con el más alto riesgo
25 de desarrollar un CRC (Vogelstein B et al. Genetic

alterations during colorectal-tumor development. NEJM 1988, 319: 525-532).

La poliposis adenomatosa familiar (FAP) humana se provoca por una mutación germinal del gen supresor de tumor de APC (Poliposis Adenomatosa Coli). La FAP se caracteriza por el desarrollo de un gran número (de cientos a miles) de adenomas en el colon. En tanto que el substrato genético favorece el inicio del proceso tumorigénico y no se puede modificar de manera farmacológica o nutricional, se puede controlar la promoción de la tumorigénesis, frustrando de este modo el progreso hacia la des-diferenciación celular y la transformación neoplástica. El objetivo principal del examen colonoscópico es el monitoreo de los adenomas y su progreso a formas avanzadas y a CRC. Además del examen colonoscópico, la necesidad clínica de intervenciones quimiopreventivas, seguras y efectivas para mantener baja la frecuencia de recurrencia de los adenomas, es ciertamente alta, pero aún está insatisfecha. La inhibición de ciclooxigenasa-2 (COX-2) por celecoxib y rofecoxib, aunque efectiva, se asocia con un riesgo insufrible de efectos secundarios cardiovasculares (*Lance P. Chemoprevention of colorectal cancer: some progress but a long way to go. Gastroenterology 2008, 134: 341-343).*

Una mayor prevalencia de CRC en hombres, y una incidencia reducida en mujeres que se someten a terapia de

reemplazo hormonal (HRT) soporta la hipótesis que los estrógenos juegan un papel protector en el proceso tumorigénico del colon. Como una prueba adicional del concepto del papel protector de los estrógenos en la mucosa intestinal, se debe citar la evidencia reciente en la regresión de los adenomas en una mujer joven, efectuada por FAP, después de terapia estro-progestínica para propósitos anticonceptivos (Giardiello F, Hyland L, Trimbath J et al. *Oral Contraceptives and Polyp Regression in Familial Adenomatous Polyposis. Gastroenterology* 2005; 128:1077-1080). Desafortunadamente, en los estudios epidemiológicos llevados a cabo en mujeres post-menopáusicas que se someten a terapia estro-progestínica, se ha observado una alta incidencia de cáncer de mama y accidentes cardiovasculares, requiriendo de este modo la terminación temprana del estudio para proteger la salud de los pacientes enroladas (Writing Group of the Women's Health Initiative Investigators. *Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA* 2002, 288: 321-333).

Los receptores de estrógenos existen en dos formas (alfa ($ER\alpha$) y beta ($ER\beta$), diferentemente localizados y expresados en los varios distritos anatómicos. El $ER\alpha$ está principalmente localizado en la mama, sistema cardiovascular, huesos, sistema nervioso urogenital y central, en tanto que se expresa $ER\beta$ en próstata, glándulas salivales, testículos,

ovarios, en el sistema inmunitario, y de manera predominante, en el intestino. El 17β -estradiol (estrógeno) es un ligando no selectivo para ambos receptores, que desempeña una actividad proliferativa en la presencia de $ER\alpha$ y anti-proliferativa en la presencia de $ER\beta$ (Koehler KF et al. *Reflections on the discovery and significance of Estrogen Receptor β* . *Endocr Rev* 2005, 26: 465-478). Los efectos benéficos observados en los estudios mencionados anteriormente en la incidencia reducida de CRC pueden depender por lo tanto específicamente de la activación de $ER\beta$.

La implicación de $ER\beta$ en la poliposis adenomatosa y en CRC se demuestra por su expresión reducida, con respecto a mucosa intestinal normal, en pacientes con polipectomía ya en la etapa pre-cancerosa (Di Leo A et al. *ER- β expression in large bowel adenomas: implications in colon carcinogenesis*. *Dig Liver Dis* 2008, C40: 260-266). De manera significativa, la reducción de $ER\beta$ se relaciona con una proliferación incrementada y una des-diferenciación celular progresiva, demostrando que está comprendida la lentificación de $ER\beta$ en el progreso tumoral. Las lesiones pre-cancerosas, colorrectales, por lo tanto son avisos tempranos del desarrollo de CRC en una parte pertinente de la población. También se observa la declinación dramática de $ER\beta$ en las etapas más avanzadas de la transformación neoplástica

(Konstantinopoulos PA et al. Oestrogen receptor beta ($ER\beta$) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumor's dedifferentiation. *Eur J Cancer* 2003, 39: 1521-1258).

5 Los estudios experimentales llevados a cabo en ratones transgénicos C57BL/6J - APC Min/+, que tienen una mutación de la línea germinal del gen de APC, soportan el papel protector de los estrógenos en la prevención de la transformación neoplásica en células epiteliales colónicas.

10 En particular, Weyant et al. (Weyant MJ et al. *Reciprocal expression of $ER\alpha$ and $ER\beta$ is associated with estrogen-mediated modulation of intestinal tumorigenesis. Cancer Res* 2001, 61: 2547-2551), usando ratones transgénicos hembras, demostró que los adenomas intestinales se desarrollan

15 espontáneamente después de la ovariectomía. La ovariectomía dio lugar a un incremento de 77 % en la neoformación de adenomas intestinales, en tanto que la administración subsiguiente de 17- β -estradiol provocó una reducción en los adenomas intestinales.

20 Además de las causas genéticas, se conoce que la exposición a carcinógenos dentro del lumen intestinal o a agentes potencialmente genotóxicos a las células epiteliales de la mucosa intestinal es un evento epigenético que facilita la inactivación de ambos alelos o la mutación somática del

25 gen de APC, tanto en la forma esporádica como de FAP. Estas

sustancias peligrosas normalmente se ingieren con la dieta, o se pueden formar en el lumen intestinal como un efecto de la digestión, en particular de grasas, o por la degradación de ácidos biliares.

5 Por esta razón, una dieta rica en fibras, que se cree que ejerce un efecto protector en la mucosa intestinal o que absorbe xenobióticos potencialmente genotóxicos, se considera útil en la prevención de CRC (*Robertson DJ et al. Fat, fiber, meat and the risk of colorectal adenomas. Am J*
10 *Gastroenterol* 2005, 100: 2789-2795). Sin embargo, las evidencias clínicas de esta eficiencia no son siempre convincentes. Esto depende frecuentemente de la pobre calidad de los varios protocolos experimentales, donde no se ponderan los efectos esperados contra la ingestión total de
15 fibra dietética, comprendida de fibras solubles e insolubles. Por lo tanto, son bastante inciertos los efectos esperados debido a la adición a la fibra de una cantidad conocida de una fibra calificada por su estructura, grado de solubilidad y digestibilidad, con sus propias propiedades de formación de
20 gel y viscosidad en el lumen intestinal. Todas las características mencionadas anteriormente (es decir, estructura, grado de solubilidad y digestibilidad, viscosidad y propiedades de formación de gel) afectan en exceso las propiedades de la fibra, así como sus efectos biológicos en
25 la fisiología intestinal de los organismos complejos (*Jones*

JR et al. Dietary reference Intake: implication for fiber labeling and consumption: a summary of the International Life Sciences Institute North America Fiber workshop, June 1-2, 2004 Washington, DC. Nutr Rev 2006, 64: 31-38).

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Breve Descripción de la Invención

De acuerdo con los inventores, se puede cumplir la necesidad terapéutica al administrar de manera local agentes capaces de actuar como agonistas selectivos al receptor-beta de estrógenos comprendido en el progreso de la adenopoliposis hacia cáncer colorrectal, reduciendo de manera significativa la absorción sistémica de estos agentes y favoreciendo de este modo su acción farmacológica en la mucosa intestinal.

Las composiciones para administración oral que comprenden una asociación de al menos un fitoestrógeno, selectivamente agonista al receptor-beta de estrógenos (ER β), o más fitoestrógenos con las propiedades anteriores, y fibras dietéticas insolubles, es decir, fibras resistentes a la digestión enzimática en el tracto intestinal, pueden satisfacer la necesidad en el campo médico de herramientas útiles para la prevención o tratamiento de la condición de adenopoliposis coli y su progreso a cáncer colorrectal.

Por lo tanto, en un primer aspecto, es un objeto de la presente invención, composiciones que comprenden al menos un fitoestrógeno o una mezcla de fitoestrógenos selectivamente agonistas del receptor-beta de estrógenos

(ER β), y al menos una fibra insoluble e indigerible o mezclas de la misma seleccionadas del grupo que consiste de polisacáridos sin almidón.

Aún, es un objeto adicional de la invención, el uso
5 de estas composiciones administradas de manera oral para el tratamiento tópico de la mucosa intestinal, y en particular el tratamiento de condiciones de adenopoliposis coli, y para la prevención de su progreso a cáncer colorrectal.

Como será fácilmente evidente a partir de la
10 descripción detallada de la invención informada en la presente, en la presencia de lesiones pre-cancerosas del colon caracterizadas por mutaciones del supresor tumoral de APC, definidas como pólipos o adenomas, las composiciones para administración oral se pueden usar de manera provechosa
15 para reducir el número y volumen de pólipos y para impedir su progreso hacia la transformación neoplástica, reduciendo su grado de displasia.

Las composiciones para administración oral, objeto de la presente invención, se pueden usar de manera provechosa
20 para restaurar la expresión de ER β sobre la mucosa de colon, de otro modo deficiente o lentificado cuando se presentan adenomas intestinales, y para restaurar una relación de expresión entre el receptor beta y alfa de estrógenos a favor de la actividad anti-proliferativa.

25 Además, la asociación de al menos un fitoestrógeno,

selectivo para el receptor-beta de estrógenos, y al menos una fibra dietética insoluble, que forman las composiciones para administración oral, objeto de la presente invención, representa un sistema preferencial de administración para el fitoestrógeno selectivo de ER β hacia el colon, donde está preferentemente localizada la adenopoliposis, con un enriquecimiento de los componentes sobre la mucosa intestinal y en los colonocitos. Las composiciones orales, objeto de la presente invención, por lo tanto son un sistema seguro y efectivo de administración para el tratamiento tópico de la mucosa intestinal, en condiciones en alto riesgo de desarrollo de adenomas y su progreso a adenocarcinoma, por lo tanto útiles en el tratamiento de sujetos afectados por adenopoliposis coli, en formas esporádicas y familiares, reclutadas a examen colonoscópico y después de polipectomía, para impedir el desarrollo de adenomas avanzados, y para impedir su transformación neoplástica potencial en adenocarcinoma, también se conoce como cáncer colorrectal (CRS).

20

Breve Descripción de las Figuras

Figura 1. La Figura muestra la expresión génica (ARN mensajero, ARNm) de receptores de estrógenos en intestino de ratón APC Min^{+/+}. Los niveles de expresión del ARNm de ER β y ER α se expresan como unidades de densidad óptica en comparación con las unidades de densidad óptica de

ARNm 18S constitutivo, con relación a una proteína estructural no modificada en las condiciones experimentales del presente estudio. Los resultados muestran: un incremento significativo del ARNm de ER β en el grupo de silimarina 200 ppm (p = 0.03), con respecto al grupo de control alimentado con dieta estándar; una disminución significativa inesperada de ARNm de ER α en el grupo alimentado con sólo fibra insoluble, lignina (p = 0.04); un incremento significativo de ER β en el grupo alimentado con la composición formada por la asociación lignina + silimarina (p = 0.04).

Figura 2. La Figura muestra la relación entre los ARNm del receptor alfa y beta de estrógenos, en el intestino de ratones APC Min^{+/+}. Por comparación con el grupo de animales tratados subcutáneamente con estrógeno (que se conoce que no son selectivamente activos en ER β y ER α), los resultados confirman la selectividad de silimarina para ER β , que es el receptor incrementado por el ligando. Además, los resultados demuestran que la asociación de silimarina (aún a bajas dosis) con la fibra insoluble lignina, por sí mismo es sorprendentemente activo en reducir la expresión de ER α que posee una actividad proliferativa, facilita la restauración de una relación de expresión entre los receptores de estrógenos a favor de la actividad anti-proliferativa.

Figura 3. La Figura muestra la relación entre el nivel de proteínas de receptor alfa y beta de estrógenos, en

el intestino de ratones APC Min^{+/+}, como se detecta por análisis Western Blot. Con respecto al grupo de control de animales alimentados sólo con dieta estándar, se observa un incremento de la proteína de ER β versus la proteína de ER α en
5 animales alimentados con diferentes dietas, este incremento que es mayor en el grupo tratado subcutáneamente con 17 β -estradiol; en el grupo alimentado con silimarina; en el grupo alimentado con lignina; en el grupo alimentado con la composición que contiene lignina + silimarina, aún a bajas
10 dosis.

Figuras 4A-4B. La Figura 4A compara la relación de ARNm de ER β /ER α en el intestino delgado de grupos tipo silvestre (WT) y APC Min^{+/+}, alimentados con diferentes dietas. La silimarina (SIL), la lignina (LIG) y silimarina + lignina
15 (SIL+LIG) restauraron el ARNm de ER β /ER α a niveles comparables con la mucosa saludable WT. También se incrementó la proteína de ER β en todos los grupos dietéticos manejados (Figura 4B), en comparación a los controles.

Figuras 5A-5D. Ensayo TUNEL (figuras 5A-5C) y
20 valoración de caspasa-3 escindida (CC-3) (figura 5D) de actividad pro-apoptótica en la mucosa no adenomatosa y adenomatosa de animales APC Min^{+/+} alimentados con dieta normal (control) o diferentes dietas (SIL, LIG, SIL+LIG), en comparación con ratones tipo silvestre (WT) alimentados a la
25 dieta estándar. En la mucosa no adenomatosa, la silimarina,

lignina, y la combinación de los dos restauró la apoptosis a niveles comparables con la mucosa saludable de ratones WT. En la mucosa adenomatosa, sólo la asociación de silimarina y lignina restauró la apoptosis a valores comparables con la mucosa saludable de ratones WT. En tanto que CC-3 disminuyó de manera notable en ratones APC Min⁺ alimentados con dieta estándar, se observó un incremento sorprendente en los grupos dietéticos manejados.

Descripción Detallada de la Invención

De acuerdo al estado de la tecnología descrita anteriormente, y considerando los efectos secundarios potencialmente experimentados con agonistas no selectivos de receptores de estrógenos, las composiciones orales objeto de la presente invención comprenden al menos un fitoestrógeno selectivamente agonista del receptor-beta de estrógenos (ER β), o mezclas de estos fitoestrógenos, soportados por un amplio uso que sostiene su clara seguridad y perfil de efectividad.

Para el propósito de la presente invención, los fitoestrógenos preferidos se seleccionan del grupo que consiste de flavolignanós, específicamente silimarina, silibinina, iso-silibinina, silidianina, silicristina, la isoflavona genisteína, el flavonoide resveratrol, cumestrol y mezclas de los mismos. El fitoestrógeno o fitoestrógenos que componen la mezcla pueden estar presentes en la composición

tanto en su forma purificada como en extracto vegetal. De manera más preferente, el fitoestrógeno es silimarina, tal como o como un extracto de cardo lechero, el más preferido que es el extracto de cardo lechero que consiste de una
5 mezcla de flavolignanos, silimarina, silibinina, iso-silibinina, silidianina y silicristina.

Se conoce que la silimarina, una mezcla estandarizada de flavolignanos extraídos de los frutos del cardo lechero, ampliamente usados en medicina tradicional
10 como hepatoprotectores, poseen un perfil documentado de seguridad y tolerabilidad, así también se han descrito propiedades de los mismos como agonista selectivo de ER β , sin ninguna actividad pertinente en ER α , (Seidlova-Wuttke D et al. *Wilymarin is a selective estrogen receptor β (ER β)*
15 *agonist and has estrogenic effect in the metaphysis of the femur but no or aniestrogenic effects in the uterus of ovariectomized (ovx) rats. J Steroid Biochem Mol Biol* 2003, 86: 179-188).

La "silimarina" es la denominación común usada para
20 un extracto vegetal que contiene una mezcla de flavolignanos: silimarina, silibinina, iso-silibinina, silidianina y silicristina en concentraciones y relaciones variables. En las composiciones, objeto de la presente invención, la "silimarina" significa la mezcla de flavolignanos que
25 componen el extracto, en diferentes concentraciones y

relaciones, con referencia particular a un extracto de cardo lechero con un perfil analítico caracterizado por un contenido de silimarina mayor que 70 % y un contenido de silibinina A+B mayor que 30 %. En general, los componentes adicionales a y/o sustitutos de silimarina en las composiciones, objeto de la presente invención, son otros fitoestrógenos, con la condición que sean selectivos para ER β y con una actividad anti-estrogénica, que no sea inductora de una actividad proliferativa significativa en tejidos que son objetivos o dianas clásicas para los estrógenos, tal como los fitoestrógenos mencionados anteriormente, en concentraciones similares como aquellas de silimarina.

El otro componente esencial de las composiciones para administración oral de la presente invención es una fibra insoluble e indigerible, es decir, resistente a digestión enzimática en el tracto gastrointestinal. La fibra insoluble puede estar presente en una forma purificada o como un componente de un extracto natural, con la condición que en las composiciones, el contenido de fibra insoluble está entre 1 % y 30 %. Esta fibra se selecciona de fibras vegetales con una naturaleza polisacárida sin almidón y entre estas fibras están preferentemente fibras seleccionadas en el grupo que consiste de lignina polifenilpropanoide (llamada más adelante también lignina), celulosa, inulina e inulina de cadena larga y mezclas de las mismas. Se da preferencia particular a la

lignina. La fibra insoluble puede estar presente como un componente purificado así como un constituyente de un extracto. En este último caso, las fibras insolubles mencionadas anteriormente pueden estar presentes como una
5 mezcla y asociadas con otras sustancias nutricionales. En particular, las fibras se pueden usar para las composiciones de la invención como sigue: en el caso de lignina, como tal en la forma polidispersa o como salvado de trigo; en el caso de celulosa, como tal o como un extracto de soya; en el caso
10 de inulina, es preferible la inulina de cadena larga, con un grado de polimerización de 10-65 y una longitud promedio de cadena de 25, como tal o como un extracto de alcachofa, extracto de achicoria o polvo de raíz de diente de león.

Las fibras presentes en las composiciones orales,
15 objeto de la presente invención, se consideran de cualquier manera aditamentos a la dieta, pero independiente de la ingestión total de fibra dietética.

Las composiciones de acuerdo a la invención han mostrado propiedades biológicas inesperadas, puesto que estas
20 composiciones, caracterizadas sustancialmente por la asociación de al menos un fitoestrógeno, selectivamente agonista al receptor-beta de estrógenos (ER β), o mezclas del mismo y al menos una fibra dietética insoluble seleccionada de fibras que tienen una naturaleza polisacarídica sin
25 almidón o mezclas de las mismas, probó tener un efecto

sinérgico en los parámetros usados para la validación experimental necesaria de su utilidad.

En realidad, la fibra dietética insoluble (en particular, lignina) probó ser activa en mantener la expresión proteica de ER β , evidencia desconocida a la fecha, y probó ser sinérgica con el fitoestrógeno, silimarina, al mantener de manera significativa bajo parámetros de control que son marcadores de la proliferación y progreso neoplástico, tal como por el ejemplo el volumen y grado de displasia de pólipos adenomatosos.

Además de que se han visto efectos sinérgicos, similares, inesperados entre los fitoestrógenos y las fibras en otros parámetros biológicos pertinentes, tal como la restauración de una actividad pro-apoptótica en la mucosa adenomatosa y de manera aún más interesante en la mucosa no adenomatosa, a nivel comparable con aquellos observados en la mucosa intestinal saludable. La relación desequilibrada de proliferación/apoptosis, observada en la mucosa intestinal no adenomatosa de ratones APC Min⁺ alimentados con dieta estándar, un modelo experimental validado para valorar tanto los mecanismos patobiológicos así como la eficiencia de intervenciones farmacéuticas y/o nutracéuticas de pertinencia clínica humana, puede explicar la recurrencia clínicamente documentada de los pólipos en pacientes introducidos al programa de colonoscopia de examen, debido a la detección

anterior de los pólipos y a la polipectomía, considerado como un riesgo incrementado del progreso y desarrollo de CRC.

Los resultados obtenidos, mencionados anteriormente soportan en resumen, ampliamente, el uso contemplado de las composiciones, objeto de la presente invención. En particular, en mamíferos, humanos o no humanos, una composición para administración oral de acuerdo a la invención puede inducir la expresión del receptor-beta de estrógenos directamente en la mucosa intestinal, cuando este receptor está pobremente expresado o ausente debido a la presencia de lesiones pre-cancerosas en la mucosa del colon, debido a una mutación del supresor tumoral del APC, tal como por (FAP) familiar y adenopoliposis esporádica.

Además, las composiciones orales de acuerdo a la presente invención pueden ser un sistema útil para seleccionar preferentemente como objetivo la administración de los fitoestrógenos al área intestinal afectada del desarrollo de pólipos, que es esta área el intestino delgado en el modelo experimental de ratón y en el colon en humanos, donde se digieren parcialmente las fibras insolubles. Las fibras insolubles contenidas en las composiciones de acuerdo a la presente invención, y en particular lignina, actúan como un escudo en el tracto gastrointestinal superior, donde de otro modo se absorben de manera preferencial los fitoestrógenos. Además, este efecto protector permite un

contacto prolongado de los fitoestrógenos comprendidos en las composiciones de acuerdo a esta invención sobre la mucosa intestinal y sobre los colonocitos. Como una prueba del concepto, en tanto que la fibra insoluble, y particularmente la lignina, fue inefectiva per se al contraatacar la neoplasia intestinal, como se valora por el número de pólipos desarrollados y su volumen, las composiciones de acuerdo a la invención, y en particular, la combinación de silimarina a la dosis efectiva más baja con lignina, determinó una reducción máxima en el número y volumen de los pólipos, comparable a la obtenida al adicionar la dieta estándar con silimarina a la dosis efectiva más alta.

Las composiciones de acuerdo a la invención, por lo tanto, se pueden emplear de manera útil, ya sea como productos farmacéuticos o como nutracéuticos, en el tratamiento de pacientes afectados por adenomas de colon, diagnosticados a través de examen colonoscópico, con la finalidad de reducir el número y volumen de los pólipos, de impedir su progreso a adenomas avanzados, y posiblemente, a adenocarcinoma. Las composiciones de acuerdo a la invención también se pueden emplear para impedir o reducir el desarrollo de nuevos pólipos y para impedir su progreso a una forma avanzada, y posiblemente a adenocarcinoma, en pacientes con polipectomía, reclutados actualmente al programa de colonoscopia de examen, en el periodo entre las colonoscopías

programadas de examen.

Las composiciones para administración oral de la presente invención pueden estar tanto en forma líquida como sólida. Independientemente del tipo de fitoestrógenos usados y de la forma de los mismos (es decir, en forma purificada o como extractos), las composiciones, los fitoestrógenos y las mezclas de los mismos están comprendidos de 0.1 % a 10 % en peso, de manera más preferente de 0.5 % a 5 %, y de manera más preferente de 1 % a 2 %.

En realidad, cuando el fitoestrógeno es silimarina, estas composiciones orales sólidas y líquidas comprenden de manera preferente de 0.1 % a 10 % en peso, de manera más preferente de 0.5 % a 5 %, y de manera más preferente de 1 % a 2 % de silimarina, independientemente del extracto usado del mismo.

En el caso del uso de genisteína como el fitoestrógeno, las composiciones sólidas y líquidas de la presente invención comprenden de manera preferente de 0.1 % a 10 % en peso, de manera más preferente de 0.5 % a 5 %, y de manera más preferente de 1 % a 2 % de genisteína, independientemente del extracto usado.

Las composiciones sólidas y líquidas, objeto de la presente invención, comprenden de manera preferente de 0.1 % a 10 % en peso, de manera más preferente de 0.5 % a 5 %, y de manera más preferente de 1 % a 2 % de cumestrol,

independientemente del extracto usado.

Las composiciones sólidas y líquidas, objeto de la presente invención, comprenden de manera preferente de 0.1 % a 10 % en peso, de manera más preferente de 0.5 % a 5 %, y de manera más preferente de 1 % a 2 % de fitoestrógenos mezclados, independientemente del extracto usado.

Con referencia al componente de fibra insoluble, las composiciones sólidas de la presente invención comprenden de manera preferente de 1 % a 30 % en peso, de manera más preferente de 5 % a 25 %, y de manera más preferente de 10 % a 20 % de la fibra dietética indigerible, independientemente del tipo de extracto usado (es decir, purificado o como tal).

Las composiciones líquidas de la presente invención, comprenden de manera preferente de 1 % a 20 %, de manera más preferente de 2 % a 15 %, y de manera más preferente de 5 % a 10 % de fibra dietética indigerible e insoluble, independientemente del tipo de extracto usado (es decir, purificado o como tal).

Las composiciones sólidas pueden estar en la forma de polvo, polvo efervescente o tabletas masticables. Las formas de polvo pueden ser a su vez en bases de múltiples dosis (botellas, recipientes, jarras) y de dosis única (sobrecitos). Las formas líquidas pueden estar en la forma de jarabes, soluciones, suspensiones, o dispersiones acuosas. En estas formulaciones, las composiciones pueden comprender

adicionalmente excipiente, diluyentes, conservadores, sabores, agentes espumantes, agentes correctores de pH, farmacéuticos o de grado alimenticio, etc., dependiendo de la forma elegida, adecuada para la administración oral.

5 Además, las composiciones de la presente invención pueden comprender además probióticos, tal como por ejemplo lactobacilos y/o bifidobacterias, tanto vivos como tinalizados, con el propósito de contribuir a la normalización de la microflora intestinal y a la fermentación
10 intestinal de la fibra, efectos que se conoce que contribuyen a la formación de un microambiente saludable en este distrito anatómico. Se adicional probióticos como adyuvantes para mantener o equilibrar la fisiología intestinal y para ayudar a metabolizar la fibra indigerible e insoluble.

15 Las composiciones de acuerdo a la presente invención se muestran en los ejemplos 1-10 de trabajo. Los ejemplos 1-7 de trabajo se refieren a formulaciones sólidas, y los ejemplos 8-10 de trabajo a formulaciones líquidas. A menos que se especifique de otro modo, las proporciones de
20 los componentes que comprenden las composiciones en los ejemplos de trabajo representan los porcentajes en peso de los componentes respectivos con relación al producto final. Los siguientes ejemplos describen y demuestran adicionalmente las modalidades dentro del alcance de la presente invención.
25 Los ejemplos se dan sólo para el propósito de ilustración, y

no se propone que limiten el alcance de la presente invención, debido a que son posibles variaciones de la presente invención sin apartarse del espíritu y alcance de la invención.

5 Formulaciones Sólidas

Ejemplo 1.- Polvo Efervescente

	Componente	% cantidad
	Extracto seco de cardo lechero, silimarina al 70 % por HPLC	2.00
	Que corresponde a silimarina	1.40
10	Lignina	20.00
	Maltodextrinas	70.90
	Citrato de sodio	4.50
	Citrato de potasio	0.50
	Ácido cítrico	1.00
15	Dióxido de silicio	0.10
	Saborizante	1.00

Ejemplo 2.- Polvo efervescente

	Componente	% cantidad
	Extracto seco de cardo lechero, silimarina al 70 % por HPLC	2.00
20	Que corresponde a silimarina	1.40
	Fibra de oligosacárido (inulina)	20.00
	Maltodextrinas	70.90
	Citrato de sodio	4.50
25	Citrato de potasio	0.50

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Componente	% cantidad
Ácido cítrico	1.00
Dióxido de silicio	0.10
Saborizante	1.00

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Ejemplo 3.- Polvo efervescente

Componente	% cantidad
Extracto seco de cardo lechero, silimarina al 70 % por HPLC	2.00
Que corresponde a silimarina	1.40
Lignina o lignina de cadena larga	20.00
Lactobacillus Acidophilus, Tindalizados	Equivalente a 10 MLD
Maltodextrinas	71.90
Bicarbonato de sodio	2.60
Ácido tartárico	1.40
Dióxido de silicio	0.10
Saborizante	2.00

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Ejemplo 4.- Polvo dispersable en agua

Componente	% cantidad
Silimarina	1.40
Polvo de raíz de diente de león	35.00
Maltodextrinas	61.50
Dióxido de silicio	0.10

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Componente	% cantidad
Saborizante	2.00

Ejemplo 5.- Polvo efervescente

5	Componente	% cantidad
	Extracto seco de cardo lechero, silimarina al 70 % por HPLC	1.00
	Que corresponde a silimarina	0.70
	Genisteína	0.70
10	Fibra dietética u oligosacarídica (Inulina)	20.00
	Maltodextrinas	70.90
	Citrato de sodio	4.50
	Citrato de potasio	0.50
	Ácido cítrico	1.00
15	Dióxido de silicio	0.10
	Saborizante	1.00

Ejemplo 6.- Polvo efervescente

20	Componente	% cantidad
	Extracto seco de AlfaAlfa, cumestrol al 10 % por HPLC	7.00
	Que corresponde a cumestrol	0.70
25	Salvado de trigo	30.00

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Componente	% cantidad
Maltodextrinas	55.90
Citrato de sodio	4.50
Citrato de potasio	0.50
Ácido cítrico	1.00
Dióxido de silicio	0.10
Saborizante	1.00

Ejemplo 7.- Polvo dispersable en agua

Componente	% cantidad
Silimarina	0.70
Genisteína	0.70
Polvo de raíz de diente de león	35.00
Maltodextrinas	61.50
Dióxido de silicio	0.10

Formulaciones líquidas

Ejemplo 8.- Jarabe

	Cantidad en 100 ml
Componentes	Gramos
Silimarina	0.90
Lignina, celulosa o inulina	10.00

	Cantidad en 100 ml
Componentes	Gramos
Sorbato de potasio	0.10
Ácido ascórbico	0.50
Ácido cítrico, anhidro	0.05
Fructosa	35.00
Saborizante	0.35
Agua	C.S.

Ejemplo 9.- Jarabe

	Cantidad en 100 ml
Componentes	Gramos
Silimarina	0.45
Genisteína	0.45
Lignina, celulosa o inulina	10.00
Sorbato de potasio	0.10
Ácido ascórbico	0.50
Ácido cítrico, anhidro	0.05
Fructosa	35.00
Saborizante	0.35
Agua	C.S.

Ejemplo 10.- Jarabe

		Cantidad en 100 ml
	Componentes	Gramos
5	Extracto seco de cardo lechero, silimarina al 70 % por HPLC	1.38
	Que corresponde a silimarina	0.90
	Polvo de raíz de diente de león	20.00
	Sorbato de potasio	0.10
10	Benzoato de sodio	1.0
	Ácido ascórbico	0.5
	Ácido cítrico	0.15
	Sorbitol	20.00
	Sacarina	0.013
15	Saborizante	0.20
	Agua purificada	C.S.

Parte Experimental

20 El ratón transgénico C57BL/6J APC Min/+ es un
excelente modelo animal para FAP, debido a que el gen mutado
de APC es homólogo al humano. Aún en la forma más común de
CRC, llamada esporádica, 85 % de los casos muestran la
mutación somática del gen supresor de tumor de APC. Por lo
tanto, el modelo de animal transgénico mutado en APC se

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reconoce como el más significativo para el estudio de los mecanismos patogenéticos que caracterizan la poliposis adenomatosa en el colon, tanto en FAP como en forma esporádica, y se considera un modelo animal validado para el establecimiento de intervenciones nutricionales y farmacológicas que tienen como finalidad controlar el desarrollo de adenomas y de prevenir su progreso a CRC (Levy DB et al. *Inactivation of both APC alleles in human and mouse tumors. Cancer Res* 1994, 54: 5953-5958; Paulsen JE et al. *Qualitative and quantitative relationship between dysplastic aberrant crypt foci and tumorigenesis in the Min/+ mouse colon*). Por esta razón, en el establecimiento de las formulaciones orales, objeto de la presente invención, y en la evaluación de su seguridad y eficiencia, los inventores han hecho uso del modelo de ratón transgénico C57BL/6J APC Min/+, mencionado anteriormente. Más adelante se describen los datos que muestran el efecto en los ratones transgénicos C57BL/6J APC Min/+, alimentados de manera oral con dietas adicionadas con silimarina, que contienen fibras solubles e insolubles en diferentes proporciones con el propósito de resaltar las propiedades diferenciativas de la fibra insoluble e indigerible.

Las tres dietas bajo examen, formuladas con la finalidad de imitar un régimen dietético de alto contenido graso, se muestran como sigue:

- Dieta 5K20 [Mucedola Srl, Settimo Milanese (MI)], que contiene 4.50 % de fibra total, compuesta de fibras solubles e insolubles;

- Dieta purificada que contiene 6.24 % de fibra insoluble, constituida por lignina;

- Dieta purificada que contiene 6.24 % de fibra soluble, constituida por cáscara de Plantago ovata.

Se adicionó a cada dieta silimarina hasta 200 mg/kg. Las dietas se administraron oralmente durante 10 semanas a 114 ratones transgénicos C57BL/6J APC Min/+ de 5-6 semanas de edad. Estos ratones tienen una mutación del gen de APC, y están propensas a desarrollar poliposis adenomatosa de colon. El objetivo del estudio fue verificar la capacidad de la composición, de acuerdo a la presente invención, en reducir de manera significativa el desarrollo de pólipos y en prevenir su progreso a formas severamente displásticas, marcadores de transformación neoplástica, en un modelo animal reconocido como comparable a la situación patológica humana. Se ha realizado el análisis estadístico por medio del Análisis unidireccional de varianza y las pruebas t de Student, considerando una $p < 0.05$ estadísticamente significativa.

Los animales se dividieron en 14 grupos, como se reporta en la Tabla 1. Cada tratamiento oral duró 10 semanas. El esquema de tratamiento se diseñó para resaltar

los efectos del tipo de fibra, asociado o no con cantidades crecientes de silimarina. Como un control positivo, se usó un grupo de ratones transgénicos alimentados con dieta estándar (5K20, Mucedola) e implantados subcutáneamente con un gránulo que contiene 1.5 mg de 17β -estradiolo (estrógeno).

Tabla 1. Número de animales por grupo (sil = silimarina, ppm = partes por millón)

	Dieta estándar	Dieta con cáscara de Plantago ovata	Dieta con lignina
10 + sil 0 ppm	15	10	10
+ sil 10 ppm	6	6	6
+ sil 50 ppm	6	6	6
+ sil 200 ppm	15	6	6
+ sil 600 ppm	7		
15 + estrógeno (s.c., control)	9		

No se reportó diferencia en la ingestión dietética diaria (aproximadamente 3 ± 0.1 g/día) entre los animales en los diferentes grupos. Durante el periodo de tratamiento, la proporción de mortalidad fue baja en los grupos alimentados con dieta estándar o dieta adicionada con las composiciones descritas en esta solicitud de patente (7.1 %), en tanto que en los grupos alimentados con dieta adicionada con cáscara de Plantago ovata la mortalidad fue muy alta (>50 %).

La Tabla 2 enlista las medias y desviaciones estándar del peso de los animales de los diferentes grupos de dieta, en el momento del sacrificio. En la admisión a la jaula del animal su peso promedio fue 19 ± 1.2 g.

5 Tabla 2. Media y desviación estándar de pesos corporales en el momento del sacrificio

	Dieta estándar	Dieta con lignina
+ sil 0 ppm	22.0 ± 3.8 g	25.0 ± 1.9 g
+ sil 10 ppm	26.0 ± 1.9 g	25.2 ± 2.3 g
10 + sil 50 ppm	24.6 ± 3.3 g	26.9 ± 2.1 g
+ sil 200 ppm	23.5 ± 2.6 g	27.9 ± 1.7 g
+ sil 600 ppm	24.3 ± 2.4 g	-
+ estrógeno (s.c., control)	24.9 ± 2.8 g	

15 De manera diferente de los grupos animales alimentados con dieta que contiene fibra soluble de cáscara de *P. ovata*, en los cuales se observó una mayor proporción de mortalidad y ninguna reducción en los números de los pólipos, la asociación de silimarina y lignina, que constituye una de las composiciones de acuerdo a la presente invención, probó ser segura y bien tolerada, y dio por resultado un mantenimiento bajo altamente efectivo del número, volumen y el grado de displasia de pólipos adenomatosos en el modelo de ratón transgénico C57BL/6J - APC Min/+. Además, la fibra insoluble dietética (en este caso lignina) probó ser activa al mantener la expresión proteica de

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ER β , evidencia desconocida a la fecha, y probó ser sinérgica con silimarina al mantener significativamente bajo control los parámetros que son marcadores de la proliferación y progreso neoplástico, tal como por ejemplo, el volumen y grado de
5 displasia de los pólipos adenomatosos.

Después de 10 semanas de tratamiento, los animales recibieron una inyección intraperitoneal de 0.75 mg de bromodesoxiuridina (BrdU), equivalente a 30 mg/kg, para verificar la migración de células de la mucosa intestinal a lo
10 largo del eje vellosa (desde el folículo en el fondo a la punta luminal). Este parámetro permite verificar la fisiología exfoliativa normal de la mucosa intestinal, a través de lo cual la mucosa se mantiene compuesta por sí misma de células saludables y vitales.

15 Para este fin, los animales se sacrificaron 24, 48 y 72 horas después de la inyección. Se tomaron intestino delgado y colon de cada animal sacrificado. El intestino delgado se dividió en tres secciones: próxima, media y distante. El tracto próximo se dividió adicionalmente en dos porciones que se
20 rasparon de manera subsiguiente. Uno de los dos se sumergió en ARNlater^{MR} y se almacenó a -20°C y el otro se almacenó en nitrógeno líquido. Las dos muestras se usaron para PCR de tiempo real y análisis Western Blot, respectivamente. Los tractos medio y distante, conjuntamente con el colon, se usaron
25 para análisis histológico. Después de un tratamiento pro-

fijativo de 3 horas con formol, los tractos intestinales bajo examen se observaron bajo un estereomicroscopio STM1, aumento de 3x. Dos observadores que no sabían nada midieron el número y tamaño de los pólipos.

5 La Tabla 3 lista la media y desviación estándar (SD) del número de pólipos observados en los animales alimentados con las diferentes dietas. Se ha percibido que la silimarina
adicionada a 200 ppm a la dieta estándar reduce
significativamente el número de los pólipos ($p = 0.005$), a un
10 nivel comparable con la reducción observada en los ratones transgénicos del grupo de control, tratados de manera subcutánea con estradiol. La lignina sola es inefectiva en este parámetro, pero la asociación de lignina y silimarina, objeto de la
presente invención, reduce de manera significativa el número de
15 pólipos, a una dosis de silimarina (50 ppm) cuatro veces menor que aquella requerida para obtener los mismos resultados con dieta estándar (200 ppm).

Tabla 3. Número de pólipos intestinales en animales alimentados con diferentes dietas

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	Dieta estándar	Dieta con Plantago	Dieta con lignina
+ sil 0 ppm	54.8 ± 20.2	43.6 ± 9.5	46.8 ± 14.0
+ sil 10 ppm	44.0 ± 21.8	45.4 ± 23.8	43.8 ± 19.0
+ sil 50 ppm	55.4 ± 26.2	51.5 ± 20.5	34.0 ± 10.7*

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	Dieta estándar	Dieta con Plantago	Dieta con lignina
+ sil 200 ppm	37.2 ± 9.0*	50.0 ± 4.7	48.5 ± 2.5
+ sil 600 ppm	52.6 ± 24.6		
Estrógeno (control)	32.33 ± 12.06*		

La Tabla 4 lista el contenido de silimarina (expresado como $\mu\text{g/g}$ de silibinina en el tejido, determinado por HPLC) en el tracto duodenal, donde se sabe que se absorbe de manera preferencial la silimarina, y en el íleo después de siete días de alimentar con 200 ppm de silimarina, o una composición que contiene 200 ppm de silimarina y una fibra polisacárido sin almidón (lignina) como un aditamento a la dieta estándar de ratones C57BL/6J (n = 6). La asociación de silimarina y fibra insoluble provocó un transporte preferencial del fitoestrógeno en la región ileal, el área intestinal donde se desarrollan típicamente los pólipos en el ratón transgénico. Además, se conoce que esta condición imita el desarrollo de adenopoliposis en el colon humano. Por lo tanto, las composiciones objeto de la presente invención pueden ser un sistema efectivo para el tratamiento tópico del área afectada por formaciones adenomatosas.

Tabla 4. Media y desviación estándar de concentración en tejido de silimarina en intestino de ratón

	Duodeno ($\mu\text{g/g}$)	íleo ($\mu\text{g/g}$)
Silimarina 200 ppm, 7 días	6.54 ± 2.34	2.84 ± 2.03
Silimarina 200 ppm + lignina, 7 días	2.47 ± 2.38	3.13 ± 2.71

La Tabla 5 lista la media y desviación estándar (SD) del volumen de pólipos intestinales. Asumiendo la forma de los pólipos como una esfera, al medir su diámetro fue posible calcular su volumen, expresado en mm^3 . De manera sorprendente, la lignina sola es capaz de reducir el volumen de los pólipos ($p = 0.0001$). La asociación de silimarina con lignina es capaz de reducir de manera significativa el volumen de los pólipos ($p = 0.0001$). Además, la asociación es sinérgica para los dos componentes, como se sugiere por el hecho que el uso de menores dosis de fitoestrógeno, se observa el mismo efecto como para las mayores dosis del componente individual. De manera sobresaliente, el estradiol probó ser inefectivo en este parámetro, demostrando que la composición, objeto de la presente invención,

que contiene un fitoestrógeno selectivamente agonista a ER β , es efectivo en este parámetro.

5 Tabla 5. Comparación de volumen de pólipos (mm³ $\pm$ SD) en el
animal alimentado con diferentes dietas

	Dieta estándar	Dieta con lignina
+ sil 0 ppm	242.1 $\pm$ 105.1	92.3 $\pm$ 61.9*
+ sil 10 ppm	145.0 $\pm$ 100.6	121.0 $\pm$ 72.6
10 + sil 50 ppm	158.4 $\pm$ 60.3	99.00 $\pm$ 53.13*
+ sil 200 ppm	120.2 $\pm$ 69.0*	138.0 $\pm$ 63.3
+ sil 600 ppm	162.07 $\pm$ 89.35	
Estrógeno (control)	141.78 $\pm$ 79.1	

15 Las preparaciones histológicas de las secciones de
 intestino elegidas al azar de cada animal de los diferentes
 grupos de tratamiento, se observaron por un patólogo que no
 sabía nada del tema, quien graduó la displasia de las
 formaciones adenomatosas presentes como menor, media o
 severa. En los animales tratados con estradiol, 70 %
 20 mostraron pólipos con displasia clasificable como severa,
 versus 21.4 % en animales alimentados con dieta estándar
 adicionada con 200 ppm de silimarina, 30 % en animales
 alimentados con dieta con lignina, y 20 % en animales
 alimentados con dieta con lignina adicionada con 50 ppm de
 25 silimarina.

La Tabla 6 lista los resultados con respecto a HLC (célula marcada más alta) en los grupos experimentales. Los datos muestran que la proporción de migración celular, y en consecuencia la proporción de exfoliación de los enterocitos en el lumen intestinal, se incrementa de manera significativa en el grupo tratado con lignina y silimarina (aún a bajas dosis).

Tabla 6.- Porcentaje de migración de colonocitos desde el folículo en el ápice del vello en animales transgénicos de los diferentes grupos

	Migración de 24 horas (%)
Dieta estándar	28 ± 2.6
+ estrógeno	47.3 ± 10.5*
+ sil 200 ppm	46.6 ± 7.0*
+ lignina	35 ± 13.8
+ (Lignina + sil 50 ppm)	44.4 ± 11.8*

Con respecto al mecanismo de acción de la asociación de silimarina y lignina, los datos demuestran por primera vez una potenciación de la expresión de ER β en la mucosa intestinal. En la Figura 1 se muestran los resultados de RT-PCR. Los niveles de expresión de ARN mensajero para ER β y ER α se reportan como relación promedio entre la densidad óptica del mensajero para cada receptor y aquella del ARNr 18S, con relación a β -actina, una proteína estructural que está sin modificar en las condiciones

experimentales del estudio.

Los resultados muestran un incremento significativo de ARNm de ER β para el grupo de 200 ppm de silimarina ($p = 0.03$), con respecto al grupo alimentado con dieta estándar.

5 De manera sorprendente, los inventores demostraron por primera vez que el ARNm de ER α se disminuye de manera significativa en el grupo tratado con la fibra insoluble ($p = 0.04$); la reducción es más pronunciada en el grupo alimentado con una composición que contiene la asociación silimarina-
10 lignina ($p = 0.04$). De la comparación con el grupo tratado subcutáneamente con estrógeno (activo tanto en ER β como en ER α), los datos confirman a la silimarina como ligando selectivo a ER β y demuestran que la asociación de silimarina (aún a bajas dosis) y lignina, esta última sorprendentemente
15 activa en reducir la expresión de ER α que se conoce que ejerce propiedades proliferativas, facilita adicionalmente en re-establecimiento de una relación entre los dos receptores de estrógenos a favor de la actividad anti-proliferativa, como se muestra en la Figura 2 y se demuestra por los datos
20 en las Tablas 3-6.

La Figura 3 muestra el nivel de expresión intestinal de los receptores de estrógenos, como la relación entre las proteínas de ER β y ER α , determinado usando el método de análisis Western Blot. En comparación al grupo de
25 animales alimentados con sólo dieta estándar, se observó el

incremento de ER β como una proteína en comparación a la proteína de ER α , y este incremento es más marcado en el grupo tratado subcutáneamente con 17 β -estradiol, confirmando de este modo el papel protector, que se tiene como hipótesis para los estrógenos; en el grupo tratado con silimarina, confirmando de este modo sus propiedades como un fitoestrógeno selectivo a ER β ; y de manera sorprendente, en ambos grupos tratados con lignina y con lignina + silimarina, aún a bajas dosis.

10 Se llevaron a cabo experimentos adicionales a fin de proporcionar evidencia de: A) actividades apoptóticas y proliferativas alteradas aún en la mucosa no adenomatosa de animales APC Min/+, en comparación con mucosa intestinal C57BL/6J tipo silvestre (WT), no mutada de APC (considerada como normal y saludable), que son ambos grupos animales alimentados con dieta estándar; B) la capacidad de las composiciones orales, objeto de la presente invención, para restaurar la expresión de ER β , la actividad proliferativa y apoptótica en ratones APC Min/+, a los niveles comparables a los observados en la mucosa normal de animales WT alimentados con dieta normal. Por lo tanto, estas evidencias soportan adicionalmente el uso de estas composiciones orales como un adjunto útil al programa de colonoscopia de examen en pacientes anteriormente polipectomizados, en riesgo incrementado de desarrollar nuevos pólipos (recurrencia).

Las Figuras 4A-4B muestran la relación entre los ARNm de ER β y ER α y el nivel de expresión de ambos receptores como una proteína, sobre la mucosa no adenomatosa de WT y de control APC Min/+ alimentados con dieta normal, o APC Min/+ alimentados con diferentes dietas. Las Figuras 5A-5D demuestran que: i) la mucosa no adenomatosa de ratones APC Min/+ alimentados con la dieta normal ha perdido control pro-apoptótica en la proliferación celular en comparación a los ratones WT alimentado con la dieta normal, como se detecta por marcadores de apoptosis, tal como el número reducido de células positivas en el ensayo TUNEL y expresión de la proteína capsasa 3 escindida, ii) las composiciones orales, objeto de la presente invención, con referencia particular a la asociación de silimarina y lignina, para restaurar el control apoptótico en la proliferación celular a niveles similares a aquellos observados en la mucosa intestinal saludable, normal de ratones WT.

REIVINDICACIONES

1. Composición, caracterizada porque comprende al menos un fitoestrógeno o una mezcla de fitoestrógenos selectivamente agonistas al receptor-beta de estrógenos y al menos una fibra insoluble e indigerible, o mezclas de la misma, seleccionadas del grupo que consiste de polisacáridos sin almidón para tratamiento tópico profiláctico o terapéutico de condiciones patológicas de la mucosa intestinal, seleccionadas del grupo que consiste de adenopoliposis y su progreso a adenomas avanzados y adenocarcinoma por administración oral.

2. Composición de conformidad con la reivindicación 1, caracterizada porque los fitoestrógenos, en forma purificada o como extractos, se seleccionan del grupo que consiste de silimarina, silibinina, iso-silibinina, silidianina, silicristina, genisteína, resveratrol, cumestrol y mezclas de los mismos.

3. Composición de conformidad con la reivindicación 1, caracterizada porque el fitoestrógeno es el extracto del cardo lechero, silimarina, que consiste de la mezcla de silimarina, silibinina, iso-silibinina, silidianina, silicristina.

4. Composición de conformidad con la reivindicación 1, caracterizada porque las fibras insolubles e indigeribles,

en forma purificada o como extracto, se seleccionan del grupo que consiste de lignina, celulosa, inulina, inulina de cadena larga y mezclas de los mismos.

5. Composición de conformidad con las reivindicaciones 2-4, caracterizada porque el fitoestrógeno es silimarina y la fibra insoluble e indigerible es lignina.

6. Composición de conformidad con las reivindicaciones 1-5, caracterizada porque los fitoestrógenos están en un intervalo comprendido de 0.1 a 10 % del peso total de la composición.

7. Composición de conformidad con la reivindicación 6, caracterizada porque los fitoestrógenos están en un intervalo comprendido de 0.5 a 5 % del peso total de la composición.

8. Composición de conformidad con la reivindicación 6, caracterizada porque los fitoestrógenos están en un intervalo comprendido de 1 a 2 % del peso total de la composición.

9. Composición de conformidad con las reivindicaciones 1-5, caracterizada porque las fibras insolubles e indigeribles están en un intervalo comprendido de 1 a 30 % del peso total de la composición.

10. Composición de conformidad con la reivindicación 9, caracterizada porque las fibras están en un intervalo comprendido de 5 a 25 % en formulaciones sólidas.

11. Composición de conformidad con la reivindicación 9, caracterizada porque las fibras están en un intervalo comprendido de 10 a 20 % en formas sólidas.

5 12. Composición de conformidad con la reivindicación 9, caracterizada porque las fibras están en un intervalo comprendido de 2 a 15 % en formas líquidas.

13. Composición de conformidad con la reivindicación 9, caracterizada porque las fibras están en un intervalo comprendido de 5 a 10 % en formas líquidas.

10 14. Composición de conformidad con cualquiera de las reivindicaciones 1-13 anteriores, caracterizada porque se adicionan adicionalmente probióticos, vivos o tinalizados.

