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Evaluación del mecanismo de acción de una fracción concentrada en lectinas de frijol Tépari (*Phaseolus acutifolius*) en cáncer de colon.

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RESUMEN

Las lectinas son estructuras proteicas que tienen la capacidad de interactuar con hidratos de carbono específicos, lo que les confiere propiedades de reconocimiento y mediadoras de la comunicación celular. En cáncer los azucares en de la membrana celular se ven alterados cuali y cuantitativamente, lo que ha permitido proponer a las lectinas como herramientas para el diagnóstico y tratamiento del diversos tipos de cáncer. Por otro lado las lectinas de frijol Tépari en especial han sido estudiadas por sus efectos citotóxicos en cultivos celulares y su baja toxicidad en animales de experimentación; lo cual lleva a el presente trabajo a determinar el efecto de una fracción concentrada en lectinas de dicho frijol en modelos de cáncer de colon *in vitro* e *in vivo,* así como a la evaluación de los mecanismos de inducción de muerte. Para tales fines se utilizaron 3 líneas celulares de cáncer de colon (HT-29, SW-480 y RKO) cada una con diferentes características genotípicas y se evaluaron mecanismo de muerte como necrosis y apoptosis, por métodos de ELISA, Citometría de flujo y RT-qPCR. Por otro lado se desarrolló un modelo animal para la generación de fosas cripticas aberrantes en ratas Sprague Dawley y se determinaron marcadores de muerte y de progresión tumoral tales como (PCNA, p53, Bcl-2, caspasa-3, Akt, p-Akt, Citocromo-c, p-caspasa-9, p-GSK3) por inmunohistoquímica. Los resultados de las pruebas *in vitro* muestran que la FCL tiene efectos sobre la proliferación y la sobrevivencia celular, activando vías apoptóticas relacionadas con la actividad de p53 y Bcl-2; por otro lado no se detectó muerte celular por necrosis. En los experimentos *in vivo* la FCL mostró de la misma forma efectos antiproliferativos en las ratas a las que se les indujo cáncer, incrementando la señal de los marcadores de muerte celular como caspasa-3, citocromo c y disminuyendo los de progresión como p-Akt, PCNA, principalmente. En conclusión la FCL es capaz de inducir la muerte por apoptosis en las células de cáncer de colon de forma dependiente de la malignidad del linaje, al igual que en un modelo animal disminuye el grado de lesión tumoral y activa vías apoptóticas.

Palabras clave: Apoptosis, cáncer de colon, lectinas, *Phaseolus acutifolius*.

SUMMARY

The lectins are protein structures that have the ability to interact with specific carbohydrates, which gives them recognition properties and mediators of cell communication. In cancer sugars in the cell membrane are altered qualitatively and quantitatively, which has allowed to propose lectins as tools for the diagnosis and treatment of various types of cancer. On the other hand Tepari bean lectins in particular have been studied for their cytotoxic effects in cell cultures and their low toxicity in experimental animals; which leads to the present work to determine the effect of a concentrated fraction in lectins of said bean in colon cancer models in vitro and in vivo, as well as to the evaluation of death induction mechanisms. For these purposes, 3 colon cancer cell lines (HT-29, SW-480 and RKO) each with different genotypic characteristics were used, and death mechanism such as necrosis and apoptosis were evaluated by ELISA, flow cytometry and RT-qPCR methods. On the other hand, an animal model for the generation of aberrant cryptic pits in Sprague Dawley rats was developed and markers of death and tumor progression were determined such as (PCNA, p53, Bcl-2, caspase-3, Akt, p-Akt, Cytochrome-c, p-caspase-9, p-GSK3) by immunohistochemistry. The results of in vitro tests show that FCL has effects on proliferation and cell survival, activating apoptotic pathways related to the activity of p53 and Bcl-2; On the other hand, no cell death was detected due to necrosis. In the in vivo experiments the FCL showed in the same way antiproliferative effects in the rats that were induced cancer, increasing the signal of cell death markers such as caspase-3, cytochrome c and decreasing the progression as p-Akt, PCNA, mainly. In conclusion, FCL is able to induce death by apoptosis in colon cancer cells in a manner dependent on lineage malignancy, as in an animal model the degree of tumor lesion decreases and active apoptotic pathways.

(**Key words**: Apoptosis, colon cancer, lectins, *Phaseolus acutifolius*)

A mi familia…

"La familia es la brújula que nos guía. Es la inspiración para llegar a grandes alturas, y nuestro consuelo cuando ocasionalmente fallamos"

(Brad Henry).

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1. INTRODUCCIÓN

El cáncer es hoy en día la principal causa de muerte por enfermedad en el mundo. Esta enfermedad varía en sus manifestaciones clínicas y en su respuesta a las medidas terapéuticas, pero que, en esencia, comparten mecanismos desencadenantes comunes (OMS, 2012). Se han descrito más de cien formas distintas de cáncer de acuerdo con el órgano o tejido de origen y el tipo de célula a partir del cual se forman. Los más frecuentes son los llamados carcinomas, que constituyen cerca del 90% de los cánceres, se generan en los epitelios o capas celulares que recubren la superficie del cuerpo y cuya frecuencia puede incrementarse entre los veinte y los sesenta años de edad. Entre ellos, los más comunes son los que afectan al pulmón, al intestino grueso, a las mamas y al cuello uterino (Rafiemanesh y col., 2016). Las leucemias y linfomas se producen a partir de las células formadoras de la sangre que residen en la médula ósea y en los tejidos linfáticos y, aunque son menos frecuentes que los carcinomas, causan un mayor impacto moral, social y económico pues afectan a niños y jóvenes reduciendo su esperanza de vida y productividad. Los sarcomas son los más raros y se originan en las estructuras de soporte como el tejido fibroso, así como en los vasos sanguíneos (Tirado-Gomez y col., 2008; Rafiemanesh y col., 2016).

Los tumores cancerosos constituyen agrupaciones de células que adquieren un comportamiento autónomo de la capacidad de dividirse y dejan de respetar las reglas del organismo, las cuales imponen a las células normales de cada tejido un crecimiento restringido para que se logre un desarrollo armonioso (Campos-Alegria y Garcia-Garrido, 1997). El cambio de una célula normal a una cancerosa se produce por etapas. En aquellos tejidos accesibles a ser estudiados como la piel o el cuello del útero se han podido observar, en primer lugar, alteraciones sutiles de algunas células. Éstas adquieren una morfología y comportamiento distintos de los de las células vecinas y constituyen lo que se conoce como metaplasias y displasias (cambios de un tipo celular en otro). El crecimiento de esas células alteradas puede

acentuarse y dar lugar a un tumor localizado que, si no invade a los tejidos vecinos, se considera “benigno”. Nuevas modificaciones en las células tumorales traen consigo la capacidad de migración e invasión hacia los tejidos aledaños, lo que da lugar a tumores malignos o cancerosos (Gatenby y Vicent, 2003). Las células viajan a través del torrente sanguíneo para ir a anidarse en otros órganos y formar nuevos tumores o metástasis. Hoy en día las terapias para combatir el cáncer son en muchos casos poco efectivas, ya que no logran erradicar el total de las células dañadas, y muy agresivas con el organismo debido a que no logran su efecto solo en células dañadas, sino atacan células en general provocando la afección de las mismas (Campo-Alegria y Garcia-Garrido, 1997).

En la lucha incesante por encontrar nuevas terapias para combatir el cáncer se han estudiado diferentes moléculas de oroigen natural, entre ellas las lectinas vegetales. Estas glicoproteínas tienen alta especificidad contra células cancerígenas a través de mecanismos específicos como la inducción de apoptosis. En nuestro laboratorio se ha determinado que la lectinas de frijol Tépari (*Phaseolus acutifolius*) presentan acción específica y diferencias sobre células cancerígenas, particularmente de cáncer de colon (Torres-Arteaga y col., 2016). Se ha observado que estas lectinas disminuyen la tumorogénesis temprana en donde se ha determinado que aumenta la actividad de caspasa 3 así como la expresión de genes apoptóticos (datos no publicados, tesis de Ferriz Martínez, 2015; tesis de López-Martínez, 2016). Por lo anterior, el presente trabajo se enfocó en determinar el mecanismo de acción de la fracción concentra en lectinas de frijol Táperi en sistemas *in vitro* e *in vivo*.

2. MARCO TEÓRICO

2.1 Cáncer de colon.

El cáncer es la principal causa de mortalidad a escala mundial. Se le atribuyen 7.6 millones de defunciones (aproximadamente el 13% del total) ocurridas en todo el mundo en 2008. Los principales tipos de cáncer son: pulmonar (1,4 millones de defunciones); gástrico (740 000 defunciones); hepático (700 000 defunciones); colorrectal (610 000) defunciones; mamario (460 000 defunciones) (Ferlay y col., 2015)

Más del 70% de las defunciones por cáncer se registraron en 2008 en países de ingresos bajos y medianos. Se prevé que el número de defunciones anuales mundiales por cáncer seguirá aumentando y pasará de 11 millones en 2030 (Globocan, 2008; Ferlay y col., 2015) Existen diversas clasificaciones de tipos de cáncer dependiendo desde su ubicación hasta de su forma de afección, el Instituto Nacional de Cáncer de E.U. los clasifica de acuerdo a la frecuencia con la que aparece siendo los más comunes cáncer de seno (mama), cáncer de próstata, cáncer de colon y recto, cáncer de pulmón, linfoma no Hodgkin, cáncer de la tiroides, cáncer de riñón (células renales), cáncer de endometrio, cáncer del hígado, leucemia, cáncer de vejiga, (Instituto Nacional de Cancerologia., 2011; Ferlay y col., 2015)

El cáncer colorrectal (CCR) presenta una incidencia anual de aproximadamente 1 millón de casos y una mortalidad anual de más de 500.000 (Ferlay y col., 2015; Rafiemanesh y col., 2016). Se prevé que el número absoluto de casos aumentará en las próximas dos décadas como resultado del envejecimiento y la expansión de las poblaciones, tanto en los países desarrollados como en los países en desarrollo (Gastroenterologia, 2007). En México, en un periodo de 10 años ente 1998 y 2007, se observó un aumento de 80% de casos registrados de cáncer de colon según un estudio realizado en el Hospital General de México en 2009 (Tirado-Gómez y Betancourt, 2008; Lagunes-Gasca y Villanueva-Herrero, 2009; Rafiemanesh y col.,

2016). Lo anterior muestra la importante creciente de este padecimiento en nuestro país y lo importante de descubrir nuevas terapias tanto preventivas como tratamientos eficientes. Las terapias actuales para combatir el cáncer es decir, la resección, la radiación y la quimioterapia, no son tan efectivas ya que muchas veces no pueden librar al paciente de la totalidad de células cancerosas (Monteverde, 2008; Silva, 2008). Entre las estrategias terapéuticas experimentales o en estudio se encuentran la inmunoterapia, la terapia génica, la inhibición de la actividad de proteínas promotoras de cáncer y la inhibición de la formación de nuevos vasos sanguíneos (angiogénesis) (Torrecillas, 2008).