15 15. Composición de conformidad con cualquiera de las reivindicaciones 1-14, caracterizada porque está en combinación con excipientes y/o diluyentes farmacéuticamente aceptables, adecuados para formas sólidas seleccionados del grupo que consiste de polvos, polvos efervescentes, tabletas masticables y tabletas.

20 16. Composición de conformidad con cualquiera de las reivindicaciones 1-14, caracterizada porque está en combinación con excipientes y/o diluyentes aceptables del grado alimenticio, adecuados para formas sólidas seleccionadas del grupo que consiste de polvos, polvos efervescentes, tabletas masticables y tabletas.

25 17. Composición de conformidad con cualquiera de

las reivindicaciones 1-14, caracterizada porque está en combinación con excipientes y/o diluyentes farmacéuticamente aceptables, adecuados para formas líquidas seleccionados del grupo que consiste de jarabes, soluciones, suspensiones y 5 dispersiones acuosas.

18. Composición de conformidad con cualquiera de las reivindicaciones 1-14, caracterizada porque está en combinación con excipientes y/o diluyentes aceptables de grado alimenticio, adecuados para formas líquidas 10 seleccionados del grupo que consiste de jarabes, soluciones, suspensiones y dispersiones acuosas.

19. Uso de las composiciones de conformidad con cualquiera de las reivindicaciones anteriores para el tratamiento tópico profiláctico o terapéutico de condiciones 15 patológicas de la mucosa intestinal, seleccionadas del grupo que consiste de adenopoliposis y su progreso a adenomas avanzados y adenocarcinoma por administración oral.

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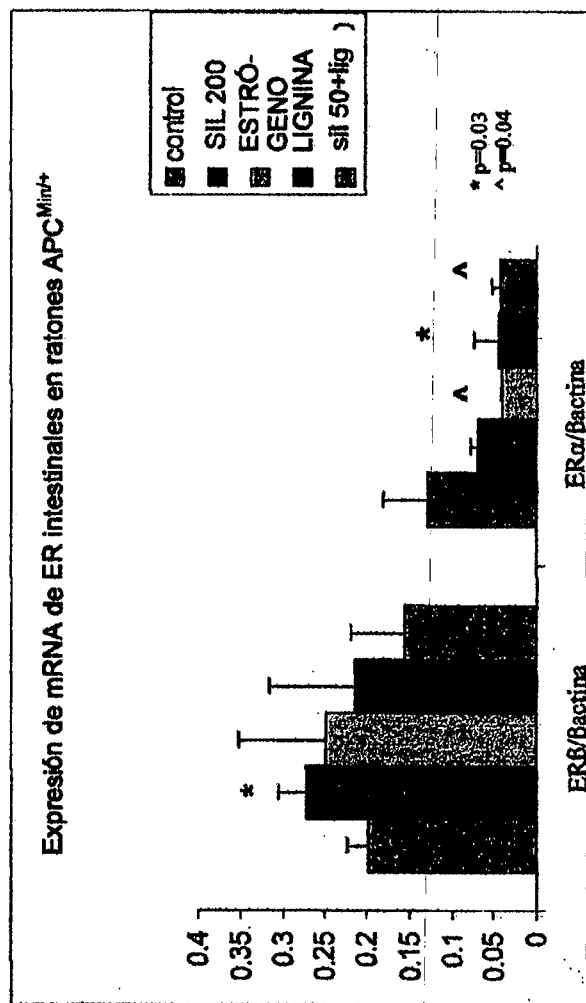
RESUMEN DE LA INVENCION

Se describen composiciones orales que comprenden una asociación de uno o de una mezcla de fitoestrógenos, selectivos para el receptor-beta de estrógenos, con fibras dietéticas. Las composiciones de los mismos se pueden usar de manera ventajosa para el tratamiento profiláctico y terapéutico en mamíferos, incluyendo humanos, de condiciones de salud caracterizadas por un alto riesgo de comienzo y 5 recurrencia de adenomas intestinales (adenopoliposis coli), y del progreso de los adenomas hacia carcinoma colorrectal. En la presencia de lesiones pre-cancerosas en el colon, caracterizadas por mutaciones del supresor de tumor de APC y definidas como pólipos o adenomas, las composiciones orales 10 descritas se pueden usar en realidad de manera ventajosa para reducir el número y volumen de los pólipos, y para impedir su progreso a transformación neoplástica, reduciendo su grado de displasia.

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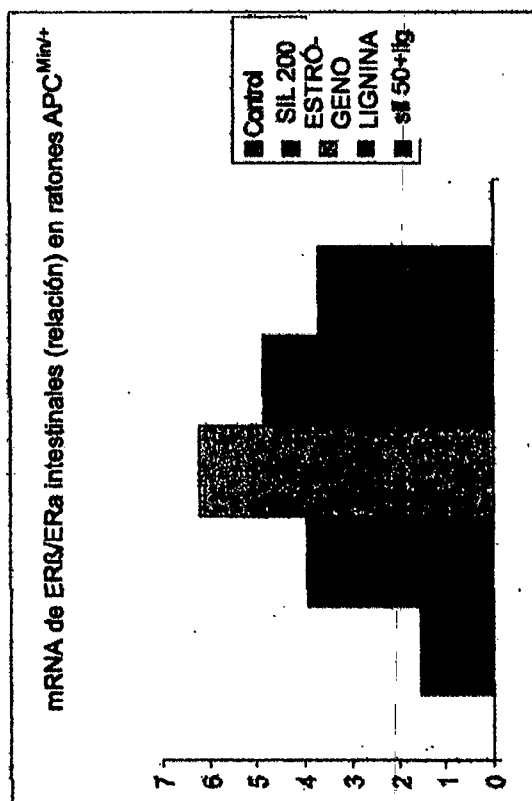
25

FIGURA 1



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FIGURA 2



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FIGURA 3

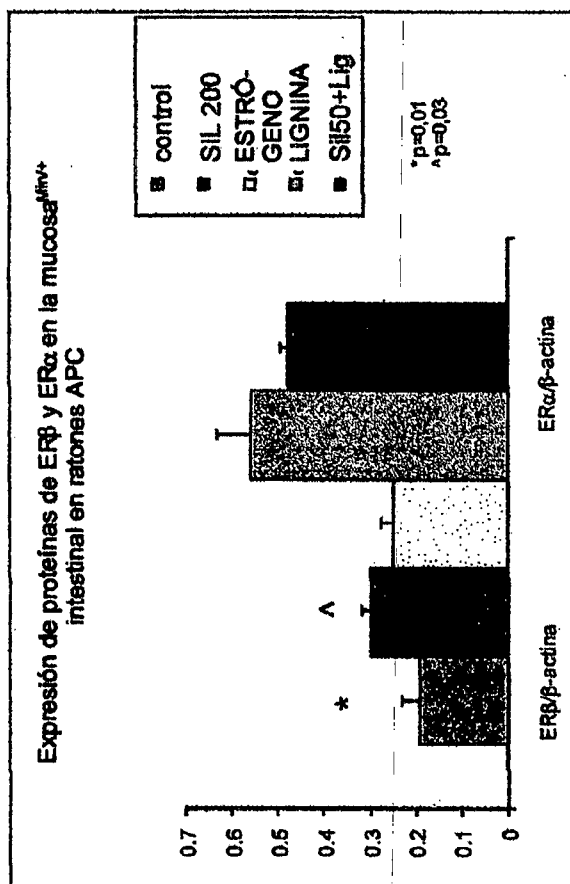


FIGURA 4

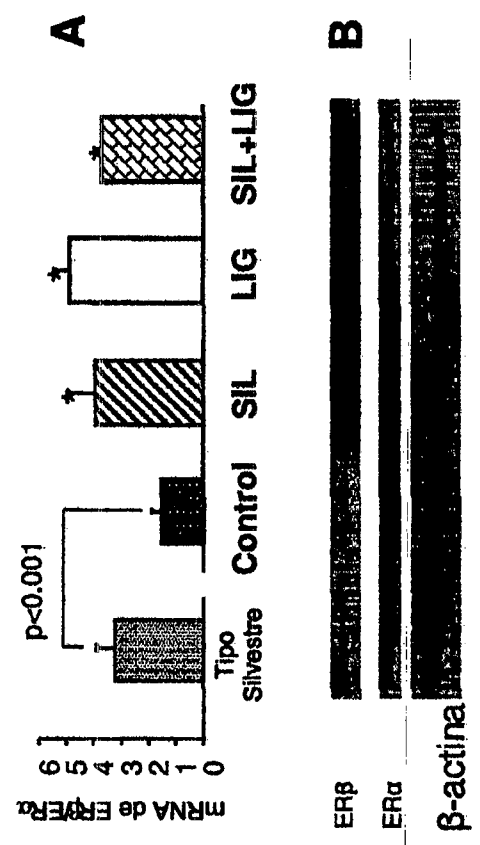
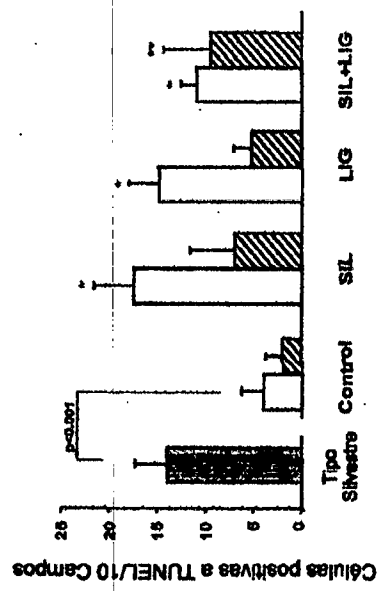
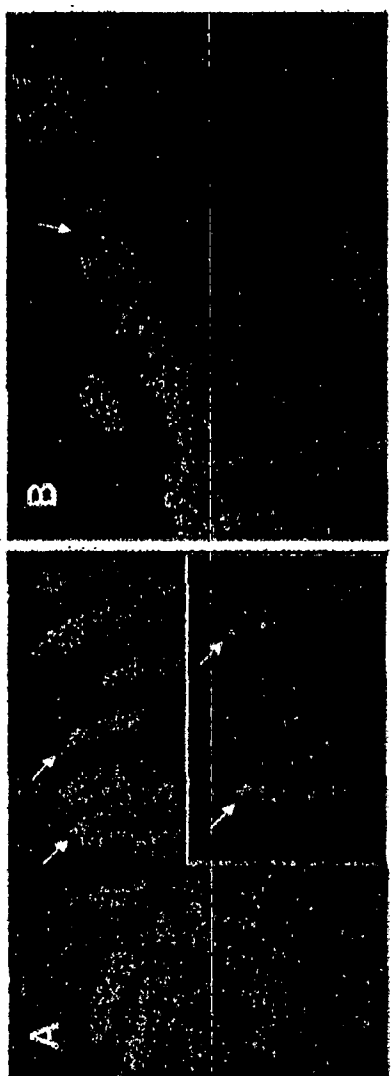


FIGURA 5



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0-2	Fecha de Presentación Internacional	20 de Febrero de 2009 (20.02.2009)
0-3	Nombre de la oficina receptora y "Solicitud Internacional de PCT	RO/EP
0-4 0-4-1	Forma – PCT/RO/101 PCT Petitorio Preparado usando	PCT Online Filing Versión 3.5.000.203 MT/FOP 20020701/0.20.5.9
0-5	Petición El abajo firmante solicita que la presente solicitud internacional sea procesada de acuerdo al Tratado de Cooperación en Materia de Patentes	
0-6	Oficina Receptora (especificada por el solicitante)	Oficina Europea de Patente (EPO) (RO/EP)
0-7	Referencia de archivo del solicitante o mandatario	9582PTWO-ca
I	Título de la invención	COMPOSICIONES QUE COMPRENDEN FITOESTRÓGENOS SELECTIVOS PARA RECEPTOR BETA DE ESTRÓGENOS Y FIBRAS DIETÉTICAS
II-1 II-1-1 II-1-2 II-1-4 II-1-5	Solicitante y/o Inventor Esta persona es: Solicitante para Nombre (APELLIDO, nombre) Dirección:	solamente solicitante para todos los estados excepto US CM&D PHARMA LIMITED Thames House Portsmouth Road Esher KT10 9AD SURREY
II-6 II-7	Estado de nacionalidad Estado de Residencia	Italia GB GB
III-1 III-1-1 III-1-2 III-1-4 III-1-5	Solicitante y/o Inventor Esta persona es: Solicitante para Nombre (APELLIDO, nombre) Dirección:	solicitante e inventor únicamente US DI LEO, Alfredo Via Filippo Turati, 1 70043 MONOPOLI
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III-2-7	Estado de Residencia	IT
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IV-6-5	Correo-e	notage@ngpatent.it
IV-6-6	Agente registro No.	

V	DESIGNACIONES		
V-1	La presentación de este petitorio se constituye de acuerdo con la Regla 4.9(a), la designación de todos los Estados Contractantes unidos al PCT en la fecha de presentación Internacional, para otorgar todos los tipos de protección disponibles y, donde se aplicable, para otorgar tanto patentes regionales como nacionales		
VI-1	Reivindicación de prioridad de la solicitud nacional anterior		
VI-1-1	Fecha de Presentación	22 de Febrero de 2008 (22.02.2008)	
VI-1-2	Número	BA2008A000008	
VI-1-3	País	IT	
VI-2	Incorporación por referencia: donde un elemento de la solicitud internacional referido en el Artículo 11(1)(III)(d) o (a) o una parte de la descripción, reivindicaciones o dibujos referidos en la Regla 20.5(a) no hay otro contenido en la solicitud internacional, pero está completamente contenido en una solicitud anterior cuya prioridad se reivindica en la fecha en la cual uno o más elementos referidos en el Artículo 11(1)(iii) son recibidos primero por la Oficina receptora, en tanto que elementos o partes son sujetos para confirmación de acuerdo con la Regla 20.6, incorporado por referencia en su totalidad para los propósitos de la Regla 20.6		
VII-1	Elección de la Autoridad de Búsqueda Internacional	Oficina Europea de Patentes (EPO) (ISA/EP)	
VIII	Declaraciones	Número de Declaraciones	
VIII-1	Declaración en cuanto la identidad del inventor	-	
VIII-2	Declaración en cuanto a la facultad del solicitante, como en la fecha de presentación internacional, para solicitud y otorgación de una patente.	-	
VIII-3	Declaración en cuanto a la facultad del solicitante, como en la fecha de presentación internacional, para reclamar de la solicitud más anterior.	-	
VIII-4	Declaración de inventividad (sólo para los propósitos de la designación de los Estados Unidos de América.	-	
VIII-5	Declaración en cuanto a las descripciones no perjudiciales a excepciones para carecer de novedad.	-	

IX	Lista de verificación	Número de Hojas	Archivo(s) electrónico(s) anexos
IX-1	Petitorio (incluyendo hojas de declaración)	5	✓
IX-2	Descripción	23	✓
IX-3	Reivindicaciones	2	✓
IX-4	Resumen	1	✓
IX-5	Dibujos	5	✓
IX-7	TOTAL	36	
	Puntos que acompañan	documentos escritos anexos	archivos electrónicos anexos
IX-8	Hoja de cálculos de tasas	-	✓
IX-18	PCT-SAFE medio físico	-	-
IX-21	Figura de los dibujos que debe adjuntarse al resumen:		
IX-21	Idioma de presentación de la solicitud internacional:	Inglés	
IX-1	Firma del Solicitante o agente	/Gemma Gervasi/	
IX-1-1	Nombre (APELLIDO, nombre)	GERVASI, Gemma	
IX-1-2	Nombre del firmante	GERVASI, Gemma	
IX-1-3	Habilidad	(Representante)	

PARA USO DE LA OFICINA RECEPTORA ÚNICAMENTE

10-1	Fecha del recibo real de la solicitud internacional alegada:	20 de Febrero de 2009 (20.02.2009)
10-2	Dibujos	
10-2-1	Recibida	✓
10-2-2	No Recibida	
10-3	Fecha corregida del vencimiento del recibo real a los documentos o dibujos recibidos posteriormente pero a tiempo que complementan la solicitud internacional alegada:	
10-4	Fecha de recepción puntual de las correcciones requeridas bajo el Artículo 11(2) del PCT	
10-5	Autoridad de Búsqueda Internacional	ISA/EP
10-6	Transmisión de copia de búsqueda retrasada hasta que la tasa de búsqueda se pague	

PARA USO DE LA OFICINA INTERNACIONAL ÚNICAMENTE

11-1	Fecha de la recepción de la copia de registro por la Oficina Internacional	
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TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES

PCT

REPORTE DE BÚSQUEDA INTERNACIONAL

(Artículo 18 y Reglas 43 y 44 del PCT)

Referencia al expediente del solicitante o del mandatario 9582PTWO-ca	PARA ACCION ADICIONAL	ver Forma PCT/ISA/220 así como, en donde aplique el inciso 5 posterior
No. de solicitud internacional PCT/EP2009/052038	Fecha de presentación internacional (día/mes/año) 20/02/2009	(Fecha de Prioridad) (La más cercana) (día/mes/año) 22/02/2008
Solicitante CM&D PHARMA LIMITED		

Este reporte de búsqueda internacional ha sido preparado por esta Autoridad de la Búsqueda Internacional y se transmite al solicitante de conformidad con el Artículo 18. Se transmite una copia a la Oficina Internacional.

Este reporte de búsqueda internacional consiste de un total de 5 hojas

☒ También está acompañado de una copia de cada documento de la técnica anterior citado en este reporte.

1. Bases del reporte

a. Con respecto al idioma, la búsqueda internacional se ha llevado a cabo en las bases de:

☒ la solicitud internacional en el idioma en el cual ha sido presentada.
☐ una traducción de la solicitud internacional _____, que está en el idioma de la traducción proporcionada para los propósitos de la búsqueda internacional (Reglas 12.3(a) y 23.1 (b)).

b. ☐ Este reporte de búsqueda internacional se ha establecido tomando en cuenta la rectificación de un error obvio autorizado por o notificado para esta Autoridad de acuerdo con la Regla 91, Regla 43.6 bis(a)

c. ☐ Con respecto a cualquier secuencia de aminoácidos y/o nucleótidos descrita en la solicitud internacional, Ver Cuadro No. I

2. ☐ Ciertas reivindicaciones no se pueden ser el objeto de una búsqueda (ver el Cuadro II).

3. ☐ Se carece de unidad de la invención (ver Cuadro III).

4. Con respecto al título,

☒ el texto se aprueba tal como lo presentó el solicitante,
☐ esta Autoridad ha establecido que el texto sea como sigue:

5. Con respecto al resumen,

☒ el texto se aprueba tal como lo presentó el solicitante.
☐ esta Autoridad ha establecido el texto, de acuerdo con la Regla 38.2(b), tal como aparece en el Cuadro IV. El solicitante puede, dentro de un mes a partir de la fecha de envío por correo de este reporte de búsqueda internacional, proporcionar comentarios a esta autoridad.

6. Con respecto a los dibujos

a. la figura de los dibujos que se va a publicar con el resumen es: La Figura No. _____

☐ como lo sugirió el solicitante.
☐ porque al solicitante le faltó sugerir una figura.
☐ porque esta figura caracteriza mejor la invención.

b. ☒ ninguna de las figuras ha sido publicada con el resumen

REPORTE DE BÚSQUEDA INTERNACIONAL

No. de Solicitud Internacional

PCT/EP2009/052038

A. CLASIFICACION DEL ASUNTO RELACIONADO			
INV.	A61K 31/357	A16K 31/352	A61K 31/05
	A61P 1/00	A61P 35/00	
De acuerdo con la Clasificación Internacional de Patentes (IPC) o tanto la clasificación nacional como la IPC.			
B. CAMPOS BUSCADOS			
Documentación mínima buscada (sistema de clasificación seguido por los símbolos de clasificación)			
A61K			
Documentación buscada diferente de la documentación mínima, al grado que estos documentos se incluyen en los campos buscados.			
Base de datos, electrónica, consultada durante la búsqueda internacional (nombre de la base de datos, donde se puede practicar, términos de búsqueda usados).			
EPO-Internal, BIOSIS, EMBASE, WPI Data			
C. DOCUMENTOS QUE SE CONSIDERAN PERTINENTES			
Categoría*	Citación del documento, con indicación, donde es apropiado, de los pasajes pertinentes		Pertinente para la reivindicación No.
X	TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 de Junio de 2003 (2003-06-01), páginas 168-171, XP009117194 ISSN: 0301-4800 página 169, columna izquierda, párrafo 1		1, 4, 5, 8, 9, 15, 18
☒ Documentos adicionales se listan en la continuación del cuadro C.		☒ Los miembros de la familia de patentes se listan en el anexo	
* Categoría especial de los documentos citados:			
"A"	documento que define el estado general de la técnica, que no se considera que es de pertinencia particular	"T"	documento posterior publicado después de la fecha de presentación internacional o la fecha de prioridad, y que no provoca conflicto con la solicitud, pero se cita para entender el principio o teoría que trata la invención
"E"	Documento anterior, pero publicado en o después de la fecha de presentación internacional	"X"	documento de pertinencia particular; la invención reivindicada no se puede considerar nueva o no se puede considerar que comprende un actividad inventiva cuando el documento se toma solo
"L"	documento que puede crear dudas de la reivindicación(es) de prioridad o que se cita para establecer la fecha de publicación de otra citación u otra razón especial (como se especifique)	"Y"	documento de pertinencia particular; la invención reivindicada no se puede considerar que comprende un actividad inventiva cuando el documento se combina con uno o más de estos documentos, estas combinaciones son obvias para una persona experta en la técnica
"O"	documento que se refiere a una descripción oral, uso, exhibición u otro medio	"&"	documento miembro de la familia de patentes
"P"	documento publicado antes de la fecha de presentación internacional, pero después de la fecha de prioridad reivindicada		
Fecha de la actual terminación de la búsqueda internacional		Fecha de envío del reporte de búsqueda internacional	
25 de Mayo de 2009		05/06/2009	
Nombre y dirección de envío de la ISA/ Oficina Europea de Patentes, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Funcionario autorizado Baurand, Petra	

REPORTE DE BÚSQUEDA INTERNACIONAL

No. Solicitud Internacional
PCT/EP2009/052038

DOCUMENTOS QUE SE CONSIDERAN PERTINENTES (continuación)

Categoría*	Citación del documento, con indicación, donde es apropiado, de los pasajes pertinentes	Pertinente para la Reivindicación No.
X	STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 01 de Septiembre de 1984 (1984-09-01), páginas 1219-1224, XP009117192 ISSN: 0008-4212 página 1220, columna izquierda, párrafo 6	1, 2, 4, 15
X	DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 09 de Junio de 2005 (2005-06-09) reivindicaciones 1,4	1, 4, 14
Y	HOH CARMEN S I ET AL.: "A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, Abril de 2006 (2006-04), páginas 752, XP001537130 resumen	1-19
Y	MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER BIOMED CENTRAL, LONDON GB, vol. 2, no. 1, 17 de Octubre de 2002 (2002-10-17), pagina 27, XP021016050 ISSN: 147-2407 página 34, columna izquierda, párrafo 1	1-19
Y	LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH) (2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, Marzo de 2004 (2004-03), páginas 736, XP001536466 resumen	1-19

REPORTE DE BÚSQUEDA INTERNACIONALNo. Solicitud Internacional
PCT/EP2009/052038**DOCUMENTOS QUE SE CONSIDERAN PERTINENTES (continuación)**

Categoría*	Citación del documento, con indicación, donde es apropiado, de los pasajes pertinentes	Pertinente para la Reivindicación No.
X	HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2 1983, paginas 189-196, XP002529043 página 193, Análisis	1-19
Y	EP 0 846 704 A (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 de Junio de 1998 (1998-06-10) reivindicaciones 1, 13	1-19

REPORTE DE BÚSQUEDA INTERNACIONAL

Información sobre los miembros de familia de patentes

Solicitud Internacional No.:

PCT/EP2009/052038

Documento de la patente citado en el reporte de búsqueda	Fecha de publicación	Miembros de la familia de patentes		Fecha de publicación
DE 102004054133 A1	09-06-2005	CZ	20033042 A3	15-06-2005
		SK	322004 A3	02-06-2005
EP 0846704 A	10-06-1998	AT	214400 T	15-03-2002
		AU	725110 B2	05-10-2000
		AU	4684697 A	04-06-1998
		CA	2223149 A1	03-06-1998
		DE	69711002 D1	18-04-2002
		DE	69711002 T2	28-11-2002
		DK	846704 T3	21-05-2002
		ES	2170340 T3	01-08-2002
		JP	10191931 A	28-07-1998
		PT	846704 E	31-07-2002
		US	6043229 A	28-03-2000

TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES

De la AUTORIDAD DE LA BÚSQUEDA INTERNACIONAL

Para:

*ver forma PCT/ISA/220***PCT**

**OPINIÓN ESCRITA DE LA AUTORIDAD
ENCARGADA DE BÚSQUEDA INTERNACIONAL
(Regla 43bis.1 de PCT)**

Fecha de envío: (día/mes/año)

ver forma PCT/ISA/210 (segunda hoja)

Referencia al expediente del Solicitante o Mandatario

*ver forma PCT/ISA/220***PARA ACCIÓN ADICIONAL****Ver Párrafo 2 más adelante**

Número de Solicitud Internacional:

PCT/EP2009/052038

Fecha de Presentación Internacional: (día/mes/año)

20.02.2009

Fecha de prioridad (día/mes/año)

22.02.2008

Clasificación Internacional de Patente (IPC) o tanto la clasificación internacional como la IPC

INV. A61K 31/357 A61K 31/352 A61K 31/05 A61K 31/716 A61K 45/06 A61P 1/00 A61P 35/00

Solicitante:

CM&D PHARMA LIMITED

1. Este reporte contiene las indicaciones que están relacionadas con los siguientes puntos:

☒ Cuadro No. I Base del reporte☐ Cuadro No. II Prioridad☐ Cuadro No. III Ningún establecimiento del reporte con respecto a la novedad, actividad inventiva, o aplicabilidad industrial☐ Cuadro No. IV Carencia de unidad de la invención☒ Cuadro No. V Declaración razonada de acuerdo con la Regla 66.2 con respecto a la novedad, actividad inventiva o aplicabilidad industrial; las citas y explicaciones que soportan tal declaración☐ Cuadro No. VI Ciertos documentos citados☐ Cuadro No. VII Ciertos defectos en la solicitud internacional☒ Cuadro No. VII Ciertas observaciones en la solicitud internacional.**2. ACCIÓN ADICIONAL**

Si se hace una solicitud para un examen preliminar internacional, esta opinión se considerará que es una opinión escrita de la Autoridad del Examen Preliminar Internacional ("IPEA") sin embargo, esto no aplica donde el solicitante elige una autoridad diferente de esta para ser la IPEA y la IPEA elegida ha notificado a la Oficina Internacional de acuerdo con la Regla 66.1bis(b) que no se considerarán las opiniones escritas de esta Autoridad Encargada de la Búsqueda Internacional.

Si esta opinión se considera, como se proporciona anteriormente, que es una opinión escrita de la IPEA, se invita al solicitante a presentar a la IPEA una contestación escrita conjuntamente, donde sea apropiado, con enmiendas, antes del vencimiento de los 3 meses desde la fecha de envío de la forma PCT/ISA/220 o antes del vencimiento de los 22 meses desde la fecha de prioridad, cualquiera que expire posteriormente.

Para opciones adicionales, ver la forma PCT/ISA/220.

3. Para detalles adicionales, ver notas a la Forma PCT/ISA/220.

Nombre y dirección de envío de la ISA:

Oficina Europea de Patentes

D-80298 Munich

Tel. + 49 89 2399 - 0

Fax: + 49 89 2399 - 4465

Fecha de terminación de esta
opinión*ver forma
PCT/ISA/210*

Oficial Autorizado

(rúbrica)

Baurand, Petra

Teléfono No. +49 89 2399-2156

Forma PCT/ISA/237 (Hoja de Cubierta) (Abril de 2005)

**OPINIÓN ESCRITA DE LA
AUTORIDAD ENCARGADA
DE BÚSQUEDA INTERNACIONAL**

No de Solicitud Internacional
PCT/EP2009/052038

Cuadro No. I Bases de la opinión

1. Con respecto al idioma, este reporte se basa en

- ☒ la solicitud internacional en el idioma en el cual se ha presentado
- ☐ una traducción de la solicitud internacional _____, que está en el idioma de la traducción proporcionada para los propósitos de la búsqueda internacional (Reglas 12.3(a) y 23.1 (b)).

2. ☐ Esta opinión se ha establecido tomando en cuenta la rectificación de una autoridad obvia por o notificada a esta Autoridad de acuerdo con la Regla 91 (Regla 43bis.1(a))

3. Con respecto a cualquier **secuencia de nucleótidos y/o aminoácidos** descrita en la solicitud internacional, y necesario a la invención reivindicada, esta opinión se ha establecido en las bases de:

a. tipo de material:

- ☐ un listado de secuencias
- ☐ tabla(s) que se refieren al listado de secuencias

b. formato del material

- ☐ en formato escrito
- ☐ en forma legible por computadora

c. tiempo de la presentación/otorgamiento

- ☐ contenido en la solicitud internacional como se presentó
- ☐ presentado junto con la solicitud internacional en la forma legible por computadora
- ☐ otorgado de manera subsiguiente a esta Autoridad para los propósitos de búsqueda

4. ☐ Además, en el caso de que más de que una versión o copia del listado de secuencias y/o tablas que se relacionan se han presentado u otorgado, las declaraciones requeridas de que la información en las copias subsiguientes o adicionales es idéntica a esto en la solicitud como se presentó o no van más allá de la solicitud como se presentó, como es apropiado, se otorgó.

5. Comentarios adicionales:

**OPINIÓN ESCRITA DE LA
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No de Solicitud Internacional
PCT/EP2009/052038

Cuadro No. V Declaración razonada de acuerdo con la Regla 43bis.1(a)(i) con respecto a la novedad, actividad inventiva, y aplicabilidad industrial; citas y explicaciones que soportan esta declaración

1. DECLARACIÓN

Novedad (N)

Si Reivindicaciones 3, 6, 7, 10-13, 16, 17, 19

No Reivindicaciones 1, 2, 4, 5, 8, 9, 14, 15, 18

Actividad
Inventiva (PI)

Si Reivindicaciones -

No Reivindicaciones 1-19

Aplicabilidad
Industrial (AI)

Si Reivindicaciones 1-19

No Reivindicaciones -

2. Citas y explicaciones

ver hoja separada

Cuadro No. VIII Ciertas observaciones en la solicitud internacional

Se hacen las siguientes observaciones en la claridad de las reivindicaciones, descripción y dibujos en la cuestión de si las reivindicaciones se soportan completamente por la descripción

ver hoja separada

**OPINIÓN ESCRITA DE LA
AUTORIDAD ENCARGADA
DE BÚSQUEDA INTERNACIONAL
HOJA SEPARADA**

No de Solicitud Internacional
PCT/EP2009/052038

Con Referencia al Punto V.

5.1 Se hace referencia a los siguientes documentos:

D1: TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 de junio de 2003 (2003-06-01), páginas 168-171, XP009117194 ISSN: 0301-4800

D2: STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 01 de septiembre de 1984 (1984-09-01), páginas 1219-1224, XP009117192 ISSN: 0008-4212

D3: DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 de junio de 2005 (2005-06-09)

D4: HOH CARMEN S I ET AL.: "A clinical pilot study of oral silibinin, a putativa colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, de abril de 2006 (2006-04), página 752, XP001537130

D5: MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 de octubre de 2002 (2002-10-17), página 27, XP021016050 ISSN: 1471-2407

D6: LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, Marzo de 2004 (2004-03), página 736, XP001536466

D7: HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, páginas 189-196, XP002529043

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HOJA SEPARADA

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D8: EP-A-O 846,704 (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 de junio de 1998 (1998-06-10)

5.2 La presente solicitud no cumple con los criterios del Artículo 33(1) del PCT, debido a que la materia de las reivindicaciones 1, 2, 4, 5, 8, 9, 14, 15 y 18 no es nueva en el sentido del Artículo 33(2) del PCT, las razones que son como sigue:

El documento D1 describe la alimentación de ratas con una dieta sólida de celulosa-isoflavona que contiene celulosa y una mezcla de daidzeína y genisteína, en donde la dieta se encontró que tiene un efecto en la microflora intestinal (página 169, columna izquierda, primer párrafo). De esta manera, se considera que es pertinente para la novedad de las reivindicaciones 1, 4, 5, 8, 9, 15 y 18.

D2 reporta ratas alimentadas con la mezcla de coumestrol + lignina (página 1220, columna izquierda, séptimo párrafo). Por lo tanto, quita la novedad de las presentes reivindicaciones 1, 2, 4 y 15.

D3 describe una composición que comprende silimarina, yacon y celulosa (reivindicaciones 1 y 4) y por lo tanto está destruyendo la novedad para la materia de las reivindicaciones 1, 4 y 14.

5.3 Con respecto a la novedad se señala que la reivindicación 1 se refiere a una composición "para" administración oral. Una reivindicación a una composición "para" un uso particular se debe considerar como que significa una composición que en realidad es "adecuada para" el uso señalado. Si una composición conocida está en una forma en la cual es adecuada para el uso señalado, aunque nunca se haya descrito para este uso, quita la reivindicación de novedad.

5.4 la presente solicitud no cumple con los criterios del Artículo 33(1) del PCT, debido a que la materia de las reivindicaciones 1-19 no comprende una actividad inventiva en el sentido del Artículo 33(3) del PCT.

Los Documentos D4-D6, que se consideran como equivalentes y representan el estado más pertinente de la técnica a la materia de la reivindicación 1, muestran que los fitoestrógenos

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tal como silimarina, silibinina, resveratrol y genisteína poseen eficiencia quimiopreventiva en carcinogénesis colorrectal (D4: resumen; D5: página 34, página izquierda, primer párrafo; D6: resumen).

La materia de la reivindicación 1 independiente difiere de estas descripciones ya que la composición reivindicada comprende una fibra insoluble e indigerible.

El problema que se va a solucionar por la presente invención por lo tanto se puede considerar como la provisión de una composición adicional eficiente en la prevención y/o tratamiento de carcinogénesis colorrectal.

Sin embargo, los documentos D7 y D8 reportan que las fibras dietéticas tal como celulosa y almidón resistente son útiles para el tratamiento de enfermedades del tracto digestivo colorrectal tal como carcinogénesis colorrectal (D7: página 193, Análisis; D8: reivindicaciones 1 y 13).

Por lo tanto, las características descritas en D4-D6 se combinarán por la persona experta con las características conocidas a partir de ya sea D7 o D8, sin ejercicio de ninguna habilidad inventiva a fin de solucionar el problema presentado. La solución propuesta en la reivindicación 1 independiente no se puede considerar de esta manera como inventiva (Artículo 33(3) del PCT).

5.5 Las reivindicaciones 2-19 dependientes no contienen ninguna característica que, en combinación con las características de cualquier reivindicación a la cual se refieran, cumplan con los requisitos del PCT con respecto a la actividad inventiva.

Con Referencia al Punto VIII.

8.1 Se señala que la reivindicación 18 no es aceptable de acuerdo al Artículo 6 del PCT. Se señala que el concepto de "terapia" o "aplicación terapéutica" incluye tratamiento de una dolencia o enfermedad particular con una sustancia química especificada o composición especificada en un sujeto humano o animal especificado en necesidad de este tratamiento. En la ausencia de la identificación de al menos (i) la dolencia o enfermedad que se va a tratar o la afección que se va a curar o (ii) la naturaleza del compuesto terapéutico usado para tratar

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o curar la enfermedad o (iii) el sujeto que se va a tratar, una característica más de proceso ("**tratamiento de la mucosa intestinal**") no se puede considerar como que especifica un método particular de tratamiento o aplicación terapéutica.

8.2 La presente reivindicación 1 se refiere a un compuesto definido por referencia a una característica o propiedad deseable, específicamente "**fitoestrógeno selectivamente agonista al receptor-beta de estrógeno**". Se hace un intento para definir el compuesto por su perfil farmacológico, volviendo oscura la protección de esta reivindicación. Se señala que un compuesto no se puede caracterizar de manera suficiente por su perfil farmacológico o su modo de acción. El uso de esta definición funcional es vaga y no clara y deja al lector en duda con respecto al significado de la característica técnica (es decir, los compuestos) a la cual se refiere.

Se dirige la atención del Solicitante al hecho que una definición funcional no puede ser permisible, sólo si se cumplen **todos** los siguientes criterios:

(a) compuestos que cumplen la función son parte del conocimiento general común (es decir, se pueden encontrar en enciclopedias, libros de texto o diccionarios),

(b) la función se puede verificar por prueba o procedimientos adecuadamente especificados en la descripción o conocidos por la persona experta, y

(c) los contenidos completos de la solicitud de patente muestran de manera inambigua que la función es causal para la solución del problema que fundamente la invención de modo que es claramente razonable que se puedan usar no sólo los ejemplos en la solicitud sino cualquier otro compuesto con la función señalada.

8.3 Adicionalmente, la reivindicación 1 se refiere a "**polisacáridos no de almidón**". En contraste, la reivindicación 4 (que es dependiente de la reivindicación 1) precisa este polisacárido no de almidón como que es "**almidón resistente**". Esta inconsistencia dentro de las reivindicaciones da por resultado una carencia de claridad, contrario al Artículo 6 del PCT.

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8.4 Se dirige la atención del Solicitante al hecho que el término "progreso de adenopoliposis a adenomas y adenocarcinomas" (reivindicación 19) no se puede considerar una aplicación terapéutica. El descubrimiento que una mezcla de sustancias impide el progreso de la adenopoliposis hacia adenomas y adenocarcinoma, aun si representa una pieza importante de conocimiento científico, aun necesita encontrar una aplicación práctica en la forma de un tratamiento real definido de cualquier condición patológica a fin de hacer una contribución técnica a la técnica y que se considere como una invención elegible para protección por patente.

TRATADO DE COOPERACION EN MATERIA DE PATENTES
P C T
REPORTE PRELIMINAR INTERNACIONAL DE PATENTABILIDAD
(Capítulo II del Tratado de Cooperación en Materia de Patentes)

(Artículo 36 y Regla 70 del PCT)

Referencia de registro del Solicitante o del Agente 9582PTWO	PARA TOMAR ACCION Véase Forma PCT/IPEA/416 ADICIONAL	
Solicitud Internacional No. PCT/EP2009/052038	Fecha de Presentación Internacional 20.02.2009	Fecha de Prioridad 22.02.2008
Clasificación de Patente Internacional (IPC) o Clasificación nacional e IPC INV. A61K 31/357		
Solicitante CM&D Pharma Limited		
1. Este reporte de examen preliminar internacional ha sido preparado por esta Autoridad de Examen Preliminar Internacional y es transmitido al solicitante de acuerdo con el Artículo 36. 2. Este REPORTE consiste de <u>7</u> hojas, incluyendo esta hoja de cubierta 3. Este reporte también está acompañado por ANEXOS, que comprenden: a. ☒ (enviar al solicitante y al Buró Internacional) un total de <u>5</u> hojas, como sigue: ☒ hojas de la descripción, las reivindicaciones y/o los dibujos, los cuales han sido enmendados y son la base para este reporte y/o las hojas que contienen las rectificaciones hechas antes de esta Autoridad. (Véase la Regla 70.16 y la Sección 607 de las Instrucciones Administrativa). ☐ hojas que reemplazan las hojas anteriores, pero que esta Autoridad considera que contienen una enmienda que va más allá de la descripción en la solicitud internacional como se presentó, como se indica en el punto 4 del Cuadro No. I y el Cuadro Complementario. b. ☐ (enviar al solicitante y al Buró Internacional) un total de (tipo indicado y número de conductor electrónico(s)) <u> </u> contienen un listado de secuencias y/o tablas que se relacionan al mismo, en forma legible por computadora únicamente, como se indica en el Cuadro Complementario Relacionado al Listado de Secuencias (ver Sección 802 de las Instrucciones Administrativas).		
4. Este reporte contiene las indicaciones que están relacionadas con los siguientes puntos: ☒ Cuadro No. I Base del reporte ☐ Cuadro No. II Prioridad ☐ Cuadro No. III Ningún establecimiento del reporte con respecto a la novedad, actividad inventiva, o aplicabilidad industrial ☐ Cuadro No. IV Carencia de unidad de la invención ☒ Cuadro No. V Declaración razonada de acuerdo con la Regla 66.2 con respecto a la novedad, actividad inventiva o aplicabilidad industrial; las citas y explicaciones que soportan tal declaración ☐ Cuadro No. VI Ciertos documentos citados ☐ Cuadro No. VII Ciertos defectos en la solicitud internacional ☐ Cuadro No. VIII Ciertas observaciones en la solicitud internacional.		
Fecha de presentación de la solicitud 21-12-2009	Fecha de terminación de este reporte 20.04.2010	
Nombre y dirección de envío de la ISA: Oficina Europea de Patentes D-80298 Munich Tel. +49 89 2399 - 0 Fax. +49 89 2399 - 4465	Oficial Autorizado (Rúbrica) Baurand, Petra Teléfono No. +49 89 2399-2156	

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Cuadro No. I Bases del reporte

1. Con respecto al idioma, este reporte se basa en

- ☒ la solicitud internacional en el idioma en el cual se ha presentado
- ☐ una traducción de la solicitud internacional en, que es el idioma de una traducción otorgada para los propósitos de:
- ☐ búsqueda internacional (de acuerdo con las Reglas 12.3 y 23.1(b))
 - ☐ publicación de la solicitud internacional (de acuerdo con la Regla 12.4)
 - ☐ examen preliminar internacional (de acuerdo con las reglas 55.2 y/o 55.3).

2. Con respecto a los elementos* de la solicitud internacional este reporte se basa en (las hojas de reemplazo que se han otorgado a la Oficina receptora en respuesta a una invitación bajo el Artículo 14 se refieren en este reporte como "presentadas originalmente" y no se anexan a este reporte puesto que no contienen enmiendas):

Descripción, páginas	1-7, 9-19, 22, 23	como se presentó originalmente
	8, 20, 21	presentada con la carta de 18-12-2009
Reivindicaciones, Números	1-19	presentada con la carta de 18-12-2009
Dibujos, Hojas.	1-5	como se presentó originalmente

☐ un listado de secuencias y/o cualquier tabla(s) que se relaciona – ver cuadro Suplementario que se Refiere al Listado de Secuencias

3. ☐ Las enmiendas han dado por resultado la cancelación de:

- ☐ la descripción, páginas
- ☐ las reivindicaciones, Números
- ☐ los dibujos, hojas/figuras
- ☐ el listado de secuencias (especificar):
- ☐ cualquier tabla(s) que se relaciona(n) al listado de secuencias (especificar):

4. ☐ Este reporte se ha establecido como si (alguna de) las enmiendas no se hayan hecho, puesto que se han considerado que van más allá de la descripción como se presentó como se indica en el Cuadro Complementario (Regla 70.2 (c)):

- ☐ la descripción, páginas
- ☐ las reivindicaciones, Números
- ☐ los dibujos, hojas/figuras
- ☐ el listado de secuencias (especificar):

5. ☐ Esta opinión se ha establecido tomando en cuenta la rectificación de un error obvio autorizado por o notificado por esta Autoridad de acuerdo con la Regla 91 (Regla 70.2 (e)).

6. ☐ Reporte de búsqueda internacional complementario de la(s) Autoridad(es) se ha recibido y tomado en cuenta en la redacción de este reporte (Regla 45bis.8(b) y (c))

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Cuadro No. V Declaración razonada de acuerdo con el Artículo 35(2) con respecto a la novedad, actividad inventiva, y aplicabilidad industrial; citas y explicaciones que soportan esta declaración.

1. DECLARACION

Novedad (N)	Si Reivindicaciones <u>3, 5, 7, 8, 11-14, 17-19</u> No Reivindicaciones <u>1, 2, 4, 6, 9, 10, 15, 16</u>
Actividad Inventiva (PI)	Si Reivindicaciones No Reivindicaciones <u>1-19</u>
Aplicabilidad Industrial (AI)	Si Reivindicaciones <u>1-19</u> No Reivindicaciones

2. Citas y explicaciones (Regla 70.7):
ver hoja separada

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Con Referencia al Punto V

Declaración razonada con respecto a la novedad, actividad inventiva o aplicabilidad industrial; citas y explicaciones que soportan esta declaración

5.1 Se hace referencia a los siguientes documentos:

D1: TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 de junio de 2003 (2003-06-01), páginas 168-171, XP009117194 ISSN: 0301-4800

D2: STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 01 de septiembre de 1984 (1984-09-01), páginas 1219-1224, XP009117192 ISSN: 0008-4212

D3: DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 de junio de 2005 (2005-06-09)

D4: HOH CARMEN S I ET AL.: "A clinical pilot study of oral silibinin, a putativa colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, abril de 2006 (2006-04), página 752, XP001537130

D5: MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 de octubre de 2002 (2002-10-17), página 27, XP021016050 ISSN: 1471-2407

D6: LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, Marzo de 2004 (2004-03), página 736, XP001536466

D7: HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, páginas 189-196, XP002529043

D8: EP-A-O 846,704 (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 de junio de 1998 (1998-06-10)

5.2 La presente solicitud no cumple con los criterios del Artículo 33(2) del PCT, debido a que la materia de las reivindicaciones 1, 2, 4, 6, 9, 10, 15 y 16 no es nueva, las razones que son como sigue:

Se señala que una reivindicación a una composición para un uso particular se debe construir como que significa una composición que en realidad es adecuada para el uso señalado. Si una composición está en una forma en la cual es

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adecuada para el uso señalado, aunque nunca se haya descrito para ese uso, despoja la reivindicación de novedad.

En el presente caso, la novedad de la reivindicación 1 dirigida a "una composición que comprende al menos un fitoestrógeno y al menos una fibra disoluble e indigerible para aplicación tópica profiláctica o terapéutica de condiciones patológicas de la mucosa intestinal" se destruye por cualquier documento que describa la composición. En consecuencia, las siguientes descripciones quitan la novedad de las reivindicaciones 1, 2, 4, 6, 9, 10, 15, 16 y 19;

D1 que describe la alimentación de ratas con una dieta sólida de celulosa-isoflavona que contiene celulosa y una mezcla de daidzeina y genisteína, en donde la dieta se encontró que tiene un efecto en la microflora intestinal (página 169, columna izquierda, primer párrafo).

D2 que reporta ratas alimentadas con la mezcla de coumestrol + lignina (página 1220, columna izquierda, séptimo párrafo).

D3 que describe una composición que comprende silimarina, yacon y celulosa (reivindicaciones 1 y 4).

5.3 con respecto a la novedad, el Solicitante parece argumentar en su carta con fecha de 18.12.2009 que la característica que confiere novedad de las reivindicaciones 1 y 19 con respecto a los documentos D1-D3 citados sería "el tratamiento tópico de las condiciones patológicas de la mucosa intestinal". La presente Autoridad le gustaría enfatizar que en la técnica relacionada, el término "tópico" se refiere en general a un tratamiento realizado en la superficie del cuerpo y de esta manera parece estar en contradicción con la expresión "por administración oral" usada en las reivindicaciones 1 y 19. Especialmente, cuando se toman en cuenta los presentes Ejemplos que describen la administración oral de la composición reivindicada hacia ratas, es claro que las reivindicaciones 1 y 19 se refieren a un tratamiento oral y no a uno tópico.

5.4 La presente solicitud no cumple con los criterios del Artículo 33(3) del PCT, debido a que la materia de las reivindicaciones 1-19 no comprende una actividad inventiva.

La actividad inventiva sólo se puede conocer cuando la materia de la reivindicación (independiente) solución de un problema técnico de una manera inesperada o sorprendente, es decir, cuando se cumplan todas las siguientes condiciones:

- (i) debe haber un problema técnico,
- (ii) el problema técnico se debe solucionar en el intervalo completo reivindicado,

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(iii) la reivindicación debe contener todas las características técnicas que conducen a la solución del problema,

(iv) la solución del problema debe ser inventiva (= inesperada/sorprendente).

El problema que se va a solucionar por la presente invención se puede considerar como que proporciona una composición efectiva en el tratamiento de la adenopoliposis y su progreso a adenomas avanzados y adenocarcinoma.

Se señala que el problema técnico no se soluciona en el intervalo completo reivindicado. Las presentes reivindicaciones 1 y 19 abarcan sustancialmente cualquier fitoestrógeno selectivamente agonista al receptor-beta de estrógenos en combinación con sustancialmente cualquier polisacárido no de almidón. En contraste, los presentes ejemplos describen el efecto de una combinación única, específicamente silimarina + lignina, en un modelo de poliposis adenomatosa familiar humana. Puesto que no parece ser creíble que se puedan extrapolar resultados obtenidos con una combinación única a sustancialmente cualquier fitoestrógeno en combinación con cualquier polisacárido no de almidón, se considera que no se cumple la condición del punto (ii) anterior.

En consecuencia, la materia de las reivindicaciones 1 y 19 carece de actividad inventiva (Artículo 33(3) del PCT).

5.5 Las reivindicaciones dependientes 2-18 no contienen ninguna característica que, en combinación con las características de cualquier reivindicación a la cual se refieren, cumplan con el requisito del PCT con respecto a la actividad inventiva.

5.6 La reivindicación 19 se refiere a materia considerara por esta Autoridad como que se cubre por las provisiones de la Regla 39.1 (iv)/67.1 (iv) del PCT. La patentabilidad puede ser dependiente de la formulación de las reivindicaciones. La EPO, por ejemplo, no reconoce como reivindicaciones patentables el uso de un compuesto para un tratamiento médico, pero puede permitir reivindicaciones a un producto, en particular sustancias o composiciones para el uso en un primero o adicional tratamiento médico.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

GERVASI, Gemma
NOTARBARTOLO & GERVASI S.P.A.
Corso di Porta Vittoria 9
I-20122 Milan
ITALIE

Date of mailing (day/month/year) 09 June 2009 (09.06.2009)	
Applicant's or agent's file reference 9582PTWO-ca	IMPORTANT NOTIFICATION
International application No. PCT/EP2009/052038	International filing date (day/month/year) 20 February 2009 (20.02.2009)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 22 February 2008 (22.02.2008)
Applicant CM&D PHARMA LIMITED et al	

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. (If applicable) The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
3. (If applicable) An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
22 February 2008 (22.02.2008)	BA2008A000008	IT	02 June 2009 (02.06.2009)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Yolaine Cussac e-mail PT05.PCT@WIPO.INT Telephone No. +41 22 338 74 05
Facsimile No. +41 22 338 82 70 Form PCT/IB/304 (October 2005)	

1/D232GUD60

From the INTERNATIONAL BUREAU

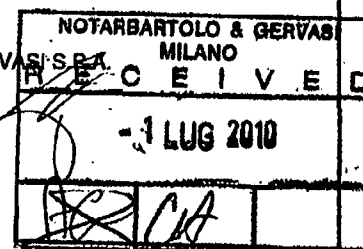
PCT

SECOND AND SUPPLEMENTARY NOTICE
INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION (TO DESIGNATED OFFICES
WHICH APPLY THE 30 MONTH TIME
LIMIT UNDER ARTICLE 22(1))

(PCT Rule 47.1(c))

To:

GERVASI, Gemma
NOTARBARTOLO & GERVASI S.R.L.
Corso di Porta Vittoria 9
I-20122 Milan
ITALIE



Date of mailing (day/month/year) 24 June 2010 (24.06.2010)		
Applicant's or agent's file reference 9582PTWO-ca		IMPORTANT NOTICE
International application No. PCT/EP2009/052038	International filing date (day/month/year) 20 February 2009 (20.02.2009)	Priority date (day/month/year) 22 February 2008 (22.02.2008)
Applicant CM&D PHARMA LIMITED et al		

1. **ATTENTION:** For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), does not apply, please see Form PCT/IB/308(First Notice) issued previously.
2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93bis.1. The International Bureau has effected that communication on the date indicated below:
27 August 2009 (27.08.2009)

AU, AZ, BY, CN, CO, DZ, EP, HU, KG, KP, KR, MD, MK, MY, MZ, NA, NG, PG, RU, SY, TM, US

In accordance with Rule 47.1(c-bis)(i), those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93bis.1:

AE, AG, AL, AM, AO, AP, AT, BA, BB, BG, BH, BR, BW, BZ, CA, CH, CR, CU, CZ, DE, DK, DM, DO, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, ID, IL, IN, IS, JP, KE, KM, KN, KZ, LA, LC, LK, LR, LS, LT, LY, MA, ME, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PH, PL, PT, RO, RS, SC, SD, SE, SG, SK, SL, SM, ST, SV, TJ, TN, TR, TT, UA, UZ, VC, VN, ZA, ZM, ZW

In accordance with Rule 47.1(c-bis)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. TIME LIMITS for entry into the national phase

For the designated or elected Office(s) listed above, the applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain of the designated or elected Office(s) listed above. For regular updates on the applicable time limits (30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

It is the applicant's sole responsibility to monitor all these time limits.

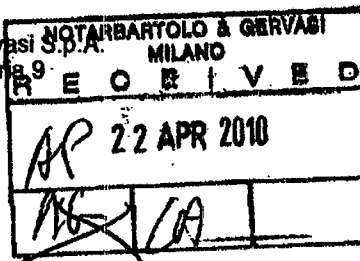
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Yolaine Cussac
Facsimile No. +41 22 338 82 70	e-mail: pt05.pct@wipo.int

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Gervasi, Gemma
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PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
REPORT ON PATENTABILITY

(PCT Rule 71.1)

Date of mailing
(day/month/year)

20.04.2010

Applicant's or agent's file reference
9582PTWO

IMPORTANT NOTIFICATION

International application No.
PCT/EP2009/052038

International filing date (day/month/year)
20.02.2009

Priority date (day/month/year)
22.02.2008

Applicant
CM&D Pharma Limited

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary report on patentability and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary report on patentability. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

The applicant's attention is drawn to Article 33(5), which provides that the criteria of novelty, inventive step and industrial applicability described in Article 33(2) to (4) merely serve the purposes of international preliminary examination and that "any Contracting State may apply additional or different criteria for the purposes of deciding whether, in that State, the claimed inventions is patentable or not" (see also Article 27(5)). Such additional criteria may relate, for example, to exemptions from patentability, requirements for enabling disclosure, clarity and support for the claims.

Name and mailing address of the international
preliminary examining authority:



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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 9582PTWO		FOR FURTHER ACTION	See Form PCT/PEA/16
International application No. PCT/EP2009/052038	International filing date (day/month/year) 20.02.2009	Priority date (day/month/year) 22.02.2008	
International Patent Classification (IPC) or national classification and IPC INV. A61K31/357			
Applicant CM&D Pharma Limited			
1. This report is the International preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36. 2. This REPORT consists of a total of <u>7</u> sheets, including this cover sheet. 3. This report is also accompanied by ANNEXES, comprising: a. ☒ sent to the applicant and to the International Bureau a total of <u>5</u> sheets, as follows: ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions). ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box. b. ☐ (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see paragraph 3b/s of Annex C of the Administrative Instructions).			
4. This report contains indications relating to the following items: ☒ Box No. I Basis of the report ☐ Box No. II Priority ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability ☐ Box No. IV Lack of unity of invention ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement ☐ Box No. VI Certain documents cited ☐ Box No. VII Certain defects in the international application ☐ Box No. VIII Certain observations on the international application			
Date of submission of the demand 2009-12-21		Date of completion of this report 20.04.2010	
Name and mailing address of the International preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4485		Authorized officer Baurand, Petra Telephone No. +49 89 2399-2156 	

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/EP2009/052038

Box No. I Basis of the report

1. With regard to the language, this report is based on
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
 - ☐ International search (under Rules 12.3(a) and 23.1(b))
 - ☐ publication of the international application (under Rule 12.4(a))
 - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))
2. With regard to the elements* of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report):*

Description, Pages

1-7, 9-19, 22, 23 as originally filed

8, 20, 21 filed with the letter of

18-12-2009

Claims, Numbers

1-19 filed with the letter of

18-12-2009

Drawings, Sheets

1-5 as originally filed

- ☐ a sequence listing - see Supplemental Box Relating to Sequence Listing.

3. ☐ The amendments have resulted in the cancellation of:
 - ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):
4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
 - ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
5. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 70.2 (e)).
6. ☐ Supplementary international search report(s) from Authority(ies) have been received and taken into account in drawing up this report (Rule 45bis.8(b) and (c)).

**INTERNATIONAL PRELIMINARY REPORT
ON PATENTABILITY**International application No.
PCT/EP2009/052038

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>3, 5, 7, 8, 11-14, 17-19</u>
	No: Claims	<u>1, 2, 4, 6, 9, 10, 15, 16</u>
Inventive step (IS)	Yes: Claims	
	No: Claims	<u>1-19</u>
Industrial applicability (IA)	Yes: Claims	<u>1-19</u>
	No: Claims	

2. Citations and explanations (Rule 70.7):see separate sheet

**INTERNATIONAL PRELIMINARY
REPORT ON PATENTABILITY
(SEPARATE SHEET)**

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Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

5.1 Reference is made to the following documents:

- D1 TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 June 2003 (2003-06-01), pages 168-171, XP009117194 ISSN: 0301-4800
- D2 STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 1 September 1984 (1984-09-01), pages 1219-1224, XP009117192 ISSN: 0008-4212
- D3 DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 June 2005 (2005-06-09)
- D4 HOH CARMEN S I ET AL: "A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, April 2006 (2006-04), page 752, XP001537130
- D5 MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 October 2002 (2002-10-17), page 27, XP021016050 ISSN: 1471-2407
- D6 LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, March 2004 (2004-03), page 736, XP001539466
- D7 HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, pages 189-196, XP002529043

**INTERNATIONAL PRELIMINARY
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International application No.

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D8 EP 0 846 704 A (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 June 1998 (1998-06-10)

5.2 The present application does not meet the criteria of Article 33(2) PCT, because the subject-matter of claims 1, 2, 4, 6, 9, 10, 15 and 16 is not new, the reasons being as follows:

It is noted that a claim to a composition for a particular use should be construed as meaning a composition which is in fact suitable for the stated use. If a known composition is in a form in which it is suitable for the stated use, though it has never been described for that use, it deprives the claim of novelty.

In the present case, novelty of claim 1 directed to "a composition comprising at least one phytoestrogen and at least one insoluble and indigestible fibre for prophylactic or therapeutic topical treatment of pathological conditions of intestinal mucosa" is destroyed by any document disclosing said composition. Consequently, the following disclosures take away novelty of claims 1, 2, 4, 6, 9, 10, 15, 16 and 19:

D1 which describes feeding of rats with a cellulose-isoflavone solid diet containing cellulose and a mixture of daidzein and genistein wherein said diet was found to have an effect on the intestinal microflora (page 169, left-hand column, 1st paragraph);

D2 which reports rats fed with the mixture of coumestrol + lignin (page 1220, left-hand column, 6th paragraph) and

D3 which discloses a composition comprising silymarin, yacon and cellulose (claims 1 and 4).

5.3 With respect to novelty the Applicant appears to argue in his letter dated 18.12.2009 that the novelty-conferring feature of claims 1 and 19 over the cited documents D1 - D3 would be "the topical treatment of intestinal mucosa pathological conditions". The present Authority would like to stress that in the related art the term "topical" generally relates to a treatment performed on the surface of the body and thus seems to be in contradiction with the expression "by oral administration" used in said claims 1 and 19. Especially when taking into account the present Examples describing oral administration of the claimed composition to rats, it is clear that claims 1 and 19 relate to an oral and not to a topical treatment.

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5.4 The present application does not meet the criteria of Article 33(3) PCT, because the subject-matter of claims 1 - 19 does not involve an inventive step.

Inventive activity can only be acknowledged when the subject-matter of an (independent) claim solves a technical problem in an unexpected or surprising manner, i.e. when all of the following conditions are fulfilled:

- (i) there must be a technical problem
- (ii) the technical problem must be solved in the whole range claimed
- (iii) the claim must contain all technical features leading to the solution of the problem
- (iv) the solution of the problem must be inventive (= unexpected/surprising)

The problem to be solved by the present invention may be regarded as providing for a composition effective in the treatment of adenopolyposis and its progression to advanced adenomas and adenocarcinoma.

It is pointed out that the technical problem is not solved in the whole range claimed. Present claims 1 and 19 encompass substantially any phytoestrogen selectively agonist to the estrogen receptor-beta in combination with substantially any non-starch polysaccharide. By contrast, the present Examples describe the effect of one single combination, namely silymarin + lignin, in a model of human familial adenomatous polyposis. Since it does not appear to be credible that results obtained with one single combination could be extrapolated to substantially any phytoestrogen in combination with substantially any non-starch polysaccharide, it is considered that the condition of point (ii) above is not fulfilled.

Consequently, the subject-matter of claims 1 and 19 lacks inventive step (Article 33(3) PCT).

5.5 Dependent claims 2 - 18 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step.

5.6 Claim 19 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 39.1 (iv) / 67.1 (iv) PCT. The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound for a medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

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different concentrations and ratios, with particular reference to a Milk Thistle extract with an analytical profile characterised by a silymarin content higher than 70% and a silyblinin A+B content higher than 30%. In general, additional components to and/or substitutes of silymarin in the compositions object of the present invention are other phytoestrogens, provided that they are selective for ER β and with a anti-estrogenic activity, that is not inducing a significant proliferative activity on tissues that are classical targets for the estrogens, such the above mentioned phytoestrogens, in similar concentrations as that of silymarin.

The other essential component of the compositions for oral administration of the present invention is an insoluble and indigestible, i.e. resistant to enzymatic digestion in the gastrointestinal tract, fibre. The insoluble fibre can be present in a purified form or as component of a natural extract, provided that in the compositions the insoluble fibre content is between 1% and 30%. Such fibre is selected from plant fibres with a non-starch polysaccharidic nature and among these fibres are preferably fibres selected in the group consisting of polyphenylpropanoid lignin (hereafter called also lignin), cellulose, inulin and long chain inulin and mixtures thereof. Particular preference is given to the lignin. The insoluble fibre can be present as a purified component as well as a constituent of an extract. In the later case, the above mentioned insoluble fibres can be present as a mixture and associated with other nutritional substances. In particular, the fibres can be used for the compositions of the invention as follows: in the case of lignin, as such in the polydisperse form or as wheat bran; in the case of cellulose, as such or as a soy extract; in the case of inulin, long chain inulin is preferable, with a polymerisation degree of 10-65 and an average chain length of 25, as such or as artichoke extract, chicory extract or Dandelion root powder.

The fibres present in the oral compositions object of the present invention are anyway to be considered add-ons to the diet, but independent from the total dietary fibre intake.

The compositions according to the inventions have shown unexpected biological properties, since said compositions, characterized substantially by the association from at least one phytoestrogen, selectively agonist to estrogen receptor beta (ER β), or mixtures thereof and at least one dietary insoluble fibre selected from

Table 3. Number of intestinal polyps in animals fed different diets

	Standard diet	Diet with Plantago	Diet with ligin
+ sil 0 ppm	54.8 ± 20.2	43.6 ± 9.5	46.8 ± 14.0
+ sil 10 ppm	44.0 ± 21.8	45.4 ± 23.8	43.8 ± 19.0
+ sil 50 ppm	55.4 ± 26.2	51.5 ± 20.5	$34.0 \pm 10.7^*$
+ sil 200 ppm	$37.2 \pm 9.0^*$	50.0 ± 4.7	48.5 ± 2.5
+sil 600 ppm	52.6 ± 24.6		
Estrogen (control)	$32.33 \pm 12.06^*$		

Table 4 lists the silymarin content (expressed as $\mu\text{g/g}$ silybinin in the tissue, determined by HPLC) in the duodenal tract, where silymarin is known to be preferentially absorbed, and in the ileum after seven days feeding 200 ppm silymarin, or a composition containing 200 ppm silymarin and a non starch polysaccharidic fibre (lignin) as an add-on to the standard diet of C57BL/6J mice ($n=6$). The association of silymarin and insoluble fibre caused a preferential transport of the phytoestrogen in the ileal region, the intestinal area where polyps typically develop in the transgenic mouse. Moreover, it is known that such condition mimics the development of adenopolyposis in human colon. Therefore, the compositions object of the present invention can be an effective system for the topical treatment of the area affected by adenomatous formations.

Table 4. Mean and standard deviation of silymarin tissue concentration in mouse

	Intestine	
	Duodenum ($\mu\text{g/g}$)	Ileum ($\mu\text{g/g}$)
Silymarin 200 ppm, 7 days	6.54 ± 2.34	2.84 ± 2.03
Silymarin 200 ppm + Lignin, 7 days	2.47 ± 2.38	3.13 ± 2.71

Table 5 lists mean and standard deviation (SD) of the volume of intestinal polyps. Assuming polyps shape as a sphere, by measuring their diameter it was possible

to calculate their volume, expressed in mm^3 . Surprisingly, lignin alone is able to reduce polyps volume ($p=0.0001$). The association of silymarin with lignin is able to significantly reduce the volume of polyps ($p=0.0001$). Moreover, the association is synergic for the two components, as suggested by the fact that the using lower doses of phytoestrogen, the same effect as per the higher doses of single component is observed. Noteworthy, estradiol proved to be ineffective on this parameter, demonstrating that the composition object of the present invention, containing a phytoestrogen selectively agonist to $\text{ER}\beta$, is effective on this parameter.

Table 5. Comparison of polyps volume ($\text{mm}^3 \pm \text{SD}$) in animal fed the different diets

	Standard diet	Diet with lignin
+ sil 0 ppm	242.1 $\pm$ 105.1	92.3 $\pm$ 61.9*
+ sil 10 ppm	145.0 $\pm$ 100.6	121.0 $\pm$ 72.6
+ sil 50 ppm	158.4 $\pm$ 60.3	99.00 $\pm$ 63.13*
+ sil 200 ppm	120.2 $\pm$ 69.0*	138.0 $\pm$ 63.3
+ sil 600 ppm	162.07 $\pm$ 89.35	
Estrogen (control)	141.78 $\pm$ 79.1	

Histological preparations of randomly chosen intestine sections of every animal from the different treatment groups were observed by a blinded pathologist, who graded the dysplasia of the adenomatous formations present as minor, mild or severe. In the estradiol-treated animals, 70% showed polyps with dysplasia classifiable as severe, versus 21.4% in animals fed standard diet added with 200 ppm silymarin, 30% in animals fed diet with lignin, and 20% in animals fed diet with lignin added with 50 ppm silymarin.

Table 6 lists the results concerning HLC (highest labeled cell) in the experimental groups. Data shows that cell migration rate, and consequently the esfoliation rate of enterocytes in the intestinal lumen, is significantly increased in the group treated with lignin and silymarin (even at low doses).

CLAIMS

1. A composition comprising at least one phytoestrogen or a mixture of phytoestrogens selectively agonists to the estrogen receptor-beta and at least one insoluble and indigestible fibre or mixtures thereof selected from the group
5 consisting of non-starch polysaccharides for prophylactic or therapeutic topical treatment of intestinal mucosa pathological conditions selected from the group consisting of adenopolyposis and its progression to advanced adenomas and adenocarcinoma by oral administration.
2. The composition according to claim 1, wherein the phytoestrogens, in
10 purified form or as extracts, are selected from the group consisting of silymarin, silybinin, iso-silybinin, silydianin, silychristin, genistein, resveratrol, coumestrol and mixtures thereof.
3. The composition according to claim 1, wherein the phytoestrogen is the Milk Thistle extract silymarin consisting of the mixture of silymarin, silybinin, iso-silybinin, silydianin, silychristin.
15
4. The composition according to claim 1, wherein insoluble and indigestible fibres, in purified form or as extracts, are selected from the group consisting of lignin, cellulose, inulin, long chain inulin and mixtures thereof.
5. The composition according to claims 2- 4, wherein the phytoestrogen is
20 silymarin and the insoluble and indigestible fibre is lignin.
6. The composition according to claims 1-5, wherein phytoestrogens are in a range comprised from 0.1 to 10 % of the total weight of said composition.
7. The composition according to claims 6, wherein phytoestrogens are in a range comprised from 0.5 to 5% of the total weight of said composition.
- 25 8. The composition according to claims 6, wherein phytoestrogens are in a range comprised from 1 to 2% of the total weight of said composition.
9. The composition according to claims 1-5, wherein the insoluble and indigestible fibres are in a range comprised from 1 to 30% of the total weight of said composition.
- 30 10. The composition according to claim 9, wherein the fibres are in a range comprised from 5 to 25% in solid formulations.

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25

11. The composition according to claim 9, wherein the fibres are in a range comprised from 10 to 20% in solid forms.
12. The composition according to claim 9, wherein the fibres are in a range comprised from 2 to 15% in liquid forms.
- 5 13. The composition according to claim 9, wherein the fibres are in a range comprised from 5 to 10% in liquid forms.
14. The composition according to any one of the preceding claims 1-13, wherein probiotics, live or tyndalized, are further added.
15. The composition according to any one of the preceding claims 1-14, in
10 combination with pharmaceutically acceptable excipients and/or diluents suitable for solid forms selected from the group consisting of powders, effervescent powders, chewable tablets and tablets.
16. The composition according to any one of the preceding claims 1-14, in
15 combination with food grade acceptable excipients and/or diluents suitable for solid forms selected from the group consisting of powders, effervescent powders, chewable tablets and tablets.
17. The composition according to any one of the preceding claims 1-14, in
20 combination with pharmaceutically acceptable excipients and/or diluents suitable for liquid forms selected from the group consisting of syrups, solutions, suspensions and aqueous dispersions.
18. The composition according to any one of the preceding claims 1-14, in
25 combination with food grade acceptable excipients and/or diluents suitable for liquid forms selected from the group consisting of syrups, solutions, suspensions and aqueous dispersions.
19. Use of the compositions according to any one of the preceding claims for the prophylactic or therapeutic topical treatment of intestinal mucosa pathological conditions selected from the group consisting of adenopolyposis and its progression to advanced adenomas and adenocarcinoma by oral administration.

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Received at the EPO on Dec 21, 2009 16:10:40. Page 9 of 21

21/12/2009

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
27 August 2009 (27.08.2009)(10) International Publication Number
WO 2009/103788 A1

(51) International Patent Classification:

A61K 31/357 (2006.01) A61K 45/06 (2006.01)
A61K 31/352 (2006.01) A61P 1/00 (2006.01)
A61K 31/05 (2006.01) A61P 35/00 (2006.01)
A61K 31/716 (2006.01)

(21) International Application Number:

PCT/EP2009/052038

(22) International Filing Date:

20 February 2009 (20.02.2009)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

BA2008A000008 22 February 2008 (22.02.2008) IT

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& GERVASI S.P.A., Corso di Porta Vittoria 9, I-20122
Milan (IT).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ,
EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LV, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG,
SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GI,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR),
OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML,
MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

(54) Title: COMPOSITIONS COMPRISING PHYTOESTROGENS SELECTIVE FOR ESTROGEN BETA RECEPTOR AND
DIETARY FIBRES

(57) Abstract: Oral compositions comprising an association of one or of a mixture of phytoestrogens, selective for the estrogen receptor- β , with dietary fibres are described. The compositions thereof can be profitably used for the prophylactic and therapeutic treatment in mammals, including humans, of health conditions characterised by a high risk of onset and recurrence of intestinal adenomas (adenopolyposis coli), and of adenoma's progression to colorectal carcinoma. In presence of pre-cancerous lesions in the colon, characterised by mutations of the APC tumor suppressor and defined as polyps or adenomas, the oral compositions disclosed can be in fact profitably used to reduce the number and volume of polyps, and to prevent their progression to neoplastic transformation, reducing their degree of dysplasia.

WO 2009/103788 A1

Compositions comprising phytoestrogens selective for estrogen beta receptor and dietary fibres

FIELD OF INVENTION

5 The present invention relates to compositions comprising one or more phytoestrogens in association with dietary fibres, insoluble and resistant to enzymatic digestion, and to the use of said compositions for the prophylactic and/or therapeutic treatment of intestinal mucosa and in particular of adenopolyposis coli, and of their progression to colorectal carcinoma.

10 **BACKGROUND OF THE INVENTION**

Adenocarcinoma, or colorectal carcinoma (CRC), is the third cause of death in the world. CRC pathogenesis includes polyps formation onto the intestinal mucosa, considered pre-cancerous lesions. When diagnosed by colonoscopy, the intestinal polyps are usually endoscopically removed. After polypectomy, the recurrence rate
15 is around 10-15 % per year. Among recurrent polyps, 10% occurs as advanced adenoma, characterised by a diameter ≥ 1 cm and histologically villous. The presence of one advanced adenoma, or the presence of two new formations after polypectomy, independently from their histological characteristics, characterise the population with the highest risk of developing a CRC (*Vogelstein B et al. Genetic alterations during colorectal-tumor development. NEJM 1988, 319: 525-532*).
20

Human familial adenomatous polyposis (FAP) is caused by a germinal mutation of the APC (Adenomatous Polyposis Coli) tumour suppressor gene. FAP is characterised by the development of a large number (hundreds to thousands) of adenomas in the colon. Whereas the genetic substrate favours the initiation of the
25 tumorigenic process and cannot be pharmacologically or nutritionally modified, promotion of tumorigenesis may be controlled, so thwarting the progression toward cell de-differentiation and neoplastic transformation. The primary goal of the colonoscopic screening is the monitoring of adenomas and their progression to advanced forms and to CRC. In addition to colonoscopic screening, the clinical
30 need for chemopreventive interventions, safe and effective in keeping low the recurrence rate of adenomas, is certainly high, but still unmet. Cyclooxygenase-2 (COX-2) inhibition by celecoxib and rofecoxib, although effective, is associated

with an unbearable risk of cardio-vascular side effects (*Lance P. Chemoprevention of colorectal cancer: some progress but a long way to go. Gastroenterology 2008, 134: 341-343*).

5 A higher CRC prevalence in males, and a reduced incidence in females undergoing hormone replacement therapy (HRT) support the hypothesis that estrogens play a protective role in colon tumorigenic process. As a further proof of concept of the protective role of estrogens in the intestinal mucosa, it must be cited the recent evidence on the regression of the adenomas in a young woman, affected by FAP, after estro-progestinic therapy for contraceptive purposes
10 (*Giardiello F, Hylind L, Trimbath J et al. Oral Contraceptives and Polyp Regression in Familial Adenomatous Polyposis. Gastroenterology 2005; 128:1077-1080*). Unfortunately, in the epidemiological studies conducted on post-menopausal women undergoing estro-progestinic therapy, a high incidence of breast cancer and cardiovascular accidents have been observed, thus requiring the early
15 termination of the study to safeguard the health of the enrolled patients (*Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA 2002, 288: 321-333*).

Estrogen receptors exist in two forms: alpha (ER α) and beta (ER β), differently
20 localised and expressed in the various anatomical districts. ER α is mainly localised in breasts, cardiovascular system, bones, urogenital and central nervous system, whereas ER β is expressed in prostate, salivary glands, testes, ovaries, in the immune system and, predominantly, in the intestine. 17 β -estradiol (estrogen) is a non selective ligand for both receptors, that performs a proliferative activity in
25 presence of ER α and anti-proliferative in presence of ER β (*Koehler KF et al. Reflections on the discovery and significance of Estrogen Receptor β . Endocr Rev 2005, 26: 465-478*). The beneficial effects observed in the above mentioned studies on the reduced CRC incidence could therefore depend specifically from the ER β activation.

30 The involvement of ER β in the adenomatous polyposis and CRC is demonstrated by its reduced expression, with respect to normal intestinal mucosa, in polypectomized patients already in pre-cancerous stage (*Di Leo A et al. ER- β*

expression in large bowel adenomas: Implications in colon carcinogenesis. *Dig Liver Dis* 2008, C40: 260-266). Significantly, ER β reduction relates with an increased proliferation and a progressive cell de-differentiation, demonstrating that ER β silencing is involved in tumour progression. The colorectal pre-cancerous lesions hence are early warnings of CRC development in a relevant part of the population. The dramatic decline of ER β is also observed in the more advanced stages of the neoplastic transformation (Konstantinopoulos PA et al. *Oestrogen receptor beta (ER β) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumor's dedifferentiation. Eur J Cancer* 2003, 39: 1251-1258).

Experimental studies conducted on C57BL/6J - APC Min/+ transgenic mice, that carry a mutation of the germinal line of the APC gene, support the estrogens' protective role in the prevention of the neoplastic transformation in colonic epithelial cells. In particular, Weyant et al. (Weyant MJ et al. *Reciprocal expression of ER α and ER β is associated with estrogen-mediated modulation of intestinal tumorigenesis. Cancer Res* 2001, 61: 2547-2551), using female transgenic mice, demonstrated that intestinal adenomas spontaneously develop after ovariectomy. Ovariectomy gave rise to a 77% increase in intestinal adenomas neoformation, whereas the subsequent administration of 17- β -estradiol caused a reduction in intestinal adenomas.

Besides genetic causes, it is known that exposure to carcinogens inside the intestinal lumen or to agents potentially genotoxic for the epithelial cells of intestinal mucosa is an epigenetic event that facilitates the inactivation of both alleles or the somatic mutation of the APC gene, both in FAP and sporadic form. These dangerous substances are normally ingested with the diet, or they can be formed in the intestinal lumen as an effect of the digestion, in particular of fats, or by degradation of bile acids.

For this reason, a diet rich in fibres, believed exerting a shielding effect on the intestinal mucosa or adsorbing potentially genotoxic xenobiotics, is considered useful in CRC prevention (Robertson DJ et al. *Fat, fiber, meat and the risk of colorectal adenomas. Am J Gastroenterol* 2005, 100: 2789-2795). However, the clinical evidences of such efficacy are not always convincing. This often depends

on the poor quality of the various experimental protocols, where the expected effects are not weighed against the total fibre dietary intake, comprised of soluble and insoluble fibres. Therefore, the expected effects due to the addition to the diet of a known amount of a fibre qualified for its structure, degree of solubility and digestibility, with its own viscosity and gelling properties in the intestinal lumen, are largely unclear. All the previously mentioned features (i.e. structure, degree of solubility and digestibility, viscosity and gelling properties) heavily affect the properties of the fibre, as well as its biological effects on the intestinal physiology of complex organisms (Jones JR et al. *Dietary reference Intake: implication for fiber labeling and consumption: a summary of the International Life Sciences Institute North America Fiber workshop, June 1-2, 2004 Washington, DC. Nutr Rev* 2006, 64: 31-38).

SUMMARY OF THE INVENTION

According to the Inventors, the therapeutic need may be met by locally delivering agents capable to act as selective agonists to the estrogen receptor-beta involved in the progression of the adenopolyposis to colorectal cancer, significantly reducing the systemic absorption of such agents and thus favouring their pharmacological action onto the intestinal mucosa.

Compositions for oral administration comprising an association of at least one phytoestrogen, selectively agonist to Estrogen Receptor Beta (ER β), or more phytoestrogens with the above properties, and insoluble dietary fibres, i.e. fibres resistant to enzymatic digestion in the intestinal tract, may meet the need in the medical field of tools useful for prevention or treatment of the adenopolyposis coli condition and its progression to colorectal cancer.

Therefore, in a first aspect it is an object of the present invention compositions comprising at least one phytoestrogen or a mixture of phytoestrogens selectively agonists to the estrogen receptor-beta (ER β), and at least one insoluble and indigestible fibre or mixtures thereof selected from the group consisting of non-starch polysaccharides.

Yet, it is a further object of the invention the use of such compositions orally administered for the topical treatment of the intestinal mucosa, and in particular

the treatment of adenopolyposis coli conditions, and for the prevention of their progression to colorectal cancer.

As it will be readily apparent from the detailed description of the invention reported hereafter, in the presence of pre-cancer lesions of the colon characterised by mutations of the APC tumour suppressor, defined as polyps or adenomas, the compositions for oral administration can be profitably used to reduce the number and volume of polyps and to prevent their progression toward the neoplastic transformation, reducing their grade of dysplasia.

The compositions for oral administration object of the present invention can be profitably used to restore ER β expression onto the colon mucosa, otherwise deficient or silenced when intestinal adenomas occur, and to restore a ratio of expression between the estrogen receptor beta and alpha in favour of the anti-proliferative activity.

Moreover, the association from at least one phytoestrogen, selective for the estrogen receptor-beta, and at least one insoluble dietary fibre forming the compositions for oral administration object of the present invention represents a preferential delivery system for the ER β selective phytoestrogen to the colon, where the adenopolyposis is preferentially located, with an enrichment of the components onto the intestinal mucosa and into the colonocytes. The oral compositions object of the present invention are therefore a safe and effective delivery system for the topical treatment of the intestinal mucosa, in conditions at high risk for the development of adenomas and their progression to adenocarcinoma, therefore useful in the treatment of subjects affected by adenopolyposis coli, in familial and sporadic forms, recruited to colonoscopic screening and after polypectomy, to prevent the development of advanced adenomas, and to prevent their potential neoplastic transformation into adenocarcinoma, also known as colorectal cancer (CRS).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. The figure shows the genic expression (Messenger RNA, mRNA) of estrogen receptors in APC Min⁺ mouse intestine. The levels of expression of ER β and ER α mRNA are expressed as optical density units in comparison with the optical density units of constitutive 18S mRNA, relative to a structural protein

unmodified in the experimental conditions of the present study. The results show: a significant increase of ER β mRNA in the silymarin 200 ppm group ($p=0.03$), with respect to the control group fed standard diet; an unexpected significant decrease of the ER α mRNA in the group fed the insoluble fibre lignin alone ($p = 0.04$); a significant increase of ER β in the group fed the composition formed by the association lignin+silymarin ($p=0.04$).

Figure 2. The figure shows the ratio between the mRNAs of estrogen receptor alpha and beta, in the intestine of APC Min⁺ mice. By comparison with the group of animals subcutaneously treated with estrogen (known to be non selectively active on ER β and ER α), the results confirm the selectivity of silymarin for ER β , being the receptor increased by the ligand. Moreover, the results demonstrate that the association of silymarin (even at low doses) with the insoluble fibre lignin, by itself surprisingly active in reducing the expression of ER α which possesses a proliferative activity, facilitates the restoration of an expression ratio between the estrogen receptors in favour of the anti-proliferative activity.

Figure 3. The figure shows the ratio between the level of estrogen receptor alpha and beta proteins, in the intestine of APC Min⁺ mice, as detected by Western Blotting. With respect to the control group of animals fed standard diet only, an increase of the ER β protein versus the ER α protein is observed in animals fed different diets, this increase being higher in the group subcutaneously treated with 17 β -estradiol; in the silymarin fed group; in the lignin fed group; in the group fed the composition containing lignin + silymarin, even at low doses.

Figure 4. Figure 4A compares ER β /ER α mRNA ratio in the small intestine of wild type (WT) and APC Min⁺ groups, fed different diets. Silymarin (SIL), lignin (LIG) and silymarin+lignin (SIL+LIG) restored ER β /ER α mRNA to levels comparable with WT healthy mucosa. ER β protein was also increased in all the dietary managed groups (Fig. 4 B), when compared to controls.

Figure 5. TUNEL assay (A-C) and cleaved caspase-3 (CC-3) (D) assessment of pro-apoptotic activity in non adenomatous and adenomatous mucosa from APC Min⁺ animals fed the standard diet (Control) or different diets (SIL, LIG, SIL+LIG), in comparison with wild type (WT) mice fed the standard diet. In non-adenomatous mucosa, silymarin, lignin, and the combination of the two restored apoptosis to

levels comparable with the healthy mucosa from WT mice. In adenomatous mucosa, only the association of silymarin and lignin restored apoptosis to values comparable with healthy mucosa from WT mice. Whereas CC-3 markedly decreased in the APC Min⁺ mice fed the standard diet, a strikingly increase was
5 observed in the dietary managed groups.

DETAILED DESCRIPTION

According to the state of the art described above, and considering the side effects potentially experienced with non selective agonists of estrogen receptors, the oral compositions object of the present invention comprise at least one phytoestrogen
10 selectively agonist to estrogen receptor beta (ER β), or mixtures of such phytoestrogens, supported by a wide use that sustains its/their clear safety and effectiveness profile.

For the purpose of the present invention, the preferred phytoestrogens are selected from the group consisting of flavolignans, namely silymarin, silybinin, iso-silybinin, silydianin, silychristin, the isoflavone genistein, the flavonoid resveratrol,
15 coumestrol and mixtures thereof. The phytoestrogen or phytoestrogens composing the mixture can be present in the composition both in their purified form and as plant extract. More preferably, the phytoestrogen is silymarin, as such or as a Milk Thistle extract, the most preferred one being the Milk Thistle extract consisting of a
20 mixture of flavolignans silymarin, silybinin, iso-silybinin, silydianin and silychristin.

It is known that silymarin, a standardized mixture of flavolignans extracted from Milk Thistle fruits, widely used in traditional medicine as hepatoprotectant, possesses a documented profile of safety and tolerability, as well as properties of the same as selective ER β agonist, without any relevant activity on ER α , have
25 been described (*Seidlova-Wuttke D et al. Silymarin is a selective estrogen receptor β (ER β) agonist and has estrogenic effect in the metaphysis of the femur but no or antiestrogenic effects in the uterus of ovariectomized (ovx) rats. J Steroid Biochem Mol Biol 2003, 86: 179-188*).

"Silymarin" is the common denomination used for a plant extract containing a
30 mixture of flavolignans: silymarin, silybinin, iso-silybinin, silydianin and silychristin in variable concentrations and ratios. In the compositions object of the present invention, "Silymarin" means the mixture of flavolignans composing the extract, in

different concentrations and ratios, with particular reference to a Milk Thistle extract with an analytical profile characterised by a silymarin content higher than 70% and a silybinin A+B content higher than 30%. In general, additional components to and/or substitutes of silymarin in the compositions object of the present invention are other phytoestrogens, provided that they are selective for ER β and with a anti-estrogenic activity, that is not inducing a significant proliferative activity on tissues that are classical targets for the estrogens, such the above mentioned phytoestrogens, in similar concentrations as that of silymarin.

The other essential component of the compositions for oral administration of the present invention is an insoluble and indigestible, i.e. resistant to enzymatic digestion in the gastrointestinal tract, fibre. The insoluble fibre can be present in a purified form or as component of a natural extract, provided that in the compositions the insoluble fibre content is between 1% and 30%. Such fibre is selected from plant fibres with a non-starch polysaccharidic nature and among these fibres are preferably fibres selected in the group consisting of polyphenylpropanoid lignin (hereafter called also lignin), cellulose, resistant starch, inulin and long chain inulin and mixtures thereof. Particular preference is given to the lignin. The insoluble fibre can be present as a purified component as well as a constituent of an extract. In the later case, the above mentioned insoluble fibres can be present as a mixture and associated with other nutritional substances. In particular, the fibres can be used for the compositions of the invention as follows: in the case of lignin, as such in the polydisperse form or as wheat bran; in the case of cellulose, as such or as a soy extract; in the case of inulin, long chain inulin is preferable, with a polymerisation degree of 10-65 and an average chain length of 25, as such or as artichoke extract, chicory extract or Dandelion root powder.

The fibres present in the oral compositions object of the present invention are anyway to be considered add-ons to the diet, but independent from the total dietary fibre intake.

The compositions according to the inventions have shown unexpected biological properties, since said compositions, characterized substantially by the association from at least one phytoestrogen, selectively agonist to estrogen receptor beta (ER β), or mixtures thereof and at least one dietary insoluble fibre selected from

fibres having a non-starch polysaccharidic nature or mixtures thereof, proved to have a synergistic effect on the parameters used for the necessary experimental validation of their usefulness.

In fact, the dietary insoluble fibre (in particular lignin) proved to be active in maintaining ER β proteic expression, evidence unknown to date, and proved to be synergic with the phytoestrogen silymarin in significantly keeping under control parameters that are markers of the proliferation and neoplastic progression, such as for example the volume and degree of dysplasia of adenomatous polyps.

In addition similar synergistic unexpected effects between phytoestrogens and fibres have been seen in other relevant biological parameters, such as the restoration of a pro-apoptotic activity on the adenomatous mucosa and even more interestingly on the non-adenomatous mucosa, to level comparable with those observed on healthy intestinal mucosa. The unbalanced proliferation/apoptosis ratio observed on the non adenomatous intestinal mucosa of APC Min⁺ mice fed the standard diet – an experimental model validated to assess both the pathobiological mechanisms as well as the efficacy of pharmaceutical and/or nutraceutical interventions of human clinical relevance – can explain the clinically documented recurrence of polyps in patients entered the screening colonoscopy program, because of previous polyp detection and polypectomy, and regarded as at increased risk of CRC development and progression.

The results obtained above mentioned in summary widely support the envisaged use of the compositions object of the present invention. In particular, in mammals, humans or non-humans, a composition for oral administration according to the invention can induce the expression of the estrogen receptor beta directly on the intestinal mucosa, when such receptor is poorly expressed or absent because of the presence of pre-cancerous lesions in the colon mucosa, due to a mutation of the APC tumoral suppressor, such as per familial (FAP) and sporadic adenopolyposis.

In addition, the oral compositions according to the present invention could be a useful system to preferentially target the delivery of the phytoestrogens to the intestinal area affected from polyp development, being this area the small intestine in the experimental mouse model and the colon in humans, where the insoluble

fibres are partially digested. The insoluble fibres contained in the compositions according to the present invention, and particularly lignin, act as a shield in the upper gastrointestinal tract, where phytoestrogens are otherwise preferentially absorbed. Moreover, this shielding effect allows a prolonged contact of the phytoestrogens comprised in the compositions according to this invention onto the intestinal mucosa and onto colonocytes. As a proof of concept, whereas the insoluble fibre, and particularly lignin, was *per se* ineffective in counterfighting intestinal neoplasia, as assessed by the number of developed polyps and their volume, the compositions according to the invention, and particularly the combination of silymarin at the lowest effective dose with lignin determined a maximal reduction in polyp number and volume, comparable with the one obtained by adding the standard diet with silymarin at the highest effective dose.

Compositions according to the invention can therefore usefully be employed, either as pharmaceutical products or nutraceuticals, in the treatment of patients affected by colon adenomas, diagnosed through colonoscopic examination, with the aim of reducing polyp number and volume, of preventing their progression to advanced adenomas and, possibly, to adenocarcinoma. Compositions according to the invention can also be employed to prevent or reduce the development of new polyps and to prevent their progression to an advanced form, and possibly to adenocarcinoma, in polypectomised patients currently recruited to screening colonoscopy program, in the period between scheduled screening colonoscopies.

The compositions for oral administration of the present invention can be in both liquid and solid form. Independently from the type of phytoestrogens used and from the form thereof (i.e. in purified form or as extract), in said compositions the phytoestrogens and the mixtures thereof are comprised from 0.1% to 10% by weight, more preferably from 0.5% to 5%, and most preferably from 1% to 2%.

In fact, when the phytoestrogen is silymarin, said solid and liquid oral compositions preferably comprise from 0.1% to 10% by weight, more preferably from 0.5% to 5%, and most preferably from 1% to 2% of silymarin, independently from the extract thereof used.

In the case of use of genistein as phytoestrogen, the solid and liquid compositions of the present invention preferably comprise from 0.1% to 10% by weight, more

preferably from 0.5% to 5%, and most preferably from 1% to 2% of genistein, independently from the extract used.

The solid and liquid compositions object of the present invention preferably comprise from 0.1% to 10% by weight, more preferably from 0.5% to 5%, and
5 most preferably from 1% to 2% of coumestrol, independently from the extract used.

The solid and liquid compositions object of the present invention preferably comprise from 0.1% to 10% by weight, more preferably from 0.5% to 5%, and
10 most preferably from 1% to 2% of mixed phytoestrogens, independently from the extract used.

With reference to the insoluble fibre component, the solid compositions of the present invention preferably comprise from 1% to 30% by weight, more preferably from 5% to 25%, and most preferably from 10% to 20% of indigestible dietary fibre, independently from the type of extract used (i.e. purified or as such).

15 The liquid compositions of the present invention preferably comprise from 1% to 20%, more preferably from 2% to 15%, and most preferably from 5% to 10% of indigestible and insoluble dietary fibre, independently from the type of extract used (i.e. purified or as such).

The solid compositions can be in form of powder, effervescent powder or
20 chewable tablets. The powder forms can in turn be multi-dose (bottles, canisters, jars) and single dose (sachets) packages. The liquid forms can be in form of syrups, solutions, suspensions or aqueous dispersions. In such formulations, the compositions can additionally comprise acceptable pharmaceutical or food grade excipients, diluents, preservatives, flavours, foaming agents, pH correcting agents,
25 etc., depending on the chosen form, suitable for the oral administration.

Moreover, the compositions of the present invention can further comprise probiotics, such as for example lactobacilli and/or bifidobacteria, both live and tyndallized, with the purpose of contributing to the normalization of the intestinal microflora and to the intestinal fermentation of the fibre, effects known contributing
30 to the formation of a healthy microenvironment in this anatomical district. Probiotics are added as adjuvants to maintain or balance the intestinal physiology and to help metabolise the indigestible and insoluble fibre.

Compositions in accordance with the present invention are shown in working examples 1-10. Working examples 1-7 refer to solid formulations, and working examples 8-10 to liquid formulations. Unless otherwise specified, the proportions of the components comprising the compositions in the working examples represent the weight percentages of the respective components relative to the final product. The following examples further describe and demonstrate embodiments within the scope of the present invention. The examples are given solely for the purpose of illustrations, and are not intended to limit the scope of the present invention, because variations of the present invention are possible without departing from the spirit and scope of the invention.

Solid formulations

Example 1: Effervescent powder

Component	Quantity %
Milk Thistle Dry Extract 70% HPLC silymarin	2.00
Corresponding to silymarin	1.40
Lignin	20.00
Maltodextrins	70.90
Sodium Citrate	4.50
Potassium Citrate	0.50
Citric Acid	1.00
Silicon Dioxide	0.10
Flavouring	1.00

Example 2: Effervescent powder

Component	Quantity %
Milk Thistle Dry Extract 70% HPLC silymarin	2.00
Corresponding to silymarin	1.40
Oligosaccharide Fibre (Inulin)	20.00
Maltodextrins	70.90
Sodium Citrate	4.50

Potassium Citrate	0.50
Citric Acid	1.00
Silicon Dioxide	0.10
Flavouring	1.00

Example 3: Effervescent powder

Component	Quantity %
Milk Thistle Dry Extract 70% HPLC silymarin	2.00
Corresponding to silymarin	1.40
Lignin or Long Chain Inulin	20.00
Lactobacillus Acidophilus, Tyndallised	Equivalent to 10 MLD
Maltodextrins	71.90
Sodium Bicarbonate	2.60
Tartaric Acid	1.40
Silicon Dioxide	0.10
Flavouring	2.00

Example 4: water-dispersible powder

Component	Quantity %
Silymarin	1.40
Dandelion Root Powder	35.00
Maltodextrins	61.50
Silicon Dioxide	0.10
Flavouring	2.00

5

Example 5: Effervescent powder

Component	Quantity %
Milk Thistle Dry Extract 70% HPLC silymarin	1.00
Corresponding to silymarin	0.70
Genistein	0.70
Dietary or Oligosaccharidic Fibre (Inulin)	20.00

Maltodextrins	70.90
Sodium Citrate	4.50
Potassium Citrate	0.50
Citric Acid	1.00
Silicon Dioxide	0.10
Flavouring	1.00

Example 6: Effervescent powder

Component	Quantity %
AlfaAlfa Dry Extract 10% HPLC coumestrol	7.00
Corresponding to coumestrol	0.70
Wheat Bran	30.00
Maltodextrins	55.90
Sodium Citrate	4.50
Potassium Citrate	0.50
Citric Acid	1.00
Silicon Dioxide	0.10
Flavouring	1.00

Example 7: water-dispersible powder

Component	Quantity %
Silymarin	0.70
Genistein	0.70
Dandelion Root Powder	35.00
Maltodextrins	61.50
Silicon Dioxide	0.10

Liquid formulations

Example 8: Syrup

	Quantity in 100 ml
Component	Grams
Silymarin	0.90
Lignin, Cellulose or Inulin	10.00
Potassium Sorbate	0.10
Ascorbic Acid	0.50
Citric Acid, Anhydrous	0.05
Fructose	35.00
Flavouring	0.35
Water	Q.S.

Example 9: Syrup

	Quantity in 100 ml
Component	Grams
Silymarin	0.45
Genistein	0.45
Lignin, Cellulose or Inulin	10.00
Potassium Sorbate	0.10
Ascorbic Acid	0.50
Citric Acid, Anhydrous	0.05
Fructose	35.00
Flavouring	0.35
Water	Q.S.

5 *Example 10: Syrup*

	Quantity in 100 ml
Component	Grams
Milk Thistle Dry Extract 70% HPLC silymarin	1.38
Corresponding to silymarin	0.90
Dandelion Root Powder	20.00

Potassium Sorbate		0.10
Sodium Benzoate		1.0
Ascorbic Acid		0.5
Citric Acid		0.15
Sorbitol		20.00
Saccharin		0.013
Flavouring		0.20
Purified Water		Q.S.

Experimental part

The C57BL/6J APC Min/+ transgenic mouse is an excellent animal model for FAP, because the mutated APC gene is homologous to the human one. Even in the most common form of CRC, called sporadic, 85% of cases show the somatic mutation of the APC tumour suppressor gene. Therefore, the APC mutated transgenic animal model is acknowledged as the most significant for the study of the pathogenetic mechanisms that characterise the adenomatous polyposis in the colon, both in FAP and sporadic form, and it is considered a validated animal model for the set up of nutritional and pharmacological interventions aimed at controlling the development of adenomas and at preventing their progression to CRC (Levy DB et al. *Inactivation of both APC alleles in human and mouse tumors. Cancer Res* 1994, 54: 5953-5958; Paulsen JE et al. *Qualitative and quantitative relationship between dysplastic aberrant crypt foci and tumorigenesis in the Min/+ mouse colon*). For this reason, in setting up the oral formulations object of the present invention, and in the evaluation of their safety and efficacy, the Inventors made use of the above mentioned C57BL/6J APC Min/+ transgenic mouse model. Hereafter are described the data showing the effect in C57BL/6J - APC Min/+ transgenic mice, orally fed silymarin added diets, containing soluble and insoluble fibres in different proportions with the purpose of highlighting the differentiative properties of the insoluble and indigestible fibre.

The three diets under examination, formulated with the aim of mimicking a fat rich dietary regimen, were as follows:

- Diet 5K20 [Mucedola Srl, Settimo Milanese (MI)], containing 4.50% of total fibre, composed of soluble and insoluble fibres;
- Purified diet containing 6.24% of insoluble fibre, constituted by lignin;
- Purified diet containing 6.24% of soluble fibre, constituted by *Plantago ovata* husk.

Silymarin up to 200 mg/kg was added to each diet. The diets were orally administered for 10 weeks to 114 male C57BL/6J - APC Min/+ transgenic mice 5-6 weeks old. These mice have a mutation of the APC gene, and are prone to develop colon adenomatous polyposis. Objective of the study was to verify the capacity of the composition according to the present invention in significantly reducing polyps development and preventing their progression to severely dysplastic forms, markers of neoplastic transformation, in an animal model recognised as comparable to the human pathologic situation. The statistical analysis has been performed by means of the One-way Analysis of Variance and the t-Student tests, considering statistically significant a $p < 0.05$.

The animals were divided into 14 groups, as reported in Table 1. Every oral treatment lasted 10 weeks. The treatment scheme was designed to highlight the effects of the type of fibre, associated or not with increasing amounts of silymarin. As a positive control, a group of transgenic mice fed standard diet (5K20, Mucedola) and subcutaneously implanted with a pellet containing 1.5 mg of 17 β -estradiol (estrogen) was used.

Table1. Number of animals per group (sil= silymarin, ppm=part per million)

	Standard diet	Diet with <i>Plantago ovata</i> husk	Diet with lignin
+ sil 0 ppm	15	10	10
+ sil 10 ppm	6	6	6
+ sil 50 ppm	6	6	6
+ sil 200 ppm	15	6	6
+ sil 600 ppm	7		
+ estrogen (s.c., control)	9		

No difference in the daily dietary intake (about 3 ± 0.1 g/day) was reported among animals in the different groups. During the treatment period, the mortality rate was low in the groups fed standard diet or diet added with compositions described in this patent application (7.1%), whereas in groups fed diet added with *Plantago ovata* husk the mortality was very high (>50%).

Table 2 list the means and standard deviations of the weight of the animals from different diet groups, at time of sacrifice. At admission to the animal house their average weigh was 19 ± 1.2 g.