El proceso de carcinogénesis es de largo tiempo y es dado por una serie de daños que afectan genes reparadores del DNA (Gatenby y Vincent, 2003). La acumulación de estas alteraciones genéticas en el epitelio colónico requiere algunos años, normalmente décadas, que coincide con la edad media de los pacientes diagnosticados de CCR (alrededor de 65-70 años) (Jackson y Loeb, 2001). Además de las mutaciones, otros cambios genéticos están implicados en la tumorogénesis: metilación de ADN, amplificaciones, sobreexpresión y deleciones (Campo Alegria y Garcia Garrido, 1997). En la Figura 1 se muestra el proceso de carcinogénesis colónica en sus diferentes etapas.

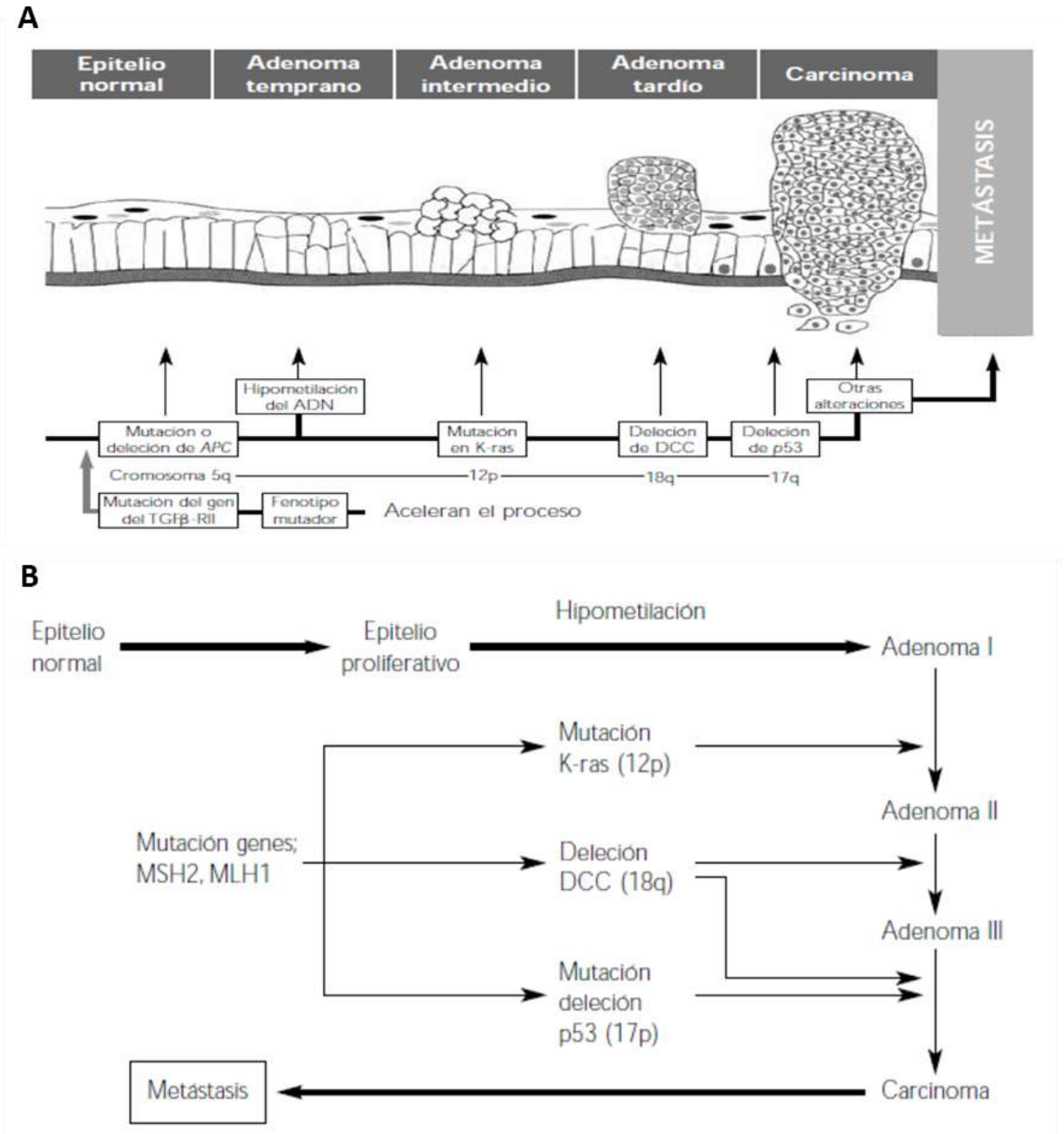

Figura 1.Esquema de la carcinogénesis colónica (Campo Alegria y Garcia Garrido, 1997). A) Representación del proceso ordenado de la carcinogénesis en colon, así como las principales mutaciones y deleciones de protoncogenes y genes supresores de tumores a través del proceso. B) Esquema del proceso de progresión del cáncer de colon**.**

2.2 Lectinas y cáncer

En la actualidad diversos estudios apuntan a la búsqueda de terapias contra el cáncer de colon, que sean más específicas y menos agresivas para el organismo (Monteverde, 2008; Silva, 2008; Torrecillas, 2008). El término Lectinas se aplica a glicoproteínas o proteínas de origen no inmune que comparten la propiedad de enlazarse de forma específica y reversible a carbohidratos, ya sean libres o que formen parte de estructuras más complejas (Goldstein y Hayes, 1978; Freed, 1999; Estrada-Martínez y col., 2017). Estas proteínas usualmente tienen al menos dos sitios de unión por molécula (Cassandra y Barroso, 2016) y, como característica particular, tienden a aglutinar a las células a las cuales se unen (Kim y col., 1974; Rüdiger y Gabius, 2001). La capacidad de estas proteínas para interactuar con células de la respuesta inmune permite que ejerzan efectos inmunosupresores, otras son tóxicas, algunas de ellas inhiben el crecimiento de células tumorales y pueden participar en la adhesión celular (Kiss y col., 1997; Lichtenstein y Rabinovich, 2013). Todas las actividades biológicas reportadas para las lectinas tienen en común el reconocimiento de un receptor oligosacarídico (Castillo-Villanueva y Abdullaev, 2005; Estrada-Martínez y col., 2017).

En los vegetales, la mayoría de las lectinas se encuentran en órganos de reserva, lo cual es una evidencia indirecta de su papel como proteínas de defensa (Mendoza y col., 2007). Una gran diversidad de lectinas han sido aisladas y caracterizadas de diferentes especies vegetales y/o animales (Fu y col., 2011). Se ha podido identificar que existe similitud estructural entre las lectinas de una misma familia y, en algunos casos, se ha demostrado que las lectinas son capaces de reconocer carbohidratos en una determinada configuración o secuencia de carbohidratos es decir, que las características de especificidad por estructuras sacarídicas también es altamente conservada entre proteínas de una misma especie (Castillo-Villanueva y Abdullaev, 2005). En la tabla 1 se muestran las principales lectinas y la especificidad a residuos de H-C o secuencias de oligosacáridos (Ghazarian y col., 2011).

Tabla 1. Lectinas y su afinidad por distintos Carbohidratos.

Nombre común	Acrónimo	Preferencias de unión a residuos de azúcares o secuencias deoligosacáridos
Dolichos biflorus	DBA	Terminal FP[a]>GalNAcα1,3GalNAc>GalNAcα1,3Gal
Glycine maximus	SBA	Terminal α,βGalNAc>--,βGal
Vicia villosa	VVA	Terminal GalNAcα1,3Gal>GalNAcα1,6Gal=GalNAc-serina
Griffonia simplicifolia	GSL-I	Terminal αGal
Arachis hypogaea	PNA	Terminal Galβ1,3GalNAc
Ricinus communis	RCA-I	Galβ1,4GlcNAc>βGal>βGal
Sophora japonica	SJA	TerminalGalβ1,3GalNAc>Galβ1,3GlcNAc>β,βGalNAc>β,βGal
Artocarpus integrifolia	JAC	Terminal Galβ1,3GalNAc>GalNAcα1,6Gal tanto en la formo mono como disialatada.
Erythrina cristagalli	ECL	Oligosacáridos triantenarios o tetraantenarios conteniendo tres o cuatro ramificaciones de N-acetillactosamina respectivamente
Phaseolus vulgariserythroagglutinin	PHA-E	Complejos bi- o tri-antenarios unidos en forma N (bisected)
Phaseolus vulgarisleukoagglutinin	PHA-L	Secuencias complejas muy ramificadas (triantenarias o más) (non-bisected)
Triticum vulgare	WGA	GlcNAc(β1,4GlcNAc)$_{1-2}$>β1,4GlcNAc>NeuAc
Succinyl WGA	S-WGA	GlCNAc(β1,4GlcNAc)$_{1-2}$
Griffonia(=Bandeiraea)simplicifolia	GSL-II	Terminal α,βGlcNAc, glucógeno
Solanum tuberosum	STL	poli-N-acetillactosamina
Lycopersiconesculentum	LEL	Oligosacáridos conteniendo poli-N-acetilactosamina
Datura stramonium	DSL	Gal β1,4GlcNAc(N-acetillactosamina)>GlcNAc
Canavalia ensiformis	Con-A	α Man> α Glc
Lens culinaris	LCA	región fucosilada del núcleo de oligosacáridos bi otriantenarios unidos en forma N-glicosídica
Pisum sativum	PSA	Igual que LCA
Galanthus nivalis	GNA	Manα1,3Man>Manα1,6Man>Manα1,2Man
Ulex europaeus	UEA-I	L-Fucα1,2Galβ1,4GlcNAcB1,6
Aleuria aurantia	AAA	L-Fuc
Maackia amurensis	MAA	NeuAca2,3GalB1,4GlcNAc
Sambucus nigra	SNA	NeuAcα2,6Gal=NeuAcα2,6GalNAc