Table 2. Mean and standard deviation of body weights at time of sacrifice

	Standard diet	Diet with lignin
+ sil 0 ppm	22.0 ± 3.8 g	25.0 ± 1.9 g
+ sil 10 ppm	26.0 ± 1.9 g	25.2 ± 2.3 g
+ sil 50 ppm	24.6 ± 3.3 g	26.9 ± 2.1 g
+ sil 200 ppm	23.5 ± 2.6 g	27.9 ± 1.7 g
+ sil 600 ppm	24.3 ± 2.4 g	-
+ estrogen (s.c., control)	24.9 ± 2.8 g	

Differently from animal groups fed the diet containing *P. ovata* husk soluble fibre, in which a high mortality rate and no reduction in polyp numbers were observed, the association of silymarin and lignin constituting one of the compositions according to the present invention proved to be safe and well tolerated, and resulted highly effective in keeping down the number, volume, and grade of dysplasia of adenomatous polyps in the C57BL/6J - APC Min/+ transgenic mouse model. In addition, the dietary insoluble fibre (in this case lignin) proved to be active in maintaining ER β proteic expression, evidence unknown to date, and proved to be synergic with silymarin in significantly keeping under control parameters that are markers of the proliferation and neoplastic progression, such as for example the volume and degree of dysplasia of adenomatous polyps.

After 10 weeks of treatment the animals received an intraperitoneal injection of 0.75 mg of Bromodeoxyuridine (BrdU), equivalent to 30 mg/kg, to verify the migration of cells of the intestinal mucosa along the villus axis (from crypt at the bottom to the luminal tip). This parameter allows to verify the normal esfoliative
5 physiology of the intestinal mucosa, through which the mucosa keeps itself composed of healthy and vital cells.

To this end, the animals were sacrificed 24, 48 and 72 hours after injection. Small intestine and colon were taken from every sacrificed animal. The small intestine was divided into three sections: proximal, medial and distal. The proximal tract
10 was further divided in two portions that were subsequently scraped. One of the two was dipped in RNAlater[®] and stored at -20°C and the other stored in liquid nitrogen. The two samples were used for Real Time PCR and Western Blotting, respectively. The medial and distal tracts, together with the colon, were used for the histological analysis. After a 3-hours pre-fixative treatment with formol, the
15 intestinal tracts under examination were observed under an STM1 stereomicroscope, 3x magnification. Two blinded observers measured the number and size of polyps.

Table 3 lists mean and standard deviation (SD) of the number of polyps observed in the animals fed the different diets. It has to be noticed that silymarin added at
20 200 ppm to the standard diet significantly reduces the number of polyps ($p=0.005$), at a level comparable with the reduction observed in the transgenic mice of the control group, subcutaneously treated with estradiol. Lignin alone is ineffective on this parameter, but the association of lignin and silymarin, object of the present invention, significantly reduces the number of polyps, at a silymarin dose (50 ppm)
25 four times lower than that required to obtain the same results with standard diet (200 ppm).

Tabella 3. Number of intestinal polyps in animals fed different diets

	Standard diet	Diet with Plantago	Diet with lignin
+ sil 0 ppm	54.8 $\pm$ 20.2	43.6 $\pm$ 9.5	46.8 $\pm$ 14.0
+ sil 10 ppm	44.0 $\pm$ 21.8	45.4 $\pm$ 23.8	43.8 $\pm$ 19.0
+ sil 50 ppm	55.4 $\pm$ 26.2	51.5 $\pm$ 20.5	34.0 $\pm$ 10.7*
+ sil 200 ppm	37.2 $\pm$ 9.0*	50.0 $\pm$ 4.7	48.5 $\pm$ 2.5
+sil 600 ppm	52.6 $\pm$ 24.6		
Estrogen (control)	32.33 $\pm$ 12.06*		

Table 4 lists the silymarin content (expressed as $\mu\text{g/g}$ silybinin in the tissue, determined by HPLC) in the duodenal tract, where silymarin is known to be preferentially absorbed, and in the ileum after seven days feeding 200 ppm silymarin, or a composition containing 200 ppm silymarin and a non starch polysaccharidic fibre (lignin) as an add-on to the standard diet of C57BL/6J mice ($n=6$). The association of silymarin and insoluble fibre caused a preferential transport of the phytoestrogen in the ileal region, the intestinal area where polyps typically develop in the transgenic mouse. Moreover, it is known that such condition mimics the development of adenopolyposis in human colon. Therefore, the compositions object of the present invention can be an effective system for the topical treatment of the area affected by adenomatous formations.

Tabella 4. Mean and standard deviation of silymarin tissue concentration in mouse intestine

	Duodenum ($\mu\text{g/g}$)	Ileum ($\mu\text{g/g}$)
Silymarin 200 ppm, 7 days	6.54 $\square$ 2.34	2.84 $\square$ 2.03
Silymarin 200 ppm + Lignin, 7 days	2.47 $\square$ 2.38	3.13 $\square$ 2.71

Table 5 lists mean and standard deviation (SD) of the volume of intestinal polyps. Assuming polyps shape as a sphere, by measuring their diameter it was possible

to calculate their volume, expressed in mm^3 . Surprisingly, lignin alone is able to reduce polyps volume ($p=0.0001$). The association of silymarin with lignin is able to significantly reduce the volume of polyps ($p=0.0001$). Moreover, the association is synergic for the two components, as suggested by the fact that the using lower doses of phytoestrogen, the same effect as per the higher doses of single component is observed. Noteworthy, estradiol proved to be ineffective on this parameter, demonstrating that the composition object of the present invention, containing a phytoestrogen selectively agonist to $\text{ER}\beta$, is effective on this parameter.

10 **Table 5.** Comparison of polyps volume ($\text{mm}^3 \pm \text{SD}$) in animal fed the different diets

	Standard diet	Diet with lignin
+ sil 0 ppm	242,1 $\pm$ 105.1	92.3 $\pm$ 61.9*
+ sil 10 ppm	145.0 $\pm$ 100.6	121.0 $\pm$ 72.6
+ sil 50 ppm	158.4 $\pm$ 60.3	99.00 $\pm$ 53.13*
+ sil 200 ppm	120.2 $\pm$ 69.0*	138.0 $\pm$ 63.3
+ sil 600 ppm	162.07 $\pm$ 89.35	
Estrogen (control)	141.78 $\pm$ 79.1	

Histological preparations of randomly chosen intestine sections of every animal from the different treatment groups were observed by a blinded pathologist, who graded the dysplasia of the adenomatous formations present as minor, mild or severe. In the estradiol-treated animals, 70% showed polyps with dysplasia classifiable as severe, versus 21.4% in animals fed standard diet added with 200 ppm silymarin, 30% in animals fed diet with lignin, and 20% in animals fed diet with lignin added with 50 ppm silymarin.

Table 6 lists the results concerning HLC (highest labeled cell) in the experimental groups. Data shows that cell migration rate, and consequently the esfoliation rate of enterocytes in the intestinal lumen, is significantly increased in the group treated with lignin and silymarin (even at low doses).

Table 6. Percentage of colonocytes migration from the crypt to the apex of the villus in transgenic animals of the different groups

	24 hours migration (%)
Standard diet	28 ± 2.6
+ estrogen	$47.3 \pm 10.5^*$
+ sil 200 ppm	$46.6 \pm 7.0^*$
+ lignin	35 ± 13.8
+ (Lignin + sil 50 ppm)	$44.4 \pm 11.8^*$

5

With regard to the mechanism of action of the association silymarin and lignin, data demonstrates for the first time a potentiation of the ER β expression on the intestinal mucosa. RT-PCR results are shown in Figure 1. The levels of expression of messenger RNA for ER β and ER α are reported as average ratio between the optical density of the messenger for each receptor and that of 18S rRNA, relative to β -actin, a structural protein that is unmodified in the experimental conditions of the study.

10

The results show a significant increase of ER β mRNA for in the silymarin 200 ppm group ($p=0.03$), with respect to the group fed standard diet.

15

Surprisingly, the Inventors demonstrated for the first time that the ER α mRNA is significantly decreased in the group treated with the insoluble fibre ($p=0.04$); the reduction is more pronounced in the group fed with a composition containing the association silymarin-lignin ($p=0.04$). From comparison with the group subcutaneously treated with estrogen (active on both ER β and ER α), data confirms silymarin as selective ligand to ER β and demonstrates that the association of silymarin (even at low doses) and lignin - the latter surprisingly active in reducing expression of ER α known to exert proliferative properties - further facilitates the re-establishment of a ratio between the two estrogen receptors in favour of the antiproliferative activity, as shown in Figure 2 and

20

25 demonstrated by the data in Tables 3-6.

Figure 3 shows the level of intestinal expression of the estrogen receptors, as ratio between ER β and ER α proteins, determined using the Western Blotting method. Compared to the group of animals fed standard diet only, an increase of ER β as a protein in comparison to ER α protein was observed, and this increase is more marked in the group subcutaneously treated with 17 β -estradiol, thus confirming the protective role hypothesised for the estrogens; in the silymarin-treated group, thus confirming its properties as a phytoestrogen selective to ER β ; and, surprisingly, in both groups treated with lignin and with lignin + silymarin, even at low doses.

Additional experimental were collected in order to provide evidence of: A) altered proliferative and apoptotic activities even on the non adenomatous mucosa of APC Min/+ animals, in comparison with syngeneic wild type (WT), no APC mutated, C57BL/6J intestinal mucosa (regarded as normal and healthy), being both animal groups fed the standard diet; B) the capability of the oral compositions object of the present invention to restore ER β expression, proliferative and apoptotic activity in APC Min/+ mice, to levels comparable to the ones observed onto the normal mucosa of WT animals fed the standard diet. Therefore, these evidences further support the use of such oral compositions as an useful adjunct to screening colonoscopy program in previously polipectomised patients, at increased risk of developing new polyps (recurrence). Figure 4 (A, B) shows the ratio between ER β and ER α mRNAs and the level of expression for both receptors as a protein, onto the non-adenomatous mucosa of WT and control APC Min/+ fed the standard diet, or APC Min/+ fed different diets. Figure 5 (A-D) demonstrates that: i) the non adenomatous mucosa of APC Min/+ mice fed the standard diet has lost pro-apoptotic control on cell proliferation when compared to the WT mice fed the standard diet, as detected by markers of apoptosis, such as the reduced number of positive cells on the TUNEL assay and cleaved capsase 3 protein expression, ii) the oral compositions object of the present invention, with particular reference to the association of silymarin and lignin, restore apoptotic control on cell proliferation to levels similar with the ones observed onto the normal, healthy intestinal mucosa of WT mice.

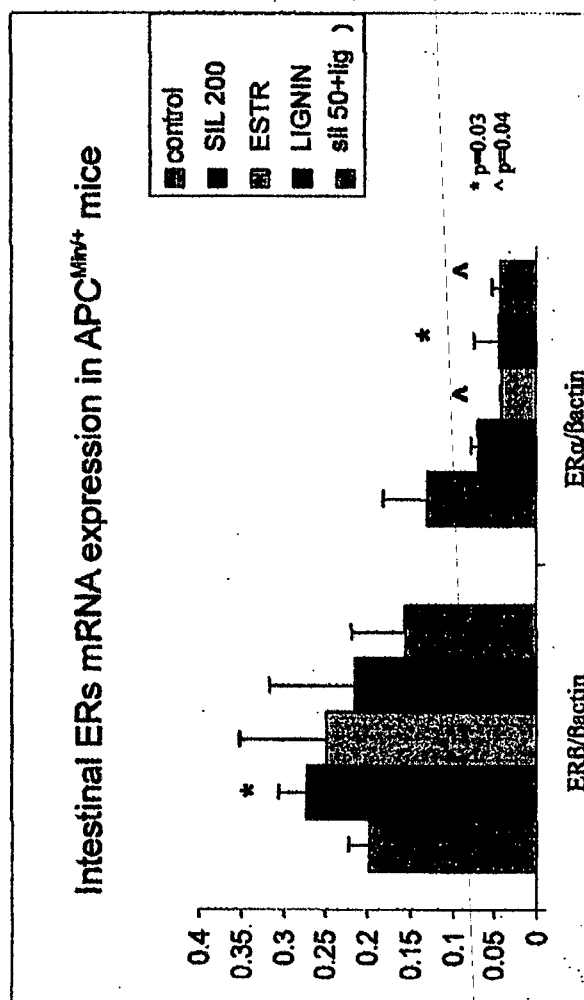
CLAIMS

1. A composition for oral administration comprising at least one phytoestrogen or a mixture of phytoestrogens selectively agonists to the estrogen receptor-beta and at least one insoluble and indigestible fibre or mixtures thereof selected from
5 the group consisting of non-starch polysaccharides.
2. The composition according to claim 1, wherein the phytoestrogens, in purified form or as extracts, are selected from the group consisting of silymarin, silybinin, iso-silybinin, silydianin, silychristin, genistein, resveratrol, coumestrol and mixtures thereof.
- 10 3. The composition according to claim 1, wherein the phytoestrogen is the Milk Thistle extract silymarin consisting of the mixture of silymarin, silybinin, iso-silybinin, silydianin, silychristin.
4. The composition according to claim 1, wherein insoluble and indigestible fibres, in purified form or as extracts, are selected from the group consisting of
15 lignin, cellulose, resistant starch, inulin, long chain inulin and mixtures thereof.
5. The composition according to claims 1-4, wherein phytoestrogens are in a range comprised from 0.1 to 10 % of the total weight of said composition.
6. The composition according to claims 5, wherein phytoestrogens are in a range comprised from 0.5 to 5% of the total weight of said composition.
- 20 7. The composition according to claims 5, wherein phytoestrogens are in a range comprised from 1 to 2% of the total weight of said composition.
8. The composition according to claims 1-4, wherein the insoluble and indigestible fibres are in a range comprised from 1 to 30% of the total weight of said composition.
- 25 9. The composition according to claim 8, wherein the fibres are in a range comprised from 5 to 25% in solid formulations.
10. The composition according to claim 8, wherein the fibres are in a range comprised from 10 to 20% in solid forms.
11. The composition according to claim 8, wherein the fibres are in a range
30 comprised from 2 to 15% in liquid forms.
12. The composition according to claim 8, wherein the fibres are in a range comprised from 5 to 10% in liquid forms.

- 13 The composition according to any one of the preceding claims 1-12, wherein probiotics, live or tyndalized, are further added.
14. The composition according to any one of the preceding claims 1-13, in combination with pharmaceutically acceptable excipients and/or diluents suitable
5 for solid forms selected from the group consisting of powders, effervescent powders, chewable tablets and tablets.
15. The composition according to any one of the preceding claims 1-13, in combination with food grade acceptable excipients and/or diluents suitable for solid forms selected from the group consisting of powders, effervescent powders,
10 chewable tablets and tablets.
16. The composition according to any one of the preceding claims 1-13, in combination with pharmaceutically acceptable excipients and/or diluents suitable for liquid forms selected from the group consisting of syrups, solutions, suspensions and aqueous dispersions.
- 15 17. The composition according to any one of the preceding claims 1-13, in combination with food grade acceptable excipients and/or diluents suitable for liquid forms selected from the group consisting of syrups, solutions, suspensions and aqueous dispersions.
18. The composition according to any one of the preceding claims for use in the
20 prophylactic or therapeutic topical treatment of the intestinal mucosa.
19. The composition according to any one of the preceding claims for use for the prevention and/or treatment of adenopolyposis and its progression to adenomas and adenocarcinoma.

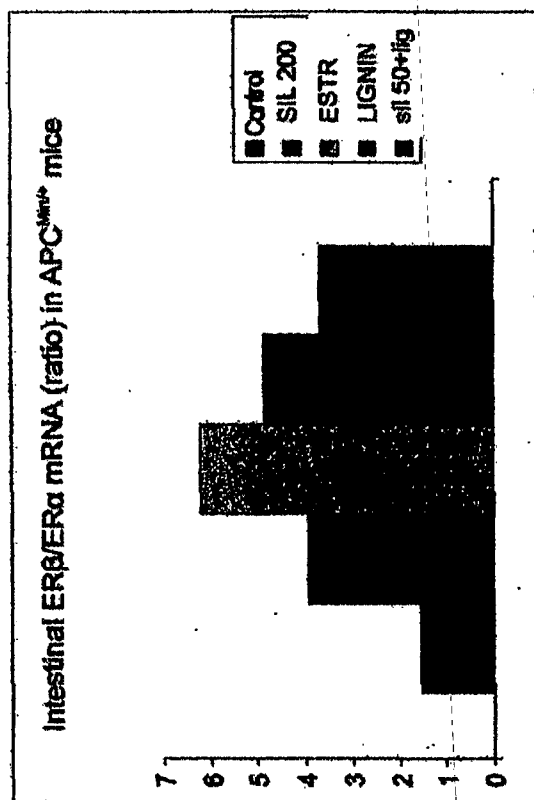
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FIGURE 1



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FIGURE 2



3/5

FIGURE 3

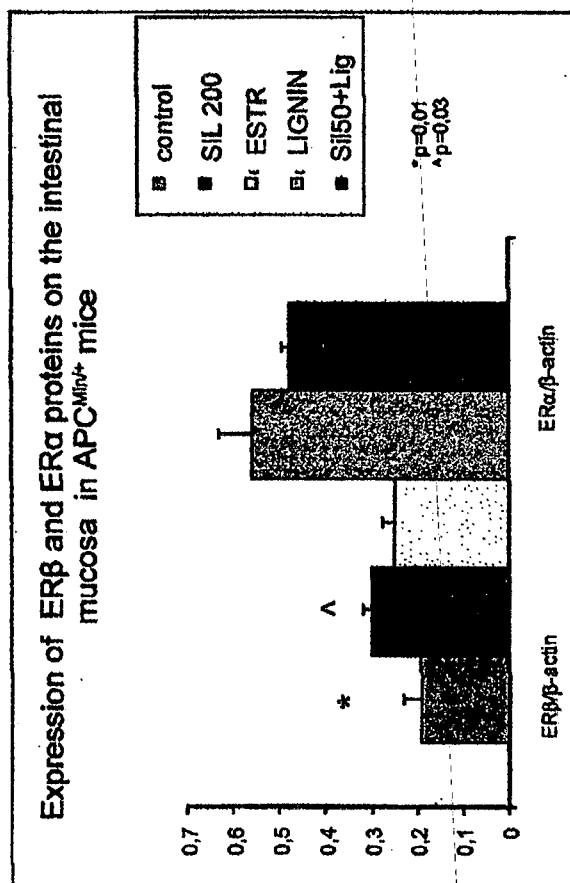


FIGURE 4

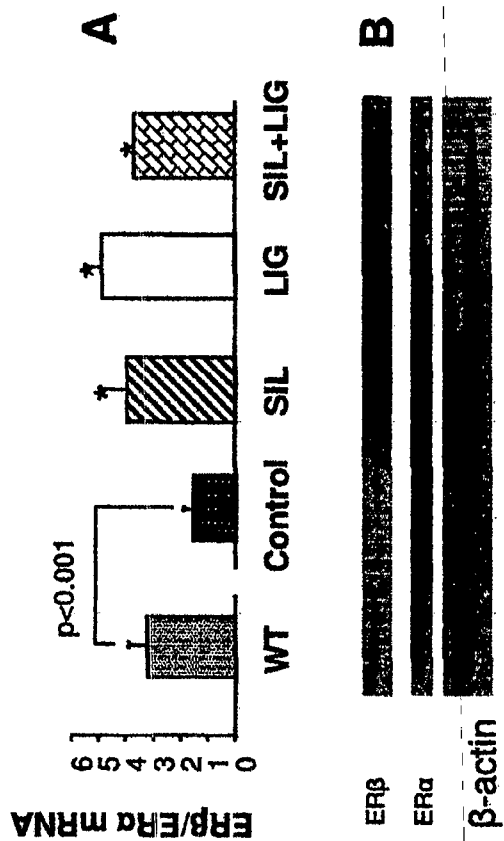
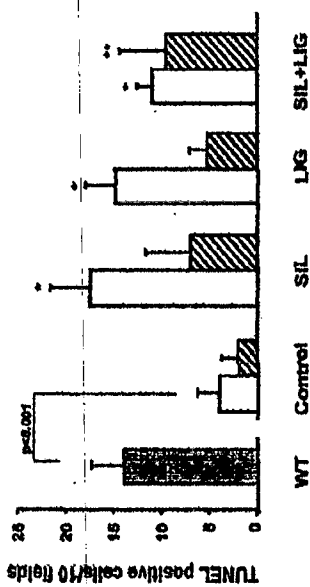
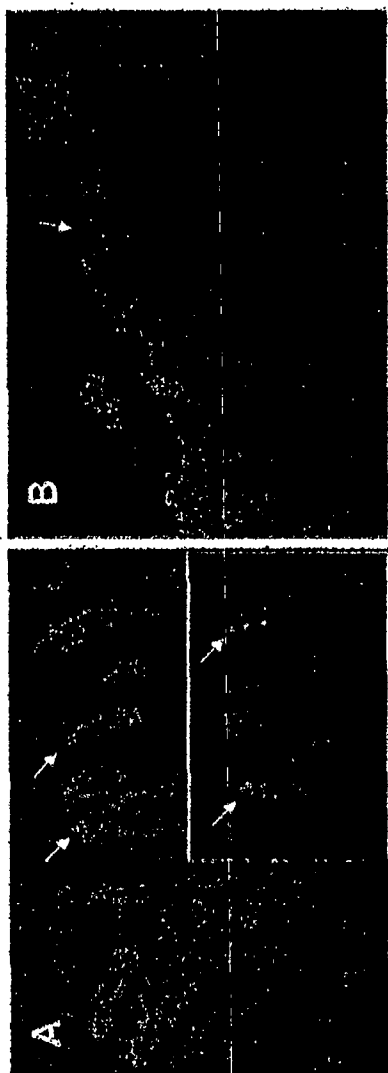


FIGURE 5



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/052038

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/357 A61K31/352 A61K31/05 A61K31/716 A61K45/06 A61P1/00 A61P35/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 June 2003 (2003-06-01), pages 168-171, XP009117194 ISSN: 0301-4800 page 169, left-hand column, paragraph 1 --- -/-	1,4,5,8, 9,15,18
☒ Further documents are listed in the continuation of Box C.		
☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
Date of the actual completion of the international search 25 May 2009		Date of mailing of the international search report 05/06/2009
Name and mailing address of the ISA/ European Patent Office, P.O. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3010		Authorized officer Baurand, Petra

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2009/052038

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 1 September 1984 (1984-09-01), pages 1219-1224, XP009117192 ISSN: 0008-4212 page 1220, left-hand column, paragraph 6	1,2,4,15
X	DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 June 2005 (2005-06-09) claims 1,4	1,4,14
Y	HOH CARMEN S I ET AL.: "A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, April 2006 (2006-04), page 752, XP001537130 abstract	1-19
Y	MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 October 2002 (2002-10-17), page 27, XP021016050 ISSN: 1471-2407 page 34, left-hand column, paragraph 1	1-19
Y	LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, March 2004 (2004-03), page 736, XP001536466 abstract	1-19

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/052038

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, pages 189-196, XP002529043 page 193, Discussion	1-19
Y	EP 0 846 704 A (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 June 1998 (1998-06-10) claims 1,13	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/052038

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 102004054133 A1	09-06-2005	CZ SK	20033042 A3 322004 A3
EP 0846704 A	10-06-1998	AT AU AU CA DE DE DK ES JP PT US	214400 T 725110 B2 4684697 A 2223149 A1 69711002 D1 69711002 T2 846704 T3 2170340 T3 10191931 A 846704 E 6043229 A
			15-06-2005 02-06-2005 15-03-2002 05-10-2000 04-06-1998 03-06-1998 18-04-2002 28-11-2002 21-05-2002 01-08-2002 28-07-1998 31-07-2002 28-03-2000

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PCT REQUEST

Print Out (Original in Electronic Form)

0	For receiving Office use only	
0-1	International Application No.	PCT/EP2009/052038
0-2	International Filing Date	20 FEB 2009 (20.02.2009)
0-3	Name of receiving Office and "PCT International Application"	RO/EP
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT Online Filing Version 3.5.000.203 MT/FOP 20020701/0.20.5.9
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	European Patent Office (EPO) (RO/EP)
0-7	Applicant's or agent's file reference	9582PTWO-ca
I	Title of invention	COMPOSITIONS COMPRISING PHYTOESTROGENS SELECTIVE FOR ESTROGEN BETA RECEPTOR AND DIETARY FIBRES
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	CM&D PHARMA LIMITED
II-5	Address	Thames House, Portsmouth Road Esher KT10 9AD SURREY Italy
II-6	State of nationality	GB
II-7	State of residence	GB
III-1	Applicant and/or inventor	
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III-1-6	State of nationality	IT
III-1-7	State of residence	IT

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III-2	Applicant and/or inventor	
III-2-1	This person is	applicant and inventor
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III-2-5	Address	Viale de Laurentis, 23/d 70124 BARI Italy
III-2-6	State of nationality	IT
III-2-7	State of residence	IT
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
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IV-1-6	Agent's registration No.	100018866
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IV-2-6	Agent's registration No.	100058067

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IV-6-4	Facsimile No.	+39 02 36216366
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IV-6-6	Agent's registration No.	

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PCT REQUEST

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V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	22 February 2008 (22.02.2008)
VI-1-2	Number	BA2008A000008
VI-1-3	Country	IT
VI-2	Incorporation by reference : where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.	
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)
VIII	Declarations	Number of declarations
VIII-1	Declaration as to the identity of the inventor	—
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	—
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	—
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	—
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	—

PCT REQUEST

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IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	23	✓
IX-3	Claims	2	✓
IX-4	Abstract	1	✓
IX-5	Drawings	5	✓
IX-7	TOTAL	36	
	Accompanying items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-18	PCT-SAFE physical media	-	-
IX-20	Figure of the drawings which should accompany the abstract		
IX-21	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative	/Gemma Gervasi/	
X-1-1	Name (LAST, First)	GERVASI, Gemma	
X-1-2	Name of signatory	GERVASI, Gemma	
X-1-3	Capacity	(Representative)	

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	20 FEB 2009 (20.02.2009)
10-2	Drawings:	
10-2-1	Received	✓
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
------	--	--

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

NOTARBARTOLO & GERVASI S.p.A.
Attn. Gervasi, Geoma
Corso di Porta Vittoria 9 R
I-20122 Milan
ITALIE

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT, AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Filing (day/month/year) 05/06/2009	
Applicant's or agent's file reference 9582PTWO-ca	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/EP2009/052038	International filing date (day/month/year) 20/02/2009
Applicant CM&D PHARMA LIMITED	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Iris Hodzic
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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 18 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

If a demand for international preliminary examination is made, the written opinion of the International Searching Authority will, except in certain cases where the International Preliminary Examining Authority did not act as International Searching Authority and where it has notified the International Bureau under Rule 68.1bis(b), be considered to be a written opinion of the International Preliminary Examining Authority. If a demand is made, the applicant may submit to the International Preliminary Examining Authority a reply to the written opinion together, where appropriate, with amendments before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later (Rule 43bis.1(c)).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see the *PCT Applicant's Guide*, Volume II.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 9582PTWO-ca	FOR FURTHER ACTION		see Form PCT/ISA/220 as well as, where applicable, Item 5 below.
International application No. PCT/EP2009/052038	International filing date (day/month/year) 20/02/2009	(Earliest) Priority Date (day/month/year) 22/02/2008	
Applicant CM&D PHARMA LIMITED			

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box No. II)

3. ☐ Unity of invention is lacking (see Box No. III)

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. _____
☐ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
- b. ☒ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

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International application No
PCT/EP2009/052038

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/357 A61K31/352 A61K31/05 A61K31/716 A61K45/06 A61P1/00 A61P35/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 June 2003 (2003-06-01), pages 168-171, XP009117194 ISSN: 0301-4800 page 169, left-hand column, paragraph 1 -/-	1,4,5,8,9,15,18
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *S* document member of the same patent family		
Date of the actual completion of the international search 25 May 2009		Date of mailing of the international search report 05/06/2009
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentleien 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax (+31-70) 340-3016		Authorized officer Baurand, Petra

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/052038

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 1 September 1984 (1984-09-01), pages 1219-1224, XP009117192 ISSN: 0008-4212 page 1220, left-hand column, paragraph 6	1,2,4,15
X	DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 June 2005 (2005-06-09) claims 1,4	1,4,14
Y	HOH CARMEN S I ET AL.: "A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, April 2006 (2006-04), page 752, XP001537130 abstract	1-19
Y	MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 October 2002 (2002-10-17), page 27, XP021016050 ISSN: 1471-2407 page 34, left-hand column, paragraph 1	1-19
Y	LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, March 2004 (2004-03), page 736, XP001536466 abstract	1-19
	----- -/-	

INTERNATIONAL SEARCH REPORT

42

International application No
PCT/EP2009/052038

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, pages 189-196, XP002529043 page 193, Discussion	1-19
Y	EP 0 846 704 A (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 June 1998 (1998-06-10) claims 1,13	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/052038

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 102004054133 A1	09-06-2005	CZ SK	20033042 A3 322004 A3
EP 0846704 A	10-06-1998	AT AU AU CA DE DE DK ES JP PT US	214400 T 725110 B2 4684697 A 2223149 A1 69711002 D1 69711002 T2 846704 T3 2170340 T3 10191931 A 846704 E 6043229 A
			15-06-2005 02-06-2005 15-03-2002 05-10-2000 04-06-1998 03-06-1998 18-04-2002 28-11-2002 21-05-2002 01-08-2002 28-07-1998 31-07-2002 28-03-2000

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/SA/220

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/EP2009/052038

International filing date (day/month/year)
20.02.2009

Priority date (day/month/year)
22.02.2008

International Patent Classification (IPC) or both national classification and IPC
INV. A61K31/357 A61K31/352 A61K31/05 A61K31/716 A61K45/06 A61P1/00 A61P35/00

Applicant
CM&D PHARMA LIMITED

1. This opinion contains indications relating to the following items:

- | | |
|--|--|
| ☒ Box No. I | Basis of the opinion |
| ☐ Box No. II | Priority |
| ☐ Box No. III | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| ☐ Box No. IV | Lack of unity of invention |
| ☒ Box No. V | Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| ☐ Box No. VI | Certain documents cited |
| ☐ Box No. VII | Certain defects in the international application |
| ☒ Box No. VIII | Certain observations on the international application |

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0
Fax: +49 89 2399 - 4465

**Date of completion of
this opinion**

see form
PCTISA210

Authorized Officer

Baurand, Petra

Telephone No. +49 89 2399-2158



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2009/052038

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☐ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☐ on paper
 - ☐ in electronic form
 - c. time of filing/furnishing:
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in electronic form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
4. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2009/052038

Box No. V Reasoned statement under Rule 43b/s.1(a)(i) with regard to novelty, Inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>3,6,7,10-13,16,17,19</u>
	No: Claims	<u>1,2,4,5,8,9,14,15,18</u>
Inventive step (IS)	Yes: Claims	<u>=</u>
	No: Claims	<u>1-19</u>
Industrial applicability (IA)	Yes: Claims	<u>1-19</u>
	No: Claims	<u>=</u>

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/EP2009/052038

Re Item V.

5.1 Reference is made to the following documents:

- D1: TAMURA MOTOI ET AL: "Effects of soy oligosaccharides on plasma and cecal isoflavones, and cecal enzyme activities in mice" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, UNIVERSITY OF TOKYO PRESS, TOKYO, JP, vol. 49, no. 3, 1 June 2003 (2003-06-01), pages 168-171, XP009117194 ISSN: 0301-4800
- D2: STANGROOM K E ET AL: "Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine" CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 62, no. 9, 1 September 1984 (1984-09-01), pages 1219-1224, XP009117192 ISSN: 0008-4212
- D3: DE 10 2004 054133 A1 (NATURPRODUKT CZ SPOL S R O [CZ]) 9 June 2005 (2005-06-09)
- D4: HOH CARMEN S I ET AL: "A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent, in patients with primary colorectal cancer and colorectal liver metastases" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 47, April 2006 (2006-04), page 752, XP001537130
- D5: MAHYAR-ROEMER MOJGAN ET AL: "Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells" BMC CANCER, BIOMED CENTRAL, LONDON, GB, vol. 2, no. 1, 17 October 2002 (2002-10-17), page 27, XP021016050 ISSN: 1471-2407
- D6: LECHNER DANIEL; CROSS HEIDE S: "Genistein prevents downregulation of 25-D-3-1 alpha-hydroxylase by 1,25-(OH)(2)-D-3 in human mammary and colorectal tumor cells: Relevance for cancer prevention" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 45, March 2004 (2004-03), page 736, XP001536466
- D7: HILLMAN L; PETERS S; FISHER A; POMARE E W: "DIFFERING EFFECTS OF PECTIN CELLULOSE AND LIGNIN ON STOOL PH TRANSIT TIME AND WEIGHT" BRITISH JOURNAL OF NUTRITION, vol. 50, no. 2, 1983, pages 189-196, XP002529043
- D8: EP-A-0 846 704 (CERESTAR HOLDING BV [NL] CERESTAR HOLDING BV) 10 June 1998 (1998-06-10)

5.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1, 2, 4, 5, 8, 9, 14, 15 and 18 is not new in the sense of Article 33(2) PCT, the reasons being as follows:

Document D1 describes feeding of rats with a cellulose-isoflavone solid diet containing cellulose and a mixture of daidzein and genistein, wherein said diet was found to have an effect on the intestinal microflora (page 169, left-hand column, 1st paragraph). Thus, it is considered to be relevant for novelty of claims 1, 4, 5, 8, 9, 15 and 18.

D2 reports rats fed with the mixture of coumestrol + lignin (page 1220, left-hand column, 6th paragraph). Hence, it takes away novelty of present claims 1, 2, 4 and 15.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/EP2009/052038

D3 discloses a composition comprising silymarin, yacon and cellulose (claims 1 and 4) and is therefore novelty-destroying for the subject-matter of claims 1, 4 and 14.

5.3 With respect to novelty it is noted that claim 1 refers to a composition "for" oral administration. A claim to a composition "for" a particular use should be construed as meaning a composition which is in fact "suitable for" the stated use. If a known composition is in a form in which it is suitable for the stated use, though it has never been described for that use, deprives the claim of novelty.

5.4 The present application does not meet the criteria of Article 33(1) PCT, because the subject matter of claims 1 - 19 does not involve an inventive step in the sense of Article 33(3) PCT.

Documents D4 - D6, which are considered as equivalent and represent the most relevant state of the art to the subject matter of claim 1, show that phytoestrogens such as silymarin, silibinin, resveratrol and genistein possess chemopreventive efficacy in colorectal carcinogenesis (D4: abstract; D5: page 34, left-hand column, 1st paragraph; D6: abstract).

The subject-matter of independent claim 1 differs from these disclosures in that the claimed composition comprises an insoluble and indigestible fibre.

The problem to be solved by the present invention may therefore be regarded as provision of a further composition efficient in the prevention and/or treatment of colorectal carcinogenesis.

However, the documents D7 and D8 report that dietary fibres such as cellulose and resistant starch are useful for the treatment of diseases of the colorectal digestive tract such as colorectal carcinogenesis (D7: page 193, Discussion; D8: claims 1 and 13).

Therefore the features disclosed in D4 - D6 would be combined by the skilled person with the features known from either D7 or D8, without exercise of any inventive skills in order to solve the problem posed. The proposed solution in independent claim 1 thus cannot be considered inventive (Article 33(3) PCT).

5.5 Dependent claims 2 - 19 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step.

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)

International application No.

PCT/EP2009/052038

Re Item VIII.

8.1 It is pointed out that claim 18 is not acceptable under Article 6 PCT. It is pointed out that the concept of "therapy" or "therapeutic application" includes treatment of a particular illness or disease with a specified chemical substance or composition in a specified human or animal subject in need of such treatment. In the absence of the identification of at least (i) the illness or disease to be treated or the ailment to be cured or (ii) the nature of the therapeutic compound used for treating or curing the disease or (iii) the subject to be treated, a mere process feature ("**treatment of the intestinal mucosa**") cannot be construed as specifying a particular method of treatment or therapeutic application.

8.2 Present claim 1 relates to a compound defined by reference to a desirable characteristic or property, namely "**phytoestrogen selectively agonist to the estrogen receptor-beta**". An attempt is made to define the compound by its pharmacological profile, rendering the protection of said claim obscure. It is pointed out that a compound cannot be sufficiently characterised by its pharmacological profile or its mode of action. The use of such a functional definition is vague and unclear and leaves the reader in doubt as to the meaning of the technical feature (i.e. the compounds) to which it refers.

The attention of the Applicant is drawn to the fact that a functional definition may be allowable, only if all of the following criteria are fulfilled:

(a) compounds fulfilling the function are part of the common general knowledge (i.e. can be found in encyclopaedias, text books or dictionaries),

(b) the function can be verified by test or procedures adequately specified in the description or known to the skilled person and

(c) the entire contents of the patent application unambiguously show that the function is causal for the solution of the problem underlying the invention so that it is reasonably clear that not only examples in the application but any other compound with the stated function may be used.

8.3 Furthermore, claim 1 refers to "**non-starch polysaccharides**". By contrast, claim 4 (which is dependent on claim 1) precises said non-starch polysaccharide as being "**resistant starch**". This inconsistency within the claims results in a lack of clarity, contrary to Art. 6 PCT.

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/EP2009/052038

8.4 The attention of the Applicant is drawn to the fact that the term "**progression of adenopolyposis to adenomas and adenocarcinoma**" (claim 19) cannot be considered as a therapeutical application. The discovery that a mixture of substances prevents the progression of adenopolyposis to adenomas and adenocarcinoma, even if representing an important piece of scientific knowledge, still needs to find a practical application in the form of a defined, real treatment of any pathological condition in order to make a technical contribution to the art and to be considered as an invention eligible for patent protection.

Possible steps after receipt of the international search report (ISR) and written opinion of the International Searching Authority (WO-ISA)

General information

For all international applications filed on or after 01/01/2004 the competent ISA will establish an ISR. It is accompanied by the WO-ISA. Unlike the former written opinion of the IPEA (Rule 66.2 PCT), the WO-ISA is not meant to be responded to, but to be taken into consideration for further procedural steps. This document explains about the possibilities.

Amending claims under Art. 19 PCT

Within 2 months after the date of mailing of the ISR and the WO-ISA the applicant may file amended claims under Art. 19 PCT directly with the International Bureau of WIPO. The PCT reform of 2004 did not change this procedure. For further information please see Rule 46 PCT as well as form PCT/ISA/220 and the corresponding Notes to form PCT/ISA/220.

Filing a demand for international preliminary examination

In principle, the WO-ISA will be considered as the written opinion of the IPEA. This should, in many cases, make it unnecessary to file a demand for international preliminary examination. If the applicant nevertheless wishes to file a demand this must be done before expiry of 3 months after the date of mailing of the ISR/ WO-ISA or 22 months after priority date, whichever expires later (Rule 54bis PCT). Amendments under Art. 34 PCT can be filed with the IPEA as before, normally at the same time as filing the demand (Rule 66.1 (b) PCT).

If a demand for international preliminary examination is filed and no comments/amendments have been received the WO-ISA will be transformed by the IPEA into an IPRP (International Preliminary Report on Patentability) which would merely reflect the content of the WO-ISA. The demand can still be withdrawn (Art. 37 PCT).

Filing informal comments

After receipt of the ISR/WO-ISA the applicant may file informal comments on the WO-ISA directly with the International Bureau of WIPO. These will be communicated to the designated Offices together with the IPRP (International Preliminary Report on Patentability) at 30 months from the priority date. Please also refer to the next box.

End of the international phase

At the end of the international phase the International Bureau of WIPO will transform the WO-ISA or, if a demand was filed, the written opinion of the IPEA into the IPRP, which will then be transmitted together with possible informal comments to the designated Offices. The IPRP replaces the former IPEA (international preliminary examination report).

Relevant PCT Rules and more information

Rule 43 PCT, Rule 43bis PCT, Rule 44 PCT, Rule 44bis PCT, PCT Newsletter 12/2003, OJ 11/2003, OJ 12/2003

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XS CPRTENFRDE

Effects of Soy Oligosaccharides on Plasma and Cecal Isoflavones, and Cecal Enzyme Activities in Mice

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(Received November 1, 2002)

Summary This paper studies the effects of soy oligosaccharides on plasma and cecal isoflavones and floral enzyme activities in adult mice. The isoflavones used in this experiment were daidzein and genistein. Male 6-wk-old mice were fed a soy oligosaccharide-isoflavone (SOI) diet or cellulose-isoflavone (CEI) diet for 10 d. Plasma and cecal isoflavones were analyzed by HPLC, and cecal bacterial β -glucosidase and β -glucuronidase activities were also measured. The plasma genistein concentration was significantly higher in the SOI diet group than in the CEI diet group, but no significant difference was observed in plasma daidzein concentration between the two dietary groups. The total amount of cecal isoflavones existing as aglycone were significantly greater in the SOI diet group than in the CEI diet group, and both cecal β -glucosidase and β -glucuronidase activities were significantly higher in the SOI diet group than in the CEI diet group. These results indicate that soy oligosaccharides have an impact on the metabolic activity of intestinal microflora, plasma concentrations of isoflavones, and amount of isoflavones in the intestine. The present study suggests that ingestion of soy oligosaccharides may influence the effect of dietary isoflavones on the host.

Key Words soy oligosaccharides, isoflavones, cecal enzyme activities

The role of dietary isoflavones in the prevention of several chronic diseases is the subject of intense research interest. Daidzein and genistein are major components of soy isoflavones, and daidzein and genistein are their aglycones. These isoflavones are known to have antioxidant actions (1). It is also reported that isoflavones might be partially responsible for the risk-lowering effects of soy as related to cardiovascular disease (2), and that genistein inhibits tyrosine kinase (3). Further reports indicate that glucosidases of intestinal microflora in the lower bowel can liberate aglycones from the glucosides and promote their absorption (4), and that soy isoflavone aglycones are absorbed faster and in higher amounts than their glycosides in humans (5). Intestinal bacteria seem to play some role in the absorption of isoflavone glucosides. In addition, intestinal perfusion studies indicate that some amount of the genistein glucuronide formed in the rat small intestine returned to the lumen (6-8) and would be hydrolyzed by the glucuronidase of the intestinal microflora in the colon (9). Thus, the bacteria in the gastrointestinal tract play an important role in determining the magnitude and pattern of isoflavone bioavailability (10), and intestinal microflora therefore seem to have an important influence on the effects of isoflavone on the host.

Stachyose and raffinose, which are the main constituents of soy oligosaccharides, are selectively utilized by bifidobacteria except *Bifidobacterium bifidum* (11). Soy

oligosaccharides seem to change the composition of intestinal flora by increasing the bifidobacteria. However, there are few reports regarding the effect of soy oligosaccharides on the plasma and cecal isoflavones. The present study was undertaken to investigate the effects of soy oligosaccharides on plasma and cecal isoflavones and enzyme activity in mice fed diets containing daidzein and genistein.

MATERIALS AND METHODS

Materials. An isoflavone aglycone mixture prepared from soybean was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The isoflavone aglycone mixture contained 44.3% (w/w) daidzein and 50.6% (w/w) genistein. Daidzein and genistein, which were used as standards for HPLC analysis, were purchased from Fujicco (Kobe, Japan). Soy oligosaccharides were kindly provided by Calpis Food Industry Co., Ltd. (Tokyo, Japan). The soy oligosaccharides contained 74.2% (w/w) stachyose and 21.7% (w/w) raffinose. β -Glucuronidase type H-5, *p*-nitrophenyl- β -D-glucopyranoside, and *p*-nitrophenyl- β -D-glucuronide were purchased from Sigma (St. Louis, MO, USA).

Treatment of animals. Male mice of the Crj: CD-1 (ICR) strains (5-wk-old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The mice were randomly divided into two groups of six animals each. The animals were housed in suspended stainless-steel cages with wire mesh bottoms, in a room kept at $24 \pm 0.5^\circ\text{C}$ and a relative humidity of 65%, with light-

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ing of alternating 12-h periods of light and dark. They were fed an AIN 76 diet for 1 wk. After 1 wk, the AIN 76 diet was replaced with a soy oligosaccharide-isoflavone (SOI) diet or cellulose-isoflavone (CEI) diet, and the mice received the SOI or CEI diet for 10 d. The composition of each diet is shown in Table 1. The CEI diet was based on the AIN 76 diet and contained a 5% cellulose and 0.2% isoflavone aglycone mixture. The SOI diet contained 5% soy oligosaccharides instead of cellulose as in the CEI diet. Body weight and food consumption were measured during the experimental period. After the experimental diet feeding period, the mice were sacrificed and blood and cecal contents were collected. Then, the blood samples were centrifuged and the plasma was stored at -80°C until HPLC analysis for isoflavones. Cecal contents were stored at -80°C until the measurement of cecal amounts of isoflavones and enzyme activities.

This study was carried out in accordance with the "Guidelines for Animal Care and Experimentation" of the National Food Research Institute and the University of Tokyo.

Analysis of plasma and cecal isoflavones. For analysis of plasma isoflavones, 200 μL of plasma was supplemented with 0.2 μg flavone for use as an internal standard and 200 μL of β -glucuronidase type H-5 solution (35 mg/mL, Sigma) in 0.2 M sodium acetate buffer (pH 5.0) was added. Next, the mixture was incubated at 37°C in a shaking water bath for 2 h, then treated with 3,600 μL of methanol/acetic acid (100/5, v/v), vortexed for 30 s, sonicated for 30 s, vortexed again for 30 s, and centrifuged for 10 min at 4°C and $5,000 \times g$. The supernatants were transferred to an eggplant-type flask and evaporated completely by a rotary evaporator. Then, the sample was dissolved with the mobile phase of the HPLC system at the same volume of plasma and filtrated by a 0.2 μm filter. For HPLC analysis, we injected 20 μL of each preparation into a 250×4.6 mm

Capcell Pak MG C18-5 μm column (Shiseido, Tokyo, Japan). The mobile phase consisted of water-tetrahydrofuran-acetic acid, 70:25:5 (v/v/v), with a flow rate 1.0 mL/min. A JASCO MD-1515 photodiode array detector (JASCO, Co., Ltd., Tokyo, Japan) was used to quantify spectral data from 200 to 400 nm for each peak. For analysis of cecal isoflavone aglycones, cecal contents were supplemented with 0.3 μg flavone as the internal standard, treated with nine volumes of methanol/acetic acid (100/5, v/v), vortexed for 30 s, sonicated for 30 s, vortexed again for 30 s, and centrifuged at $5,000 \times g$ for 5 min. The supernatant was then transferred to the eggplant-type flask. The same volume as the first extraction of methanol/acetic acid (100/5, v/v) was added to the sediment, and the same procedure was repeated. The supernatant was added to the first supernatant fraction. The supernatants were allowed to evaporate completely in a rotary evaporator. Then, the sample was dissolved with 0.3 mL of the mobile phase of the HPLC system and filtrated by a 0.2 μm filter. We used the same HPLC analytical method as the plasma samples described above.

Measurement of enzyme activity. We measured enzyme activities as previously described (12). A 1:100 cecal suspension was prepared in 0.1 M phosphate buffer (pH 7.0), and nonbacterial debris was removed by centrifugation at $700 \times g$ for 2 min. The supernatant fluid was used immediately for the β -glucosidase and β -glucuronidase assays. β -Glucosidase activity was measured with *p*-nitrophenyl- β -D-glucopyranoside as the substrate. β -Glucuronidase activity was measured with *p*-nitrophenyl- β -D-glucuronide.

Statistics. The data were expressed as the mean $\pm$ SE. The Student's *t*-test was used to determine the statistical significance of the differences between mean values.

RESULTS

Body weight, food consumption, and cecal contents

No significant difference was observed in body weight (g) or food consumption (g/d) between the mice fed the SOI and CEI diets. Cecal contents of the SOI diet group (0.38 ± 0.04) were significantly heavier than those of

Table 1. Compositions of the experimental diets.

Ingredient	Soy oligosaccharides-isoflavone diet (%)	Cellulose-isoflavone diet (%)
Casein	20	20
L-Methionine	0.3	0.3
Corn starch	15	15
Sucrose	49.8	49.8
Corn oil	5	5
Cellulose powder	—	5
Soy oligosaccharides	5	—
Mineral mixture ¹	3.5	3.5
Vitamin mixture ²	1	1
Choline bitartrate	0.2	0.2
Isoflavone aglycone mixture	0.2	0.2

¹ Mineral mixture was prepared according to the AIN-76 formulation.

² Vitamin mixture was prepared according to the AIN-76 formulation.

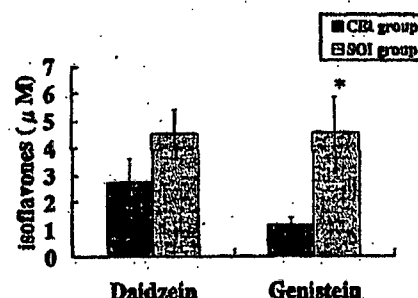


Fig. 1. Plasma isoflavones (aglycones+metabolites) of the mice fed the SOI diet or CEI diet for 10 d. Values are means $\pm$ SE. * Significantly different ($p < 0.05$) from the CEI diet group.

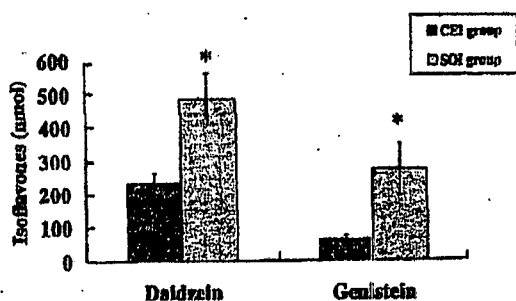


Fig. 2. The total amount of isoflavones existing as aglycone in the cecum of the mice fed the SOI diet or the CBI diet for 10 d. Values are means $\pm$ SE. * Significantly different ($p < 0.05$) from the CBI diet group.

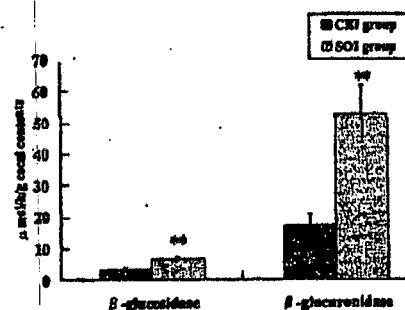


Fig. 3. Cecal β -glucosidase and β -glucuronidase activities of the mice fed the SOI diet or the CBI diet for 10 d. Values are means $\pm$ SE. ** Significantly different ($p < 0.01$) from the CBI diet group.

the CBI diet group (0.26 ± 0.03) ($p < 0.05$).

Plasma isoflavones and cecal isoflavone aglycones

The plasma genistein concentration was significantly higher in the SOI diet group as compared to the CBI diet group (Fig. 1). Though the plasma daidzein concentration tended to be high in the SOI diet group, no significant difference was observed in the plasma daidzein concentration between the two dietary groups. The total amount of isoflavones existing as aglycone in the cecum were significantly greater in the SOI diet group as compared to the CBI diet group (Fig. 2).

Cecal enzyme activity

There were significant differences in cecal enzyme activities between the SOI diet group and the CBI diet group. Both cecal β -glucosidase and β -glucuronidase activities were significantly higher in the SOI diet group as compared to the CBI diet group (Fig. 3).

DISCUSSION

It has been reported that isoflavone aglycones are absorbed from the stomach in rats (13). It has also been reported that dietary isoflavones undergo enterohepatic circulation in humans (14).

A recent report has demonstrated that genistein is highly bioavailable in rats, and because of its enterohepatic circulation, may accumulate within the gastrointestinal tract (15). When $4\text{-}^{14}\text{C}$ -genistein is infused into the duodenum, it is rapidly absorbed from the intestine, taken up by the liver, and excreted into the bile as its 7- α - β -glucuronide conjugate (15). Intestinal perfusion studies indicate that some amount of the genistein glucuronide formed in the rat small intestine returns to the lumen (6–8). Cecal β -glucuronidase activity was also significantly higher in the SOI diet group as compared to the CBI diet group. As intestinal microflora appear to be the major source of β -glucuronidase in the gut (16), the higher floral β -glucuronidase activity in the SOI diet group would cause efficient re-absorption of the isoflavones in the lower intestine by hydrolyzing the bile excretory genistein-glucuronide and genistein-glucuronide formed in the small intestine and returned to the lumen. Higher β -

glucuronidase in the SOI diet group would contribute to the higher plasma genistein concentration in the SOI diet group.

Both cecal genistein and daidzein were significantly greater in the SOI diet group as compared to the CBI diet group. Higher β -glucuronidase activity in the SOI diet group would also contribute to the greater amounts of cecal daidzein and genistein by hydrolyzing their glucuronides.

On the other hand, it has been reported that soy oligosaccharides are selectively utilized by the bifidobacteria (11), and that the bifidobacteria have β -glucosidase activity (17). In our experiment, both cecal β -glucosidase and β -glucuronidase activities were significantly higher in the SOI diet group as compared to the CBI diet group. The cecal environment in the SOI diet group might be different from that in the CBI diet group. It is reported that human intestinal bacteria metabolize and degrade ingested soybean isoflavone to a great extent (10). Metabolism and degradation of daidzein and genistein in the cecum might be suppressed in the SOI diet group as compared to the CBI diet group as the result of a change in the cecal environment.

It has been reported that soy isoflavone aglycones are absorbed faster and in higher amounts than their glycosides in humans (5). The greater part of isoflavones in soybean are glucosides. The β -glucosidase activity of the intestinal bacteria is thought to be important for the hydrolysis of glucoside compounds in the intestine (4). Higher β -glucosidase activity in the SOI diet group would be useful for digesting and absorbing the glucoside of isoflavones.

Much evidence supports the hypothesis that adequate isoflavone intake reduces cancer risk (18). For this effect, maintenance of a constant level of plasma genistein might be important. The present study suggests that the ingestion of soy oligosaccharides may influence the effects of dietary isoflavones on the host by having an impact on the metabolic activity of the intestinal microflora.

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Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine^{1,2,3}

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Received January 9, 1984

STANGROOM, K. E., and T. K. SMITH. 1984. Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine. *Can. J. Physiol. Pharmacol.* 62: 1219-1224.

Experiments were conducted to determine the mechanism by which dietary alfalfa can protect against zearalenone toxicosis. Female weanling rats were fed semipurified diets containing whole alfalfa meal, fractionated alfalfa meal (fiber, solvent extract, and water extract), and purified components of alfalfa (coumestrol, saponin, lignin, coumestrol + lignin, and saponin + lignin) with and without 250 mg zearalenone/kg of diet. All ingredients were provided for 2 weeks at levels corresponding to those found in diets containing 15 and 25% alfalfa. Yorkshire gilts were fed 15 and 25% alfalfa meal with and without 10 mg zearalenone/kg of diet for 4 weeks. The feeding of zearalenone to rats reduced growth and food consumption but this was overcome by 25% alfalfa. Zearalenone also increased the activity of hepatic 3 α -hydroxysteroid dehydrogenase (3 α -HSD), the enzyme believed to metabolize zearalenone to α - and β -zearalenols. Dietary alfalfa did not overcome this effect. Alfalfa fiber was the only fraction to partially overcome the growth-depressing effects of zearalenone while the other fractions had no beneficial effects and 3 α -HSD was not affected by diet. None of the purified components affected growth parameters or 3 α -HSD. The enzyme was also not affected by zearalenone or alfalfa in swine diets. Coumestrol, α -zearalenone, and β -zearalenone were shown to be competitive inhibitors of 3 α -HSD in rat liver. It was concluded that the fiber fraction of alfalfa protects against zearalenone toxicity, and that this effect is not dependent on coumestrol or saponin and is not likely mediated through 3 α -HSD.

STANGROOM, K. E., et T. K. SMITH. 1984. Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine. *Can. J. Physiol. Pharmacol.* 62: 1219-1224.

Ces expériences visaient à déterminer par quel mécanisme l'alfalfa alimentaire peut protéger de la toxicose au zéaralénone. Des rats femelles nouvellement sevrés reçurent des diètes semipurifiées contenant un repas complet de luzerne, un repas fractionné de luzerne (fibre, extrait de solvant et extrait d'eau), et des composantes purifiées de luzerne (coumestrol, saponine, lignine, coumestrol + lignine et saponine + lignine) avec et sans diète de 250 mg zéaralénone/kg. Les ingrédients furent ingérés pendant 2 semaines à des taux correspondant à ceux de diètes contenant 15 et 25% d'alfalfa. Des porcs Yorkshire reçurent un repas de 15 et 25% de luzerne, avec et sans 10 mg zéaralénone/kg pendant 4 semaines. L'administration de zéaralénone aux rats réduisit la croissance et la consommation de nourriture, mais ceci fut neutralisé par 25% de luzerne. En outre, zéaralénone augmenta l'activité de 3 α -hydroxystéroïde déshydrogénase (3 α -HSD), l'enzyme censé métaboliser zéaralénone en zéaralénoles α et β . La luzerne alimentaire ne renversa pas cet effet. La fibre de luzerne fut la seule fraction capable de neutraliser les effets réducteurs de croissance de zéaralénone, alors que les autres fractions n'eurent pas d'effets bénéfiques et que la diète n'affecta pas le 3 α -HSD. Aucune des composantes purifiées n'affecta les paramètres de croissance ni le 3 α -HSD. L'enzyme ne fut affectée ni par le zéaralénone ni par la luzerne contenus dans les diètes des porcs. Dans le foie de rat, le coumestrol, les zéaralénoles α et β s'avérèrent être des inhibiteurs compétitifs de 3 α -HSD. On a conclu que la fraction fibre de luzerne protège contre la toxicité du zéaralénone, que cet effet n'est fonction ni du coumestrol ni de la saponine et qu'il n'est vraisemblablement pas médié par le 3 α -HSD.

[Traduit par le Journal]

Introduction

Zearalenone is a uterotrophic mycotoxin that can be produced when cereal grains are stored in the presence of *Fusarium roseum* under conditions of greater than 25% moisture and temperatures of 12-14°C (Caldwell and Tuke 1970). Many different species are susceptible to zearalenone toxicosis, although swine are among the most sensitive. The feeding of zearalenone at levels as low as 1-5 mg/kg diet has been shown to cause hyperestrogenism in prepubertal gilts (Mirocha and Christensen 1974). Mammary gland enlargement, tumefaction of the vulva, and vaginal and rectal prolapse are seen in immature females (Change *et al.* 1979), while immature males can suffer swelling of the prepuce and enlargement of mammary glands (Christensen *et al.* 1972). Zearalenone consumption by

mature female swine can alter reproduction resulting in increased neonatal mortality, and a decrease in fertility, lactation, and litter size (Chang *et al.* 1979).

The interrelationships between diet and zearalenone toxicosis and metabolism have been investigated in rats and swine (Smith 1980a, 1980b, 1982; James and Smith 1982). Diets containing dehydrated alfalfa meal have been shown to be among the most effective treatments for zearalenone toxicosis. James and Smith (1982) observed that the inclusion of 25% alfalfa meal in purified rat diets largely overcame growth depression, owing to zearalenone and reduced hepatic residues of zearalenone and α -zearalenone, a major metabolite. The feeding of alfalfa to gilts has been shown to reduce uterine enlargement caused by zearalenone (James and Smith 1982).

The mechanism by which dietary alfalfa protects against zearalenone toxicosis has not been determined. It has been proposed that alfalfa may bind zearalenone in the intestinal lumen to reduce absorption of ingested toxin and also limit reabsorption of toxin excreted in bile (Smith 1982). The adsorptive properties of alfalfa may be related to the presence of surface-active saponins as suggested by Oakenfull and Peawick (1978). It has also been speculated that some components of

¹Supported by the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food.

²Presented in part at the 26th annual meeting of the Canadian Federation of Biological Societies, Ottawa, Ont., 1983.

³Described in part in the M.Sc. thesis presented by K. E. Stangroom to the Faculty of Graduate Studies, University of Guelph, 1983.

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alfalfa may alter the metabolism of zearalenone by hepatic 3 α -hydroxysteroid dehydrogenase (3 α -HSD), thereby altering its toxicity (Smith 1982). The physiological activity of zearalenone may be influenced by coumestrol, a phytoestrogen present in alfalfa. Coumestrol can cause hyperestrogenism in several species if ingested at appropriately high levels (Newsome and Kitts 1980; Kincaid and Browning 1981).

The current studies were undertaken to determine which components of alfalfa meal can protect against zearalenone toxicosis. The effect of these components on the activity of 3 α -HSD was measured to further determine any role of this enzyme in the alleviation of zearalenone toxicosis by alfalfa.

Materials and methods

Experimental animals and control diets

Female weanling Wistar rats (initial weight of 50–55 g, Woodlyn Labs Ltd., Guelph, Ont.) were used in all rat experiments. They were housed in individual stainless steel cages and fed a commercial stock diet for the first 3 days. A semipurified control diet (Table 1) with various modifications was then fed to the rats for 14 days. Feed consumption and weight gain were measured every 3 days and water was supplied *ad libitum*. At the end of the experimental period all rats were killed and various tissues were excised and stored at -65°C for subsequent assay of 3 α -HSD activity.

Yorkshire gilts (8–10 kg initial weight) were used in swine experiments. Animals were housed in individual concrete pens and fed modifications of a corn-soy control diet (Table 1) for 4 weeks. All animals were then killed and tissues were excised and stored at -65°C for subsequent assay of 3 α -HSD activity.

Rats fed alfalfa meal

A total of 60 rats were fed diets containing 0, 15, or 25% dehydrated alfalfa meal with and without 250 mg zearalenone/kg diet (10 rats/diet). Alfalfa was added to diets at the expense of corn starch, casein, and corn oil to keep all diets isocaloric and isonitrogenous (James and Smith 1982).

Rats fed fractionated alfalfa meal

Rats were separated into 14 groups of 10 each and fed the control diet, alfalfa fiber, alfalfa solvent extract, and alfalfa water extract. The various alfalfa fractions were fed at levels corresponding to those found in diets of 15 and 25% alfalfa meal. All diets were supplied with and without 250 mg zearalenone/kg diet and were again kept isocaloric and isonitrogenous.

Rats fed purified components of alfalfa meal

Six experimental diets were fed with and without 250 mg zearalenone/kg diet to a total of 120 rats (10 per diet). These included the control diet, and at levels corresponding to those found in 25% alfalfa meal diets: saponin (Pederson and Wang 1971) (United States Biochemical Corp., Cleveland, OH), coumestrol (Bickoff *et al.* 1969) (Eastman Kodak Co., Rochester, NY), lignin (Smith 1980a) (Rood Paper Co., Québec, Qué.), saponin + lignin, and coumestrol + lignin. It is recognized that these purified compounds were not necessarily derived from alfalfa and may not exactly duplicate the biological activity of the same compounds found in alfalfa.

Swine fed alfalfa meal

Swine were fed diets containing 0, 15, or 25% alfalfa meal with and without 10 mg zearalenone/kg diet. Forty-eight swine were used, eight per diet. Alfalfa was added to the diets at the expense of corn and soybean meal to ensure that all diets were isocaloric and isonitrogenous.

Characterization of 3 α -HSD

The activity of 3 α -HSD was measured in liver, uterus, kidney, and leg muscle of both rats and swine. Initial characterization of 3 α -HSD was conducted on pooled samples of tissue obtained from two groups of 10 rats fed either the control diet or the control diet plus zearalenone. A similar procedure was used for swine. Subsequent

TABLE 1. Composition of control diets (%)

Ingredient	Rats	Swine
Casein (vitamin free) ^a	16.3	—
Corn oil ^b	6.0	—
Corn starch ^b	67.8	—
Mineral mixture	3.5 ^c	0.1 ^d
Vitamin mixture	1.0 ^c	0.25 ^d
Cellulose ^e	5.2	—
DL-Methionine	0.2	—
Corn	—	64.05
Soybean meal (49%)	—	33.0
Iodized salt	—	0.5
Limestone	—	0.9
Dicalcium phosphate	—	1.2

^aTeklad Test Diets, Madison, WI.

^bSt. Lawrence Starch Co., Port Credit, Ont.

^cSmith 1981.

^dSmith 1984.

^eCelufil, United States Biochemical Corp., Cleveland, OH.

assays were conducted on samples from individual animals. Approximately 2 g of tissue was homogenized for 30 s at 0°C in 30 mL of 0.1 M potassium phosphate buffer. The homogenate was then centrifuged at $25\,000 \times g$ for 15 min at 0°C . The supernatant, which contained both soluble and microsomal enzymes, was stored on crushed ice prior to assay. The spectrophotometric method of Koide (1969) was used to determine pH optimum, optimum substrate concentration, cofactor dependency, substrate specificity, and kinetic parameters for the enzyme.

A modification of the radioisotopic assay of Thouvenot and Morfin (1980) was employed to determine the activity of 3 α -HSD in rat liver in the presence of various potential inhibitors. The reaction mixture included potassium phosphate buffer (660 μL of 0.1 M, pH 8.0), 34 μL 0.1 M NAD in buffer, 10 μL ($2-5 \times 10^5$ disintegration per minute) [$1,2-^3\text{H}$]androsterone (New England Nuclear, Boston, MA), 10 μL androstosterone in methanol (at various concentrations), 10 μL inhibitor in methanol, and an additional 40 μL methanol to ensure steroid solubility. All reaction mixtures were incubated for 5 min at 37°C and the reactions were then initiated by the addition of 34 μL of supernatants. The reactions were terminated after 15 min by the addition of 5 mL ethyl acetate. The phases of the incubation mixture were allowed to separate over 12 h and the ethyl acetate layer was removed. The aqueous phase was extracted twice more with 5 mL ethyl acetate and the extracts were combined and evaporated to dryness under nitrogen. The residues were dissolved in methanol and aliquots were applied to chromatographic plates coated with silica gel G (Analtech Uniplate, Fisher Scientific Ltd., Toronto, Ont.). Plates were developed in benzene:ethyl acetate (3:1). The positions of the substrate ($[1,2-^3\text{H}]$ androsterone) and the product ($[1,2-^3\text{H}]$ androstanedione) were determined by spraying the plates with anisaldehyde: H_2SO_4 : glacial acetic acid (1:2:100) and heating at 100°C for 10 min. Identification of spots was made by comparison with known standards. Spots corresponding to the product were scraped from the plate and radioactivity was quantified by liquid scintillation spectrophotometry.

Substances tested as inhibitors included coumestrol and α - and β -zearalenone. The latter two compounds were purified from a racemic mixture as described by James *et al.* (1982).

Fractionation of alfalfa meal

Approximately 1 kg of alfalfa meal was extracted with acetone by refluxing for 72 h in a soxhlet apparatus. Acetone was separated from the solvent-extract by distillation. The residue remaining after the acetone extraction was further refluxed for 24 h with boiling water. The residue was then centrifuged at $8000 \times g$ for 5 min and dried. This yielded the alfalfa fiber fraction. A water extract was obtained by refluxing whole alfalfa meal for 24 h with boiling water as described

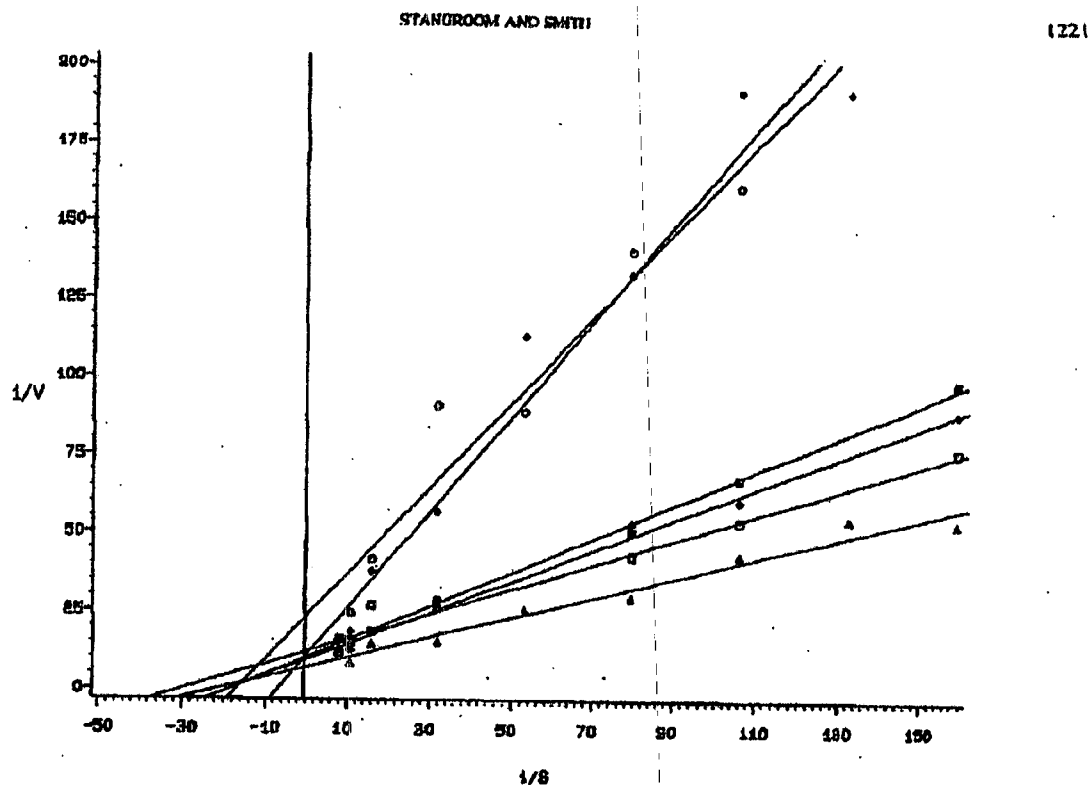


FIG. 1. Inhibition of 3α -hydroxysteroid dehydrogenase activity (androsterone as substrate) by α - and β -zearalenone and coumestrol. Female rat liver supernatant ($25\,000 \times g$ fraction) was incubated with $[1,2\text{-}^3\text{H}]$ androsterone in the absence (Δ) or presence of 1.0 mM α -zearalenol ($\diamond$), 0.1 mM α -zearalenol ($\circ$), 1.0 mM β -zearalenol ($\blacksquare$), 0.1 mM β -zearalenol ($\square$), or 0.1 mM coumestrol ($\circ$). $1/s = 1/\text{concentration of androsterone } (1/M \times 10^3)$ and $1/v = 1/\text{velocity } (1/\mu\text{mol } [1,2\text{-}^3\text{H}] \text{ androsterone produced per minute per milligram protein})$.

above. The supernatant remaining following centrifugation was concentrated by lyophilization.

Statistical analyses

Values for 3α -HSD, K_m , and V_{max} as obtained from double reciprocal plots of enzyme activity at varying substrate concentrations were compared by *t*-test (Steel and Torrie 1960). The type of enzyme inhibition caused by inhibitors was determined by analysis of covariance and subsequent *t*-tests performed on individual slopes and *y*-intercepts (Steel and Torrie 1980). Data from rat and swine feeding experiments were analyzed by analysis of variance appropriate for factorial designs (Ostle and Mensing 1975). Group comparisons were made as orthogonal contrasts where necessary.

Results

Characterization of 3α -HSD

The pH sensitivity of hepatic 3α -HSD in rats fed the control diet was not influenced by the feeding of zearalenone. Optimal activity occurred at pH 8.0 and 9.0 and the former was chosen for assays to be conducted following feeding experiments. Assays of liver homogenates from swine fed the control diet indicated maximal activity at pH 8.2 and 9.0.

The K_m for androsterone in liver of rats fed the control diet ($43\text{ }\mu\text{M}$) was greater ($P < 0.025$) than that of rats fed the same diet containing zearalenone ($26\text{ }\mu\text{M}$). V_{max} was not affected by zearalenone feeding in rats or swine ($P > 0.05$). Hepatic K_m of swine fed the control diet ($8.8\text{ }\mu\text{M}$) did not differ from K_m of those animals fed zearalenone ($7.7\text{ }\mu\text{M}$). The use of NAD as

cofactor resulted in greater activity under saturating conditions than NADP, therefore, NAD was used as the cofactor in all subsequent assays. The K_m for NAD in liver of rats and swine were 470 and 200 μM , respectively.

The K_m for androsterone in rat kidney was much lower ($8.3\text{ }\mu\text{M}$) than that observed in liver. The V_{max} , however, was only 15% of that seen in liver. No activity was detected in muscle from either rat or swine. Only a trace of activity was noted in rat uterus and none was seen in swine uterus.

Inhibition of rat liver 3α -HSD by α - and β -zearalenol is indicated by the double reciprocal plot (Fig. 1). Comparison of the slopes and the *y*-intercepts revealed that while each of the slopes for α -zearalenol (1.0 and 0.1 mM initial concentration), β -zearalenol (1.0 and 0.1 mM initial concentration), and coumestrol (0.1 mM initial concentration) was different from the control plot ($P < 0.025$), *y*-intercepts did not differ ($P > 0.05$). Apparent K_m values for 3α -HSD were 45.8, 146.0, 58.7, 78.1, 44.2, and 109.8 μM in the presence of no inhibitor, 1.0 mM α -zearalenol, 0.1 mM α -zearalenol, 1.0 mM β -zearalenol, 0.1 mM β -zearalenol, and 0.1 mM coumestrol, respectively.

Rats fed alfalfa meal

The addition of zearalenone to the control diet reduced final body weight, food consumption, and gain/food ratios ($P < 0.01$, Table 2). The feeding of zearalenone also increased the activity of hepatic 3α -HSD ($P < 0.05$). The addition of 25%

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TABLE 2. Effect of alfalfa meal and zearalenone on final body weight, food consumption, gain/food ratio, and hepatic 3 α -hydroxysteroid dehydrogenase activity in rats

	Zearalenone (mg/kg)	Alfalfa (%)		
		0	15	25
Final body weight (g) ^a	0	134 ^b	142	140
	250	116**	121**	133
Food consumption (g) ^a	0	194	211	208
	250	171**	178**	194
Weight gained/food consumed ^a	0	0.38	0.38	0.40
	250	0.35**	0.33**	0.38
Activity of 3 α -hydroxysteroid dehydrogenase ^{a,c}	0	0.0611	0.0558	0.0573
	250	0.0645*	0.0624*	0.0603*

^aSD (standard deviation) is ± 12.4 .^bMean for 10 rats over a 14-day period.^cSD is ± 16.3 .^dSD is ± 0.05 .^eMicrosome NADH formed per minute per milligram of protein.^fSD is ± 0.0079 .^gSignificantly different from same diet devoid of zearalenone, $P < 0.05$.^hSignificantly different from same diet devoid of zearalenone, $P < 0.01$.

alfalfa meal to the diets overcame the effects of zearalenone on growth and food consumption but the activity of 3 α -HSD was not affected ($P > 0.05$).

Rats fed fractionated alfalfa meal

Rats fed the control diet supplemented with zearalenone again had lower final body weights, food consumption, and gain/food ratios compared with rats fed the same diet without toxin (Table 3). Alfalfa fiber overcame both the growth-depressing effects of zearalenone and the depressing effects of zearalenone on food consumption when fed at levels corresponding to those found in 25% alfalfa meal (Table 3). Alfalfa solvent extract and alfalfa water extract (Table 4) had no such effects ($P > 0.05$). Alfalfa fiber (Table 3) and alfalfa solvent extract (Table 4) increased gain/food ratios compared with alfalfa water extract (Table 4) when a three-factor analysis was employed ($P < 0.01$).

Rats fed purified components of alfalfa meal

The effects of zearalenone in reducing final body weight, food consumption, and gain/food ratio were not overcome by the feeding of purified coumestrol or saponin (Table 5). Purified lignin and combinations of coumestrol + lignin and saponin + lignin also had no beneficial effect (Table 6). None of these dietary treatments significantly influenced hepatic activity of 3 α -HSD ($P > 0.05$).

Swine fed alfalfa meal

The activity of hepatic 3 α -HSD was not significantly affected ($P > 0.05$) by the addition of zearalenone or alfalfa meal to the diets of swine.

Discussion

The objective of the characterization of 3 α -HSD in tissues of rats and swine was to establish conditions for maximal activity for use in analyzing tissues from the subsequent feeding trials. A pH of 8.0 and saturating concentrations of substrate and

TABLE 3. Effect of alfalfa fiber and zearalenone on final body weight, food consumption, gain/food ratio, and hepatic 3 α -hydroxysteroid dehydrogenase activity in rats

	Zearalenone (mg/kg)	Alfalfa fiber (equivalent %) ^a		
		0	15	25
Final body weight (g) ^a	0	146 ^b	150	147
	250	110**	127**	136
Food consumption (g) ^a	0	196	211	205
	250	149**	174**	200
Weight gained/food consumed ^a	0	0.40	0.39	0.38
	250	0.29**	0.36**	0.35**
Activity of 3 α -hydroxysteroid dehydrogenase ^{a,c}	0	0.0637	0.0672	0.0634
	250	0.0645	0.0667	0.0665

^aLevels fed were equivalent to the levels found in these percentages of whole dehydrated alfalfa meal.^bSD (standard deviation) is ± 10.5 .^cMean for 10 rats over a 14-day period.^dSD is ± 18.8 .^eSD is ± 0.06 .^fMicrosome NADH formed per minute per milligram of protein.^gSD is ± 0.0071 .^hSignificantly different from same diet devoid of zearalenone, $P < 0.01$.

cofactor were used following their determination. Koide (1969), using the same buffer as that employed in the current studies, noted maximal activity at pH 7.5, 8.0, and 9.5 in rat liver. Data in the literature are not available for the optimal pH in liver of swine.

Activity of 3 α -HSD was assayed in the livers of animals tested in the feeding trials, since liver was determined to have the highest activity of all tissues examined. Ueno *et al.* (1983) have reported that female rat liver has a much greater capacity to metabolize zearalenone to the major metabolite α -zearalenone (Ueno and Tashiro 1981) than kidney, spleen, or uterus. NADH has been demonstrated to be a more efficient hydrogen donor in this reaction than NADPH (Tashiro *et al.* 1983). The data in the current studies also showed maximal activity in the presence of NADH. It is not possible to compare K_m values obtained in this study with those reported previously in the literature because different conditions for analysis were used.

The K_m and specific activity of the swine enzyme were much smaller than those measured in rats. This may indicate a higher affinity of the enzyme for the substrate (androsterone) in the former species; however, maximal activities were only 15% of those seen for rat liver. This may be due to differences in the relative sexual maturity of 3-month-old swine and weanling rats. The specific activity of 3 α -HSD has been shown to decrease with age in the testes of male rats (Peng *et al.* 1979). Olsen and Kietzling (1983) have observed further species differences and reported that female swine liver has a higher capacity to form both α - and β -zearalenol than cow or hen.

Statistical analyses of the enzyme activity in the presence of α - and β -zearalenol and coumestrol indicated that all three of these compounds competitively inhibit 3 α -HSD. The concept that zearalenols can serve as competitive inhibitors of 3 α -HSD is supported by Thourvenot and Morfin (1980) who reported that zearalenone and zearalenols caused significant increases in apparent K_m but no difference in V_{max} or 3 α -HSD in hyper-

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TABLE 4. Effect of alfalfa solvent extract, alfalfa water extract, and zeaxalenone on final body weight, food consumption, gain/food ratio, and hepatic 3 α -hydroxysteroid dehydrogenase activity in rats

	Zeaxalenone (mg/kg)	Solvent extract (equiv. %)*		Water extract (equiv. %)*	
		15	25	15	25
Final body weight (g) ^a	0	136 [*]	143	143	140
	250	113**	111**	113**	108*
Food consumption (g) ^a	0	176	182	205	222
	250	148**	142**	175**	165**
Weight gained/ food consumed ^a	0	0.37	0.42	0.37	0.33
	250	0.30**	0.32**	0.29**	0.26**
Activity of 3 α - hydroxysteroid dehydrogenase ^{a,b}	0	0.0618	0.0684	0.0671	0.0621
	250	0.0715	0.0697	0.0678	0.0667

*Levels fed were equivalent to the levels found in these percentages of whole dehydrated alfalfa meal.

^aSD is ± 10.5 .^bMean for 10 rats over a 14-day period.^cSD is ± 18.8 .^dSD is ± 0.06 .^eMicromoles NADH formed per minute per milligram of protein.^fSD is ± 0.0071 .^gSignificantly different from same diet devoid of zeaxalenone, $P < 0.05$.^hSignificantly different from same diet devoid of zeaxalenone, $P < 0.01$.TABLE 5. Effect of purified coumestrol, saponin, and zeaxalenone on final body weight, food consumption, gain/food ratio, and hepatic 3 α -hydroxysteroid dehydrogenase activity in rats

	Zeaxalenone (mg/kg)	Control	Coumestrol (25 μ g/g)	Saponin (3.5%)
Final body weight (g) ^a	0	114 [*]	118	118
	250	98**	96**	95**
Food consumption (g) ^a	0	167	168	174
	250	149**	145**	137**
Weight gained/ food consumed ^a	0	0.41	0.43	0.41
	250	0.35**	0.35**	0.36**
Activity of 3 α - hydroxysteroid dehydrogenase ^{a,b}	0	0.0628	0.0656	0.0637
	250	0.0737	0.0651	0.0658

^aSD is ± 9.5 .^bMean for 10 rats over a 14-day period.^cSD is ± 15.0 .^dSD is ± 0.04 .^eMicromoles NADH formed per minute per milligram of protein.^fSD is ± 0.009 .^gSignificantly different from same diet devoid of zeaxalenone, $P < 0.01$.

plastic human prostate.

The expected consequence of this inhibition would be an increase in normal levels of the androgenically potent precursor dihydrotestosterone and a resulting masculinization. Zeaxalenone administration, however, results in hyperestrogenism. There is extensive evidence that zeaxalenone and the zeaxalenols bind to estradiol 17- β receptors in rat uterus, mammary gland, and liver (Kiang *et al.* 1978; Katzenellenbogen

TABLE 6. Effect of purified lignin, lignin + coumestrol, lignin + saponin, and zeaxalenone on final body weight, food consumption, gain/food ratio, and hepatic 3 α -hydroxysteroid dehydrogenase activity in rats

	Zeaxalenone (mg/kg)	Lignin (4.2%)	Lignin + coumestrol	Lignin + saponin
Final body weight (g) ^a	0	112 [*]	117	118
	250	93**	93**	93**
Food consumption (g) ^a	0	156	158	166
	250	133**	142**	142**
Weight gained/ food consumed ^a	0	0.42	0.45	0.44
	250	0.35**	0.33**	0.35**
Activity of 3 α - hydroxysteroid dehydrogenase ^{a,b}	0	0.0681	0.0656	0.0635
	250	0.0680	0.0641	0.0617

^aSD is ± 9.5 .^bMean for 10 rats over a 14-day period.^cSD is ± 15.0 .^dSD is ± 0.04 .^eMicromoles NADH formed per minute per milligram of protein.^fSD is ± 0.009 .^gSignificantly different from same diet devoid of zeaxalenone, $P < 0.01$.

et al. 1979; Boyd and Wutliff 1978). The metabolic effects of such binding likely negate any opposing influence of 3 α -HSD inhibition.

In the present study the activity of 3 α -HSD in rats *in vivo* was significantly increased by zeaxalenone. This increase was not significantly altered by the feeding of alfalfa meal but tended to be lessened by the feeding of lignin + saponin. Because the assays were conducted at saturating substrate concentrations to determine the maximal enzyme activity, any competitive inhibition by low residual levels of zeaxalenone or zeaxalenols would not be detected. The increased activity of 3 α -HSD when zeaxalenone was fed is in contrast with earlier reports (James and Smith 1982). The earlier studies, however, were not conducted under optimal reaction conditions.

The lack of measurable effect of zeaxalenone or alfalfa meal on the activity of 3 α -HSD in swine indicates that the enzyme is not likely a major factor in zeaxalenone toxicosis in this species. The low activity seen in the swine tissues, however, complicates interpretation of these data.

It has recently been suggested by Ueno *et al.* (1983) that 3 α -HSD may not be the major enzyme catalyzing zeaxalenone metabolism. This argument is based on the assumption that 3 α -HSD is cytoplasmic, while zeaxalenone reductase activity was found to be mainly microsomal. This is in contrast with various reports that zeaxalenone is metabolized by 3 α -HSD (Thouvenot and Morfin 1980; Klessling and Pottersson 1978; Olsen *et al.* 1981). The most recent work of Olsen and Klessling (1983), moreover, cites literature to support the concept that 3 α -HSD is not compartmentalized within the cell but is both cytoplasmic and microsomal.

The current studies indicate that the protective effect of alfalfa meal against zeaxalenone toxicosis is due mainly to the fiber fraction. The extensive extraction of the meal with acetone and hot water would remove any of the surface-active saponins and phytoestrogenic coumestrol. Effects of purified coumestrol on activity of 3 α -HSD or any interference with the binding of zeaxalenone to estrogen receptors does not appear to

be of physiological significance. Adsorptive properties of saponins also do not seem to contribute to the protective action of alfalfa meal.

It is likely that the unique physical configuration of fibrous components in alfalfa was still present after the extensive extraction enabling the fiber to retain the ability of alfalfa meal to adsorb compounds such as bile acids (Calvert and Yeats 1982). The data of Smith (1980b) indicate that bile may be a major excretory route for zearalenone in the rat and the ability of the alfalfa fiber to bind bile constituents is likely important in overcoming zearalenone toxicosis. Lignin is a major constituent of alfalfa fiber, but the lack of effect of feeding purified lignin indicates that other fiber constituents are also necessary for the beneficial effects of alfalfa. The lack of effect of both purified lignin and alfalfa fiber on the activity of 3 α -HSD indicates that the fiber portion of alfalfa is not likely altering the metabolism of zearalenone. Such an effect has been reported for dietary fiber in the metabolism of pesticides (Chadwick *et al.* 1978).

The most significant characteristic of alfalfa in overcoming zearalenone toxicosis is, therefore, the prevention of zearalenone reaching target tissues such as uterus, mammary glands, and liver. This is supported by the studies of Smith (1982), which indicated that while alfalfa meal reduced tissue residues of zearalenone and zearalenols, there was no significant change in the relative amounts of parent compound and metabolites.

Acknowledgments

The technical assistance of Mr. Donald Saunders, Miss Suzanne Cadden, and Mrs. Margaret Rich is gratefully recognized. Dr. Ian McMillan is thanked for his excellent statistical advice. Purified zearalenone was the gift of IMC Chemical Group Inc., Terre Haute, IN, U.S.A.

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PREVENTION RESEARCH 6

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roboration with functional p53 is very significant and is a first time observation. Studies are in progress to see a similar effect in cancer cells with mutated p53. The biological and functional significance of the enhanced expression of HSPs induced by the combined actions of DHA and celecoxib and their role during apoptosis and differentiation process in the presence of wild-type p53 are discussed (Supported by NCI CA106296-01, and CA107813-01 grants).

#3189 Fenretinide and six novel phenylretinamides - Inhibition of the cyclooxygenase pathway and its relevance for growth inhibition and induction of cell death of oral carcinoma cells. Stewart Sale, Elizabeth Moss, Katherine Eddolls, Angela Hague. *University of Bristol, Bristol, United Kingdom*

Cyclooxygenase-2 (COX-2) has been shown to be up-regulated in numerous tumor types including oral carcinomas. COX-2 catalyses the formation of prostaglandins from arachidonic acid, and chemopreventive agents that suppress its expression or activity could be considered beneficial for preventing cancer development. The retinoid analogue, fenretinide (4-HPR, 4-hydroxyphenyl retinamide), has potential for oral cancer chemoprevention, since topical treatment reduces the recurrence of oral leukoplakia. The aim of this study was to determine whether fenretinide and six novel fenretinide analogues modulated the cyclooxygenase pathway in oral carcinoma cells. We investigated whether any of these retinoids suppressed the expression of COX-1 and COX-2 in human oral squamous carcinoma cell lines H376 and H400 and whether they were able to inhibit the activity of the COX enzyme by using *in vitro* and cell free systems. In addition, the ability of the retinoids to induce growth inhibition and/or apoptosis in oral carcinoma cells was investigated. Although expression of COX-1 or COX-2 protein was not reduced by retinoid treatment, both fenretinide and the analogue 2-CPR at 5 and 10 μ M and the analogues 2-HPR and 3-CPR at 10 μ M inhibited synthesis of prostaglandin E2 (PGE₂) by over 50%. Interestingly levels of two other prostaglandins (PGF₂ α and PGI₂) were not altered in either cell line. Growth inhibition was obtained in response to all retinoids tested (IC₅₀ values for fenretinide and the six analogues, ranging from 5.9-9.2 μ M). Fenretinide did not significantly induce apoptosis above vehicle control, however, interesting findings were achieved with the 6 novel analogues. At the highest concentration of 20 μ M three of the agents (3-HPR, 3-CPR (3-carboxyphenyl retinamide) and 4-CPR) caused significant induction of necrosis whereas the other three agents (2-HPR, 2-CPR and 4-MPR (4-methoxyphenyl retinamide)) induced varying degrees of apoptosis without any necrosis. Taken together these data reveal that fenretinide, 2-CPR, 2-HPR and 3-CPR are all able to inhibit prostaglandin formation in oral cancer cells. To determine the relevance to cancer chemoprevention, additional studies of functionality are warranted to determine the mechanism of action of these novel analogues. Their abilities to induce varying degrees of necrosis and apoptosis would suggest different mechanisms of action, some of which maybe in part dependent on reduced autocrine PGE₂ survival signalling.

#3190 Prevention of esophageal cancer from targeting a single molecule to a molecular pathway. Xiaochun Xu, Tsung-Teh Wu, Jie Huang, Jaffer A. Ajani, Scott M. Lippman. *UT M. D. Anderson Cancer Center, Houston, TX*

Esophageal cancer has become a significant health problem in the US and accounts for the seventh cancer death in American men. Tobacco smoke and gastro-esophageal reflux are the most important risk factors in esophageal carcinogenesis. Over past 10 years, our group has accumulated extensive experiences working on esophageal cancer. Our objective is to develop novel strategies to prevent esophageal cancer through understanding of the molecular mechanisms. Our data have demonstrated that loss expression of RAR- β 2 and RRIG1 in premalignant and malignant esophageal tissues as compared to the normal tissues. BPDE (a carcinogen present in tobacco smoke and environment pollution) and tumor-promoting bile acid suppressed expression of RAR- β 2 and induced expression of EGFR, AP-1, COX-2, and phosphorylated Erk1/2 in premalignant and malignant esophageal cells. Induction of RAR- β 2 expression inhibited tumor cell growth and colony formation *in vitro* and tumor formation in nude mice, which were correlated with reduced expression of EGFR, AP-1, COX-2, and phosphorylated Erk1/2. However, esophageal cancer cells that do not express RAR- β 2 were resistant to retinoid treatment. Moreover, we have cloned a novel retinoid receptor-induced gene-1, RRIG1, which is a downstream gene of RAR- β 2 in regulating the effects of RAR- β 2. RRIG1 binds to and inhibits a small GTPase RhoA activity. Induction of RRIG1 inhibits RhoA activation and in turn, reduces tumor cell colony formation, invasion, and proliferation, which were correlated with inhibition of Erk1/2 phosphorylation, COX-2, and CyclinD1 expression. These data have described a novel molecular pathway involving RAR- β 2-induced RRIG1 expression and suppression of RhoA/Erk1/2/AP-1/COX2. Our further study has shown that targeting this gene pathway by using different chemopreventive agents like EGCG, Curcumin, Selenine, and Erk1/2 inhibitor U0126 can effectively control growth and induce apoptosis of esophageal cancer cell lines. These agents can also significantly mod-

ulate expression or activity of these genes. In conclusion, our data have shown that targeting of this gene pathway could translate into better control of esophageal cancer and these genes should be further evaluated as intermediate biomarkers for clinical study of these agents in esophageal cancer.

XP-001537130

#3191 A clinical pilot study of oral silibinin, a putative colorectal cancer preventive agent in patients with primary colorectal cancer and colorectal liver metastases. Carmen Si Moh, David P. Berry, Giuseppe Garcia, David Hemingway, Andrew S. Miller, Kevin West, Ashley R. Dennison, Rajinder Singh, David J. Bootock, Timothy H. Marczylo, Hong Cai, Will P. Steward, Andreas J. Geischer. *University of Leicester, Leicester, United Kingdom and University Hospitals of Leicester NHS Trust, Leicester, United Kingdom*

Silibinin, a flavonolignan from milk thistle, and the crude milk thistle extract silymarin, which contains mainly silibinin, possesses cancer chemopreventive efficacy in rodents including models of colorectal carcinogenesis. Silymarin preparations are used clinically as hepatoprotectants against alcohol- and drug-related damage. Silibinin is a strong antioxidant and modulates the insulin-like growth factor (IGF) system *in vivo* by increasing circulating levels of IGF binding protein 3 (IGFBP-3). We wished to test the hypothesis that consumption of silibinin furnishes pharmacologically active levels of agent in blood, liver and colorectum. Twelve patients with confirmed colorectal carcinoma and twelve with colorectal liver metastases received silibinin formulated with phosphatidylcholine ('silipide' Indena Spa, Milan) at total daily doses (divided into three equal portions) of 360, 720, or 1440 mg silibinin for seven days. Blood and normal and malignant colorectal or liver tissue were obtained before and after silibinin intervention. Levels of silibinin were quantified using a previously developed and validated HPLC-UV method. Plasma metabolites were identified by liquid chromatography-mass spectrometry (negative electrospray ionization mode, selected ion monitoring). Levels of IGFBP-3 and IGF-1 in serum and of the oxidative DNA damage pyrimidopurine adduct of deoxyguanosine (M₁dG) in leukocytes were determined as potential pharmacodynamic markers of silibinin efficacy. Repeated administration of silipide was well tolerated. Silibinin levels recovered from plasma between 1-4 h post final silipide dose were 0.3-4.0 μ M. Silibinin monoglucuronide (*m/z* 657), silibinin diglucuronide (*m/z* 833), silibinin monosulphate (*m/z* 561) and silibinin glucuronide sulphate (*m/z* 737) were identified as silibinin metabolites in the plasma. Silibinin concentrations in liver and colorectal tissues obtained by resection 3-6 h post last silipide dose were 0.3-2.5 and 20-141 nmol/g^{wt}, respectively. Intervention with silipide did not affect blood levels of IGF-1, IGFBP-3 or M₁dG. The results suggest that ingestion of silibinin at safe doses can achieve detectable levels of agent in plasma and liver, and that silibinin concentrations reached in the colorectal tract are of an order of magnitude which has been shown to elicit pharmacological effects in cells *in vitro*. The results support the further development of silibinin as a potential human colorectal cancer chemopreventive agent.

#3192 Silibinin inhibits ligand-induced EGFR and Akt phosphorylation, and causes cell cycle arrest and apoptosis in human colorectal carcinoma SW480 cells. Koral Raima, Suchitra Katiyar, Chitra Agarwal, Rajesh Agarwal. *University of Colorado at Denver and Health Science Center, Denver, CO*

Colorectal cancer (CRC) is the second most common invasive malignancy and cause of cancer-related deaths in the United States; however, limited success is achieved in its prevention and/or intervention. Silymarin (milk thistle extract) and silibinin (active agent in silymarin) are potent anti-cancer and cancer chemopreventive agents in several *in vivo* and *in vitro* pre-clinical cancer models. Regarding CRC, dietary feeding of silymarin prevents AOM-induced CRC in rats, and silibinin inhibits HT29 human CRC cell growth and causes cell cycle arrest and apoptotic death. Since epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are potent mitogens that regulate proliferation and survival of CRC cells via autocrine and paracrine loops involving both ERK1/2- and Akt-mediated signaling, here we assessed the effect of silibinin on these mitogens-stimulated signaling and associated biological responses in human CRC SW480 cells. Treatment of serum-starved SW480 cells with EGF or IGF1 resulted in a strong phosphorylation of ERK1/2 and Akt, and pre-treatment with silibinin at 100 μ M dose resulted in a strong inhibition of these mitogens-induced Akt phosphorylation; however, silibinin further increased ERK1/2 phosphorylation. These opposite effects of silibinin were further assessed in detail where treatment of starved cultures with silibinin alone was sufficient to cause ERK1/2 phosphorylation, which was inhibited by pre-treatment with NAC suggesting an oxidative effect of silibinin under these conditions. Silibinin also caused an early ERK1/2 phosphorylation in cells cultured under serum conditions, but showed an inhibitory effect on constitutive ERK1/2 phosphorylation following its prolonged treatment. Upstream of ERK1/2 regarding EGFR, silibinin strongly inhibited EGF-induced EGFR phosphorylation at Tyr845 and Tyr1068, but not at Tyr1173. As phosphorylation of EGFR at Tyr845 is in the



(19)
Bundesrepublik Deutschland
Deutsches Patent- und Markenamt

(10) DE 10 2004 054 133 A1 2005.06.09

(12)

Offenlegungsschrift

(21) Aktenzeichen: 10 2004 054 133.7

(22) Anmeldetag: 08.11.2004

(43) Offenlegungstag: 09.06.2005

(51) Int. Cl.⁷: A23L 1/30

(30) Unionspriorität:
PV 2003-3042 10.11.2003 CZ

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Erfinder wird später genannt werden

Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

(54) Bezeichnung: Nahrungsergänzungsmittel auf der Basis von Silymarin

(57) Zusammenfassung: Nahrungsergänzung auf der Basis von Silymarin, die zu einer Steigerung der Diätqualität mit positiver Wirksamkeit auf die Reduzierung des Risikos einer Zivilisationserkrankung beiträgt und deren Wesen darin besteht, dass diese als Wirkungskomponenten ein Gemisch von 10 bis 90 Gewichts-% der standardisierten Flavonolignanfraktion aus dem Extrakt der Mariendistel (*Cardui mariae extractum siccum*) und 90 bis 10 Gewichts-% des galenisch zubereiteten getrockneten Yaconkollens (*Smallanthus sonchifolius*) enthält. Dabei sind deren Wirkungskomponenten in einer vorteilhaften Ausführung ggf. mit einer bis zu 20-fachen Gewichtsmenge eines physiologisch unschädlichen Vehikels vermischt.

Beschreibung

Erfindungsbereich

[0001] Die Erfindung betrifft die Zusammensetzung eines Nahrungsergänzungsmittels auf der Basis von Silymarin, d.h. einer standardisierten Fraktion von Flavonolignanen aus dem Samenextrakt der Mariendistel (*Silybum marianum*), die zu einer Erhöhung der Diätqualität mit positiver Wirksamkeit auf die Verminderung des Risikos einer Zivilisationserkrankung, wie Diabetes mellitus und Hyperlipidämie, beiträgt und ggfs. den gesamten Gesundheitszustand des Organismus von Risikogruppen der Bevölkerung günstig beeinflusst.

Stand der Technik

[0002] Die „*Silybum marianum* (L.) Gaertner“ (Mariendistel, Asteraceae) findet in der europäischen Traditionsmedizin wegen ihrer hepatoprotektiven Wirkung seit Jahrhunderten Verwendung. Deren Wurzeln, Blätter und Samen wurden als Nahrung zubereitet (Morazzoni und Bombardelli, 1995). Die Bezeichnung Silymarin wird für den Extrakt aus dem Samen der „*S. marianum* (*Cardui mariae extractum siccum*)“ verwendet, der 70–80% des Silymarinkomplexes, d.h. eines Gemischs aus auf Silibinin standardisierten Flavonolignanen und 30–20% einer chemisch nicht definierten Fraktion, enthält.

[0003] Der „*S.-marianum*“-Extrakt und Silibinin schützen die Leber vor der Wirkung hepatotoxischer Stoffe (s. DE-A-19 23 082 und DE-A 35 37 656).

[0004] Der „*S.-marianum*“-Extrakt und Silibinin verhindern die von kupferhaltigen Ionen induzierte Oxidation der menschlichen LDL-Lipoproteine *in vitro* (s. Škottová et al., 1999).

[0005] Der Extrakt und die Flavonolignane der „*S. Marianum*“, insbesondere das Silibinin bishemisukcinat sind als unterstützende Therapie bei der Behandlung mit Zytostatika (s. Lentzen et al., 1998) gut wirksam.

[0006] In der Tschechischen Republik bildet der „*S.-marianum*“-Extrakt einen aktiven Bestandteil von Medikamenten, die als Hepatoprotektiva klassifiziert sind, z.B. FLAVOBION®, SILYGAL®, LAGOSA® oder LEGALON® (der Extraktgehalt in einer Tablette ist 100–204mg, d.h. 70–150mg Silymarinkomplex), das Phytopräparat SIMEPAR® enthält den „*S.-marianum*“-Extrakt in der Kombination mit dem Vitamin B-Komplex, das HEPABEN® mit dem „*Fumaria-officinalis*“-Extrakt und das HEPATOFALK® PLANTA mit dem „*Chelidonium-majus*“-Extrakt. Die Indikation dieser Präparate ist um die cholekinetische und choleretische Wirkung erweitert. Bei den Nahrungsergänzungsmitteln beträgt die Extraktmenge in einer Tablette 15–100mg, d.h. 10–70mg Silymarinkomplex.