2.3 Aplicaciones de las Lectinas

Las lectinas usualmente tienen al menos dos sitios de unión por moléculas, lo que les confiere la capacidad para interactuar con células teniendo efectos tóxicos o permitiendo inhibir el crecimiento de células tumorales. Todas las actividades biológicas reportadas para las lectinas tienen en común el reconocimiento de un receptor glicosilado (Castillo-Villanueva y Abdullaev, 2005). Entre los efectos biológicos descritos para lectinas vegetales se encuentran:

Efectos insecticidas. Las lectinas de plantas han sido utilizadas ampliamente debido a sus selectos mecanismos en el reconocimiento celular y se han evaluado sus efectos naturales como insecticida, debido a que son moléculas naturales en la defensa de plantas (Micussi y Camps, 1987). Lectinas de *Allium sativum* incrementan la mortalidad del gorgojo de la lenteja (*Acyrthosiphon pisum)* (Fitches y col., 2008), lectinas de *Arisaema intermedium* y *Arisaema wallichianum* aumentan el periodo de desarrollo e inhiben la pupación y eclosión de la mosca del melón (*Bactrocera cucurbitae*) (Kaur y col., 2009), lectinas de *Gracilaria cornea* (alga roja) disminuye el peso de la garrapata de ovinos y sus huevos (*Boophilus microplus*) (Lima y col., 2005), lectina del alga roja *Gracilaria ornate* retrasa el desarrollo del gorgojo del frijol (*Callosobruchus maculatus*) (Marques Leite y col., 2005), lectina de *Myracrodruon urundeuva* aumenta la mortalidad en *Aedes aegypti* (mosquito del dengue) (Sá y col., 2009), la lectina de *Xerocomus chrysenteron* incrementa la mortalidad en el gorgojo del melocotón (*Myzus persicae*) (Jaber y col., 2007; Jaber y col., 2008). Estos efectos se estudian para la implementación de estrategias en donde las lectinas puedan ser utilizadas como insecticidas naturales y específicos de ciertas especies de insectos.

Efectos fungicidas. Diversos estudios han probado la actividad de las lectinas de plantas en hongos patógenos de diversas especies y, aunque solo pocas lectinas han mostrado tener efectos, se muestra la selectividad al tipo de hongo agresor (Yan y col., 2005; Lam y col., 2009; Lam y Ng, 2010; Yang y col., 2010).

Efectos antivirales. Las lectinas de las algas *Gerardia savaglia,* fueron las primeras en ser estudiadas por sus propiedades en la prevención de la infección por virus de inmodeficiencia humana (VIH). Se ha reportado que la mayor actividad en esta patología se da por lectinas que enlazan estructuras que contienen el sacárido manosa (Lam y Ng, 2010; Lam y Ng, 2011). Estas mismas lectinas tienen efecto también sobre el síndrome de resfriado agudo (Keyaerts y col., 2007) y lectinas quiméricas recombinantes son potentes inhibidores de la Influenza tipo A (Wei-Chuan y col., 2011).

Efectos anticancerígenos. Diversos estudios muestran el efecto citotóxico de las lectinas vegetales sobre líneas de células cancerígenas, demostrando su alta especificidad por carbohidratos de las membranas celulares (Fu y col., 2011). La acción de las lectinas varía desde la especificidad de unión a azúcares hasta el mecanismo de acción a nivel molecular (Ferriz-Martínez y col., 2010; Estrada-Martínez y col., 2017). La especificidad sobre las diferentes líneas celulares tumorales podría reflejar distintas vías de progresión de estas últimas (Castillo-Villanueva y Abdullaev, 2005). Algunos autores afirman que el anclaje de lectinas a los ligandos de la superficie celular inicia la internalización de las lectinas a la célula, lo cual a veces lleva a la muerte celular por medio de la inactivación ribosomal o encendiendo cascadas de señalización que conducen a la apoptosis (Fu y col., 2011; Han y col., 2015; Cassandra y Barroso, 2016). Sin embargo, se describen muchos posibles mecanismos de acción de las lectinas, entre los más congruentes con los resultados obtenidos en experimentos de distintos grupos de investigadores están los siguientes tres: 1) Metabólicamente se describe una secuencia de eventos desde que las lectinas entran al tracto digestivo: unión a linfocitos, liberación de citocinas en la sangre, activación y liberación de linfocitos del bazo en la circulación, activación de células NK y macrófagos, producción de antiangiogénicos, combinación de hiperplasia intestinal y efecto antiangiogénico reduciendo la disponibilidad de nutrientes para el tumor y el efecto citotóxico sobre las células (González de Mejía y Prisecaru, 2005; Timoshenko y col., 2014). 2) A nivel

bioquímico y molecular se proponen diferentes mecanismos de acción. Un mecanismo describe la unión de lectinas a moléculas de adhesión de la superficie (epCAM) que participan en una gran variedad de señales de transducción que son importantes para la regulación celular. Un segundo mecanismo sugiere que la lectina se internaliza en la célula y afecta el proceso celular fundamental para la división celular. La lectina se une a una forma trunca de Orp150 la cual está directamente involucrada en el proceso del importe nuclear dependiente de NLS (Endo y col., 1987; Fang y col., 2011; Deepa y col., 2012). 3) Un tercer mecanismo explica que la lectina induce apoptosis por diversas vías: dependiente de la activación intracelular de la caspasa 8/FLICE, lo que requeriría la internalización de la lectina e involucraría su actividad inhibitoria ribosomal y de la síntesis de proteínas (Bantel y col., 1999; Fu y col., 2011; Delebinski y col., 2012; Zhang y col., 2015); a través de la activación de la caspasa-3 y la ruptura de PARP (Lyu y col., 2002; Hostanska y col., 2003; Deepa y col., 2012; Lichtenstein y Rabinovich, 2013; Shi y col., 2014); por la activación de Bax (acelerador de apoptosis) y la inhibición tanto de Bcl-2 (supresor de apoptosis) como de la telomerasa (Khwaja y col., 2008; Fu y col., 2011; Kabir y col., 2013; Kabir y Reza, 2014). Es importante notar que no es requisito indispensable que la lectina sea internalizada ya que los efectos apoptóticos pueden se provocados por la interacción de la lectina con receptores de la membrana celular. En la Figura 2 se muestran algunos de los principales efectos de lectinas y en la Tabla 2 se muestran las alteraciones moleculares conocidas desencadenadas por los tratamientos con lectinas y sus principales efectos (Fu, 2011).

Tabla 2. Alteraciones moleculares mediadas por lectinas.

Origen	Lectina	Alteraciones moleculares	Efectos en cáncer
Muérdago Europeo	ML-I	TNF-a, Caspasa 2, 3, 8 y 9, JNK, MAPK (ERK y P38), NF-κB, Akt, XIAP	**Inducción de apoptosis y efectos antiproliferativos**
	ML-II	SAPK/JNK y P38	
Muérdago Chino	CM-I	miR-135a&b ----> APC	
Regaliz americano	Abrina	caspasa-3, Bcl-2	
Canavalia ensiformis	ConA	caspasa 9/3, Citocromo c, Akt, MMP-9, MAPK, IKK/NF-κB, Bax/Bcl-2, Foxo1a-Bim	
Phaseolus coccineus	PHCL	Caspasa 3/9	
Polygonatum cyrtonema	PCL	ROS-p38-p53, Ras-Raf y PI3K-Akt, Caspasa 3 y 9	
Phaseolus vulgaris	PHA	**No hay dato**	
Frijol Rojo Oscuro	PHA-E		**Efectos antiploriferativos**
Plátano	Lectinas de Plátano		
Soya	Lectinas de Soya		
Ejote	Letinas de Ejote		
Astragalus mongholius	AMML		
Sophora flavescens	SFL		

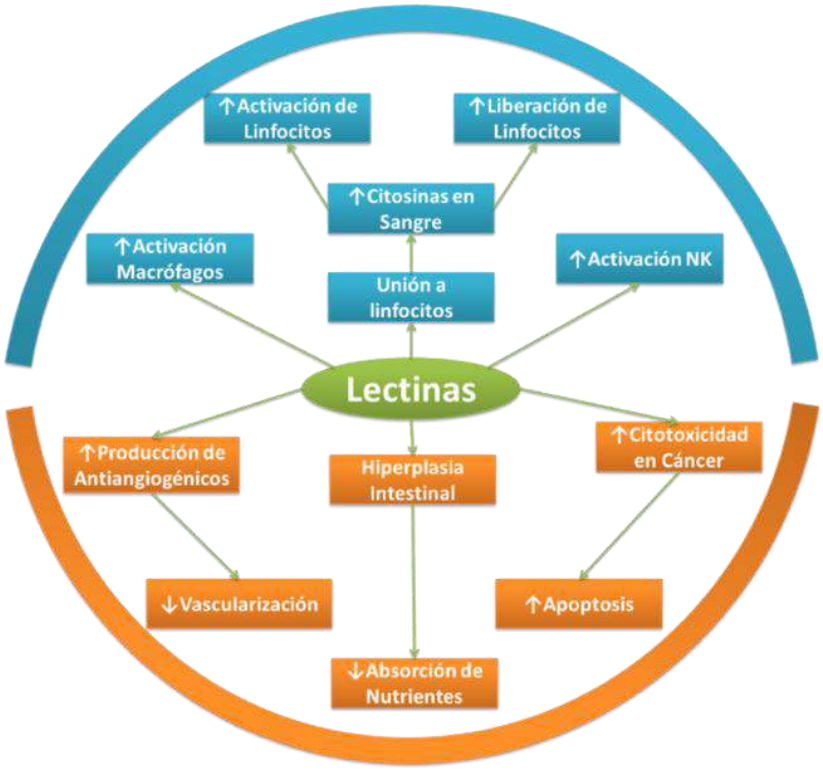

Figura 2. Procesos en los que se ven involucradas las lectinas al interaccionar con células.
En azul efectos de inmunomodulación, en Naranja efectos anticáncer.