[0007] Beschrieben ist ebenfalls ein Nahrungsergänzungsmittel mit Wirkung auf Plasmalipide und die Antioxidationskapazität des Bluts gemäß der CZ PV 2000-4404, die als Wirkungskomponenten ein Gemisch des Mariendistelextrakts und mindestens eines n-3- und n-6- polyungesättigte Fettsäuren enthaltendes Pflanzenöls enthält. Dieses Präparat hat eine positive Wirkung auf die Cholesterinparameter, die Lebersteatose und den Antioxidationszustand des Organismus, besitzt jedoch keine präbiotische Wirkung.

[0008] Im Zusammenhang mit der Suche nach neuen Naturquellen mit antidiabetischen, Nährwert-, Immunstimulierenden und anderen Eigenschaften wurde experimentell festgestellt, dass verarbeitete Knollen des Yacon (*Smallanthus sonchifolius*) eine präbiotische Wirkung aufweisen, was z.B. in den Artikeln von Romin Pedreschi et al.: „Andean Yacon Root (*Smallanthus sonchifolius* Poepp. Endl) Fructooligosacharides as a Potential Novel Source of Prebiotics“, Journal of Agricultural and Food Chemistry, 2003, oder Aleš Lebeda et al.: „Biologická a chemická variabilita maky a jakonu“, Chem. Listy 97, Seiten 548–556, 2003, beschrieben ist.

Aufgabenstellung

[0009] Das Bestreben der vorliegenden Erfindung ist daher, ein Nahrungsergänzungsmittel zu schaffen, das die bekannte Wirkung des Silymarin mit der neu entdeckten Wirkung des Yacon in sich vereint.

Zusammenfassung der Erfindung

[0010] Eine neue Wirksamkeit gegenüber dem bisherigen Stand weist das Nahrungsergänzungsmittel auf der Basis von Silymarin auf, das zu einer Erhöhung der Diätqualität mit positiver Wirksamkeit auf die Verminderung des Risikos einer Zivilisationserkrankung beiträgt, wobei die Erfindung darin besteht, dass das Nahrungs-

gänzungsmittel als Wirkungskomponenten ein Gemisch aus 10 bis 90 Gewichts-% der standardisierten Flavonolignanfraktion aus dem Extrakt der Mariendistel (*Cardui mariae extractum siccum*) und 90 bis 10 Gewichts-% der galenisch zubereiteten, getrockneten Yaconknolle (*Smallanthus sonchifolius*) enthält.

[0011] Ein weitere Ausbildung der Erfindung besteht darin, dass die Wirkungskomponenten des Nahrungsergänzungsmittels ggf. mit einer bis zu 20-fachen Gewichtsmenge eines physiologisch unschädlichen Vehikels vermischt sind, das in einer vorteilhaften Zusammensetzung 0,1 bis 80 Gewichts-% an inertem Füllstoff und 0,05 bis 10 Gewichts-% an Schmierstoffen enthält.

[0012] Gemäß einer weiteren Ausbildung der Erfindung ist der inerte Füllstoff aus einer Stoffgruppe ausgewählt, die mikrokristalline Zellulose und/oder Siliziumoxid und/oder Kartoffelstärke und/oder Maisstärke und/oder modifizierte Stärke enthält, und sind die Schmierstoffe aus einer Stoffgruppe ausgewählt, die Talkum und/oder hydratisiertes Siliziumoxid und/oder Stearinsäure und/oder Kalziumstearat enthält.

[0013] Die Wirksamkeit des Nahrungsergänzungsmittels der genannten Zusammensetzung äußert sich darin, dass sich die Kombination des Silymarins mit Yacon als Vorbeugung gegen chronische Zivilisationserkrankungen, die durch ein atherogenes Lipoproteinprofil, die Lebersteatose und einen verschlechterten Antioxidationszustand des Organismus gekennzeichnet sind, positiv auswirkt. Das Nahrungsergänzungsmittel zeichnet sich ferner durch eine nachweisbar präbiotische Wirksamkeit aus, da Yacon Zucker enthält, der die nichtpathogene Darmflora unterstützt, welche die Tätigkeit des Verdauungssystems günstig beeinflusst.

Ausführungsbeispiel

Ausführungsbeispiele der Erfindung

[0014] Die Möglichkeiten einer konkreten Anwendung der Erfindung sind aus den folgenden Beispielen ersichtlich:

Beispiel 1

[0015] Die Wirksamkeit der ausgewählten Zusammensetzung des Nahrungsergänzungsmittels, d.h. des Nutrazeutikums, wurde an einer Gruppe von 44 Freiwilligen, sog. Probanden, der metabolischen Beratungsstelle eines der Krankenhäuser der Olmützer Region getestet, wobei das Kriterium für deren Eingliederung in die Studie der Befund des metabolischen Syndroms, d.h. der nachweisbaren Hyperinsulinämie, Hypertonie, Hypertriglyzeridämie und Fettleibigkeit waren. Ihr Einverständnis zur Studiendurchführung gab die ethische Kommission des entsprechenden Krankenhauses. Die Probanden wurden in drei Gruppen eingeteilt:

- 1) PLACEBO: 15 Probanden, d.h. Patienten, dieser Gruppe wurde mikrokristalline Zellulose P als Placebo in einer Tagesdosis von 3,2g gereicht, wobei eine Kapsel 200mg Zellulose P enthielt.
- 2) SILYMARIN: 14 Probanden wurde Silymarin in einer Tagesdosis von 0,8g gereicht, wobei eine Kapsel 134mg Silymarin enthielt.
- 3) SILYMARIN-YACON: 15 Probanden dieser Gruppe wurde ein kombiniertes Präparat in einer Tagesdosis von 0,8g Silymarin und 2,4g Yacon gereicht, wobei 1 Kapsel 50mg Silymarin und 150mg der galenisch zubereiteten Yaconknolle enthielt.

[0016] Die klinische Studie wurde im Verlauf des Jahres 2002 durchgeführt, wobei die Präparate den Probanden während einer Dauer von 90 Tagen gereicht wurden. Anschließend wurden die Ergebnisse der Studie mittels des statistischen Verfahrens der Streuungsanalyse, der sog. ANOVA, eingeschätzt. Die Eingangsergebnisse zu Beginn des 1. Monats des Studienverlaufs und die Endergebnisse am Ende des 3. Monats des Studienverlaufs sowie deren Differenz wurden statistisch ausgewertet und mit den Werten der Placebos verglichen. Die Ergebnisse sind aus der Tabelle 1 ersichtlich.

[0017] Die resultierende Wirkung des nur Silymarin enthaltenden Nutrazeutikums äußerte sich in einer statistisch bedeutenden Verminderung des Spiegels der Triglyzeride. Bei der Kombination von Yacon und Silymarin konnte eine statistisch bedeutende Verminderung des Spiegels der Triglyzeride und der Glukose im Blut der Patienten festgestellt werden. Es konnten dabei keinerlei statistisch bedeutende, negative Einflüsse beider gereicher Nutrazeutika auf die Parameter des Sicherheitslabors festgestellt werden.

Tabelle 1

Der Einfluss des Silymarin (SIL) + Yacon (YAC) auf die Triglyzeride (TAG), das Cholesterol und die Glukose bei Probanden mit einem metabolischen Syndrom (Durchschnitt $\pm$ S.D. *p < 0,05).

Parameter	Gruppe	1. Tag	90. Tag	Nach 90 Tagen	Trend	Signif.
TAG	SIL	2,16 $\pm$ 1,10	1,88 $\pm$ 0,69	- 0,28 $\pm$ 0,93		0,029*
Mmol.l ⁻¹	Placebo	1,94 $\pm$ 0,89	2,78 $\pm$ 1,91	+ 0,84 $\pm$ 1,56		
TAG	SIL+JAK	2,24 $\pm$ 0,97	1,92 $\pm$ 0,78	- 0,32 $\pm$ 0,66	^	0,017*
Mmol.l ⁻¹	Placebo	1,94 $\pm$ 0,89	2,78 $\pm$ 1,91	+ 0,84 $\pm$ 1,56		
Cholesterol	SIL	6,49 $\pm$ 1,13	6,05 $\pm$ 1,17	- 0,44 $\pm$ 1,01	$\leftrightarrow$	0,927
Mmol.l ⁻¹	Placebo	6,56 $\pm$ 2,53	6,17 $\pm$ 1,21	- 0,39 $\pm$ 1,75		
Cholesterol	SIL+JAK	5,43 $\pm$ 1,11	5,55 $\pm$ 1,07	+ 0,13 $\pm$ 1,01	$\leftrightarrow$	0,327
Mmol.l ⁻¹	Placebo	6,56 $\pm$ 2,53	6,17 $\pm$ 1,21	- 0,39 $\pm$ 1,75		
Glucose	SIL+JAK	6,93 $\pm$ 1,56	6,50 $\pm$ 1,78	- 0,43 $\pm$ 0,83	^	0,041*
Mmol.l ⁻¹	SIL	6,79 $\pm$ 1,99	7,05 $\pm$ 2,63	+ 0,26 $\pm$ 0,90		

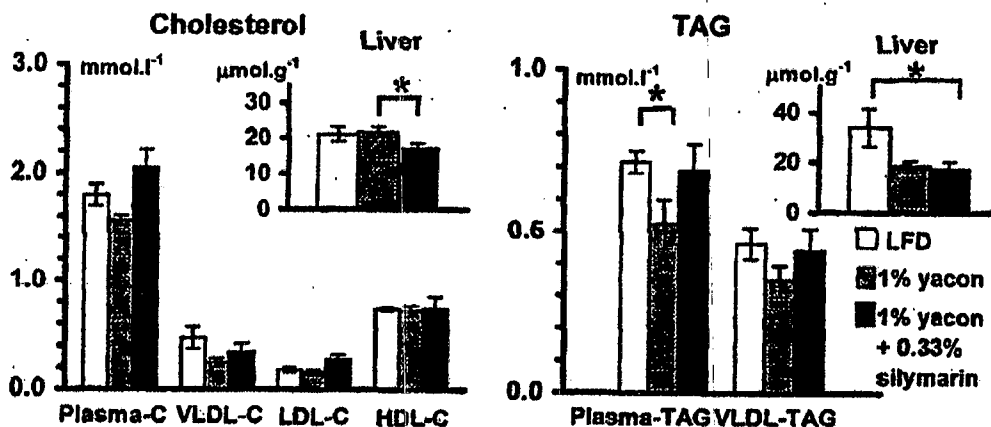
Beispiel 2

[0018] Die hypolipidemischen und Antioxidationseffekte des Yacon, des Silymarins und der Kombination dieser Drogen wurden an Ratten an zwei Experimentalmodellen der diätinduzierten Veränderungen im Lipidmetabolismus und dem Antioxidationszustand der Ratten studiert. Beide Diäten enthielten 1% Cholesterin und 10% qualitativ unterschiedliches Fett. Zum Hervorrufen der Lebersteatose mit Fibrose und proatherogenen Änderungen im Lipoproteinprofil wurde Schweinefett verwendet, das reich an gesättigten und monounsättigten Fettsäuren ist (lard fat diet = LFD), und zur Sensibilisierung des Oxidationszustands der Ratten wurde Johannisbeeröl verwendet, das reich an polyungesättigten Fettsäuren ist (black currant oil diet = BCOD). Yacon und Silymarin wurden 3 Wochen lang als Bestandteil dieser Diäten in einer Konzentration von 0,1, 0,5 und 1,0% gereicht. Bei der Drogenkombination wurden Diätkonzentrationen von 1% Yacon (mikronisierter Staub aus getrockneten Knollen) und 0,33% Silymarin gewählt.

[0019] Die Kombination von 1% Yacon und 0,33% Silymarin (Fig. 1) führte zur Verminderung des VLDL-Cholesterins und verhinderte das Ansteigen der Plasma- und der VLDL-Triglyzeride. In der Leber äußerte sich die Wirkung der Drogenkombination als Verminderung des Cholesteringehalts und TAG bei der Fettdiät (LFD). Die Oxidationsparameter wurden durch die Drogenkombination bei der Fettdiät nur unbedeutend beeinflusst, aber im Falle der Diät mit polyungesättigten Fettsäuren (BCOD) stieg nach der Drogenkombination der Spiegel des Blut-GSH an. Diese Ergebnisse deuteten die potentielle hypotriglyzeridemische und die VLDL-vermindernde Wirkung der Yaconknollen an. In der Kombination des Yacon mit Silymarin, das vor allem die Cholesterinparameter und das GSH positiv beeinflusst, wurde außerdem eine Verminderung der Lipide in der Leber und eine Erhöhung des GSH im Blut erzielt. Die Kombination von Yacon und Silymarin erweist sich deshalb als vielversprechend bei einer positiven Beeinflussung und der Vorbeugung von Zivilisationserkrankungen, die sich durch ein atherogenes Lipoproteinprofil, die Lebersteatose und eine Verschlechterung des Antioxidationszustands des Organismus auszeichnen.

Figur 1

Wirkung eines Gemisches von Yacon + Silymarin auf das Profil der Lipoproteide und der Leberlipide bei Ratten
(Durchschnitt $\pm$ S.E., n = 6, *p < 0,05).



Beispiel 3

[0020] Als optimale, den Anforderungen an die Stabilität und Homogenität nachkommende Medikamentenform wurde nach klinischer Erprobung eine 300mg-Gelatinekapsel mit folgender, beispielhafter Zusammensetzung gewählt:

• gemahlene getrocknete Yaconknollen	150mg
• mikronisierter Silymarinkomplex	70mg
• Siliziumstearan	8mg
• Maisstärke	37mg
• mikrokristalline Zellulose	37mg

Industrielle Verwendbarkeit

[0021] Das Nahrungsergänzungsmittel auf der Basis von Silymarin kann zur Vorbeugung von Zivilisationserkrankungen, wie z.B. Diabetes mellitus und Hyperlipidämie, ggfs. als präventives Nahrungsergänzungsmittel, das den Gesamtzustand des menschlichen Organismus günstig beeinflusst, verwendet werden.

Patentansprüche

1. Nahrungsergänzungsmittel auf der Basis von Silymarin, das zu einer Erhöhung der Diätqualität mit positiver Wirkung auf die Verminderung des Risikos einer Zivilisationserkrankung beiträgt, dadurch gekennzeichnet, dass das Nahrungsergänzungsmittel als Wirkungskomponenten ein Gemisch aus 10 bis 90 Gewichts-% der standardisierten Flavonolignanfraktion aus dem Extrakt der Mariendistel (*Cardui mariae extractum siccum*) und 90 bis 10 Gewichts-% der galenisch zubereiteten, getrockneten Yaconknolle (*Smallanthus sonchifolius*) enthält.
2. Nahrungsergänzungsmittel nach Anspruch 1, dadurch gekennzeichnet, dass seine Wirkungskomponenten ggfs. mit einer bis zu 20-fachen Gewichtsmenge eines physiologisch unschädlichen Vehikels vermischt sind.
3. Nahrungsergänzungsmittel nach Anspruch 2, dadurch gekennzeichnet, dass das physiologisch unschädliche Vehikel 0,1 bis 80 Gewichts-% an inertem Füllstoff und 0,05 bis 10 Gewichts-% an Schmierstoffen enthält.
4. Nahrungsergänzungsmittel nach Anspruch 3, dadurch gekennzeichnet, dass der inerte Füllstoff aus einer Stoffgruppe ausgewählt ist, die mikrokristalline Zellulose und/oder Siliziumoxid und/oder Kartoffelstärke und/oder Maisstärke und/oder modifizierte Stärke enthält.
5. Nahrungsergänzungsmittel nach Anspruch 3, dadurch gekennzeichnet, dass die Schmierstoffe aus ei-

ner Stoffgruppe ausgewählt sind, die Talkum und/oder hydratisiertes Siliziumoxid und/oder Stearinsäure und/oder Kalziumstearat enthält.

Es folgt kein Blatt Zeichnungen

Research article

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Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells

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Published: 17 October 2002

Received: 25 June 2002

BMC Cancer 2002, 2:27

Accepted: 17 October 2002

This article is available from: <http://www.biomedcentral.com/1471-2407/2/27>

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Abstract

Background: The natural plant polyphenol resveratrol present in some foods including grapes, wine, and peanuts, has been implicated in the inhibition, delay, and reversion of cellular events associated with heart diseases and tumorigenesis. Recent work has suggested that the cancer chemoprotective effect of the compound is primarily linked to its ability to induce cell division cycle arrest and apoptosis, the latter possibly through the activation of pro-apoptotic proteins such as Bax.

Methods: The expression, subcellular localization, and importance of Bax for resveratrol-provoked apoptosis were assessed in human HCT116 colon carcinoma cells and derivatives with both bax alleles inactivated.

Results: Low to moderate concentrations of resveratrol induced co-localization of cellular Bax protein with mitochondria, collapse of the mitochondrial membrane potential, activation of caspases 3 and 9, and finally, apoptosis. In the absence of Bax, membrane potential collapse was delayed, and apoptosis was reduced but not absent. Resveratrol inhibited the formation of colonies by both HCT116 and HCT116 bax^{-/-} cells.

Conclusion: Resveratrol at physiological doses can induce a Bax-mediated and a Bax-independent mitochondrial apoptosis. Both can limit the ability of the cells to form colonies.

Background

Cancers claim at least six million lives *per annum* world-wide, and the common and frequently therapy-resistant colon cancers are among the most notorious. Accordingly, natural food constituents capable of inhibiting, delaying or reversing events associated with tumor initiation, promotion, and progression have attracted much attention. The pharmacologically active *trans* form of the polyphenolic,

antifungal phytoalexin resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is produced by more than 70 plant species and is reaching particularly high levels in grapes, peanuts and wine [1]. A large number of publications in recent years has shown that physiological quantities of resveratrol can modulate multiple cellular pathways relevant for tumorigenesis, among them phase II drug metabolizing, cyclooxygenase, nitric oxide, DNA-synthesis, and

inflammatory response, as well as cell survival, cell death and cell cycle pathways [2]. The consensus of the many *in vitro*- and a limited number of *in vivo*-studies seems to be that micromolar quantities of the compound can not only counteract tumor initiation through anti-oxidant activities and the inhibition of enzymatic carcinogen conversion [2-4], or counteract tumor promotion through cyclooxygenase and hydroperoxidase inhibition [5,6], but can affect tumor cell proliferation and survival through the inhibition of cell cycle, stimulation of differentiation, and induction of apoptosis [2-4]. The latter effects may be of particular relevance for the anti-tumorigenic activity of resveratrol [2], not least because the cell cycle/cell death machinery is specifically altered in many tumor cells and the targeting of this machinery by resveratrol opens up the possibility to specifically affect tumor cells and spare normal proliferating tissue. The compound indeed seems to possess some specificity for tumor cells [7].

At the level of individual proliferating cells, resveratrol appears to act primarily through the inhibition of the cell cycle [8-14]. Although apoptosis may be provoked in normal, non-transformed proliferating cells exposed to high resveratrol concentrations [11], a temporary, reversible cell cycle arrest in G1 and G2 phase has been reported to result at low to moderate concentrations from activation of the p53 tumor suppressor and, subsequently, from the activation of the p21Cip1 inhibitor of cyclin-dependent kinases [14,15]. In tumor cells however, p53 function is frequently lost, and consequently, cells usually arrest in S-phase instead of G1 or G2, probably due to the inhibition of DNA synthesis by resveratrol [16,17]. Although this cell cycle arrest is also reversible, cells with altered cell cycle checkpoints can be more prone to apoptotic death [14,15,18].

The precise mechanisms through which resveratrol induces apoptosis are unknown. Together, the available data point to the existence of several apoptotic pathways that can be triggered by the compound, and to the existence of survival pathways that can be inhibited by it. For example, resveratrol has been documented to be able to induce apoptosis through the activation of p53 [19], but also through other mechanisms in wild-type p53-deficient tumor cells [8]. Furthermore, the compound has been shown to inhibit the activation of NFkB transcription factor which serves as a survival factor in some cell types [20,21].

Apoptosis following resveratrol treatment has been reported to be mediated, in dependence on cell type, through the death receptor [22] and mitochondrial pathways [14,23]. Recent findings make the involvement of the death receptor pathway questionable though [24,25]. For instance, dominant-negative inhibition of this path-

way failed to block apoptosis induction by a resveratrol analog [24]. The pro-apoptotic Bax protein is one of the major players in the mitochondrion form of apoptosis [26], and mitochondria-mediated cell death induced by resveratrol may involve stimulation of the expression of the *bax* gene by p53 or other transcription factors [10,14,27,28]. However, mitochondria-mediated cell death may also involve down-modulation of Bax-antagonists such as Bcl-X_L or Bcl-2 [29], or the translocation of Bax from the cytosol to mitochondria ([30] and discussion therein). Since Bax was activated moderately or weakly under resveratrol in some cell types [10,14,27], or was activated only at high drug concentrations although apoptosis was observed at low concentrations as well [14], the present work was designed to address the role of Bax in colon tumor cell apoptosis more directly by studying the effect of the drug on the human HCT116 colon carcinoma cell line and a derivative in which both *bax* alleles were disrupted by spontaneous frameshift mutation and targeted homologous recombination [31].

Methods

Reagents and cell culture

Resveratrol was purchased from Alexis (San Diego, CA). JC-1 and MitoTracker Red were obtained from Molecular Probes (Eugene, OR). ADR, 5-FU, and PI were provided by Sigma (St. Louis, MO). Rabbit polyclonal antibodies Bax N-20, Bcl-XL S-18, Bcl-2 N-19, and cytochrome b were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal caspase 3 antibody and mouse monoclonal caspase 8 and Bax 6A7 antibodies were purchased from Transduction Laboratories/Pharmingen (San Diego, CA). The rabbit polyclonal caspase 9 antibody detecting a 36 kDa cleavage product of pro-caspase 9 was from BioVision (Mountain View, CA). Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies, the β -actin and FITC-labeled anti-mouse monoclonal antibodies, and a liquid alkaline phosphatase detection kit were from Sigma. Stock solutions of resveratrol and MitoTracker Red were prepared in DMSO; JC-1 was dissolved in methanol; ADR, 5-FU, and PI stocks were prepared in water. The HCT116 cells and derivatives were cultured as monolayers at 37°C in a humidified 7% CO₂ atmosphere in McCoy's 5A medium supplemented with 10% FCS. HT29 cells were maintained in DMEM plus 10% FCS.

Immunoblotting and subcellular fractionation

Cells were seeded in 10 cm dishes to approx. 50% confluence at 24 h before resveratrol treatment. Protein extracts were prepared by lysing the cultures in 150 μ l of lysis buffer heated to 90°C and containing 50 mM Tris-HCl (pH 6.8); 100 mM DTT, 2% SDS, and 20% glycerol. Samples containing 15 or 30 μ g of total cellular protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA).

Membranes were then incubated overnight with antibodies directed against β -actin (1:5,000), Bax or cytochrome b (1:500), and Bcl-XL, Bcl-2, or one of the caspases (1:200, respectively). For signal detection, the secondary anti-mouse antibody was used at a dilution of 1:5,000, and the secondary anti-rabbit antibody at 1:1,000. For the preparation of subcellular fractions, at least 10^7 cells were scraped off the dishes, washed with PBS, suspended in 0.5 ml fractionation buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10 μ g/ml each of leupeptin, aprotinin, and pepstatin A; supplemented with 250 mM sucrose), and homogenized by 10 strokes with a Dounce homogenizer. Nuclei and residual unlysed cells were pelleted at 750 g for 5 min (4°C). The high membrane (HM) fraction containing the mitochondria was harvested by centrifugation at 10,000 g for 15 min (4°C), and the supernatant was collected as the cytoplasmic fraction. The quality of all fractions was routinely tested by Western blotting with antibodies specific for nuclear proteins (anti-PCNA, p53, Rb), cytoplasmic proteins (anti-eIF4A, γ -tubulin), and mitochondrial proteins (anti-cytochrome oxidase, cytochrome b, c).

Immunofluorescence and membrane potential changes

To stain mitochondria (MT), cells were incubated with 250 nM Mitotracker Red in pre-warmed full medium for 30 min at 37°C and washed twice with full medium. For immunofluorescence, cells were washed in pre-warmed full medium and fixed for 15 min at 37°C in full medium containing 3.7% formaldehyde. The cells were then washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After repeated washes with PBS, cells were incubated with the primary anti-Bax antibodies (1:100) or appropriate controls for 1 h, washed twice with PBS, exposed to the secondary FITC-labeled anti-rabbit antibody (1:50), and finally examined with a Leitz fluorescence microscope. For membrane potential ($\Delta\psi$ m) analysis, 2×10^5 cells were trypsinized, washed in pre-warmed medium, resuspended in McCoy's 5A medium supplemented with 0.2% FCS and JC-1 at 10 μ g/ml, and incubated for 30 min at 37°C. The MT membrane potential is reflected by the quotient of red vs. green fluorescence measured by FACScanning; observation wavelengths were 530 nm for the green fluorescence and 585 nm for the red fluorescence.

FACS analysis and colony formation assay

Cells were seeded in 6-well dishes to approx. 20 % confluency 24 h prior to resveratrol treatment. Asynchronously growing cultures were harvested by trypsinization, combined with the cells floating in the medium, washed with PBS, resuspended in 100 μ l of 0.9% NaCl, squeezed through a 23.5 gauge needle into 0.9 ml of methanol and fixed for 30 min at -20°C. Cells were then repeatedly

washed with PBS, resuspended in PBS supplemented with RNase A (25 μ g/ml) at approx. 10^6 cells/ml, and stained with PI (25 μ g/ml) for at least 1.5 h at 4°C in the dark. DNA fluorescence was measured with a Becton Dickinson FACScan (Bedford, MA); the Cell Quest software (Becton) was employed for data acquisition and analysis. Cells with a less-than-2n DNA content were apoptotic. For colony formation assays, initially $1.5 \times$, $3 \times$, and 4.5×10^3 live cells, counted with a CASY cell counter, were seeded in 25 cm^2 tissue culture flasks and incubated for approx. 10 days until colonies formed. Since untreated bax -/- cultures were able to produce approx. 5 $\times$ more colonies than the parental HCT116 cells, the numbers of seeded live cells were adjusted to produce equal colony numbers for the drug treatment experiments. Colonies were fixed with 0.25 % glutaraldehyde and stained with crystal violet.

Results

Human colorectal adenocarcinoma HCT116 is a poorly differentiated, growth factor-insensitive cell line deficient for hMLH1, a homologue of the bacterial Mut L protein and crucial component of DNA mismatch repair. The present study has made use primarily of HCT116 and the isogenic cell lines HCT116 p53-/- and HCT116 bax-/- derived from the parental line by targeted disruption of the p53 alleles or - in the case of the bax alleles - by a combination of spontaneous frameshift mutation and targeted disruption [31,32]. HCT116 cells are responsive to a variety of stresses including DNA damage and spindle disruption, in part owing to the integrity of the wild-type p53 tumor suppressor pathway. For this reason, and since deregulated colon epithelial cells are the supposedly primary targets of natural cancer chemopreventive food constituents, this cell line has been frequently employed for the study of the molecular mechanisms of drug action. We have recently reported that the polyphenol resveratrol present in some foods can increase the steady-state levels of pro-apoptotic Bax protein and induce a mitochondria-mediated apoptosis in HCT116 cells independently of p53 [14]. However, although Bax overproduction in response to 100 μ M resveratrol was weak, and at lower drug doses (< 40 μ M), undetectable (Figure 1A), doses of 10 to 20 μ M were able to provoke significant apoptotic cell death (typically 9 to 25 % within 48 h vs. 5 % in mock-treated cultures, Figure 1B). We therefore asked what role, if any, Bax might have in the resveratrol-induced apoptosis of HCT116 cells.

Previous work has documented that subtle shifts in the expression levels of Bax-interacting anti-apoptotic members of the Bcl-2 family of proteins can influence cell survival even when Bax levels are unchanged [29]. We failed to detect changes of the steady-state levels of anti-apoptotic Bcl-XL in response to resveratrol (Figure 1A), and Bcl-2 was not detectably produced in HCT116 cells, indicating

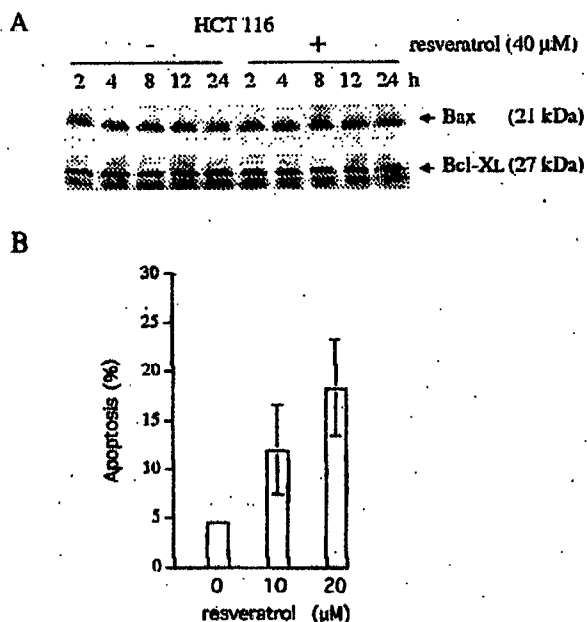


Figure 1

Expression of Bax and Bcl-X_L and apoptosis in human HCT116 colon carcinoma cells treated with low doses of resveratrol. Panel A: Total protein extracts (15 μg) of HCT116 cultures treated with 40 μM resveratrol or only DMSO (the solvent) were prepared at different times and analyzed by immunoblotting, employing the anti-Bax N20 (1:500) and anti-Bcl-X_L antibodies (1:200). Note that at this low drug concentration, neither the level of Bax nor of Bcl-X_L changed significantly. Panel B: FACS analyses on exponentially growing HCT116 cultures either mock-treated or treated with low doses of resveratrol for 48 h exhibited significant apoptosis in the drug-treated cultures. Error bars depict standard deviations of the means of five experiments.

that the apoptotic sensitivity to resveratrol is not regulated via alterations in the Bax/Bcl-XL ratio. Recent work has furthermore shown that Bax levels do not always increase following apoptotic stimuli but that, instead, the occluded N-terminus of Bax may become exposed and the protein may then co-localize with the mitochondria ([30] and discussion therein). When mock-treated formaldehyde-fixed HCT116 cells, p53-deficient HCT116 p53^{-/-} cells, or HCT116 bax^{-/-} cells were stained with anti-Bax antibody 6A7 (Pharmingen/Transduction Labs) or an antibody raised against the conformation-sensitive N-terminus of Bax (N20, amino acids 11 to 30, Santa Cruz), both antibodies recognized a protein in the bax-positive

HCT116 cells and produced a diffuse immunofluorescence signal (see Figure 2, mock-treated cultures), whereas no fluorescence was produced in the bax^{-/-} or isotype controls (not shown), indicating that staining was specific and that the N-terminal epitope of at least a fraction of the Bax molecules was exposed in untreated HCT116 cells after standard fixation.

Treatment of the cells with 100 μM resveratrol for 24 h resulted in the redistribution of Bax, regardless of p53-status, producing a distinctive, punctuate staining pattern in the cytosol with perinuclear concentration, resembling the pattern produced by the mitochondria-specific Mitotracker Red dye (Figure 2A). Confocal imaging confirmed that a fraction of the Bax immunofluorescence merged with the mitochondrial staining; however, a proportion of Bax appeared not to co-localize with the mitochondria (Figure 2B). Furthermore, when cultures were treated with resveratrol and were separated into cell fractions after 24 h, Bax protein accumulated in the high membrane fractions enriched for mitochondria, again regardless of p53-status (Figure 2C). Finally, when we studied the response to 100 μM resveratrol of human HT29 colon carcinoma cells expressing the dysfunctional p53-273H mutant, HT29 like HCT116 cells showed co-localization of Bax with mitochondria and underwent apoptotic cell death upon drug treatment (data not shown), demonstrating that this effect is not limited to one cell line and is indeed wild-type p53 independent. Together, these results demonstrate that active cellular Bax in colon cancer cells co-localizes with the mitochondria in response to resveratrol exposure, independently of the p53 tumor suppressor.

Active Bax located at the mitochondria can mediate apoptosis [26], and we have recently reported that resveratrol can indeed trigger the mitochondrial form of death in HCT116 cells [14]. Mitochondria-mediated apoptosis is often, though not always, associated with the collapse of the mitochondrial transmembrane potential $\Delta\Psi_m$ as the result of leakiness of the inner mitochondrial membrane [26]. To study whether the translocation of Bax correlates with $\Delta\Psi_m$ changes, exponentially growing cultures of bax-positive and bax-negative cells were treated with 100 μM resveratrol or mock-treated for various times and were then incubated with the J aggregate-forming lipophilic cation JC-1, which as a monomer emits green fluorescence and in a reaction driven by $\Delta\Psi_m$ assembles into a red fluorescence-emitting dimer. In accord with the sensitivity of HCT116 cells to resveratrol-induced apoptosis, and paralleling the co-localization of Bax to the mitochondria, $\Delta\Psi_m$ rapidly dissipated, as indicated by the incremental increase in JC-1 green fluorescence and the lack of a corresponding increase in red fluorescence (Figure 3A). Remarkably, although stable for the first eight hours of drug

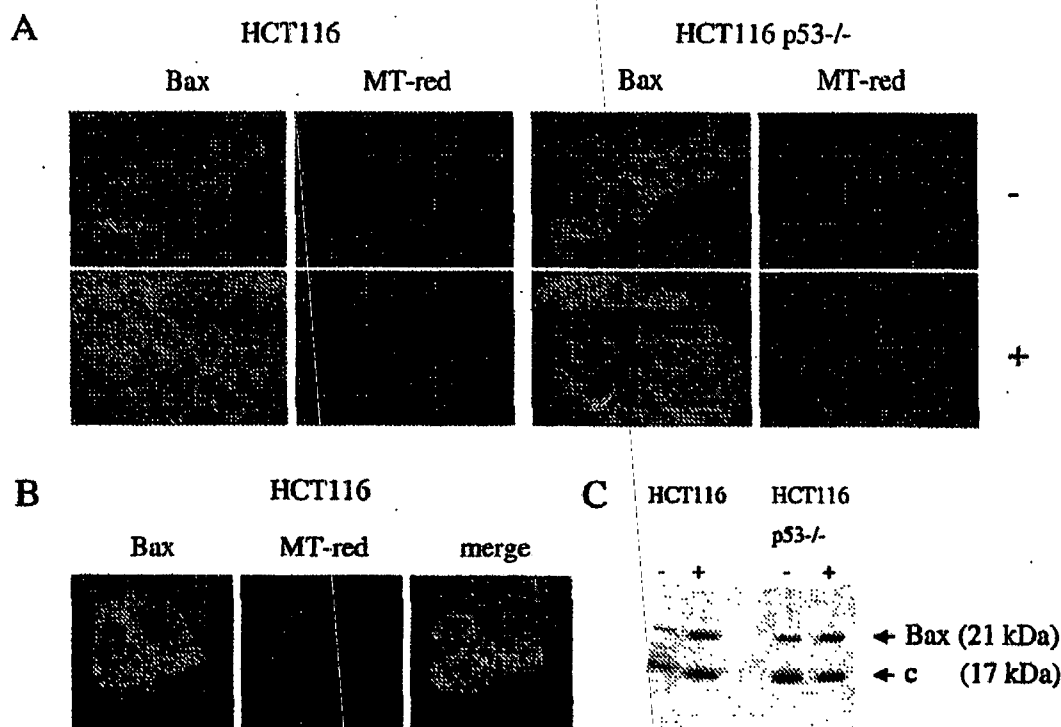


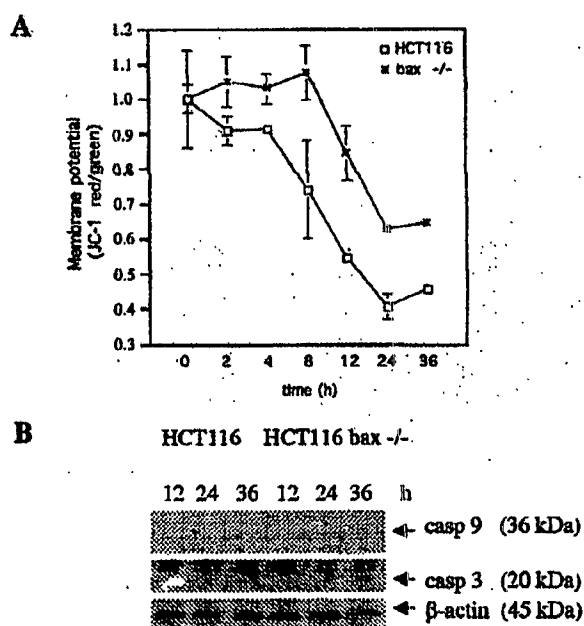
Figure 2

Subcellular localization of Bax in drug-treated HCT116 cells and the p53^{-/-} derivatives. Panel A: HCT116 and HCT116 p53^{-/-} cultures were treated with 100 μ M resveratrol (+) or mock-treated (-) and, after 24 h, stained with the mitochondria-specific dye Mitotracker Red (MT-red) and anti-Bax N-20 antibody (1:100). Note the punctuate, cytoplasmic stain with perinuclear concentration produced by the anti-Bax antibody in cells exposed to the drug, resembling the pattern produced by MT-red. Panel B: Confocal microscopy of HCT116 cells treated with resveratrol for 24 h confirmed the co-localization of part of the Bax-produced fluorescence with the MT-red fluorescence. Panel C: Immunoblot analysis on 15 μ g of total protein from the high membrane (mitochondrial) fraction of cells mock-treated (-) or drug-treated (+) for 24 h. Staining with Bax and cytochrome b (control) antibody (1:500) revealed the increase in Bax levels in the mitochondria fraction of resveratrol-treated cells. The co-localization of Bax with the mitochondria was not dependent on the presence of p53.

treatment, the membrane potential of the bax^{-/-} cells eventually also collapsed, indicating that a Bax-independent, mitochondria-mediated death with delayed kinetics can be triggered in HCT116 cells by resveratrol. To study this further, Western blot analyses with antibodies directed against caspases 3 and 9 were performed. Mitochondria-mediated apoptosis involves the cleavage of pro-caspase 9 into the 36 kDa active caspase 9, which then cleaves and thereby activates pro-caspase 3. Resveratrol treatment (100 μ M) resulted in the increase of active caspase 9 levels, and concomitantly, in the cleavage of pro-caspase 3, regardless of Bax-status (Figure 3B), supporting the suggestion that resveratrol can provoke both Bax-de-

pendent and -independent, mitochondria- and caspase-mediated forms of apoptosis in HCT116 cells. As reported before [14], we failed to establish a role of caspase 8 in the resveratrol-provoked cell death, as neither caspase 8 activation in Western blot analyses nor inhibitory effects of the caspase 8 inhibitor IETH-CHO could be observed.

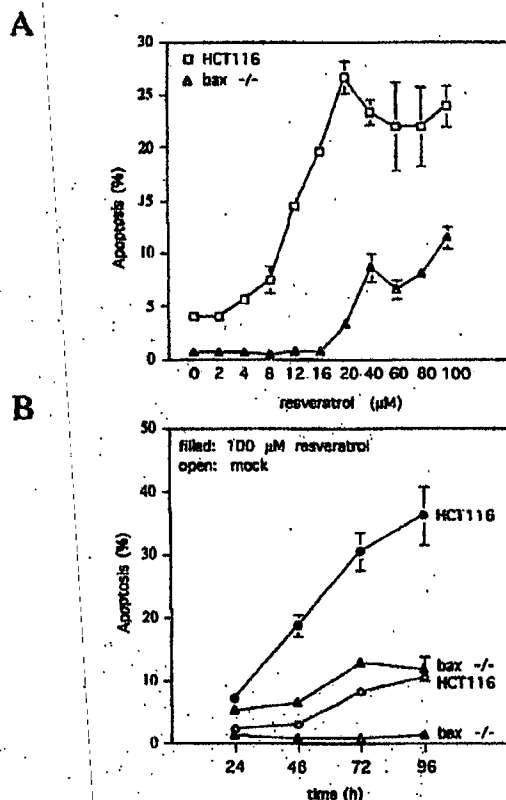
We next asked how the survival of HCT116 and HCT116 bax^{-/-} cultures is affected by resveratrol. When cells were incubated for 48 h in the presence of increasing concentrations of resveratrol and subsequently FACS-scanned for cell size and DNA content, a dose-dependent induction of apoptosis was observed in both cultures (Figure 4A). In

**Figure 3**

Mitochondrial membrane potential changes and caspase activation. Panel A: Quotient of JC-1 red/green fluorescence as an indicator for the membrane potential $\Delta\Psi_m$ in HCT116 cells and the *bax*^{-/-} derivatives at different times after exposure to 100 μ M resveratrol. Note that the absence of Bax delays but fails to completely inhibit membrane potential collapse. Standard deviations were derived from 4 experiments. Panel B: Detection of cleaved (active) caspases (casp) 9 and 3 in total protein extracts from parental and *bax*-deficient cells exposed to resveratrol. The anti-caspase antibodies were used at a dilution of 1:200. Staining with anti- β -actin is to show equal protein loading.

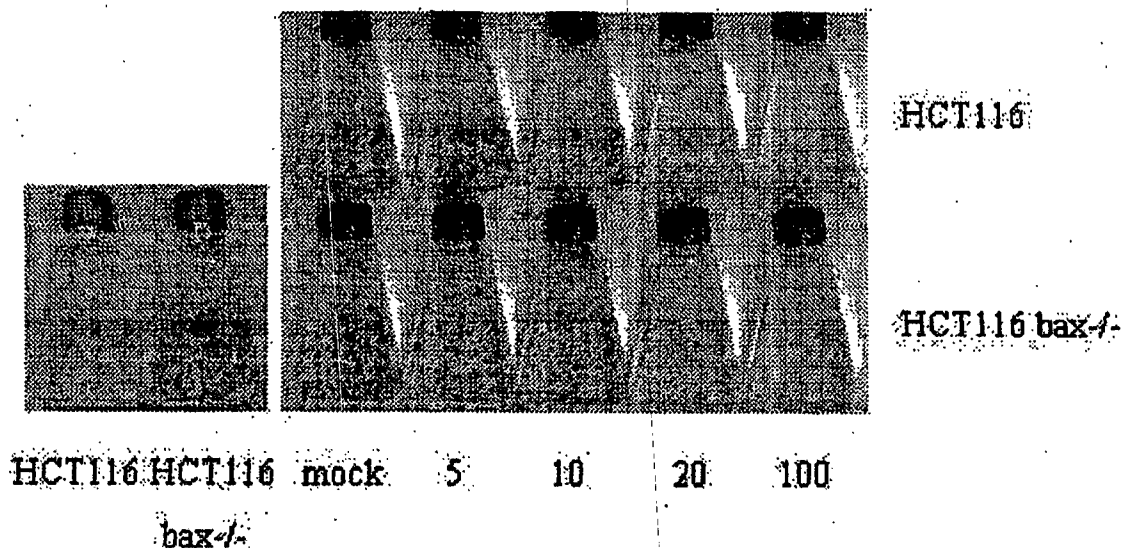
HCT116 cultures, the rate of apoptosis increased to approximately 25 % at 20 μ M resveratrol and plateaued thereafter. Although the *bax*-deficient cultures showed a similar dose-dependence of apoptosis, the rate of cell death was significantly lower. A time course over 96 h confirmed that resveratrol at 100 μ M can provoke apoptosis in both cell lines, but much less efficiently in the *bax*^{-/-} cells (Figure 4B), documenting that resveratrol-induced apoptosis in HCT116 cells is in part, but not entirely, dependent on Bax.

To assess whether the difference in FACS analysis in the apoptosis sensitivity between *bax*^{+/+} and *bax*^{-/-} cells is meaningful, standard colony formation assays were per-

**Figure 4**

Effect of resveratrol on the survival of the parental HCT116 cells and the *bax*^{-/-} derivatives in culture. Panel A: Treatment of exponentially growing cultures with different doses of resveratrol for 48 h and quantitation of the numbers of cells with a sub-2n-DNA content by flow-cytometry revealed a dose-dependent increase of apoptosis in both cell lines and a much stronger apoptotic response in the cells expressing Bax, especially at low drug doses. Panel B: Time course of apoptosis in exponentially growing cultures either mock-treated or treated with 100 μ M resveratrol, documenting the time-dependence of cell death induction. Note the reduced basal level of apoptosis in the *bax*^{-/-} cultures. Error bars denote standard deviations of the means of four experiments.

formed. In a first set of tests, it was revealed that a short initial exposure of the cultures to 100 μ M resveratrol for up to 6 h and removal of the drug thereafter, had no deleterious effect on colony formation (data not shown). This contrasted with the effects of damaging drugs such as adriamycin (0.34 μ M) or 5-fluorouracil (375 μ M), which inhibited colony formation under these conditions, and suggested that resveratrol acts primarily through the reversible inhibition of cellular factors. In the further study,

**Figure 5**

Representative colony formation assay with HCT116 and HCT116 *bax*^{-/-} cells. When 1.5×10^3 live cells, counted in a CASY cell counter, were seeded into 25 cm² tissue culture flasks and were incubated for 10 days in the absence of drug, *bax*^{-/-} cells produced more colonies (left figure), indicating the survival advantage *per se* of *bax*-deficiency. Right figure: 7.5×10^3 live HCT116 cells and 1.5×10^3 *bax*^{-/-} cells were seeded into flasks and exposed to the solvent DMSO (mock) or resveratrol dissolved in DMSO, 24 h later. Colonies were stained with crystal violet.

cultures were therefore exposed to resveratrol for the duration of the assay. When equal numbers of live *bax*^{+/+} and *bax*^{-/-} cells were seeded and mock-treated, *bax*^{-/-} cells produced more colonies (Figure 5, left figure), consistent with the much lower basal level of cell death in these cultures (see Figure 4). To be able to compare the effect of resveratrol on colony formation in *bax*^{+/+} and *-/-* cultures, we therefore seeded numbers of live cells that gave rise to similar colony numbers and incubated all cultures for ten days in the presence of different concentrations of resveratrol. The drug decreased the numbers of colonies, and at the highest resveratrol concentration (100 µM), no colonies formed, regardless of Bax-status (Figure 5). At lower drug concentration (20 µM) however, more colonies formed consistently in the *bax*^{-/-} cultures when compared with the *bax*^{+/+} cultures (62 ± 15 vs. 24 ± 11; average ± SD from 10 flasks each), and the colonies were generally larger. We interpret this to reflect the lack of inhibition of colony formation by the Bax-dependent form of apoptosis in the *bax*^{-/-} cultures. In summary, our data thus show that resveratrol at concentrations present in some foods can induce in HCT116 cells i) Bax-dependent, mitochondria-mediated apoptosis, regardless of p53-status and involving Bax co-localization with mitochondria, and ii) Bax-independent, mitochondria-mediated death. Both forms of

apoptosis may independently limit the ability of the cells to produce colonies.

Discussion

Resveratrol has been documented to cause apoptosis in a large number of tumor cell lines *in vitro* [8,14,19,22,33], in a rat model of azoxymethane induced colon carcinogenesis [10], and in a mouse model of familial adenomatous polyposis [34]. The effects of the drug on cell survival are mediated through the activation of downstream effectors such as caspase 3 via two major initiator pathways, the death receptor and the mitochondrial pathway. Although the Fas death receptor pathway has been implicated in some forms of resveratrol-induced apoptosis [22,35], many solid tumors seem to be affected exclusively through the mitochondrial pathway [14,23], which intimately involves the Bcl-2 protein family members of cell survival regulators including the anti-apoptotic Bcl-2 and Bcl-X_L and the pro-apoptotic BH-123 protein Bax [26]. Recent work has shown that Bax can be an important mediator of anticancer drug-induced cell death. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) cause Bax-dependent apoptosis in human HCT116 colon carcinoma cells [31]. In this system, the NSAID-provoked cell death was associated with a downregulation of Bax-antag-

onist Bcl-X_L. In other model systems, Bax was overproduced in response to drugs, including resveratrol [10,14]. We have shown in the present paper that a further Bax-dependent mechanism of resveratrol-induced apoptosis may be active - aside from alterations of the Bax:Bcl-X_L or Bax:Bcl-2 ratios by Bax overproduction or antagonist downregulation: the subcellular redistribution of present Bax protein. Since Bax expression was stimulated only at high resveratrol concentrations while Bax translocation and mitochondria-mediated cell death were observed also at low concentrations, we asked what relevance Bax might have for the chemopreventive activity of the compound.

Our results show that the lack of Bax in HCT116 colon carcinoma cells attenuates but fails to completely inhibit the resveratrol-induced mitochondrial form of apoptosis. This finding is in accord with a recent report showing that stable overexpression of Bax-antagonist Bcl-2 in U937 cells reduces, but does not prevent, resveratrol-provoked death [36]. Along the same line, HCT116 bax^{-/-} cells exhibited a reduced sensitivity to apoptosis induction by the anticancer drug 5-FU, but were not completely resistant like they were in response to the NSAID sulindac [31]. One function of Bax and the related BH-123 protein Bak is the induction of mitochondrial outer membrane permeability, which eventually results in the activation of apoptosis-executing caspases. Cells from mice deficient for both Bax and Bak, but not cells deficient for one or the other only, are almost completely resistant to mitochondria-mediated apoptosis [37], suggesting that these proteins have redundant functions in this pathway. Bak is robustly expressed in HCT116 cells, and recent work has shown that bax-deficient HCT116 cells are more sensitive to apoptosis-provoking drugs such as ceramide than other bax-deficient cells [38]. Whether Bak complements the lack of Bax in HCT116 bax^{-/-} cells treated with resveratrol remains to be seen; however, recent observations with isolated rat mitochondria indicate that the drug is also able to contribute directly to the opening of the mitochondrial permeability transition pore complex and thereby induce the collapse of $\Delta\Psi_m$ without involving the Bax/Bak proteins [23]. Combined, the available data thus indicate that the chemopreventive agent resveratrol can affect the survival of tumor cells through several different pathways.