2.4 Lectinas sobre cánceres digestivos

Lectinas sobre cáncer de esófago y estómago. Las lectinas se han utilizado desde hace ya algunos años como marcadores diferenciales en la detección de cáncer de esófago y estómago. Éstas se han conjugado con marcadores fluorescentes para su fácil identificación (Mechref y col., 2009; Fardini y col., 2013). El cáncer de estómago es la 5 causa de muerte por cáncer en el mundo, mientras que el esófago es la sexta. El cáncer invasivo de esófago y estómago es un proceso progresivo de múltiples etapas que implica la conversión de epitelio normal a hiperplasia de células basales (HCB), displasia (DIS) o carcinoma *in situ* (CIS), y luego a carcinoma de células escamosas invasivas (Labenz y col., 2015). Recientemente, se ha prestado cierta atención a la expresión del antígeno TF en carcinomas y se la ha propuesto como un posible marcador en cáncer de esófago y estómago (Kannan y col., 2003). Las lectinas han sido utilizadas como herramientas diagnósticas del cáncer esofágico y cáncer gástrico, dado que se ha demostrado que existen alteraciones en la expresión del antígeno TF en el caso del esófago y de N-Glicanos en el caso del estómago, lo cual facilita el reconocimiento por lectinas (Aricigil y Pryme; Singh y col., 2006; Medina y Quesada, 2014; Yau y col., 2015). Estas lectinas son utilizadas principalmente como marcadores para inmunohistoquímica, ya sea con fluorocromos o moléculas de reacción enzimática para colorimetría, dando excelentes resultados (Baintner y col., 2000; Castaño-Rodríguez y col., 2014).

Lectinas sobre cáncer de intestino delgado y colon. Los tumores malignos del intestino delgado son raros, suponiendo solamente el 2% de todos los tumores del aparato gastrointestinal y menos del 0.4% de todos los tumores malignos. Por tal razón no existe una evaluación de lectinas en dicho órgano es en base a la etiología del cáncer de colon o gástrico según sea el tipo y ubicación afectada (Bowcutt y col., 2014). Diversos estudios indican el efecto las lectinas de distintas plantas sobre la inhibición del crecimiento, proliferación y promoción de la muerte celular en líneas celulares de cáncer de colon (Sasaki y col., 2002; Singh y col., 2006; Ma y col., 2008; García-Gasca y col., 2012). Actualmente se sabe que existen diferencias en

las glicosilaciones de diversas estructuras de las membranas celulares normales (NCs) y cancerígenas en colon (CCs), tanto en lípidos (Shida y col., 2009), como en glicoproteínas (Kim y col., 1974), tales como ciertos receptores (Hung y col., 2014); lo que hace a las CCs más susceptibles al reconocimiento por las lectinas. En 1999 Jordinson reportó que lectinas de haba (VFA) aumentan la diferenciación celular de distintas líneas de CCs disminuyendo el fenotipo maligno (Jordinson y col., 1999). Tal es el caso de lectinas de algunos hongos como *Agrocybe aegerita* (AAL), *Algaricus bisporus* (ABL) así como de plantas como *Arisaema helleborifolium* (AHL), *Arisaema tortuosum* (ATL), *Arachis hypogaea* (PNA), *Viscum album* (VAL, VAA, VAA-1), *Sauromatum venosum* (SVA), *Phaseolus acutifolius* (García-Gasca y col., 2012; Valadez-Vega y col., 2014), *Phaseolus vulgaris* L. (PVA) (Cruz-Bravo y col., 2011), *Vicia faba* (VFA) principalmente (Fu y col., 2011; (Ferriz-Martinez y col., 2010).

Lectinas sobre cáncer de hígado y páncreas. Globalmente, el cáncer de hígado representa más de un millón de casos diagnosticados cada año en todo el mundo, mientras que se estima que cada año se diagnostican unos 233.000 nuevos casos de cáncer de páncreas, el 60% de ellos en países desarrollados (Europa, América y Australia) (OMS, 2011). Las lectinas han sido utilizadas en el tratamiento y diagnóstico de dichas patologías, en este sentido se demostró que lectinas recombinantes de *Viscum album* inducen apoptosis en células de carcinoma hepático (Yang y col., 2012). Por otro lado, se ha mostrado que las lectinas de cacahuate (PNA) son capaces de diagnosticar cáncer de páncreas con una sensibilidad del 77% y una especificidad del 82% comparando con un radioinmunoensayo. Por su parte, lectinas de germen de trigo (WGA) resultaron ser toxicas sobre cáncer de páncreas en comparación con otras lectinas como Concavalina A y lectinas de frijol común (PHA), que resultaron tener un efecto dosis respuesta varias veces menor (Schwarz y col., 1999).

En el mismo sentido las lectinas de frijol Tépari (FCL) han sido estudiadas en experimentos *in vitro* e *in vivo.* En células en cultivo ha mostrado tener un efecto

citotóxico diferencial entre células no trasformadas (3T3-L1) y células transformadas con un fenotipo maligno (3T3-L1/Vmos); de la misma forma que las lectinas mostraron tener efectos citotóxicos diferenciales entre diversos tipos de cáncer (Mama, Cervix y Colon), siendo más efectivas en cáncer de colon (Garcia-Gasca y col., 2012). Por otro lado, en estudios *in vivo*, se observaron bajos efectos tóxicos relacionados con ligera pérdida de peso (10% aproximadamente), aumento en la cantidad de granulocitos sanguíneos, disminución de linfocitos; todo esto sin alterar marcadores hepáticos y renales del estado nutricio (Ferriz-Martinez y col., 2015). De la misma forma recientemente se evaluó el daño resultante tras la administración de FCL en las vellosidades intestinales y tejidos relacionados con el sistema inmune; resultando afectados durante el tratamiento pero logrando la recuperación tras la suspensión del mismo (A la Torre-Cruz y col., 2018)

Si bien es incierto el mecanismo específico mediante el cual las lectinas actúan metabólica y molecularmente, es muy cierto que tienen gran capacidad para afectar ciertas células cancerígenas. Nuestro grupo de trabajo ha determinado que FCL, reconoce de forma diferencial a células cancerígenas de sus contrapartes no cancerígenas así como entre células de diferentes tipos de cáncer (García-Gasca y col., 2012).

3. JUSTIFICACIÓN

El cáncer de colon es una patología que afecta al mundo entero y cobra principal importancia epidemiológica en los países en vías de desarrollo como producto de los cambios en los estilos de vida; aumento en el estrés y cambios en los hábitos de alimentación principalmente. En México el cáncer de colon ha pasado de ser el cuarto de mayor importancia al tercer puesto en 5 años, lo que denota la importancia de desarrollar investigación para la prevención, diagnóstico y tratamiento en nuestro país. Actualmente las terapias más utilizadas para combatir el cáncer de colon son la quimioterapia, la radioterapia y la resección quirúrgica; dichos tratamientos resultan ser poco específicos a las células malignas afectando a todas las células de crecimiento rápido del organismo, tales como, células epiteliales y células del sistema inmune, causando graves afecciones al organismo. En este sentido las lectinas de diversos organismos (animales, plantas, bacterias y hongos) están siendo estudiadas por sus capacidades de reconocimiento, dándoles la especificidad necesaria en un tratamiento contra cáncer. Es por tal motivo y dados los efectos citotóxicos *in vitro,* la baja toxicidad mostrada en experimentos *in vivo*; que el presente trabajo propone la evaluación de los efectos anticancerígenos, así como, indagar en los posibles mecanismos de muerte involucrados tras el tratamiento de una fracción concentrada en lectinas de frijol Tépari en células de cáncer de colon y en un modelo animal de cáncer de colon.

4. HIPÓTESIS

La fracción concentrada en lectinas de frijol Tépari induce muerte celular por apoptosis en células de cáncer de colon y en un modelo animal de inducción cáncer de colon con azoximetano.

5. OBJETIVOS

5.1 Objetivo General:

Evaluar los mecanismos de acción de una fracción concentrada en lectinas de frijol Tépari en cáncer de colon.

5.2 Objetivos Particulares:

- Determinar la expresión de genes importantes en CCR afectados por el tratamiento con FCL en cáncer de colon *in vitro*.
- Determinar las proteínas de vías de señalización de cáncer de colon afectadas por el tratamiento con FCL *in vitro.*
- Evaluar marcadores de inflamación y apoptosis en tejido de cáncer de colon *in vivo.*
- Evaluar proteínas de vías de señalización afectados por el tratamiento con FCL tejido de cáncer de colon *in vivo.*

6. MATERIALES Y MÉTODOS

6.1 Obtención de la fracción concentrada en lectinas de frijol Tépari.

La fracción concentrada en lectinas (FCL) se obtuvo de acuerdo al método descrito por García–Gasca y col. (2012). Brevemente, se molieron semillas de frijol para obtener la harina la cual fue desgrasada. Se obtuvo el extracto acuoso y se realizó una precipitación selectiva al 40 y 70% con sulfato de amonio, se dializó, se separó por cromatografía de exclusión de peso molecular y se realizaron pruebas de aglutinación para obtener la FCL.

6.2 Experimentos *in vitro*

6.2.1 Cultivo Celular

Células HT-29, RKO y SW-480 de cáncer de colon se obtuvieron de la American Type Culture Collection (ATCC®). Las células fueron descongeladas en cajas de 60 mm de diámetro con medio de Eagle modificado por Dulbecco DMEM (GIBCO, NY, EU) suplementado al 10% de suero fetal bovino (SFB), e incubadas a 37º C, bajo atmósfera de CO_2 al 5% saturada de agua, con cambios de medios cada dos días hasta confluencia.

6.2.2 Evaluación del Efecto Citotóxico:

Se sembraron 3x104 células por pozo en cajas de 24 pozos en medio DMEM (GIBCO, NY, EU) al 10% de suero fetal bovino (SFB) (Biowest, Nuaillé, Francia); hasta confluencia. Después de 48 h las células se sincronizaron con DMEM al 2% SFB por 24 h, tiempo seguido se expusieron a las distintas concentraciones de los tratamientos (0, 0.1, 1, 5, 10, 50, 100, 150 mg de proteína/mL de la FCL en DMEN al 0.5% de SFB por 24 h. Las células se cosecharon con una incubación de 5 min en tripsina/EDTA (0.15 mM/0.5 M) y se contaron utilizando una cámara de Newbauer. Se calculó la concentración letal media (CL50) graficando el logaritmo de la concentración contra el porcentaje de sobrevivencia**.**

$$\boldsymbol{x = (y - b)/m}$$

Donde:

x = ln de la concentración

y= % de sobrevivencia

b= intersección en cero

m= pendiente

6.2.3 Evaluación de muerte celular por Anexina V

Para confirmar la CL50 e indagar en el tipo de muerte celular se realizó el ensayo de Anexina V utilizando el Kit Muse® Annexin V and Dead Cell Assay (Milipore cat. No: MCH 100105) para las tres líneas celulares (HT-29, RKO y SW-480). Se sembraron $3x10^5$ células en cajas de cultivo de 60 mm de diámetro, en medio DMEM con SFB al 10% y se mantuvieron en incubación a 37° C y en una atmósfera húmeda con 5% de CO_2. Cuando los cultivos alcanzaron el 70% de confluencia, se dividieron en 3 grupos, grupo Control (-), el cual se incubó con DMEM al 0.5% de albumina sérica bovina (ASB); grupo de tratamiento (CL50 de FCL= 0.402 mg/mL) en DMEM al 0.5% de ASB y grupo Control (+) (Camptotecina 5 µM) en DMEM 0.5% de ASB, durante 8 h. Las células se colectaron por tripsinización y se concentraron por centrifugación (6000 g durante 5 min), posteriormente se realizó un lavado con PBS al 1 mM de EDTA para disgregar las células aglutinadas por la FCL. Las células de cada grupo se ajustaron a la densidad celular a $1X10^6$ células por mL en el medio de cultivo. Se procedió con las indicaciones del proveedor. Los experimentos se realizaron por triplicado y los datos se promediaron para su representación gráfica.

6.2.4 Evaluación morfológica de células HT-29

Las células HT-29 se cultivaron bajo sus condiciones específicas hasta confluencia de un 70%, las cajas de cultivos se separaron en dos grupos Control (-) y tratadas con FCL, se realizaron micrografías con microscopio invertido de la marcar ZEISS

a diferentes tiempos de los tratamientos (0, 1, 2, 4, 6, 8, 12, 16 y 20 h) con un aumento de 10X para poder observar la panorámica de la placa de cultivos.

6.2.5 Evaluación del tipo de muerte celular.

- Ensayo de Lactato deshidrogenasa (LDH). El ensayo de lactato LDH se emplea para evaluar la necrosis celular y se utilizó para descartar un efecto necrótico en las células HT-29. Se utilizaron placas de 24 pozos tal como se describió en el ensayo de proliferación celular, las células se dividirán en 3 grupos, grupo Control (-), el cual se incubó con DMEM al 0.5% de ASB; grupo de tratamiento (CL50 de FCL= 0.402 mg/mL) en DMEM al 0.5% de ASB y se incubaron por 8 h y Control (+), al cual se le agregó Tritón 100X (JT Baker, cat N° X198-07) al 1% en DMEM 0.5% de ASB y se incubaron a 37° C durante 30 min. Se obtuvieron los medios condicionados y se utilizó el kit LDH-Cytotoxicity Assay (Biovision, cat. N° K311- 400) para evaluar el efecto citotóxico de acuerdo a las instrucciones del fabricante. Las muestras fueron analizadas en un espectrofotómetro a 492 nm para determinar su densidad óptica. El porcentaje de citotoxicidad se calculó con base en la actividad enzimática de LDH, de acuerdo a la siguiente fórmula:

$$\%\boldsymbol{Citotoxicidad} = \mathbf{Tratamiento} - \mathbf{Control}\ (-)\ \boldsymbol{x}\ \frac{\mathbf{100}}{\boldsymbol{Control}\ (+)} - \boldsymbol{Control}\ (-)$$

- Ensayo de multicaspasas. Con la finalidad de determinar la inducción de apoptosis, se determinó la actividad de caspasas. Las células se cultivaron, trataron y cosecharon tal como se indicó en el ensayo de Anexina V. Las células de cada grupo se ajustaron a la densidad celular a $1X10^6$ células por mL en el medio de cultivo. Se procedió con las indicaciones del proveedor del Kit Muse® MultiCaspase Assay (Milipore cal. No.: MCH 100109), los experimentos se realizaron por triplicado y los datos se promediaron para su representación gráfica.
- Ensayo de actividad de Caspasa 3. El ensayo de Caspasa 3 se utilizó para determinar el efecto apoptótico de la FCL, para el cual se empleó el kit de caspasa

3/CPP32 Colorimetric Assay Kit (BioVision, cat. N° K106-100). Se utilizó la misma metodología que en el ensayo de Anexina V y muerte celular. Una vez colectadas las células se obtuvo el extracto citosólico tal como indica el kit de Caspasa 3/CPP32 (BioVision®). Los extractos obtenidos se analizaron en el espectrofotómetro a 400 nm y se determinó la actividad de caspasa 3 de la manera siguiente:

$$\boldsymbol{Actividad\ de\ Caspasa\ 3\ por\ célula} = \frac{\boldsymbol{Absorbancia}}{\mu\boldsymbol{g\ de\ Proteína\ total}} \Big/ \frac{\boldsymbol{Numero\ de\ Células}}{\mu\boldsymbol{g\ de\ Proteína\ total}}$$

6.2.6 Evaluación de la expresión génica

Células HT-29 control (-) y tratadas (CL50 de FCL, 0.402mg/mL) de acuerdo al ensayo de proliferación celular durante 8 h se colectaron para la extracción de RNA: La purificación de RNA se realizó añadiendo 400 µL de Trizol (InvitrogenTM, CA, EU) por cada caja de 60 mm de diámetro, se homogenizó cada muestra, posteriormente se utilizó el kit de extracción Direct-zol™ RNA MiniPrep (Zymo Reseach, Cat. No.: R2052). Las muestras se resuspendieron en agua libre de nucleasas, posteriormente se cuantificó el RNA total y se determinó la pureza por espectrofotometría con el espectrofotómetro NanoDrop™ 2000/2000c (Thermo Scientific). Para sintetizar el cDNA se agregaron 2 µg de RNA en microtubos de 200 µL y posteriormente se procedió de acuerdo a las indicaciones del proveedor para el Kit, Maxima H Minus First Strand cDNA Synthesis (Thermo Scientific, Cat. No.: K1652) para un volumen de 40 µL y con un ciclo de 25º C por 5 min, un ciclo de 65º C por 30 min y un ciclo de 85º C por 15 min.

Para diseñar los oligos se seleccionaron genes de vía de señalización de la carcinogénesis colorrectal, posteriormente se analizaron las secuencias genéticas en el UCSC Genome Bioinformatircs (**https://genome.ucsc.edu/**), para posteriormente realizar los oligos en el programa online primer3 (**http://primer3.ut.ee/**), procurando una TM de 60±2º C, de 20±2 pb, un tamaño de

producto de 100-250 pb y %CG ≥ 50%. Las secuencias obtenidas se mandaron a sintetizar a los laboratorios SIGMA ALDRICH México:

APC (fw ttgatagctacaaatgaggacca, rv acaaagttccacatgcattactg)

β-CTNN (fw tggacttgatattggtgccca, rv gccacccatctcatgttcca)

DCC (fw cccctgaagtgtctgaggag, rv agctgcttcatgagtccttcc)

PI3K (fw tggagctgacccaaatccat, rv ttcaaaggcagggttactcc)

GSK3 (fw ctccatccaaccgtctctca, rv ggtaggtgtggcatcggtc)

CAS9 (fw caagagtggctcctggtacg, rv tccctttcaccgaaacagca)

BAD (fw cggaggatgagtgacgagtt, rv caagttccgatcccaccagg)

PTEN (fw gccgtcaaatccagaggcta, rv ggatcagagtcagtggtgtca)

AKT (fw ccttcaagccccaggtcac, rv Cgctcgctgtccacacac)

TP53 (fw ccaacaacaccagctcctct, rv tcaggaagtaacaccatcgtaag)

BCL-2 (fw gactgagtacctgaaccggc, rv ggccaaactgagcagagtct)

KRAS (fw tgtgatttgccttctagaacagt, rv Acaccctgtcttgtctttgct).

La reacción de qPCR se llevó a cabo en una placa para PCR de 96 pozos en donde se colocaron, por cada reacción, 3.4 µL de agua libre de nucleasas, 5 µL de SYBR® Select Master Mix for CFX (applied biosystems, Cat. No. 4472942) y 1µL de cDNA template. Se utilizó el equipo CFX96 BioRad, con un programa: 10min a 95° C, (15s a 95° C, 30s a 60° C, 30s a 72° C) x 40 ciclos y posteriormente a 16° C por tiempo indefinido.

6.3 Experimentos *in vivo*

6.3.1 Animales de experimentación e inducción de cáncer.

En el presente estudio se utilizaron muestras de tejidos de ratas macho Sprague Dawley de 5 semanas de edad adquiridas del bioterio del Instituto de Neurobilogía de la Universidad Nacional Autónoma de México. El estudio se llevó previamente por López-Martínez (2016). Brevemente, los animales se mantuvieron con alimentación y agua *ad libitum*, con un ciclo circadiano a 12 h de luz y 12 h de oscuridad a una temperatura aproximada de 25º C: Después de una semana de adaptación se formaron 4 grupos de estudio; como inductor de cáncer se utilizó Azoxi-metano (AOM, Sigma Aldrich, St Louis, E.U) (10 mg/kg de peso corporal) para la generación de fosas cripticas aberrantes, tal como lo muestra la Figura 3.. Los animales fueron sacrificados en la semana 14 después de iniciado el experimento; los procedimientos anteriores se llevaron dentro de los lineamientos de la Norma Oficial Mexicana (NOM-062-ZOO-1999) para el uso de animales de laboratorio bajo las consideraciones éticas correspondientes y se contó con la autorización del Comité de Bioética de la Facultad de ciencias Naturales–UAQ. Los órganos obtenidos fueron conservados en formalina 10%.

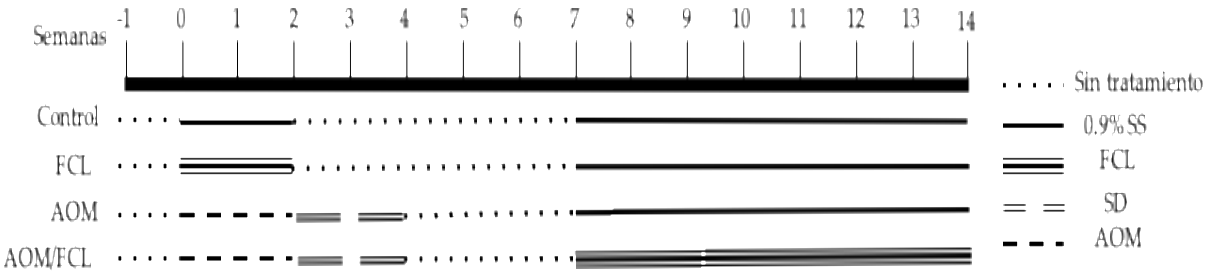

Figura 3. Esquema de tratamientos del modelo experimental. Grupo Control, administrado con solución salina (0.5 mL de NaCl 0.9%) vía intraperitoneal una dosis semanal. Grupo FCL, se administró con 0.5ml de NaCl 0.9 1 vez por semana durante 2 semanas, y posteriormente durante 6 semanas dos dosis semanales de FCL (50mg/kg de peso corporal). Grupo AOM, se administró vía intraperitoneal con 10mg/kg de peso de AOM, una dosis semanal durante 2 semas,

posteriormente durante 2 semanas se les administro Sulfato de Sodio Dextran (SSD) al 2% en el agua de bebida *ad libitum*; posteriormente se le administraron dos dosis semanales durante 6 semanas de 0.5ml de NaCl 0.9% vía intragástrica. Grupo AOM/FCL, se administró vía intraperitoneal con 10mg/kg de peso de AOM, una dosis semanal durante 2 semas, posteriormente durante 2 semanas se les administro Sulfato de Sodio Dextran al 2% en el agua de bebida *ad libitum*, posteriormente se administraron con dos dosis semanales durante 6 semanas de FCL (50mg/kg de peso corporal).