Most colorectal tumor cell lines apparently show decreases in the ratio of the pro-apoptotic Bax and the anti-apoptotic Bcl-X_L, and recent observations suggest that mutational inactivation of the bax gene may be frequent in the HNPCC-predisposition to colorectal cancer and may render these cancers resistant to important cancer chemopreventives such as the NSAIDs [31]. The natural plant polyphenol resveratrol, in contrast, can trigger several different mitochondrial apoptosis pathways, and has actually been demonstrated to prevent the formation of

colon tumors and reduce the formation of small intestinal tumors by 70% in a mouse model of familial adenomatous polyposis [34]. One might therefore consider resveratrol or derivatives of it, possibly in combination with other drugs, as a chemopreventive approach to the management of patients with hereditary predispositions to colorectal cancer.

Conclusions

Low to moderate doses of the natural cancer chemopreventive ingredient of some foods, resveratrol, induce mitochondrial apoptosis but fail to stimulate Bax expression or alter the Bax:Bcl-X_L ratio in human HCT116 colon carcinoma cells. Instead, the compound induces co-localization of active Bax protein with the mitochondria. HCT116 cells with both bax alleles inactivated undergo mitochondrial apoptosis in response to resveratrol less efficiently than the parental cells, yet cell death through the mitochondrial pathway is not completely inhibited. Thus, resveratrol can activate Bax-dependent and -independent forms of mitochondrial apoptosis. Both forms can independently limit the formation of colonies.

Abbreviations

ADR: Adriamycin, a potent DNA-damaging anticancer drug; 5-FU: 5-fluorouracil, an anticancer drug affecting DNA and RNA metabolism; MT: mitochondria; $\Delta\Psi_m$: mitochondrial transmembrane potential, can break down upon transient or permanent permeabilization of inner MT membrane; HNPCC: hereditary non-polyposis colorectal cancer, the commonest form of hereditary predisposition to colorectal cancer.

Competing interests

None declared.

Authors' contributions

MM-R conducted the protein expression, subcellular localization and apoptosis studies. HK participated in the design and coordination of the study. KR performed the colony formation assays and drafted the manuscript.

Acknowledgements

We thank Bert Vogelstein for the HCT116 cell lines. This research was supported by a grant from the German Research Foundation (DFG) to K.R.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2407/2/27/prepub>

PREVENTION RESEARCH 6

ceptor subtypes (EP1-4) that are coupled to distinct signaling pathways. EP1 is coupled to Ca^{2+} mobilization, whereas both EP2 and EP4 stimulate adenylate cyclase. In contrast, EP3 commonly inhibits adenylate cyclase on most cells, although activation of this pathway through EP3-signaling has been described. We examined COX-2 and EP1-4 mRNA expression in human breast tumors and found that all four receptor subtypes are expressed in breast cancer. Expression levels of COX-2 were positively correlated with expression of each EP subtype in individual tumors suggestive of autocrine or paracrine signaling potential. Examination of murine breast tumor cell lines differing in tumorigenic and metastatic capacities revealed complex patterns of EP expression at the protein level. In general, murine mammary cancer cells expressed either EP4 or high levels of EP2 receptor subtypes, but not both receptors simultaneously. In contrast, all cell lines expressed comparable levels of EP1 and EP3 receptor protein. Receptor activation with specific EP agonists led to increases in intracellular cAMP and enhanced migration. Selective EP receptor antagonists inhibited both cellular responses. Studies in progress will define the role of each receptor subtype in tumor cell behavior.

#3177 Selective inhibitor of cyclooxygenase-2 induces cell-cycle G1 arrest and apoptosis through elevating cyclin-dependent kinase inhibitor (CDKI) p21 in the human oral carcinoma cancer cell lines. Young Eun Kwark, Eunja Lee, Jong I. N. Yook, Jin Kim. *Department of Oral Pathology, Oral Cancer Research Institute, BK21-Project for Medical Science, Yonsei University College of Dentistry, Seoul, Republic of Korea.*

Chemoprevention is a promising strategy to inhibit carcinoma before invasive tumors develop, but a new molecular target is desirable. Cyclooxygenase-2 (COX-2) expression is a critical part of inflammation and plays a major role in defending against exogenous stimuli, while its over expression causes cells to exhibit tumor phenotypic change. Several reports have shown that NSAIDs inhibit the growth of head and neck carcinoma cell lines. In this study, we investigated the growth inhibitory mechanism of a selective COX-2 inhibitor in human oral carcinoma cancer (OCC) cell lines by studying cell growth, cell cycle distribution and the evidence of apoptosis. Celecoxib inhibited the growth of human OCC cell lines in a time and dose dependent manner and the inhibitory effects were independent of the level of COX-2 expressions. Celecoxib caused cell cycle arrest in the S phase with a reduction in cell numbers and cell accumulation in G0-G1 phase at 24h. G0-G1 arrest induced by celecoxib was accompanied by up-regulation of cyclin-dependent kinase inhibitor p21. The up-regulation of p21 in celecoxib treated cell lines might be mediated by a p53-independent pathway. In addition, increasing concentrations of celecoxib induce morphological and biochemical changes related to apoptotic cell death. Celecoxib triggers apoptosis through a mechanism that involve direct targeting of Bcl-2/x proteins. The current *in vitro* study indicates that inhibition of proliferation and induction of apoptosis in human oral cancer cell lines by cyclooxygenase-2 specific inhibitor celecoxib exerts its anticancer effect partly through COX-2-independent mechanisms in addition to the known primary function of COX-2 inhibition. (This study was supported by BK21 project for Medical Science and a grant of the National Cancer Control R&D program 200A. Ministry of Health & Welfare, Republic of Korea.)

#3178 Genistein prevents downregulation of 25-D₃-1 α -hydroxylase by 1,25-(OH)₂-D₃ in human mammary and colorectal tumor cells: Relevance for cancer prevention. Daniel Lechner and Heide S. Cross. *University of Vienna Medical School, Vienna, Austria.*

Extensive studies have confirmed the anti-neoplastic and pro-differentiating effect of 1,25-(OH)₂-D₃ (1,25-D₃) in different tissues. Two key enzymes are involved in vitamin D-metabolism: the 1,25-D₃-synthesizing 1 α -hydroxylase (CYP27B1) and the 24-hydroxylase (CYP24) responsible for its degradation. We, and others, have found that expression of these enzymes is not confined to the classical 1,25-D₃-producing organ, the kidney, but also occurs at extra-renal tissues, therefore enabling them not only to synthesize 1,25-D₃ from its precursor 25-OH-D₃ but also to catabolize it. Increasing levels of 1,25-D₃ lead to inhibition of CYP27B1 and stimulation of CYP24, thus preventing further accumulation. Because this is limiting for preventive and therapeutic strategies with most vitamin D-metabolites, degradation of 1,25-D₃ has to be inhibited and synthesis promoted. When compared to the Western industrialized world, Far Eastern countries present with a remarkably lower risk of developing e.g. breast and colon cancer. This is - at least in part - attributed to the traditionally high nutritional intake of phytoestrogens which are especially abundant in soy food and have already been shown to exert dose-dependent anti-proliferative effects. We therefore evaluated possible effects of the very potent phytoestrogen genistein on parameters of vitamin D-metabolism in cancer cell lines derived from mammary (MCF-7) and colonic tumor tissue (Caco-2) using RT-PCR, immunoblotting, and HPLC. Genistein doses ranged from 0.01 μ M to 10 μ M which are concentrations easily reached by frequent dietary ingestion of soy. After administration of genistein, Caco-2 cells presented with a

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biphasic reaction showing a clear increase of CYP27B1 activity at low concentrations (0.1 μ M and 1 μ M) and a strong decrease at higher doses. In MCF-7 cells CYP27B1 activity, but also mRNA and protein expression rose consistently in a dose-dependent manner. In both Caco-2 and MCF-7 cells, after incubation with 1,25-D₃, HPLC analysis revealed a distinct increase of 1 α -hydroxylated metabolites by genistein. This may be due to partially conserved CYP27B1 activity which was - at least in MCF-7 cells - paralleled or even exceeded by 0.01 μ M 17 β -estradiol, pointing to a possible involvement of estrogen receptors (ER). However, Caco-2 cells - as determined by RT-PCR - express ER α and ER β at much lower levels than MCF-7 cells. In conclusion, we suggest two possible protective mechanisms by genistein against mammary and colon tumor progression: by stimulating CYP27B1 expression on the one hand, and by partially preventing CYP27B1 downregulation by endogenously produced 1,25-D₃ on the other hand.

#3179 Cancer-preventive selenocompounds inactivate protein kinase C in precursor prostate epithelial cells and induce cell death: Selectivity to precursor cells promoted by peroxides. Rayudu Gopalakrishna and Usha Gundimeda. *USC Keck School of Medicine, Los Angeles, CA.*

Although cancer-preventive agent selenium is known to inhibit tumor promotion by interfering with cell signaling, it is not clear how this agent selectively interfere with cell signaling in precursor cells but not in normal cells. Methylselenol was previously postulated as the active metabolite that mediates cancer-preventive actions of selenium. Nevertheless, it is a volatile compound and its mechanism of action, especially that occurring at the tissue available low concentrations, is not known. We have previously shown that redox-active selenocompounds directly inactivate protein kinase C (PKC) by inducing sulphydryl oxidation. Since selenium is required at low concentrations to inhibit tumor promotion, it is possible that the oxidant present in promoting cells may enhance cellular retention and the preventive actions of volatile methylselenol. Methylselenol and benzene-selenol at catalytically low concentrations (100 to 500 nM) induced an inactivation of PKC only in the presence of peroxides such as arachidonic acid hydroperoxide and t-butyl hydroperoxide. By reducing peroxides, selenol is oxidized to seleninic acid, which in turn oxidized protein sulphydryls and thus reduced back to selenol. Although this peroxidic redox-cycling process nonspecifically oxidizes many thiols including GSH, the lipophilic selenols and organic peroxides present in the membrane can selectively oxidize sulphydryls of key membrane-bound enzymes such as PKC. Application of sublethal doses of these peroxides and organic selenols to RWPE human prostate epithelial cells resulted in inactivation of PKC α isoenzyme, induction of apoptosis, and inhibition of cell transformation. Conceivably, reaction of selenols with tumor-promoting peroxides provides one of the specific mechanisms by which cancer-preventive selenocompounds selectively interfere with signaling in the promoting cells to block their escape from the peroxide-induced cell death.

CARCINOGENESIS 8: Chemical Carcinogenesis

#3180 Susceptibility of rats to PhIP-induced mammary gland cancer varies with age and is associated with differential gene expression. Liang Shan, Manhu Yu, Herman A. Schut, Elizabeth G. Snyderwine. *National Cancer Institute, Bethesda, MD and Medical College of Ohio, Toledo, OH.*

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a carcinogen found in the human diet, induced a 50% incidence of mammary cancer in Sprague-Dawley rats when administered orally during mammary gland development (43-55 days of age). However, mature virgin rats at 150-days of age were resistant to PhIP-induced mammary carcinogenesis. To explore the possible mechanisms for age-related differences in susceptibility, PhIP-DNA adduct levels, mutations, and gene expression were examined in glands from 43-day and 150-day old PhIP-treated rats given identical dosage regimens. Three hours after PhIP, PhIP-DNA adduct levels in the mammary gland (detected by the postlabeling assay) were not statistically different between 43-day and 150-day old rats. By 6 weeks after dosing, adduct levels had declined in rats of both ages (1.1 $\pm$ 0.1 and 2.7 $\pm$ 0.8, mean $\pm$ S.E., relative adduct labeling $\times 10^4$, respectively). Mutations in the mammary gland were evaluated in the lacI gene of BigBlue transgenic rats at 6 weeks. Mutant frequency (mean $\pm$ S.E. ($\times 10^{-4}$), n=4) in 43- and 150-day old rats were not statistically different [195 $\pm$ 12 and 221 $\pm$ 25, respectively]. Therefore initial DNA adduct levels, adduct removal, and mutation burden did not appear to account for age differences in susceptibility. To further examine possible mechanisms, the profile of gene expression was examined in 43- and 150-day old rats 6 weeks after PhIP treatment. Expression was examined on a NCI 10K cDNA microarray using analysis tools available from the NCI microarray database. Mammary gland RNA from PhIP-treated rats was arrayed against age-matched control. In glands from PhIP-

XP-002529043

British Journal of Nutrition (1983), 50, 189-195

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Differing effects of pectin, cellulose and lignin on stool pH, transit time and weight

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(Received 5 October 1982 - Accepted 7 March 1983)

1. Randomized cross-over studies were undertaken to determine the effects of daily dietary supplements of pectin (12 g/d), cellulose (15 g/d) and lignin (12 g/d) on stool characteristics of healthy volunteers.
2. Detailed dietary records were kept throughout the study. Stool collections over 48 h were used to determine mean stool pH and weight. The single stool transit time was measured using radio-opaque markers.
3. Pectin did not significantly alter the mean stool pH, transit time or 24 h wet weight.
4. Cellulose lowered the mean stool pH from 6.38 to 6.12, decreased mean stool transit time by 27% and increased mean wet stool weight by 57%.
5. Lignin lowered the mean pH from 6.34 to 6.25, decreased the stool transit time by 20% and increased stool weight by 27% but these changes were not statistically significant.
6. These findings have shown that individual fibre components have different colonic metabolic effects and support the view that associations between dietary fibre intakes and diseases such as colorectal cancer should be evaluated with regard to the type of fibre components consumed.

The possible relationship between dietary fibre ingestion and colorectal cancer is complex and has recently been reviewed by Spiller & Freeman (1981). The most convincing evidence of a protective effect of fibre comes from the International Agency for Research on Cancer (IARC) Intestinal Microbiology Group (1977), who studied two Scandinavian populations and demonstrated that a lower incidence of colorectal cancer in rural Finland was associated with a significantly higher intake of total dietary fibre. Stool weights were also significantly heavier in the population with the lower incidence of colorectal cancer. More recent studies show that this inverse association is true over a wider range of populations and that there is a trend whereby the intake of dietary pentosans and cellulose, but not hexose or uronic acid (pectin) fibre fractions, is related to a lower incidence of colorectal cancer (IARC, unpublished results). This inverse association of colorectal cancer with certain components of fibre has also been shown in Britain (Bingham *et al.* 1979). Animal colonic carcinogenesis studies support the epidemiological information. In rats, the ingestion of cellulose has been shown to have a protective effect on 1,2-dimethyl hydrazine-induced colonic neoplasms (Freeman *et al.* 1980), whilst pectin does not exert a protective effect and may even increase carcinogenesis (Bauer *et al.* 1981).

It has been proposed (Thornton, 1981) that the effects of fibre on carcinogenesis may be mediated by alterations in stool pH. Although eating six oranges daily significantly lowers the stool pH (Walker *et al.* 1979), the ingestion of wheat bran does not appear to exert this effect (Stephen & Cummings, 1981). The present study was performed in order to investigate the effects of the individual fibre components, pectin, cellulose and lignin, on stool pH, transit time and wet weight.

METHODS

Subjects

Three groups of ten subjects each were investigated over an 8 week period. All subjects were healthy volunteers who gave their informed consent to participate in the study, which was

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approved by the Wellington Hospital Board Ethical Committee. The pectin group comprised two male medical students and eight female postgraduate nurses (mean age 30.9 (SD 7.9) years). The cellulose group was made up of four male medical students, two female medical students and four female postgraduate nurses (mean age 23.9 (SD 1.9) years). The lignin group consisted of two male medical students, two female medical students, five female postgraduate nurses and one female technical officer (mean age 28.3 (SD 7.8) years).

Experimental design

For each group, a cross-over design consisting of two periods of 4 weeks was used. Each subject acted as his (her) own control taking either pectin, cellulose or lignin for one period only (test period) and consuming his (her) normal diet during the other period (control period). Subjects were paired according to sex and occupation, then randomly allocated to the groups and periods, so that the number of males and females receiving the supplement first was the same as the number entering the control period first and the number of students and nurses was similarly distributed.

Diets

Subjects were instructed to maintain their normal diet throughout the study and not to take any medication. Detailed dietary records as recommended by Bingham & Cummings (1980) were kept over the 8 week period. Food scales, measuring cylinders and flasks were distributed and every item of food or drink taken was weighed or measured and entered in a note book. The dietary records were supervised by a dietitian and analysed to ensure that there was no discrepancy in food patterns between the control and the test periods. Baseline dietary fibre intakes for the two periods were obtained using food tables (Holloway *et al.* 1977; Paul & Southgate, 1978; Southgate, 1978). Subjects, wearing light indoor clothing, were weighed immediately before the study and at the end of both the control and test periods on the same scales.

Pure pharmaceutical citrus pectin with a methoxyl content of 93 g/kg dry weight (Yakhin Canning Company Ltd, Tel Aviv) and 99.5% pure α -cellulose fibre (Sigma Chemical Company, Missouri) were used for the study. Pure Aspen autohydrolysis lignin was supplied by Professor Morris Wayman and Stake Technology Ltd, Ontario. Before the test period, each subject was given a bag containing 420 g of the supplement and instructed to take a total of 15 g/d (a measure of 5 g with each meal) for 28 d. The pectin, cellulose or lignin was to be mixed with the usual food or drink taken at mealtimes in any way acceptable to the subjects. Any supplement left over at the end of the test period was collected and weighed. If the supply was exhausted before the end of the test period, more was distributed. The amounts of pectin, cellulose and lignin taken by each subject and the side-effects experienced are shown in Table 1. Subjects experienced great difficulty in taking the full amount of pectin because of its gelling properties, whilst lignin proved to be extremely unpalatable. One subject in the pectin group ingested a mean daily supplement of 3.4 g only and another subject in the lignin group took a mean of 6.7 g supplement/d. Exclusion of these subjects from the analyses did not alter the results. Cellulose was well tolerated and the lowest mean daily supplement taken by a subject was 11.2 g.

Stool measurements

At the end of both the control and test periods, the single stool transit time, using the radio-opaque marker pellet method described by Cummings & Wiggins (1976) was measured. A 48 h stool collection was made into plastic bags, stored in a tin at 4-6°, and a 24 h wet stool weight obtained. Faecal pH was determined by immersion of a combination pH electrode (Orion) into three regions of each stool and taking a mean of the measurements

Stool effects of fibre components

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Table 1. Mean amounts of pectin, cellulose and lignin taken (g/d) by individual human subjects and reported side-effects

Pectin				Cellulose				Lignin			
Subject				Subject				Subject			
Sex	Age (years)	Mean amount	Reported side-effects	Sex	Age (years)	Mean amount	Reported side-effects	Sex	Age (years)	Mean amount	Reported side-effects
♂	21	15.0	—	♂	21	16.0	—	♀	22	14.2	Laxative
♂	21	11.0	—	♂	23	16.3	—	♀	24	11.2	—
♀	26	15.0	—	♂	25	8.8	—	♀	43	6.7	Flatulence
♀	28	9.0	Laxative	♂	23	16.0	—	♀	42	14.2	—
♀	30	15.0	Constipation	♀	21	11.2	—	♂	24	9.8	—
♀	42	12.3	Constipation	♀	23	16.4	—	♂	23	16.0	—
♀	41	12.9	—	♀	25	13.9	Nausea, retching, Cramps, urgency	♀	23	14.2	Constipation
♀	27	3.4	'Intolerable'	♀	25	13.9	—	♀	28	9.3	Constipation
♀	40	15.0	Nausea	♀	26	15.0	—	♀	28	14.5	Flatulence, diarrhoea
♀	33	10.4	Heartburn	♀	21	19.7	—	♀	26	12.2	Satiety
Mean		11.9				14.7				12.2	
SD		3.7				3.0				2.9	

using a Radiometer pH meter. Stools were not homogenized or diluted. The coefficient of variation for ten probes with this method was 2%.

Statistics

An analysis of variance for a two-period cross-over design was used to calculate the *F*-values with their respective degrees of freedom. Correlation coefficients were calculated and their statistical significance was determined using a two-tailed *t* test. A two-tailed *t* test was also used to test for inter-group and inter-sex differences. The level of significance used for all tests was $P < 0.05$.

RESULTS

Body-weight and dietary fibre intake

Neither the mean body-weight nor the mean basal dietary fibre intakes differed significantly between the control and test periods for any of the groups. The mean (SD) basal dietary fibre intakes for the control and test periods respectively were: pectin group 18.0 (3.5) g, 22.9 (8.4) g; cellulose group 21.3 (6.6) g, 23.9 (15.9) g; lignin group 21.5 (5.1) g, 20.2 (5.5) g.

Comparison of groups and sexes

There was no statistically significant difference in the mean stool pH, transit time or weight for the control periods of the pectin, cellulose or lignin groups. Over-all comparison of the sexes did not show a statistically significant difference between males and females for stool pH or transit time, but revealed a highly-significant difference ($P < 0.01$) between the 24 h wet stool weights of males (mean 188.7 (SD 41.5) g) and females (mean 115.7 (SD 54.0) g). This may reflect the statistically significant ($P < 0.001$) difference in daily energy intake between males (mean 11.6 (SD 3.0) MJ) and females (mean 7.5 (SD 2.0) MJ) and, in particular, the mean carbohydrate intake, which was 301.4 (SD 102.9) g for males and 200.0 (SD 59.2) g for females. There was no significant difference between the sexes in basal dietary fibre intakes.

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Table 2. *Stool pH, transit time and weight for control and test periods for groups of human subjects receiving pectin, cellulose and lignin*
(Mean values with their standard deviations)

Experimental period...	Pectin		Cellulose		Lignin	
	Control	Test	Control	Test	Control	Test
Stool pH						
Mean	6.51	6.53	6.38	6.12*	6.34	6.25
SD	0.57	0.42	0.25	0.43	0.30	0.44
Single stool transit time (h)						
Mean	51.1	57.4	55.3	40.4**	50.1	40.5
SD	23.5	14.3	19.7	12.7	22.1	17.4
24 h wet stool wt (g)						
Mean	134.9	118.7	132.6	207.8*	138.7	176.7
SD	66.5	79.9	65.6	94.2	53.6	78.0

Mean values were significantly different from control values * $P < 0.05$; ** $P < 0.01$.

Table 3. *Correlation coefficients between stool pH and stool transit time, stool pH and stool weight, and stool transit time and stool weight*

Experimental period... Fibre component	Stool pH v. single stool transit time (h)		Stool pH v. 24 h wet stool wt (g)		Single stool transit time (h) v. 24 h wet stool wt (g)	
	Control	Test	Control	Test	Control	Test
Pectin	0.37	0.21	0.24	-0.33	-0.12	-0.11
Cellulose	0.80*	0.56	-0.71*	0.24	0.75*	-0.14
Lignin	0.65*	0.31	-0.15	-0.33	-0.36	-0.47

Values were statistically significant: * $P < 0.05$.

Stool pH

As shown in Table 2, pectin did not alter the mean stool pH significantly whereas cellulose resulted in a fall in stool pH in nine of the ten subjects. Analysis of variance revealed a carry-over effect whereby a significantly lower mean pH was present at the end of the control period if this period followed the test period rather than being the initial period. Lignin lowered the mean pH but the change was not statistically significant.

Stool transit time

The effect of the fibre components on mean stool transit time is given in Table 2. For pectin, the transit time was raised but the change was not statistically significant. In contrast, all subjects but one showed a more rapid stool transit time after the ingestion of cellulose, with a decrease in the mean of 27% which was significant ($P < 0.01$). There was also a carry-over effect observed as the mean transit time at the end of the control period was still significantly shorter if the control period followed the test period rather than vice versa. Lignin decreased the mean stool transit time by 20% but this change was not statistically significant.

Stool weight

When compared with controls, increasing the consumption of pectin did not significantly increase stool weight. However, the increase in stool weight of 57% caused by cellulose was significant ($P < 0.05$). The order of the two periods (test, control v. control, test) had no significant effect on stool weight. Lignin resulted in an increase of 27% in the 24 h wet stool weight, but this change was not statistically significant (Table 2).

Correlations between stool characteristics

Correlation coefficients between stool pH and transit time, stool pH and weight, and stool transit time and weight for both the control and test periods are given in Table 3. Significant correlations were observed in the control periods between stool pH and transit time for both the cellulose and lignin groups, but only for the cellulose group between stool pH and weight, and transit time and weight. After fibre component supplementation for 4 weeks, no significant correlations were observed.

DISCUSSION

The present study shows that purified α -cellulose had significant effects on stool characteristics whilst highly methoxylated citrus pectin and Aspen autohydrolysis lignin did not. The mean daily addition of either 12 g pectin or 12 g lignin to the diet did not result in any statistically significant change in stool pH, transit time or weight, whereas cellulose at a mean dose of 15 g/d caused significant changes in all these measurements. The amounts of pectin and lignin taken were the maximum tolerated by the subjects and although it could be argued that the differences between the pectin and lignin and the cellulose effects were due to the lower doses of the first two components, this seems unlikely as the difference in dosage was small, and analysis of the results after exclusion of the subjects taking the lowest doses of fibre supplement did not alter the outcome. It could also be argued that the cellulose group differed from the pectin and lignin groups as there were four males in the cellulose group compared with two in each of the other groups. Despite males having over-all a significantly greater stool weight in the control periods compared with females, this could not bias the results of the study as each subject acted as his or her own control and gender was taken into account before allocation to periods.

The authors are not aware of any previous human studies on the faecal effects of lignin. Previous studies with pectin have produced conflicting results. No significant change in faecal mass, even after the addition of 40–50 g pectin/d, was reported by Miettinen & Tarpila (1977), whilst Durrington *et al.* (1976) demonstrated a significant increase in wet stool weight but no alteration of stool transit time at a dose of 12 g/d. Jenkins *et al.* (1978) reported that pectin delayed small bowel transit time. Cellulose, in comparison, has been shown previously to increase wet stool weight (Eastwood *et al.* 1973; Slavin & Marlett 1980; Spiller *et al.* 1980) and to decrease transit time (Spiller *et al.* 1980). The effect of these components on stool pH has not been reported except in hamsters, in which lignin did not alter the pH (Rotstein *et al.* 1980). Faecal pH can be lowered, however, by the addition of six oranges daily to the diet (Walker *et al.* 1979), which would correspond to 8–12 g cellulose, given that a peeled orange comprises 14 g cellulose/kg edible fruit (Holloway *et al.* 1977). A 30 g supplement of wheat bran/d would only contribute 3 g cellulose, which may explain the inability of bran to lower stool pH (Stephen & Cummings, 1981) if dietary cellulose is an important determinant of stool pH.

The contrasting effects of pectin, cellulose and lignin on stool characteristics may be related to the different digestion of the fibre components in the gut. Pectin is completely

digested (Cummings *et al.* 1979) whilst 22.4% of ingested cellulose is excreted unchanged in the faeces (Holloway *et al.* 1978). In contrast, over 80% of lignin can be recovered from the faeces (Kelsay *et al.* 1981). Residual cellulose could exert a water-holding effect and hence alter stool transit time and weight, but this would not explain the change in stool pH. If the response were related to the production of volatile short chain fatty acids, then totally fermentable components such as pectin would be expected to cause even greater changes (Cummings, 1981) though perhaps the slower, more prolonged digestion of cellulose in the colon may be important. However, Stephen & Cummings (1980) have shown that cabbage fibre alters colonic function by stimulating microbial growth. If this were true for cellulose, then the carry-over effect seen in the present study, whereby the effects of cellulose on stool pH and transit time persisted for at least 28 d after taking the supplement, could be explained in terms of altered bacterial flora.

An inverse relationship between daily stool weights and transit times has been documented previously (Burkitt *et al.* 1972), although the IARC Intestinal Microbial Group study (1977) did not find the expected correlation. In the present study there was no consistent, statistically significant correlation between any of the stool characteristics. Although the number of subjects studied was small, this may indicate that stool pH, transit time and weight can vary independently. It is interesting that the correlation coefficient between stool pH and transit time after cellulose supplementation was 0.57, which just failed to reach statistical significance ($P = 0.09$), considering that cellulose exerted a prolonged effect on both factors.

As individual fibre components have differing effects on stool characteristics, these factors should be taken into consideration when evaluating the possible role of dietary fibre in colorectal disease. Analysis of the types of dietary fibre components consumed may be pertinent to our understanding of the aetiology of colorectal cancer.

This study was supported by a Medical Research Council of New Zealand Grant (no. 81/34). The authors wish to thank Professor Morris Wayman and Stake Technology Ltd, Ontario, for the supply of lignin.

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Printed in Great Britain

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 846 704 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
10.06.1998 Bulletin 1998/24

(51) Int Cl.⁸: C08B 30/12, C12P 19/16,
A23L 1/09

(21) Application number: 97309720.7

(22) Date of filing: 02.12.1997

(84) Designated Contracting States:
AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
NL PT SE
Designated Extension States:
AL LT LV MK RO SI

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(54) **Highly fermentable resistant starch**

(57) The present invention discloses that retrograded starch having more than 55% resistant starch with > 50% chains of DP 10 - 35 gives rise to a significantly higher amount of n-butyrate production under condi-

tions simulating the human colon. It is expected that such an increased n-butyrate production will diminish the development of colon diseases notably of colon cancer.

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Description**Technical field**

5. The present invention relates to a starch composition containing a high proportion of so-called "resistant starch" (RS). The composition of the resistant starch is further characterised by a specific chain length distribution of the RS-fraction and by a relatively low specific Differential Scanning Chromatography (DSC) melting peak temperature. The composition furthermore shows a specific fermentation pattern resulting in an increased level of n-butyrate.

10 **Background of the invention**

- It has been known for some years that a part of the starch contained in the human diet can pass the small intestine without being digested. This fraction of the food starch is called resistant starch. Different forms of starch have been found to be resistant to digestion. A classification of resistant starches has been given by Englyst and Cummings (Am. J. Clin. Nutr. (1987) 45 423-431). These authors distinguish between three types of resistant starches:

- Type 1. Physically indigestible starch e.g. partially milled grains and seeds,
 Type 2. Resistant starch granules e.g. raw potato, green banana,
 Type 3. Retrograded starch e.g. cooled-cooked potato, bread, and cornflakes.

- 20 Effective enrichment of food with RS is possible by addition of processed starch containing a large percentage of retrograded structures. Starch is composed of amylose and amylopectin. The extent of retrogradation is known to be a function of the amylose content. Heating and cooling of amylose gives rise to resistant starch. Due to the branched structure of amylopectin the amount of resistant starch which is formed is decreasing with an increase in the amount of amylopectin in starch. The amount of RS can however be increased by debranching the amylopectin prior to heating. In view of the above high amylose (maize) starches have been chosen as the primary source of resistant starch for the first commercial high RS-products.

- Carbohydrates which are not enzymatically digested in the small intestine reach the colon where they are fermented by the anaerobic microflora. Such carbohydrates include non-starch polysaccharides, resistant starch (RS), indigestible oligosaccharides and endogenous polysaccharides from mucus. The undigested starch fraction reaches the colon where it becomes a substrate for microbial fermentation. Besides gas production (H_2 , CH_4 , CO) different short chain fatty acids (SCFA) are formed depending on the type of carbohydrate.

- The major end products of bacterial carbohydrate breakdown are short-chain fatty acids (SCFA: acetate, propionate and n-butyrate). SCFA are rapidly taken up by the colonic epithelial cells. Propionate and acetate are released by the basolateral membrane to the portal circulation and may have an effect far from their production site. n-Butyrate serves as energy yielding substrate in the colonocytes and additionally affects several cellular functions e.g. proliferation, membrane synthesis and sodium absorption.

- Acetate, propionate and n-butyrate are the main SCFA produced from indigestible oligo- and polysaccharides the relative amounts of these fatty acids depend on the type of carbohydrate. SCFA are produced in the proximal colon in an average ratio of acetate : propionate : n-butyrate equivalent to 60:25:10 and in amounts of mmol/L. This ratio however is not constant but is determined by the kind of substrate fermented. It has been shown both *in vitro* and *in vivo* that the fermentation of starch yields high levels of n-butyrate. The observations that caecal SCFA levels are decreased by raw potato starch (Mallett et al. (1988) Brit. J. Nutr. 60, 597-604; Levrat et al. (1991), J. Nutr. Biochem. 2, 31-36; Mathers et al., (1991) Brit. J. Nutr. 66, 313-329) but increased by high amylose corn starch underline that different forms of RS have different effects in terms of n-butyrate production in the colon.

- According to Wyatt and Horn (1988) J. Sci. Food Agric. 44, 281-288, RS-fractions of retrograded pea and corn starch respectively show quantitative differences in *in vitro* fermentation but without qualitative changes in SCFA composition. Six different raw starches also showed different *in vitro* fermentation kinetics. At the same time the molar n-butyrate proportion was not altered. Several independent *in vivo* animal studies confirm this. Thus the source of RS is important for the fermentability and hence for the amount of n-butyrate obtained but apparently not for the relative amount.

- Compared with indigestible polysaccharides such as arabinogalactan, xylan and pectin, RS produces a significantly larger molar amount of n-butyrate (Englyst et. al. (1987) in I.D. Morton: "Cereals in a European Context", Chichester, UK, Ellis Horwood Ltd., pp. 221-223). This is considered important because of the general acceptance that n-butyrate plays a major role in the prevention of intestinal cancers (e.g. colorectal cancer) as recently summarised by Smith and German (Food Technology, (1995 November) 87-90). n-Butyrate appears to be a preferred substrate for normal colonocytes and assists in the maintenance of colonic integrity.

- n-Butyrate inhibits growth of colon cancer cell lines. At the molecular level, n-butyrate causes histone acetylation,

favours differentiation, induces apoptosis and regulates the expression of various oncogenes. *In vivo* n-butyrate increases immunogenicity of colon cancer cells.

Only indigestible polysaccharides which are associated with production of high n-butyrate concentrations in the distal large bowel (wheat bran, retrograded high amylose starch (type 3 RS)) were found to be protective against colorectal cancer in a rat model system wherein rats were treated with 1,2-dimethylhydrazine (McIntyre et al. (1993), *Gut* 34, 386-391; Young et al. (1996), *Gastroenterology* 110(2): 508-514). Oat bran, guar gum, raw potato starch (type 2 RS), cellulose and starch-free wheat bran have no protective effect in this model of colorectal cancer (McIntyre et al. (1993), *Gut* 34, 386-391, Young et al. (1996), *Gastroenterology* 110(2): 508-514).

From the above studies it appears that the amount of n-butyrate produced in the colon is important. What is needed for a maximal physiological benefit is not only a starch product with a high amount of RS, but a well fermentable RS-fraction producing high amounts of SCFA with an elevated n-butyrate level. Methods for the preparation of resistant starch have for example been disclosed in the following publications. European patent application EP 688 972 discloses a method for obtaining increased levels of resistant starch. It is demonstrated that the highest amounts of RS are obtained when after enzymatic digestion, retrogradation is performed for a prolonged period of time and at a relatively low temperature. The maximum amount of RS which could be obtained was 51.8 % (example 3 therein).

International patent application WO 91/07106 discloses a method for obtaining resistant starch wherein a retrogradation step is followed by enzymatic hydrolysis. The retrogradation step is performed at low temperature for amylose at 4 °C and for starch at 8 °C as mentioned on page 13. Moreover the process starts with undegraded starch which may be prior treated by a debranching enzyme.

European patent application EP 564893 discloses a method for obtaining resistant starch starting from a non-degraded high amylose starch. The DSC melting peak temperature of this product is mentioned to be in the range of 115 - 135 °C and the amount of resistant starch is below 51% and is correlated with the percentage of amylose used in the starting product.

There exists a need for a starch-based product which is highly fermentable and which gives rise to an increased amount of n-butyrate in the colon. The present invention provides such a starch-based product.

Summary of the invention

The present invention discloses a starch-based composition which is characterised in that it contains a high amount of resistant starch. The composition consists of partially degraded starch which has undergone a retrogradation process and contains at least 55% (w/w) pancreatine resistant starch. Preferably, the amount of resistant starch is at least 60%. The resistant starch fraction is characterised by a degree of polymerisation of predominantly between 10 and 35 and a DSC peak temperature of below 115°C, preferably between 90 and 114°C.

The partially degraded starch can be obtained by partial amylolytic or acid hydrolysis of starch followed by enzymatic debranching. A preferred partially degraded starch which is used as a starting product is a maltodextrin with a dextrose equivalent (DE) below 10 obtained by partial alpha-amylase degradation and additionally treated with iso-amylase. The present invention also discloses a method for obtaining the starch-based compositions. The method comprises the following steps:

- a) thinning of the starch,
- b) enzymatic debranching of the thinned starch,
- c) inactivation of the enzyme,
- d) drying of the composition.

Step b) is preferably accompanied by retrogradation.

Preferably the high amount of resistant starch is obtained without a separate retrogradation step at low temperature.

The present invention further discloses the use of the partially degraded retrograded starches in the preparation of food or feed compositions and food or feed compositions containing the starch-based composition.

Finally, the invention discloses the use of partially degraded retrograded starch composition to prevent or treat diseases of the colorectal digestive tract.

Description of the figures

Figure 1 is an example of a DIONEX chromatogram of the resistant starch fraction of the present invention obtained by exhaustive pancreatine digestion of debranched potato maltodextrin (IRP) (measured according to Carbohydr. Res. 215 (1991) 179-192).

Figure 2 shows the change of pH in time during *in vitro* fermentation of the RS fractions of Novolose™, Eureka and IRP.

- Figure 3 shows the formation of short-chain fatty acids during in vitro fermentation of Novelose, Euresta and IRP.
 Figure 4 shows the formation of n-butyrate during in vitro fermentation of Novelose, Euresta and IRP.
 Figure 5 shows DSC curves of milk drink residues obtained after pancreatine digestion for standard milk and milk with added IRP.

Detailed description of the invention

The present invention discloses a starch-based composition which is characterised in that it contains a high amount of resistant starch. The composition consists of partially degraded starch which has undergone a retrogradation process and contains at least 55% (w/w) pancreatine resistant starch. Preferably, the amount of resistant starch is at least 60%. The resistant starch fraction is characterised by containing alpha-glucans with a degree of polymerisation of predominantly between 10 and 35 and a DSC peak temperature below 115°C, preferably between 90 - 114°C. The partially degraded starch can be obtained by amylolytic or acid degradation of starch followed by enzymatic debranching. A preferred partially degraded starch is a maltodextrin obtained by partial alpha-amylase degradation and treated with a debranching enzyme.

The partially degraded starches for use as a starting material of the present invention are obtainable from any suitable starch source. Useful starches are obtained from potato, wheat, tapioca and maize high amylose starches which are converted to maltodextrins have also been used.

The present invention also discloses a method for obtaining the starch-based compositions of this invention. The method comprises the following steps:

- a) thinning of the starch,
- b) enzymatic debranching of the thinned starch,
- c) inactivation of the enzyme,
- d) drying of the composition.

The debranching is achieved by using a suitable enzyme such as isoamylase or pullulanase, preferably by isoamylase.

Step b) is preferably accompanied by retrogradation. Alternatively the starch may be retrograded after enzyme inactivation.

A preferred process for obtaining the products of the present invention contains the following steps:

- a) maltodextrins (DE<10, preferably DE<5) are dissolved in water,
- b) the pH is adjusted and the solution is cooled to a optimum temperature for the activity of a debranching enzyme,
- c) the debranching enzyme is added and the mixture is incubated,
- d) the enzyme is inactivated,
- e) the mixture is spray-dried
- f) the resistant starch is collected and optionally grinded.

Preferably the maltodextrin is potato or tapioca maltodextrin.

The process of the present invention starts from a partially degraded starch product. Contrary to known processes it was found that no separate retrogradation step is required. Retrogradation occurs at the same time as debranching. This results in a more economical process as the retrogradation used to be performed during a prolonged period (up to 48 hours) at a low temperature. The process is therefore faster and cheaper.

Furthermore the product obtained by the invented process contains a relatively high amount of resistant starch. It was found that this product gives a higher production of n-butyrate both relatively with respect to the other short chain fatty acids and in absolute terms than other known products.

The present invention further discloses the use of the partially degraded retrograded starches in the preparation of food or feed compositions and food or feed compositions containing the starch-based composition. The resistant starch product is added to the food or feed composition in an amount of up to 20% (w/w), preferably of up to 10%. Food preparations to which the starch-based composition of the present invention is added include, biscuits, toast, milk desserts up to 10% of the starch-based composition of claim 1.

It is shown that heating during the preparation of the food product does not significantly destroy the product. This means that sufficient RS survives the treatment of food preparation including UHT treatment and baking at 195 °C.

The invention also discloses the use of debranched/retrograded maltodextrins in the prevention of diseases of the colorectal digestive tract. On the basis of the finding that n-butyrate plays a major role in the prevention of intestinal cancers (e.g. colorectal cancer) as recently summarised by Smith and German (Food Technology, 1995 (November) 87-90) the maltodextrins of the present invention are expected, due to their production of a high amount of n-butyrate,

to assist in the maintenance of colonic integrity.

The present RS product having a specific chain length distribution range of the retrograded structures is not only fermented more easily but produces, in absolute and relative terms, significantly higher amounts of n-butyrate than RS products derived from the conventional high amylose starches.

In order to obtain the RS structures (after pancreatine treatment) having more than 50% of the specified chain length of 10-35 AGU, a suitable starting material is needed. We have found that a low DE potato maltodextrin after enzymatic debranching and retrogradation forms more than 30% RS, more preferably more than 40% RS. The RS structures after pancreatine digestion consist for more than 50% of linear chains of 10-35 alpha-glucans. Other debranched/retrograded low DE maltodextrins (e.g. from tapioca, maize, wheat starch) can be used for this purpose too as can maltodextrins obtained from high amylose starches. Starches degraded by other methods (e.g. acid thinned) followed by debranching / retrogradation are also suited for this purpose.

Finally, the invention discloses the use of partially degraded retrograded starch composition to prevent or treat diseases of the colorectal digestive tract.

The invention is illustrated by the following examples.

Example 1 shows a method for obtaining the resistant starch of the present invention. A commercial potato maltodextrin was dissolved in water at an elevated temperature, after cooling and pH adjustment the maltodextrin was debranched with isoamylase. Following incubation the material was spray-dried. As shown in Figure 1 the product had a chain length distribution wherein the majority of the pancreatine resistant chains was between DP 10 and DP 35. The resistant starch content was determined to be 56%. This product is further indicated as IRP.

The experiment was repeated using tapioca maltodextrin and on a larger scale. Starting with 4200 kg tapioca maltodextrin about 3500 kg spray-dried product was obtained which contained 66% resistant starch had a DSC melting temperature of 112 °C and contained 65% material having a DP between 10 and 35.

Example 2 shows chemical and physical data for the resistant starch of the present invention IRP in comparison with Eureka-RS and Novelose™ (National Starch & Chemical Comp.). This example demonstrates that IRP has a significantly higher content of saccharides with DP 10-35 and much lower melting temperature of the RS residues than the two other products.

Example 3 describes in vitro fermentation tests with three different starch-based compositions; IRP (obtained according to Example 1), Eureka and Novelose™ (National Starch & Chemical Comp.). The pH of the fermentation medium between the three RS products was found to be different after 4 h of fermentation. The reduction of pH was more pronounced for IRP than for Eureka and Novelose products. A slight difference persisted after 8 h of fermentation. After 24 h of fermentation pH values were identical (see Figure 2). This indicates that IRP is better fermentable than the other products. SCFA and n-butyrate production were also followed. Figures 3 and 4 show the amounts of SCFA and of n-butyrate formed during the fermentation of the three samples. It appears that the IRP containing faeces gave the highest amount of both SCFA and n-butyrate.

Example 4 describes the addition of resistant starch (IRP) to a milk drink. It is demonstrated that after Ultra-High-Temperature treatment RS can still be determined in the milk. It can be concluded that RS can be applied in the normal food production processes without the need of adaptation of this process.

The invention is further illustrated by the following non-limiting examples.

Example 1

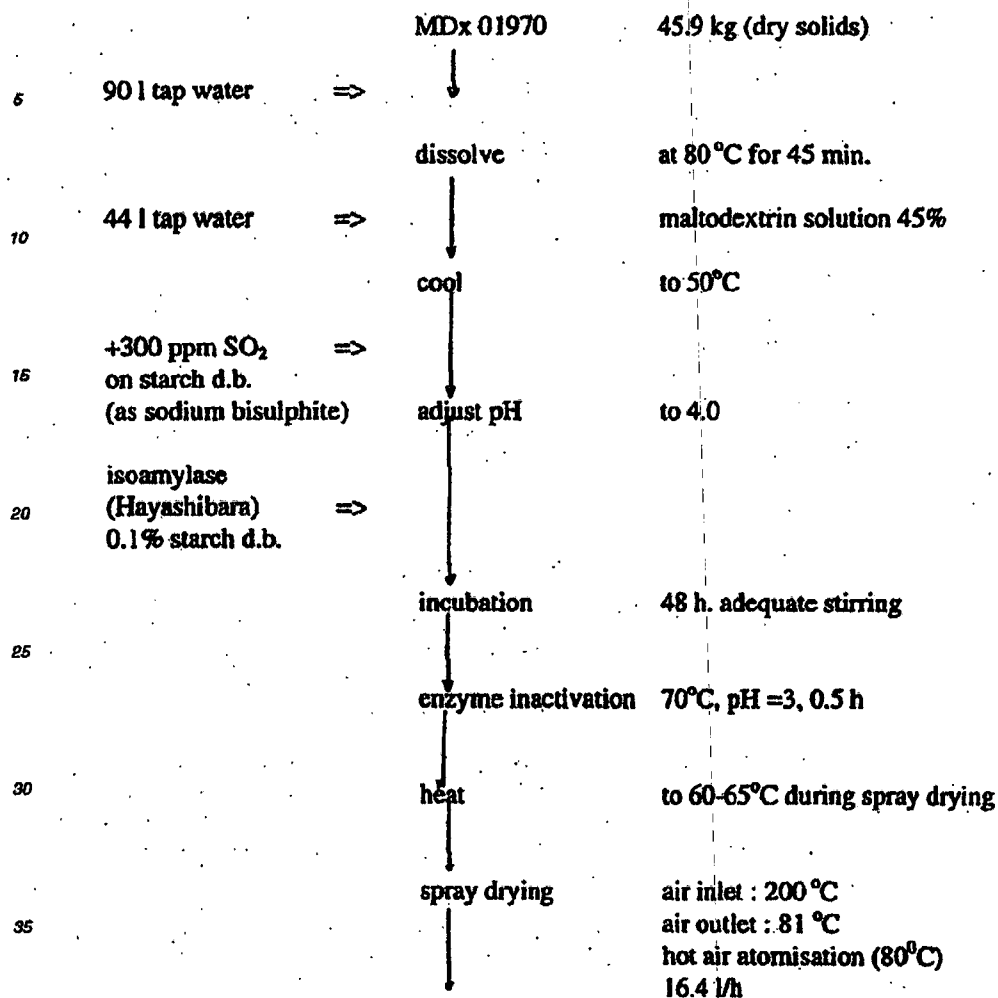
Preparation of debranched, retrograded low DE maltodextrins

A) From potato maltodextrin

The reaction sequence for the production of the resistant starch from potato maltodextrin according to the present invention is given below.

Potato maltodextrin (MDx 01970 (DE 3) from Cerestar) was used as a starting material.

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Resistant Starch enriched product (IRP) 35 kg (94.5% d.s.)

It is evident that one does not have to start from a dried product a wet product may directly be used in the same process. The enzymatic debranching conditions corresponded to the conditions given by the supplier for almost total debranching, about 59 units of enzyme activity/ g starch were used.

The product of the Indicated process was characterised as follows:

- a resistant starch content of 58 %,
- a Mw of 11340,
- a DSC melting peak temperature of the RS residue of 105°C
- the chain length distribution after pancreatic digestion of IRP is shown in Figure 1.

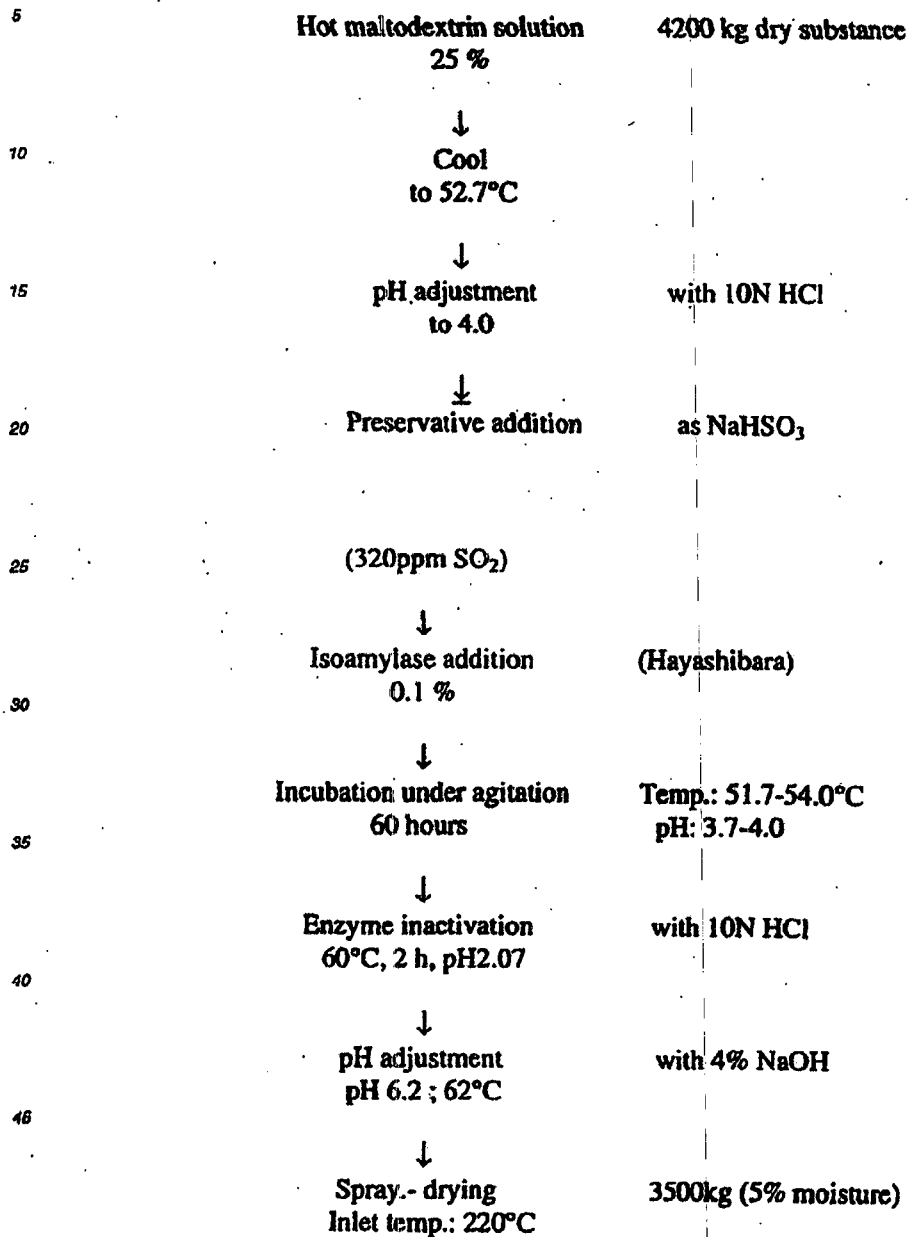
The major part of the resistant starch product consisted of alpha-glucans with a DP between 10 and 35.