6.3.2 Análisis histopatológicos.

Los tejidos de colon se deshidrataron y se incluyeron en parafina con ayuda del equipo Histoquinete Leica TP1020. Posteriormente se realizaron los cortes finos a un grosor aproximado de 5 µm y fueron montados en un porta-objetos adheridos con una fina capa de gelatina en agua caliente. Posteriormente los cortes se rehidrataron y a la tiñeron con hematoxilina y tinción de eosina (HE). Los tejidos se deshidrataron nuevamente con un tren de alcoholes y se sellaron con entellán y cubreobjetos. Posteriormente se realizó el análisis al microscopio (Zeiss-Axio Vert A1) con aumento de 5x a 10x, bajo la supervisión de un histopatólogo veterinario.

6.3.3 Análisis inmunohistoquímico (IHQ).

Se utilizó para la detección de proteínas específicas en el tejido colónico. Las muestras deshidratadas se incluyeron en bloques de parafina y se cortaron con micrótomo a 5 µm de espesor en porta objetos cargados positivamente de la marca Thermo Fisher. Posteriormente las muestras fueron rehidratadas, para el desenmascaramiento de epitopos se utilizó un baño maría (100º C) durante 20 min en solución de ácido cítrico al 0.1 M y pH ajustado a 6.0 con NaOH, consecutivamente se inactivaron las peroxidasas endógenas con una solución de H_2O_2 al 2% en PBST durante 30 min. Tiempo seguido se realizó el bloqueo con ASB al 1% en PBST durante 1 h. Pasado el bloqueo se incubaron los anticuerpos primarios de la marca Santa Cruz Biotecnology, PCNA (sc.9857), p53 (sc-101764), Bcl-2(sc-7382), caspasa-3 (sc-7272), Akt (sc-8312), p-Akt (sc-33437), Citocromo-c (sc-23982), p-caspasa-9 (sc-81650), p-GSK3 (sc-7291) durante toda la noche (16 h

aprox.) a 18º C. Los anticuerpos secundarios se incubaron durante 2 h a 18º C, el revelado se realizó con diaminobencidina (DAB) y H_2O_2 y se detuvo la reacción utilizando dH_2O. Se utilizó hematoxilina como contraste y las muestras se deshidrataron y montaron. Finalmente, las muestras se observaron al microscopio, se fotografiaron y se realizó un análisis de densitometría óptica con ayuda del programa Image J®. Los datos se reportaron como unidades arbitrarias ± D.E.

6.3.4 Análisis de la expresión génica.

El tejido fijado en formalina se rehidrató con un tren de alcoholes. El RNA se extrajo según la Referencia Gouveia y col. (2014). El cDNA fue sintetizado usando el Kit TaqMan® para transcripción inversa (Applied Biosystems, Foster City, CA, EE. UU., Cat. N ° N8080234) y se realizó una q-PCR usando los siguientes cebadores:

TP53: fw AGTGGGAATCTTCTGGGACG; rv TCTTTTGCTGGGGAGAGGAG

Bcl-2: fw TTCTTTGAGTTCGGTGGGGT; rv CAGCCTCCGTTATCCTGGAT

Caspase 9: fw GATGCTGTCCCATACCAGGA; rv TCTCGATGTACCAGGAACCG

GSK-3: fw CTCAAGGCTCTCCCCACTAG; rv GTCTTGGCCAGTCTGAGTCT

AKT: fw CAAAGGATGAAGTCGCCCAC; rv TGCAAGTACTCCAGAGCTGA

Se normalizó en base al gen de hipoxantina-guanina fosforibosil transferasa (HPRT) y se representaron los datos con base al control.

4.4 Análisis estadístico.

Se realizaron comparaciones de medias por t de student y análisis de variancia (ANOVA) de una vía, con análisis *pos hoc* de Tuckey y Dunnett, con una significancia estadística de 0.05. Los resultados se representaron ± la desviación estándar (D.E.).

7. RESULTADOS Y DISCUSIÓN

7.1 Experimentos *in vitro*

Las lectinas vegetales han sido ampliamente estudiadas por sus efectos anticancerígenos diferenciales, tal como se muestra en los trabajos realizados por Choi y col. en el 2004, donde se observó que la línea celular A253 del epitelio glandular salival humano, disminuyó su viabilidad de forma dependiente a la concentración y el tiempo de exposición a la lectina de muérdago (VCA). La viabilidad disminuyó desde las 6 h con su máximo efecto a las 24 h del inicio del tratamiento con VCA. Este mismo efecto dosis y tiempo dependiente fue reportado por Lee-Yong y col. (2007) al exponer a células de adenocarcinoma de colon (COLO) con VCA. En ambos trabajos se reportó que el efecto en la viabilidad de las líneas celulares de cáncer podría estar regulado por la actividad apoptótica (Choi y col., 2004; Khil y col., 2007). Los experimentos realizados en el presente trabajo muestran que la dosis de 40.2 µg/mL de FCL indujo el 50% de muerte (CL50) de células HT-29, mientras que el mismo efecto se observó con dosis inferiores en RKO (34.7µg/mL) y SW-480 (4.5 µg/mL) (Figura 3). Cabe destacar que los genotipos de las tres líneas celulares son diferentes, posicionándolas en distintos grados de malignidad de acuerdo a sus mutaciones (Tabla 2).

Tabla 3. Alteraciones genéticas de importancia en tres líneas de cáncer de colon

Células CCR	Alteraciones Genéticas
SW-480	TP53, β-CTNN
HT-29	TP53, APC, PI3K, K-RAS, FAM123B
RKO	u-PAR

Este efecto describe un descenso de la proliferación dependiente de la concentración y del tipo de célula, lo cual muestra que la FCL es capaz de tener un efecto citotóxico dosis-dependiente y a su vezcitotóxico diferencial, aún entre células de un mismo linaje y misma patología, pero con diferente grado de progresión. Dicho efecto diferencial se mostró anteriormente entre células transformadas (3T3-Vmos) y no transformadas (3T3-L1), así como entre diversos linajes celulares de cáncer (García-Gasca y col., 2012).

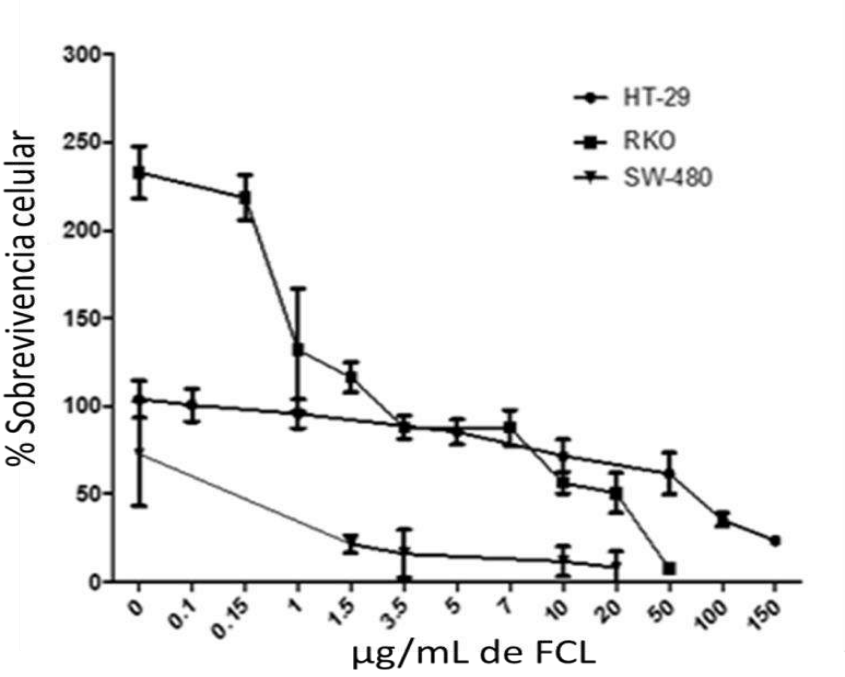

Figura 4.**Efecto citotóxico de FCL en células de cáncer de colon.**
Curva dosis respuesta de (•) Células HT-29, (■) células RKO y (▼) células SW-480. Tratadas con distintas dosis de FCL en DMEM al 0.5 de ASB. En el eje de las Y se muestra el porcentaje de sobrevivencia tras los tratamientos.

Por otra parte, los resultados obtenidos por citometría de flujo permitieron observar cambios significativos (t student, $p<0.05$) para la señal de anexina V, indicando un incremento en la actividad apoptótica del grupo tratado con la FCL en un 36.6% respecto al control negativo (Figura 4). De la misma forma lectinas de *Astragalus membranaceus* (AML) indujeron apoptosis en células de leucemia humana (K562)

en un 46% después de 48 h de tratamiento (Huang y col., 2012). Resultados similares han sido observados en el caso de una línea celular de adenocarcinoma de cuello de útero humano (HeLa) tratada con una lectina de unión a lactosa de la esponja marina *Cinachyrella Apión* (CaL), probablemente por apoptosis vía mitocondrial (Rabelo y col., 2012). Fukuda y col. en el 2006 analizaron la lectina de *Euchema serra* (ESA), un alga roja marina con especificidad a cadenas ricas en manosa. El efecto obtenido por ESA fue la inducción de muerte celular en línea de cáncer colon Colon26 a través de apoptosis (Sugahara y col., 2001). Haciendo una comparativa entre efectos de lectinas de un origen similar a la estudiada por nuestro grupo de trabajo, encontramos que lectinas de *Phaseolus vulgari*s inducen apoptosis en el 49.3% de células de hepatocarcinoma (Hep G2) (Fang y col., 2011), lo cual indica que el efecto de las lectinas de diversas plantas tienen la propiedad de inducir apoptosis por mecanismos similares.

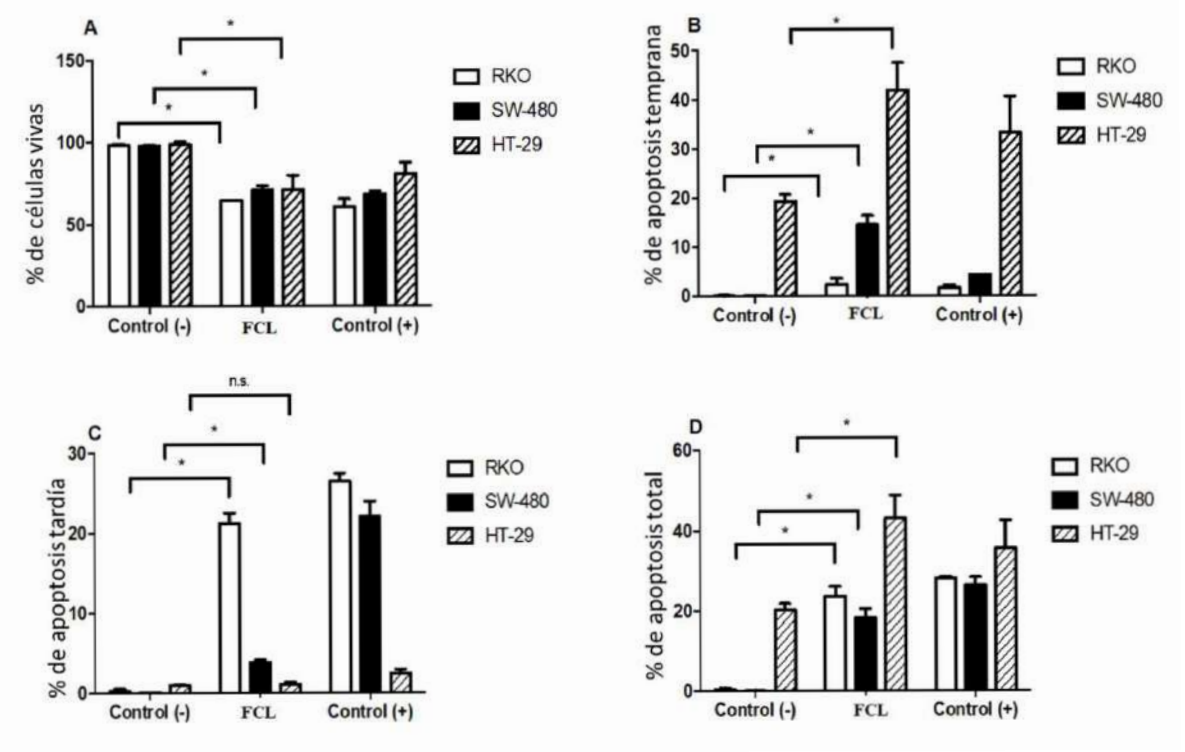

Figura 5. Evaluación del efecto apoptótico con Anexina V.

A) porcentaje de células vivas después del tratamiento ; B) porcentaje de células en apoptosis temprana después del tratamiento; C) porcentaje de células en apoptosis tardía después del tratamiento. D) Total de células en apoptosis después del tratamiento. Control (-) [DMEM al 0.5% de ASB]; FCL [40.2 µg/mL]; Control (+) [Camptotecina 5mM]. (*) Indican diferencia estadística significativa (t de student) ±D.E.

Los resultados de la evaluación de necrosis por medio de la determinación en medio de cultivo de LDH indicaron que la CL50 de la FCL no indujo muerte celular por necrosis sobre células HT-29 (Figura 5). Los resultados concuerdan con los efectos de otras lectinas como la de *Phaseolus coccineus*, donde se observó solo un incremento del 11% de LDH a las 24 h en fibroblastos L929 (Chen y col., 2009). De la misma forma, lectinas de la flor oriental (*Clematis montana*) fueron probadas en células L929 dando resultados muy parecidos a los reportados de las lectinas de *Phaseolus coccineus* (Peng y col., 2009).

En el presente trabajo se evaluó la actividad de caspasa 3, para determinar si la muerte celular inducida por el tratamiento con FCL es de tipo apoptótica y mediada por caspasas, tal como se muestra en los trabajos de Deepa y col. (2012) donde miden la actividad de caspasa 3 en células de mama (MCF-7) y de colon (HCT-15) tratadas con lectinas de hojas de morera, *Morus alba L.* (MLL), dicha actividad mostró diferencia significativa comparada con el control negativo y valores similares de la actividad de caspasa 3 al control positivo (Cisplatino) (Deepa y col., 2012a), así como lectinas de Nagaimo inducen un efecto en células MCF-7 (Chan y Ng, 2013). De la misma forma, el tratamiento con lectinas de muérdago, *Viscum album* L. (VLL), promueve la activación de caspasa 3 en células de leucemia (ALL) (Delebinski y col., 2012). El mismo efecto se ve reproducido por lectinas de la planta medicinal china, *Astragalus membranaceus,* en un modelo similar de leucemia, células K562. Estudios más recientes muestran que otra lectina ampliamente estudiada por sus efectos sobre diversos procesos cancerígenos, Concavalina A (Con A), induce muerte celular mediada por caspasas y la actividad de caspasa 3 resulta ser dosis dependiente en células MCF-7 de cáncer de mama, al igual que las lectinas del arbusto chino *Sophora flavescens* (SFL) (Shi y col., 2014).

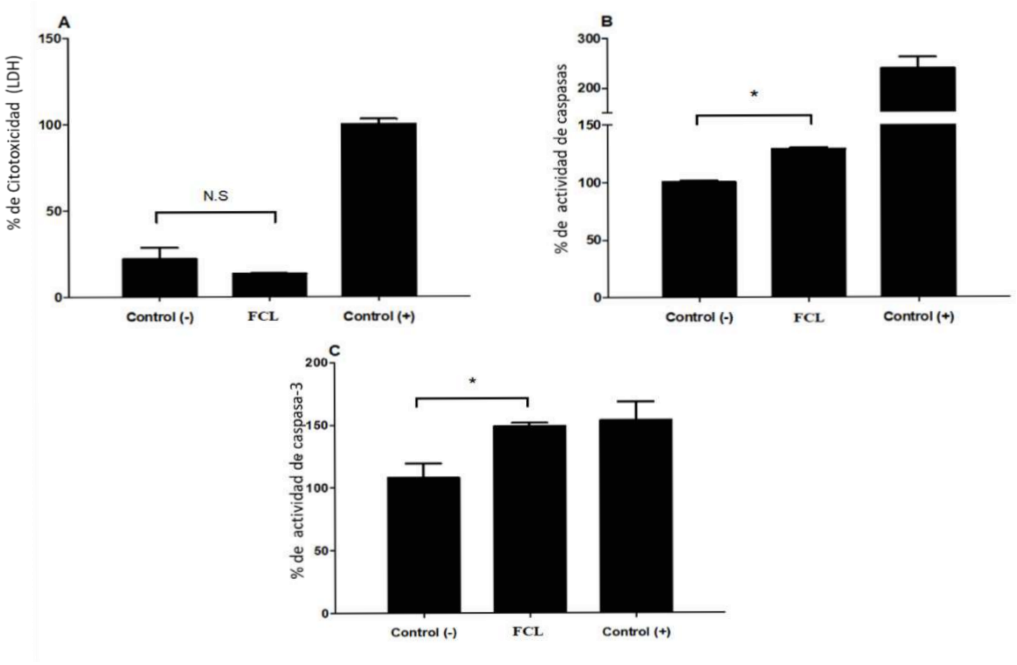

Figura 6. Evaluación citotóxica de FCL sobre células HT-29.
A) se muestra el porcentaje de LDH en medio de cultivo después de 8 h de tratamiento con CL50 de FCL, Control (-) corresponde a ASB, Control (+) corresponde a Tritón al 1%. B) se muestra el porcentaje de actividad de las caspasas totales de las células tratadas durante 8 h con FCL, el Control (-) corresponde a ASB, el Control (+) a Camptotecina (5 mM). C) actividad citosólica de caspasa-3 en células tratadas durante 8 h con FCL, el Control (-) corresponde a ASB, el Control (+) a Camptotecina (5mM). (*) Indican diferencia estadística significativa (t de student) ±D.E.

Lo anterior muestra que el efecto letal de las lectinas de diversas plantas probadas en cultivos celulares, no es por medio de la inducción de necrosis, sino que induce la muerte por mecanismos más ordenados que evitan la liberación del contenido citosólico en el medio de cultivo. En este sentido se llevó a cabo la evaluación de marcadores apoptóticos específicos, para lo que se determinó el efecto de la FCL en la expresión de genes relacionados con apoptosis. En trabajos anteriores se observaron cambios significativos en la expresión de caspasa 3, caspasa 8 y caspasa 9 ($p<0.05$) (Ferríz-Martínez., 2015)Figura. Las caspasas 3 y 9 mostraron una sobreexpresión del 35 y 78%, respectivamente en comparación con sus controles. Lo anterior concuerda con los experimentos de Ouyang y col., (2014) al

probar lectinas de *Polygonatum odoratum* (POL), en células MCF-7 de cáncer de mama, donde se observó un aumento significativo de actividad de caspasas 3 y 9 dependiente del tiempo de exposición a las lectinas. De la misma forma, se encontró un aumento de la actividad de caspasas 3 y 9 en células NALM-6 al ser tratadas con VLL y resultando la expresión dependiente de la dosis después de 24 h del tratamiento (Delebinski y col., 2012). La expresión de caspasas 3 y 9 medida en células MCF-7 también se vio aumentada al exponerlas al tratamiento con lectinas de *Abelmoschus esculentus* (AEL) (Monte y col., 2014), los mismos resultados fueron obtenidos en dichas células al ser tratas con Con A y SFL (Shi y col., 2014). Lo anterior muestra que el efecto letal de las diversas lectinas sobre células cancerígenas se da por medio de la activación de la apoptosis mediada por caspasas. La expresión de caspasa 8 en células HT-29 tratadas con la CL50 de la FCL disminuyó en un 22% lo que sugiere que la muerte por apoptosis se encuentra mediada por mecanismos que no involucran la activación de receptores de muerte y podría estar orquestada por la activación de la vía mitocondrial, tal como lo proponen los resultados de Bantel y col. (1999). Adicionalmente, se observó que la expresión del gen del promotor de apoptosis, Bad, no mostró diferencias con el control, lo que se contrapone a los resultados obtenidos por Shi y col. (2014) donde observaron un efecto dosis dependiente de la expresión de Bid y Bax, proteínas pro-apoptóticas de la misma familia de Bad. Lo anterior sugiere que las lectinas de la FCL tienen un mecanismo de acción distinto al de la Con A. La proteína Bad puede ser activada por la desfoforilación de Akt la cual se ha observado que es afectada por lectinas de muérdago VCL, induciendo a su vez apoptosis (Choi y col., 2004; Khil y col., 2007).