B) From tapioca maltodextrin

This example shows the large scale production of resistant starch from a low DE tapioca (cassava) maltodextrin.

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(DE 2.5) is described. The debranching process was performed in a 20 m³ double wall reactor. A freshly prepared maltodextrin was used after dilution to 25% d.s.. The reaction scheme with more details is shown below:



The analysis of the 3.5 tons of spray-dried debranched maltodextrin gave the following results:

- a resistant starch content of 66%
- a Mw of 7230
- a DSC melting temperature of the RS residue of 112°C
- a chain length distribution with 65% in the range between DP 10 and 35

Example 2Comparison of composition and properties of IRP with other RS products based on retrograded high amylose starches

The following table describes the RS content, the chain length distribution and the DSC melting peak temperature of IRP, Novelse and Eureka RS.

Sample	RS-content** (%)	DSC* - T _{peak} (°C)	DP < 10*** (%)	DP 10 - 35*** (%)	DP > 35*** (%)
IRP	56	105	7,0	58,7	34,3
Novelse	57	128	4,4	35,0	60,6
Eureka	36	141	5,1	26,1	68,8

*DSC measurement: 20-30 mg of starch was brought into a stainless-steel DSC-pan and water was added to give a 20% (w/w) system. The closed pan was heated in a SETARAM DSC 111 from 20-160°C at a rate of 3°C/min. The enthalpy change was continuously recorded and the characteristic transition temperatures were registered.

**For RS determinations the following procedure was used:

A 5% (w/w) suspension of the retrograded starch product is thoroughly homogenised in an acetate buffer solution. The acetate buffer is made by dissolving 8.2g anhydrous sodium acetate in 250ml of a saturated aqueous solution of benzoic acid, adding 4ml of 1M calcium chloride and making up to 800 ml with distilled water before adjusting the pH to 6.2 with acetic acid, and finally making up to 1000 ml with distilled water. 25 ml of the suspension are incubated with 1 ml pancreatic solution for 16 hours at 37°C in a shaking water bath. The incubated suspension is next stirred into 118 ml of 95% ethanol, filtered, the filter cake washed twice with 80% ethanol and dried in an oven at 105°C. The RS content was calculated as follows:

$$RS (\%) = \frac{\text{Starch content of the residue}}{\text{Starch content of the suspension before incubation (on dry basis)}} \times 100\%$$

The pancreatic solution is made by stirring 2g pancreatine with 12 ml distilled water for 10 min, centrifuging and using the supernatant as the pancreatic solution.

*** The saccharide distribution was analysed as described in *Carbohydr. Res.* 215 (1991) 178-192 this method only measures saccharides below DP 85. The content of saccharides DP > 85 was characterised by size exclusion chromatography and the relative amounts of the different fractions were calculated after normalisation.

Example 3In vitro fermentation of partially degraded, retrograded starch (IRP) in comparison with Novelse and EurekaExperimentalA. Starting material

IRP, (Ceresstar) was prepared according to Example 1. The product was recovered by spray-drying. The product contained 56 % RS and had a DSC melting peak temperature of 105°C.

Eureka-RS: retrograded starch was produced by cooling and storage of extrusion-cooked amylo maize starch (Amylo maize VII, American Maize Products Comp.). Amylo maize containing 50 % water was extrusion-cooked at 100°C, followed by 4 days storage at 4°C, then dried and milled. The product contained 36 % RS and had a DSC melting peak temperature of 141°C.

Novelse: This starch is a modified amylo maize starch (National Starch and Chemical Corp.), enriched in RS. It contains 57 % RS and had a DSC melting peak temperature of 128°C.

B. Method used for predigestion of RS products

The purification of the RS for *in vitro* fermentation has been performed by extensive digestion of the starch with pancreatic α -amylase (Sigma, A-3176).

42.5 g IRP, 72.7 g Eureka or 30.7 g of Novelse were suspended in sterile phosphate buffer pH 6.9 (300 ml, 400 ml and 700 ml respectively) and brought into a dialysis tube and α -amylase (10 mg/g of sample) was added. The tubes were then plunged in 1 L water at 37°C and kept overnight. The following day, the same amount of α -amylase activity was added again and a second digestion took place overnight. Samples were centrifuged (10 min, 3000 rpm) and washed several times. The sediment (RS) was freeze-dried.

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C. Method used for the *in vitro* fermentations

The method has been extensively described elsewhere (Barry et al., Estimation of fermentability of dietary fibre *in vitro*: a European interlaboratory study. Br. J. Nutr. (1995), 74, 303-322).

C1. General schedule

All experiments were conducted in an *in vitro* batch system. Fermentations were performed in vials using inoculum made from fresh faeces collected from healthy young volunteers. The volunteers usually ingested a normal diet, presented no digestive disease and had not received antibiotics for at least three months. Fermentation variables were measured in vials in which fermentation was stopped at various times.

C2. Inoculum

Faeces from two non-methane producer volunteers were collected in an insulated bottle previously warmed for about 5 min with hot tap water (approximately 65°C). To eliminate O₂, the bottle was flushed for 5 min with CO₂ at a flow of 100 ml/s and faeces were then collected. When the insulated bottle was received at the laboratory, CO₂ was flushed inside. The weight of faeces was then determined. The inoculum was produced in the insulated bottle by adding five parts of a warmed (37°C) nutritive buffer to one part of faeces (v/w). The nutritive medium was made from carbonate-phosphate buffer solution containing (g/l): NaHCO₃ 9.240, Na₂HPO₄·12H₂O 7.125, NaCl 0.470, KCl 0.450, Na₂SO₄ 0.100, CaCl₂ (anhydrous) 0.055, MgCl₂ (anhydrous) 0.047, urea 0.400, with added trace elements (10 ml of the following solution (mg/l) per litre of final solution: FeSO₄·7H₂O 3680, MnSO₄·7H₂O 1900, ZnSO₄·7H₂O 440, CoCl₂·6H₂O 120, CuSO₄·5H₂O 98, Mo₇(NH₄)₆O₂₄·4H₂O 17.4). Before use, and during preparation of the inoculum, continuous bubbling of CO₂ maintained anaerobiosis and ensured a constant pH. The slurry was mixed using a Stomacher (Laboratory Blended, Seward Medical, London) apparatus for 2 min and then filtered through six layers of surgical gauze. The inoculum was maintained in a water bath at 37°C and continuously bubbled with CO₂.

C3. Fermentation experiments

Fermentation was conducted in duplicate using 50 ml polypropylene vials (Falcon, Biorock). Except for blanks (B), 100 mg (dry-matter basis) of well-homogenised experimental substrate was weighed into each vial and 10 ml inoculum added. Air was displaced by flow of O₂-free N₂. After the cap was screwed on, the vial was placed horizontally (time 0) in a shaking bath. Fermentation was then performed at 37°C and the results studied at 0, 4, 8 and 24 h. Two blanks were used for each experimental time. At each experimental time, fermentation in corresponding vials was stopped by instantaneous freezing (dry ice).

C4. Sample preparation

The pH was immediately measured and 10 ml distilled water added. Sample was then centrifuged 10 min at 3000 g. Two samples of 1 ml supernatant were taken for SCFA determinations. Samples were mixed with 100 µl HgCl₂-H₃PO₄ (1%/5%) solution. Samples for SCFA determination and pellets for starch determinations were kept at -20°C until analysis.

SCFA were quantified by the gas chromatographic method as described by Jouany J.P. (Dosage des acides gras volatils (AGV) et des alcools dans les contenus digestifs, les jus d'ensilage, les cultures bactériennes et les contenus des fermenteurs anaérobies. Sci. Alim., (1982), 2, 131-144).

Remaining starch was quantified by the method of Faisant et al. (Resistant starch determination adapted to products containing high resistant starch. Sci. Alim., (1995), 15, 83-89).

C5. Calculation of short chain fatty acid in slurries

The production of P_i of each SCFA was calculated as follows for each experimental time:

$$P_i = (S_i - S_0) - (B_i - B_0)$$

where S_i and S_0 are SCFA concentration values in vials containing substrates at time i and 0 respectively, and B_i and B_0 are SCFA concentration values for blank at time i and 0 respectively.

For each experimental time, total SCFA production was calculated as the sum of individual production of acetic,

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propionic and n-butyric acid.

Results and conclusion of *in vitro* fermentations

5 Kinetics of fermentation are determined by measuring pH and SCFA production, in duplicate. For all parameters and each product the same pattern of fermentation was observed upon comparing the duplicate measurements.

A) Evolution of pH

10 The pH of the fermentation medium between the three RS products differed after 4 h of fermentation. The reduction of pH was more pronounced for IRP than for Eureka and Novalose products. A slight difference persisted after 8 h of fermentation. After 24 h of fermentation pH values were identical (see Figure 2).

B) SCFA and n-butyrate production

15 Figures 3 and 4 show the amount of SCFA and of n-butyrate formed during the fermentation of the three samples. It appears that the IRP containing faeces gave the highest amount of both SCFA and n-butyrate. IRP gives rise to a faster production of n-butyrate according to Figure 4 more than 10 mMol/L was produced within 4 hours. Figure 3 shows that also the amount of the other SCFA is increased.

Example 4

Preparation of UHT milk drinks with resistant starch

25 This example describes the use of the debranched retrograded maltodextrin of example 1 in a UHT vanilla milk drink. The standard recipe used for the preparation of milk drink is given below.

Standard recipe:	
whole milk	1000ml
Satro mix*	12g
Sucrose	20g
Dextrose	20g

*Carrageenan, vanilla, colour

30 To this standard formula in one case 30g/l and in the second case 60g/l of IRP (see example 1) were added. The ingredients were mixed and homogenised at 50 bar. The UHT treatment was done with plate heating at 137°C for 5 seconds. The products were aseptically filled into 250 ml bottles.

40 After cooling to ambient temperature the products were characterised:

Product	RS-content (%)	Taste and mouthfeel
Standard	0,0	very liquid, no off-taste, no sandiness
+ 30g IRP/l	1,0	somewhat more mouthfeel, more creamy, no off-taste, no sandiness
+ 60g IRP/l	1,95	most mouthfeel, no sandiness

50 The results show that the major RS part survives even UHT-processing and is detectable in the final product using the method as mentioned in example 3. This is further confirmed by the DSC measurement (for method see example 2) of the residues obtained after pancreatic digestion (see fig. 5). The sample prepared with 60g/l IRP shows a strong endothermic transition with a peak temperature around 96°C whilst the standard product does not show any significant transition in this temperature range. The use of IRP does not only increase the RS content but improves the organoleptic properties to a significant extent. Due to the small particle size there is no sandiness and the use of IRP causes the impression of a higher fat content. IRP can therefore be used with advantage in low (no) fat products in order to improve the sensorial properties.

Claims

1. A starch-based composition characterised in that it contains more than 55 % (w/w) pancreatine resistant starch (RS), and wherein the resistant starch consists predominantly of alpha-glucans with a DP between 10 and 35 and has a DSC melting peak temperature below 115°C.
2. A starch-based composition according to claim 1 wherein the resistant starch has a DSC peak melting temperature between 90 and 114 °C.
3. A starch-based composition according to claim 1 further characterised in that it produces a high amount of short chain fatty acids, in particular n-butyrate, when fermented by micro-organisms normally found in the human hind gut.
4. A composition according to claim 1 characterised in that it is obtained from a partially degraded starch, which has been debranched and retrograded.
5. A composition according to claim 1 characterised in that it is retrograded debranched maltodextrin with a dextrose equivalent below 10.
6. A composition according to claim 5 characterised in that the maltodextrin is derived from potato starch or tapioca.
7. A composition according to claim 5 characterised in that the maltodextrin is derived from a starch selected from the group consisting of a high amylose starch, maize, wheat, tapioca and pea starch.
8. A composition according to claim 1, 2 or 3 consisting of a debranched retrograded acid thinned starch.
9. A composition according to claim 8 obtained from a starch selected from the group consisting of potato, tapioca, maize, pea or wheat starch.
10. A method for obtaining a starch composition according to any one of claims 1 to 9 characterised by the following steps :
 - a) thinning of starch,
 - b) enzymatic debranching of the thinned starch,
 - c) inactivation of the enzyme,
 - d) drying of the composition.
11. A method according to claim 10 containing the following steps:
 - a) a maltodextrin with a DE below 10 is dissolved in water,
 - b) the pH is adjusted and the solution is cooled to a optimum temperature for the activity of debranching enzymes,
 - c) enzyme is added and the mixture is incubated,
 - d) the enzyme is inactivated,
 - e) the mixture is spray-dried
 - f) the resistant starch is collected and optionally grinded
12. A food preparation characterised in that it contains up to 10% of a starch-based composition according to claim 1.
13. Use of a product according to claim 1 in food preparations in order to keep colon/rectum in a healthy state and to prevent diseases of the colorectal digestive tract
14. Use of a product according to claim 1 in pharmaceutical preparations for the prophylaxis or the treatment of colorectal diseases

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Fig. 1

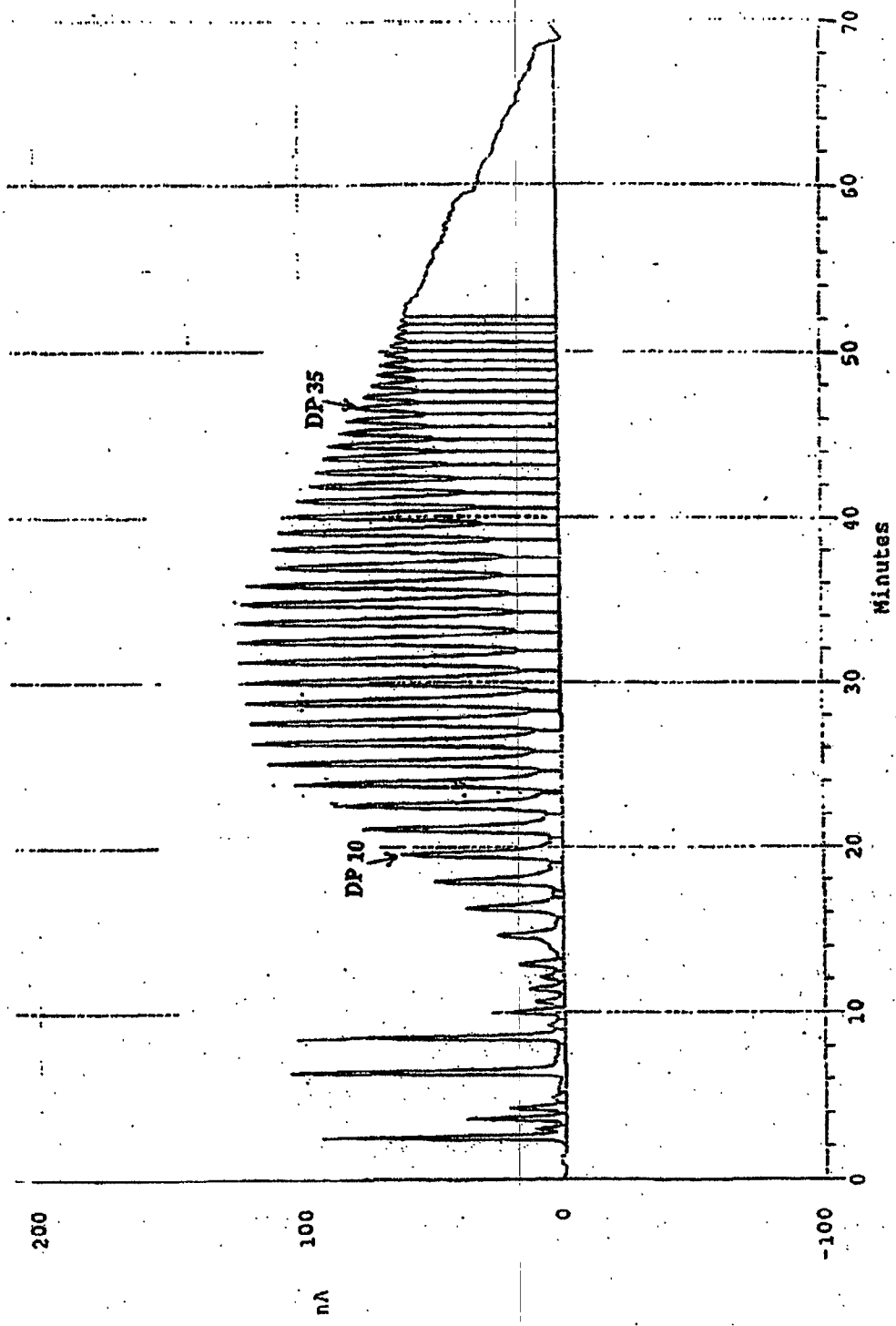


Fig. 2

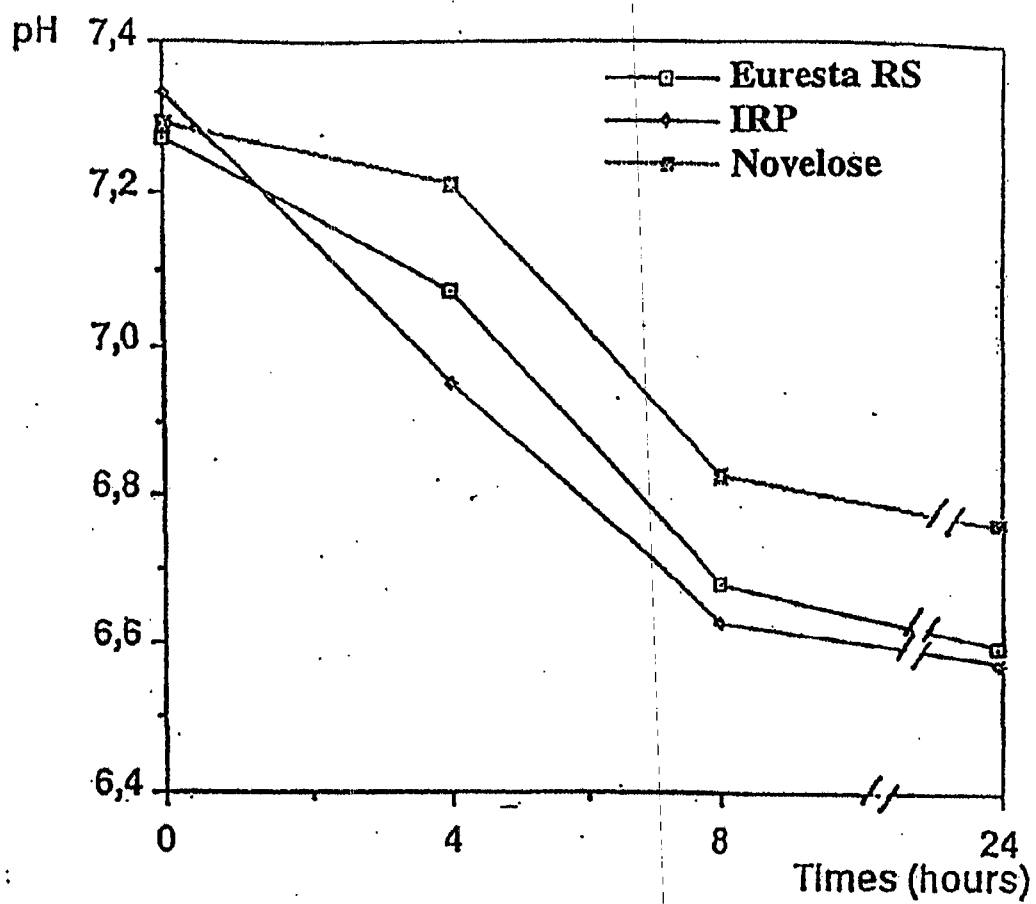
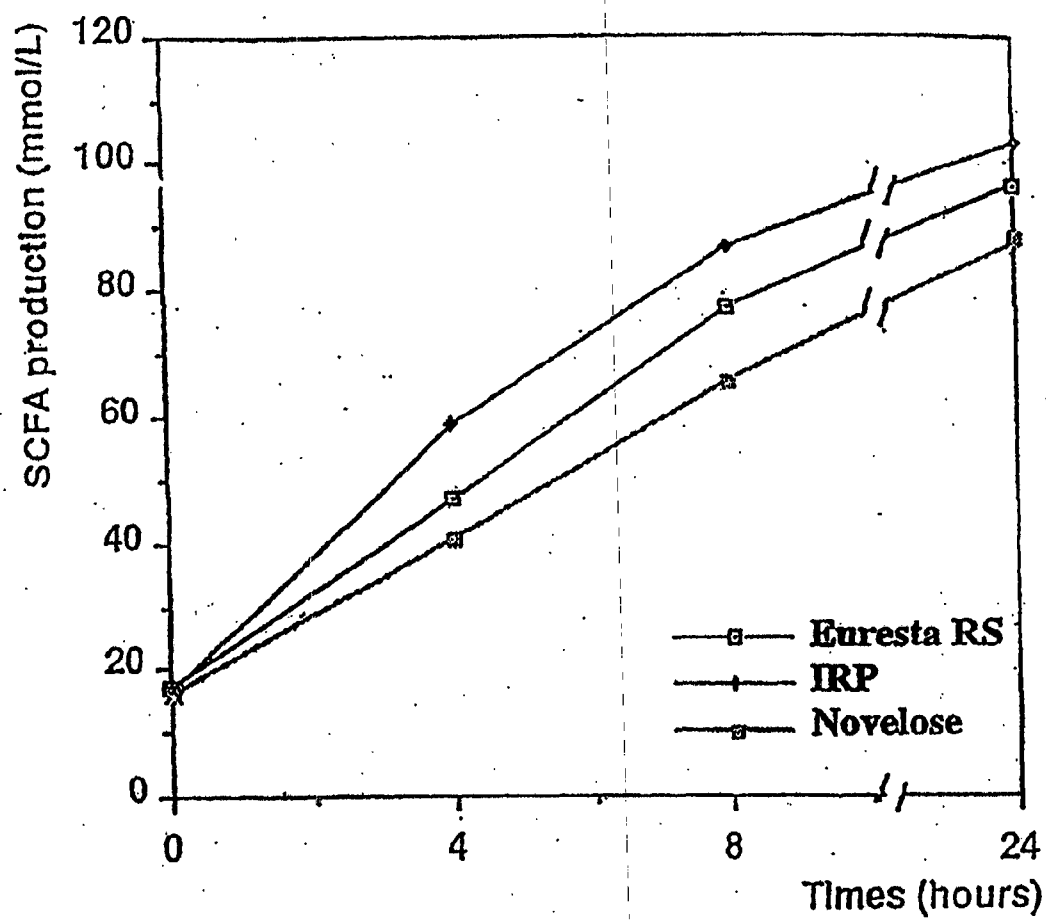
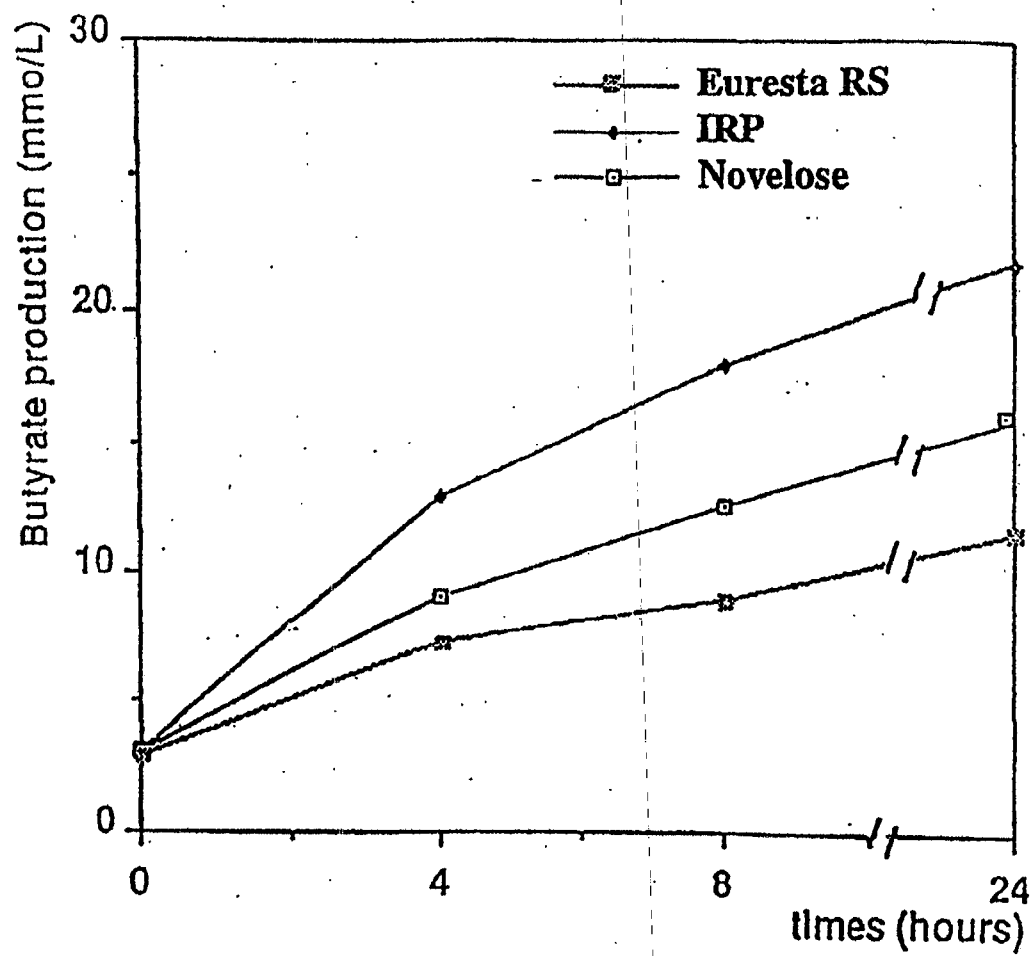


Fig. 3



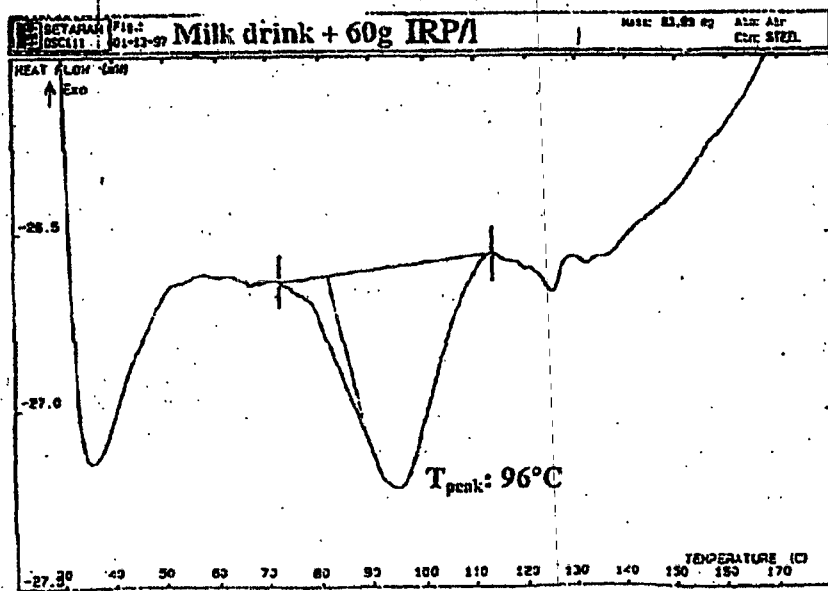
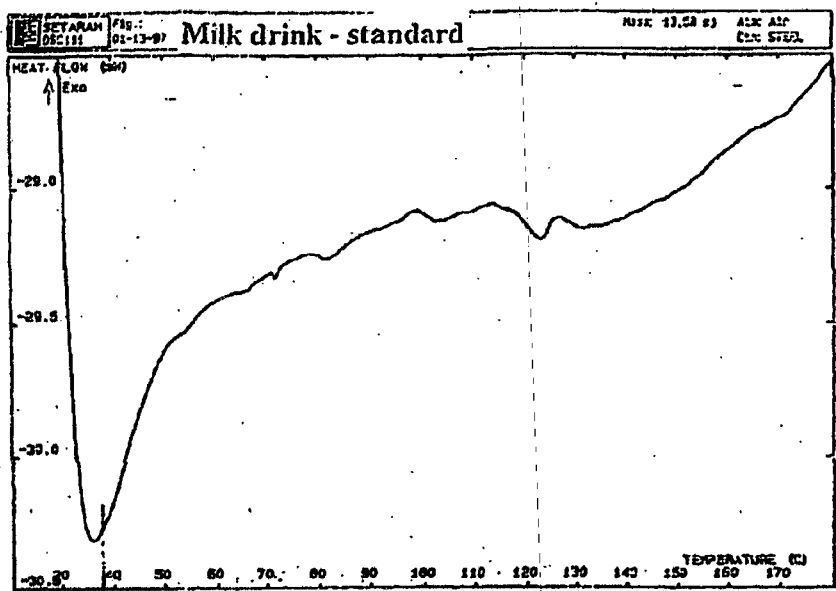
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Fig. 4



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Fig. 5: DSC curves of milk drink residues obtained after pancreatin digestion



(19)



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(11)

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(12)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
17.06.1998 Bulletin 1998/25

(51) Int Cl.⁶: **C08B 30/12, A23L 1/0522,
A61K 31/715**

(43) Date of publication A2:
10.06.1998 Bulletin 1998/24

(21) Application number: **97308720.7**

(22) Date of filing: **02.12.1997**

(84) Designated Contracting States:
**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
NL PT SE**
Designated Extension States:
AL LT LV MK RO SI

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(54) **Highly fermentable resistant starch**

(57) The present invention discloses that retrograded starch having more than 55% resistant starch with > 50% chains of DP 10 - 35 gives rise to a significantly higher amount of n-butyrate production under condi-

tions simulating the human colon. It is expected that such an increased n-butyrate production will diminish the development of colon diseases notably of colon cancer.

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European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 97 30 9720

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (INCL.6)
X	EP 0 688 872 A (CERESTAR HOLDING) 27 December 1995 * example 3 *	1-12	C08B30/12 A23L1/0522 A61K31/715
D,X	EP 0 564 893 A (NATIONAL STARCH AND CHEMICAL INVESTMENT HOLDING CORPORATION) 13 October 1993 * claims 8,15 *	1-14	
Y	US 5 051 271 A (IYENGAR ET AL.) * column 6 - column 8 * * column 4, line 30 - line 44 *	1-12	
D,Y	WO 91 07106 A (ENZYTECH) * page 7, line 22 - line 27 *	1-12	
Y	US 5 470 391 A (MALLEE ET AL.) 28 November 1995 * figure 2; example 4 *	1-12	
A	F.BORNET: "Technological treatments of cereals." CARBOHYDRATE POLYMERS, vol. 21, no. 2/3, 1993, BARKING GB, pages 195-203, XP000387697		TECHNICAL FIELDS SEARCHED (INCL.8) C08B C12P A23L A61K
P,X	WO 97 35889 A (OPTA FOOD INGREDIENTS) 2 October 1997 * page 7 - page 8 *	1-12	
P,X	EP 0 747 397 A (NATIONAL STARCH AND INVESTMENT) 11 December 1996 * table 1 *	1-12	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 24 April 1998	Examiner Leisen, H
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date O : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



(19)
Federal Republic of Germany
German Patent and Trademark Office

(10) DE 10 2004 054 133 A1 2005.06.09

Patent Claim

(21) File reference: **10 2004 054 133.7**
(22) Date of application: **08 November 2004**
(43) Publication date: **09 June 2005**

(51) Int. Cl.⁷: **A23L 1/30**

(30) Priority right: PV 2003-3042 10 November 2003 CZ (71) Applicant: Naturprodukt CZ spol. s.r.o., Praha – Vychod, Sestajovice, CZ	(74) Representative: Jeck · Fleck · Herrmann, Patent Attorneys, 71665 Vaihingen (72) Inventor: Inventor will be named at a later date.
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The following information has been extracted from the documents filed by the applicant

(54) Description: **Nutritional supplement on the basis of silymarin**

(57) Summary: Nutritional supplement on the basis of silymarin that contributes to an improvement in the quality of the diet with a positive effect on the reduction of the risk of diseases of civilization, and that is characterized by the fact that it contains as active ingredients a mixture of 10 to 90 percent by weight of the standardized flavonolignans fraction from the extract of blessed milk thistle (*Cardui mariae extractum siccum*) and 90 to 10 percent by weight of galenically prepared dried yacon root bulb (*Smallanthus sonchifolius*).
Thereby, the active ingredients are mixed in a beneficial implementation with an amount of up to 20 times the weight of a physiologically harmless vehicle, if applicable.

DE 10 2004 054 133 A1 2005.06.09

Description**Field of the Invention**

[0001] The invention relates to the composition of a nutritional supplement on the basis of silymarin, i.e. a standardized fraction of flavonolignans from the seed extract of the blessed milk thistle (*Silybum marianum*), which contributes to an improvement in the dietary quality with a positive effect on the reduction of the risk of a disease of civilization, such as diabetes mellitus and hyperlipidemia, and which potentially has a favorable influence on the entire state of health of the organism of at-risk groups of the population.

State of the Art

[0002] "*Silybum marianum* (L.) Gaertner" (blessed milk thistle, Asteraceae) has been used in traditional European medicine for centuries because of its hepato-protective properties. Its roots, leaves, and seeds were prepared as food (Morazzoni and Bombardelli, 1995). The designation silymarin is used for the extract from the seed of "*S. marianum* (*Cardui mariae extractum siccum*)", which contains 70-80% of the silymarin complex, i.e. a mixture of the flavonolignans standardized to silibinin and 30-20% of a chemically non-defined fraction.

[0003] "*S. marianum*" extract and silibinin protect the liver from the effects of hepatotoxic substances (s. DE-A-19 23 082 and DE-A 35 37 656).

[0004] "*S. marianum*" extract and silibinin prevent oxidization induced by copper-containing ions in human low-density lipoproteins in vitro (s. Skottová et al., 1999).

[0005] The extract and the flavonolignans of "*S. marianum*", particularly bilibinin [sic.] bishemisuccinate show good effectiveness as supporting therapy during treatment with cytostatic agents (s. Lentzen et al., 1998).

[0006] In the Czech Republic, "*S. marianum*" extract constitutes an active component of medications that are classified as hepato-protective agents, e.g. FLAVOBION[®], SILYGAL[®], LAGOSA[®], or LEGALON[®] (the extract content of a tablet is 100-204mg, i.e. 70-150mg of silymarin complex), the phyto preparation SIMEPAR[®] contains "*S. marianum*" extract in a combination with the vitamin B complex; HEPABEN[®] with "*Fumaria officinalis*" extract, and HEPATOFALK[®] PLANTA with "*Chelidonium majus*" extract. The indication for these preparations is broadened to include the cholekinetic and choleric effects. For the nutritional supplement, the extract amount in one tablet is 15-100mg, i.e. 10-70mg of silymarin complex.

[0007] Also described is a nutritional supplement with an effect on plasma lipids and the anti-oxidation capacity of blood according to CZ PV 2000-4404, which contains as active ingredients a mixture of blessed milk thistle extract and at least one plant oil containing n-3 and n-6 poly-unsaturated fatty acids. This preparation has a positive effect on cholesterol parameters, liver steatosis, and the anti-oxidation status of the organism; however, it does not have a prebiotic effect.

[0008] In connection with the search for new natural sources with anti-diabetic, nutritional, immuno-stimulating and other properties, it was determined through experiments that processed root bulbs of the yacon plant (*Smallanthus sonchifolius*) possess a prebiotic effect, which is described, for example, in the articles of Romin Pedreschi et al.: Andean Yacon Root (*Smallanthus sonchifolius* Poepp. Endl) Fructooligosaccharides as a Potential Novel Source of Prebiotics", Journal of Agricultural and Food Chemistry, 2003, or Ales Lebeda et al.: "Biologická a chemická variabilita maky a jakonu", Chem. Listy 97, pages 548-556, 2003.

Conceptual Formulation

[0009] It is thus the aim of the invention on hand to create a nutritional supplement that combines the known effect of silymarin with the newly discovered effect of yacon.

DE 10 2004 054 133 A1 2005.06.09

Summary of the Invention

[0010] The nutritional supplement on the basis of silymarin exhibits a new effectiveness in comparison with the state of the art up until now; contributing to an improvement of the quality of the diet with a positive effect on the reduction of the risk of a disease of civilization, whereas the invention lies in the fact that the nutritional supplement contains as active ingredients a mixture of 10 to 90 percent by weight of the standardized flavonolignans fraction from the extract of blessed milk thistle (*Cardui mariae extractum siccum*) and 90 to 10 percent by weight of galenically prepared dried yacon root bulb (*Smilax szechuanensis*).

[0011] Another design feature of the invention is the fact that the active ingredient components of the nutritional supplement are mixed with an amount of up to 20 times the weight of a physiologically harmless vehicle, if applicable, which contains a beneficial combination of 0.1 to 80 percent by weight of an inert filler substance and 0.05 to 10 percent by weight of lubricants.

[0012] According to another design feature of the invention, the inert filler substance has been chosen from a substance group that contains microcrystalline cellulose and/or silicon oxide and/or potato starch and/or cornstarch and/or modified starch, and the lubricants have been chosen from a substance group that contains talcum and/or hydrated silicon oxide and/or stearic acid and/or calcium stearate.

[0013] The effectiveness of the nutritional supplement in the described composition manifests itself in the fact that the combination of silymarin with yacon positively influences the prevention of chronic diseases of civilization characterized by an atherogenic lipoprotein profile, liver steatosis, and a deteriorated anti-oxidation status of the organism. Furthermore, the nutritional supplement also distinguishes itself with evidenced prebiotic effectiveness, because yacon contains a type of sugar that supports the non-pathogenic intestinal flora, which has a positive effect on the functioning of the intestinal system.

Implementation Example

Examples for the implementation of the invention

[0014] The possibilities of a concrete application for the invention are evident from the following examples:

Example 1

[0015] The effectiveness of the selected composition of the nutritional supplement, i.e. the nutraceutical was tested on a group of 44 volunteers, so-called probands, of the counseling center for metabolic issues of one of the hospitals in the Olmütz region, whereas the criterion for the inclusion in the study was the finding of a metabolic syndrome, i.e. of evidenced hyperinsulinemia, hypertension, hypertriglyceridemia, and obesity. The ethics committee of the respective hospital gave its consent to conduct the study. The probands were divided into three groups:

1. PLACEBO: 15 probands, i.e. patients of this group were administered a daily dose of 3.2g of microcrystalline cellulose P as a placebo, whereas one capsule contained 200mg of cellulose P.
2. SILYMARIN: 14 probands were administered a daily dose of 0.8g of silymarin, whereas one capsule contained 134mg of silymarin.
3. SILYMARIN-YACON: 15 probands of this group were administered a daily dose of a combination preparation of 0.8g of silymarin and 2.4g of yacon, whereas 1 capsule contained 50mg silymarin and 150mg of galenically prepared yacon root bulb.

[0016] The clinical study was conducted during the course of 2002, whereas the preparations were administered to the probands for a time period of 90 days. Subsequently, the results of the study were evaluated by means of the statistical procedure of analysis of variance, the so-called ANOVA. The initial results at the beginning of the 1st month of the course of the study and the final results at the end of the 3rd month of the course of the study, as well as their discrepancy, were statistically analyzed and compared with the results from the placebos. The results are displayed in table 1.

[0017] The resulting effectiveness of the nutraceutical that only contained silymarin manifested itself in a statistically significant reduction in the level of triglycerides. A statistically significant reduction in the level of triglycerides and glucose in the blood of the patients was noted with the combination of yacon and silymarin. No statistically significant negative effects whatsoever of both administered nutraceuticals on the parameters of the safety lab were detected.

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Table 1

The effect of silymarin (SIL) + yacon (YAC) on triglycerides (TAG), cholesterol, and glucose on probands with a metabolic syndrome (average $\pm$ S.D. *p < 0.05)

Parameter	Group	1 st Day	90 th Day	After 90 Days	Trend	Signif.
TAG	SIL	2.16 $\pm$ 1.10	1.88 $\pm$ 0.69	- 0.28 $\pm$ 0.93		0.029*
Mmol.l ⁻¹	Placebo	1.94 $\pm$ 0.89	2.78 $\pm$ 1.91	+ 0.84 $\pm$ 1.56		
TAG	SIL+JAK	2.24 $\pm$ 0.97	1.92 $\pm$ 0.78	- 0.32 $\pm$ 0.66	*	0.017*
Mmol.l ⁻¹	Placebo	1.94 $\pm$ 0.89	2.78 $\pm$ 1.91	+ 0.84 $\pm$ 1.56		
Cholesterol	SIL	6.49 $\pm$ 1.13	6.05 $\pm$ 1.17	- 0.44 $\pm$ 1.01	↔	0.927
Mmol.l ⁻¹	Placebo	6.56 $\pm$ 2.53	6.17 $\pm$ 1.21	- 0.39 $\pm$ 1.75		
Cholesterol	SIL+JAK	5.43 $\pm$ 1.11	5.55 $\pm$ 1.07	+ 0.13 $\pm$ 1.01	↔	0.327
Mmol.l ⁻¹	Placebo	6.56 $\pm$ 2.53	6.17 $\pm$ 1.21	- 0.39 $\pm$ 1.75		
Glucose	SIL+JAK	6.93 $\pm$ 1.56	6.50 $\pm$ 1.78	- 0.43 $\pm$ 0.83	*	0.041*
Mmol.l ⁻¹	SIL	6.79 $\pm$ 1.99	7.05 $\pm$ 2.63	+ 0.26 $\pm$ 0.90		

Example 2

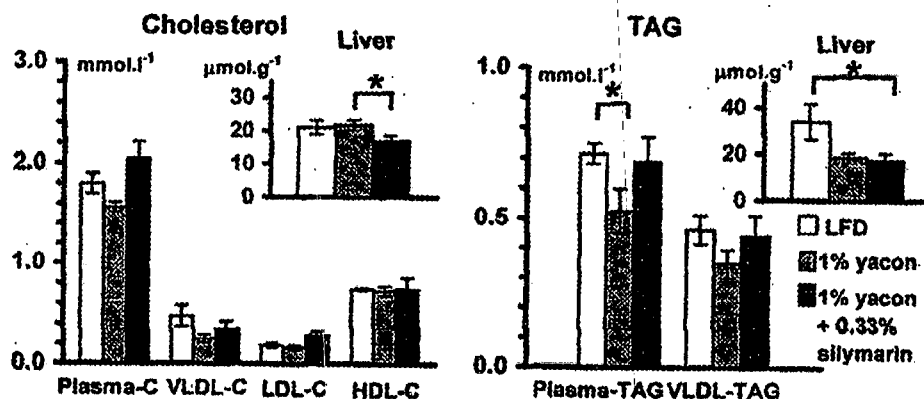
[0018] The hypolipidemic and anti-oxidation effects of yacon, silymarin, and the combination of these drugs were studied on rats in two experimental models of diet-induced changes in the lipid metabolism and the anti-oxidation status of the rats. Both diets contained 1% cholesterol and 10% of qualitatively different fat. In order to induce liver steatosis with fibrosis and proatherogenic changes in the lipoprotein profile, lard was used, which is rich in saturated and mono-unsaturated fatty acids (lard fat diet = LFD), and in order to sensitize the oxidation status of the rats, black currant oil was used, which is rich in poly-unsaturated fatty acids (black currant oil diet = BCOD). Yacon and silymarin were administered for 3 weeks as a component of these diets at a concentration of 0.1, 0.5, and 1.0%. For the drug combination, dietary concentrations of 1% yacon (micronized dust from dried root bulbs) and 0.33% silymarin were chosen.

[0019] The combination of 1% yacon and 0.33% silymarin (figure 1) led to a reduction of VLDL cholesterol and prevented an increase in plasma and VLDL triglycerides. In the liver, the effect of the drug combination manifested itself in the reduction of the cholesterol level and TAG in the case of the fatty diet (LFD). The oxidation parameters were only insignificantly influenced by the drug combination in the case of the fatty diet; however, in the case of the diet with poly-unsaturated fatty acids (BCOD), the level of blood GSH increased after administration of the drug combination. These results hint towards a potential hypotriglyceridemic and VLDL-reducing effect of the yacon root bulb. Furthermore, in the combination of yacon with silymarin, which mostly has a positive effect on cholesterol parameters and GSH, a reduction of lipids in the liver and an increase of GSH in the blood were also achieved. Therefore, the combination of yacon and silymarin proves to be promising in terms of a positive effect on and the prevention of diseases of civilization, which are characterized by an atherogenic lipoprotein profile, liver steatosis, and a deterioration of the anti-oxidation status of the organism.

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Figure 1

The effect of a mixture of yacon + silymarin on the profile of lipoproteins and liver lipids in rats
(Average $\pm$ S.E., $n = 6$, * $p < 0.05$).



Example 3

[0020] As an optimal medication form that complies with the requirements for stability and homogeneity, a 300mg gelatin capsule with the following composition, for example, was chosen after clinical trial:

• ground dried yacon root bulbs	150mg
• micronized silymarin complex	70mg
• silicon stearate	6mg
• cornstarch	37mg
• microcrystalline cellulose	37mg

Industrial usability

[0021] The nutritional supplement on the basis of silymarin can be used for the prevention of diseases of civilization, such as diabetes mellitus and hyperlipidemia; potentially as a preventative nutritional supplement that has a positive effect on the overall status of the human organism.

Patent Claims

1. Nutritional supplement on the basis of silymarin that contributes to an improvement in the quality of the diet with a positive effect on the reduction of the risk of diseases of civilization, and that is characterized by the fact that it contains as active ingredients a mixture of 10 to 90 percent by weight of the standardized flavonolignans fraction from the extract of blessed milk thistle (*Cardui mariae extractum siccum*) and 90 to 10 percent by weight of galenically prepared dried yacon root bulb (*Smallanthus sonchifolius*).

2. Nutritional supplement according to claim 1, characterized by the fact that its active ingredients are mixed with an amount of up to 20 times the weight of a physiologically harmless vehicle, if applicable.

3. Nutritional supplement according to claim 2, characterized by the fact that the physiologically harmless vehicle contains 0.1 to 80 percent by weight of an inert filler substance and 0.05 to 10 percent by weight of lubricants.

4. Nutritional supplement according to claim 3, characterized by the fact that the inert filler substance has been chosen from a substance group that contains microcrystalline cellulose and/or silicon oxide and/or potato starch and/or cornstarch and/or modified starch.

5. Nutritional supplement according to claim 3, characterized by the fact that the lubricants were

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chosen from a substance group that contains talcum and/or hydrated silicon oxide and/or stearic acid and/or calcium stearate.

There is no page of drawings in the following



Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-116255- -00000-0000

Fecha: 2010-09-20 16:43:47 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 411 PRESENTACIÓN Folios: 210

PATENTE DE INVENCION ☐

MODELO DE UTILIDAD ☐

☐	Indicación que se solicita una patente.
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Descripción de la invención
☐	Dibujos de ser estos pertinentes
☐	Comprobante de pago de las tasas establecidas (De ser el caso formato de descuento)
Completa ☐	Incompleta ☐

PATENTE DE INVENCION PCT ☒

MODELO DE UTILIDAD PCT ☐

Art.33 Decisión 486/00, Circular Única

☒	Indicación que se solicita una PCT
☒	Copia de la solicitud en español, tal como fue presentada inicialmente (capítulo descriptivo, reivindicatorio, resumen)
☒	Dibujos de ser estos pertinentes
☒	Comprobante de pago de las tasas establecidas (de ser el caso formato de descuento)
Completa ☒	Incompleta ☐

DISEÑO INDUSTRIAL ☐

(Art. 119 Decisión 486/00)

☐	Indicación que se solicita Diseño industrial
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Representación gráfica y fotográfica del Diseño industrial o muestra del material que incorpora el diseño
☐	Comprobante de pago de las tasas establecidas
Completa ☐	Incompleta ☐

ESQUEMA DE TRAZADO ☐

(Art. 92 Decisión 486/00)

☐	Indicación que se solicita un esquema de trazado
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Representación gráfica de un esquema de trazado
☐	Comprobante de pago de las tasas establecidas
Completa ☐	Incompleta ☐



Bogotá D.C.,
2020

Doctor(a)
SALZBURG MAGDA
Apoderado(a) y/o Representante de
CM & D PHARMA LIMITED

REFERENCIA	Radicación No.	10 116255
	Solicitud internacional	PCT/EP2009/052038
	Fecha inicio fase nacional	22/09/2010
	Trámite	11
	Evento	379
	Actuación	430
	Oficio No.	

13143

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.

2. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante, conforme a lo establecido por el artículo 26 literal k) de la Decisión 486 de la Comisión de la Comunidad Andina. Cuando se trate de documentos otorgados en el extranjero, estos deberán legalizarse de conformidad con lo dispuesto por los artículos 259 y 260 del Código de Procedimiento Civil. Cuando se trate de las declaraciones a que hace referencia la Regla 4.17 ii) del Tratado de Cooperación en Materia de Patentes PCT., el alcance de las tales declaraciones se determinará según indiquen claramente una cesión de derechos del inventor al solicitante o su causante, de conformidad con lo dispuesto en el numeral 5.2.15 del Capítulo Quinto, Título X de la Circular Única de Superintendencia de Industria y Comercio.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Continúa en la página siguiente...

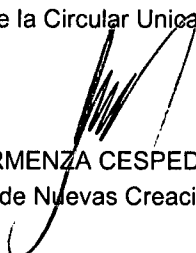


Continuación radicado No: 10 116255

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Industria y Comercio
SUPERINTENDENCIA

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Unica No.10 de 2001.


ALIX CARMENZA CESPEDES DE VERGEL
Directora de Nuevas Creaciones (c)

08 OCT. 2010

El anterior requerimiento fue notificado por fijación en lista de fecha _____.
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