En el presente trabajo se ha evaluado la expresión de genes relacionados con la cascada de señalización relacionada con el cáncer de colon (Figura 6) esto con la finalidad de mostrar los blancos moleculares afectados por el tratamiento con la FCL, curiosamente los principales afectados resultaron ser los mRNA que codifican para p53 y Bcl-2, incrementando los niveles de p53 (Wawryk-Gawda y col., 2014, la

cual se encuentra ampliamente relacionada con la reparación celular e inducción de muerte celular (Zhao y col., 2005) y disminuyendo los niveles de Bcl-2 la cual se relaciona con proceso de sobrevivencia (Zhao y col. 2005; Khwaja y col., 2014). Fu y col., (2011) muestra las distintas vías de señalización activadas por lectinas de leguminosas en comparación con lectinas tipo ricino, y muestra claramente la interacion de las lectinas de leguminosas con las vías p53 y Bcl-2. De la misma forma Liu y col., (2010) de muestran la actividad apoptótica de lectinas de *Polygonatum cyrtonema* mediadas por la vía PI3K/Akt y regulada por p53.

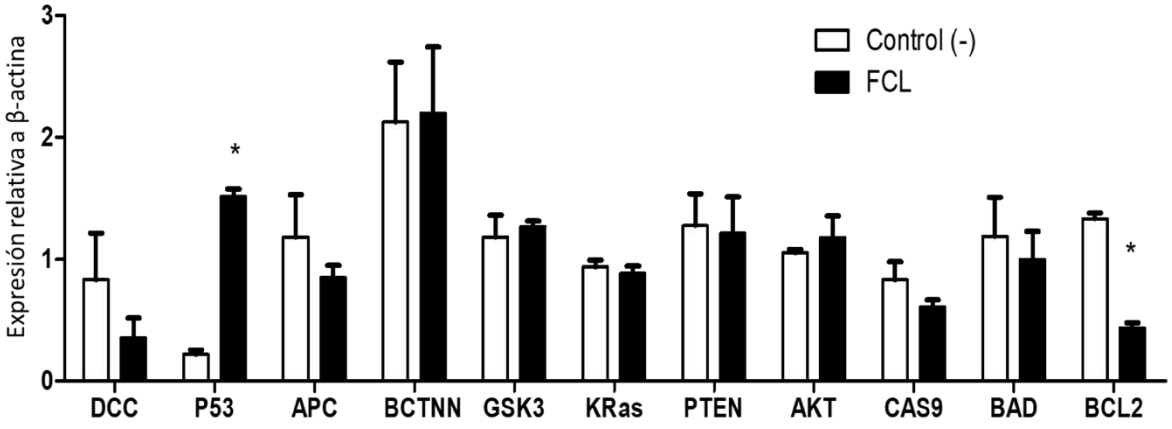

Figura 7. Cambios en la expresión génica inducidos por FCL.

Las barras claras muestran a células tratas con el vehículo de los tratamientos (ASB), Control (-). Las barras oscuras muestran la expresión en células tratadas con FCL (40.2µg/mL). (*) Indican diferencia estadística significativa (t de student) ±D.E.

7.2 Experimentos *in vivo*

7.2.1 Evaluación de efectos tóxicos generales

Se ha reportado que diversas lectinas han resultado ser tóxicas en animales de experimentación, provocando severos problemas antinutricios y en ocasiones la muerte (Duranti y Gius, 1997; Reynoso-Camacho y col., 2003). En algunas ocasiones se ha detectado inactividad ribosomal como en el caso de las lectinas tipo ricino (Fu y col., 1996; Garrosa y col., 2015). En este sentido, estudios de toxicidad aguda y subcrónica han demostrado que la FCL presenta baja toxicidad en ratas Sprague Dawley administradas vía oral. Los efectos adverdos se han observado con dosis por arriba de los 50 mg/kg de peso de FCL, de este modo se estableció la dosis más alta sin efectos adversos de 50 mg/kg de peso corporal (Ferriz-Martínez y col., 2015). En el presente trabajo las ratas no presentaron efectos adversos y aunque hubo una reducción de la ganancia de peso corporal en los animales administrados con la FCL no fue significativo ni rebasó el 10% que indicaría desnutrición de los animales (Figura 7). No se observaron diferencias estadísticas significativas respecto al control para ninguno de los grupos, lo cual es consistente con el estudio previamente llevado a cabo y con diversos trabajos reportados (Reynoso-Camacho y col., 2003; Kabir y col., 2013; Ferriz-Martínez y col., 2015; Garrosa y col., 2015). De la misma forma, el consumo de alimento no cambió a través del tiempo entre tratamientos siendo consistente con estudios posteriores y con los trabajos antes mencionados.

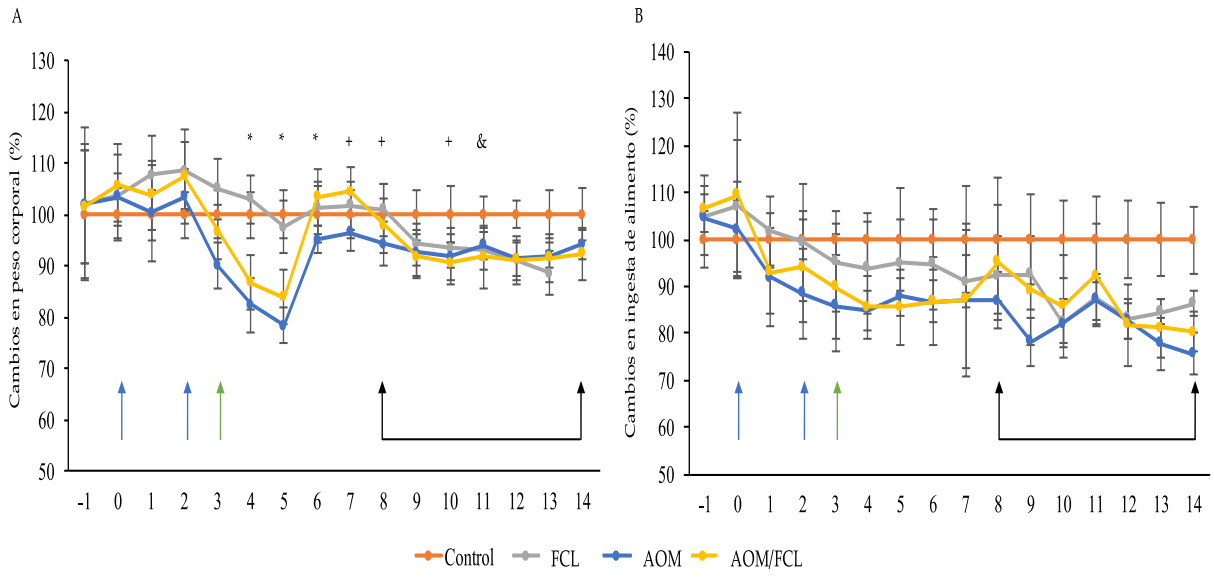

Figura 8. Cambios en el Peso y consumo de alimento.
Las ratas se administraron por vía intraperitoneal durante dos semanas con AOM (flechas azules) y una semana más con sulfato de sodio dextran (SSD) en agua potable diaria (flecha verde) para desarrollar fosa crípticas aberrantes (FCA). A continuación, se les administró FCL (50 mg/kg) en días alternos durante seis semanas mediante una cánula intragástrica (flechas negras). A) registro semanal del peso corporal con respecto al grupo de control. B) Promedio de cambios en la ingesta de alimento por semana con respecto al grupo de control. Se realizó ANOVA de una vía (post hoc Dunnett) las diferencias estadísticas significativas se representan como: (*) p≤ 0.05, (&) p≤ 0.01 y (+) p≤ 0.001.

7.2.2 Evaluación Histopatológica

Los resultados histopatológicos en colon mostraron una disminución de la longitud y fusión de las vellosidades colónicas, adelgazamiento del tejido e infiltración linfocitaria leve en los grupos que fueron tratados con la FCL (Figura 8). Los resultados concuerdan con otros estudios donde se alimentó a truchas (*Oncorhynchus mykiss*) con alimento a base de frijol rojo (*Phaseolus vulgaris* L.) y se observó aumento de la infiltración linfocitaria en el tejido intestinal, así como un acortamiento y ensanchamiento de las vellosidades intestinales (Daprà y col., 2005). Por otro lado, en un estudio realizado con harina del mismo frijol en, lechones se observó un efecto similar, provocando un aumento en el peso del estómago, aumento en la maduración intestinal y mejorando la absorción de algunos

nutrimentos (Rådberg y col., 2001). Lo anterior podría deberse a un proceso adaptativo dado la adherencia natural de las lectinas a estructuras glicosiladas (Cassandra y Barroso, 2016). Las ratas tratadas con AOM presentaron fosas crípticas aberrantes en un 100% mientras que las ratas administradas con AOM y tratadas con FCL mostraron disminución de las fosas cripticas aberrantes en 50% aproximadamente y disminución de la infiltración linfocitaria (Figura 8). Lo anterior muestra que la FCL presenta efectos sobre la tumorogénesis temprana, resultados consistentes con lo observado en estudios previos (datos no publicados, tesis de Ferriz Martínez, 2015). Resultados similares han sido observados con otras lectinas como Con A en otros modelos animales (Fu y col., 2011; Shi y col., 2014) y las de *Phaseolus vulgaris* (PHA) en un modelo similar (Fu y col., 1996; Thaker y col., 2012; Hong y col., 2015). Con la finalidad de presentar de una forma más graficas los efectos de la FCL sobre las FCA, se muestra un arreglo de micrografías (Figura 8B) donde se pue observar en primer lugar gran incremento de la actividad linfocitaria en el intestino del grupo AOM y dicha se ve contenida en el grupo AOM/FCL (cuadro rojo), lo cual denota el efecto de la FCL sobre la progresión de la inflamación causada por el AOM. En segundo lugar se puede observar que la arquitectura del

tejido no se ve tan alterada en el grupo AOM/FCL comparada con el grupo AOM (flecha roja), lo que indica que existe una disminución del daño causado por el AOM.

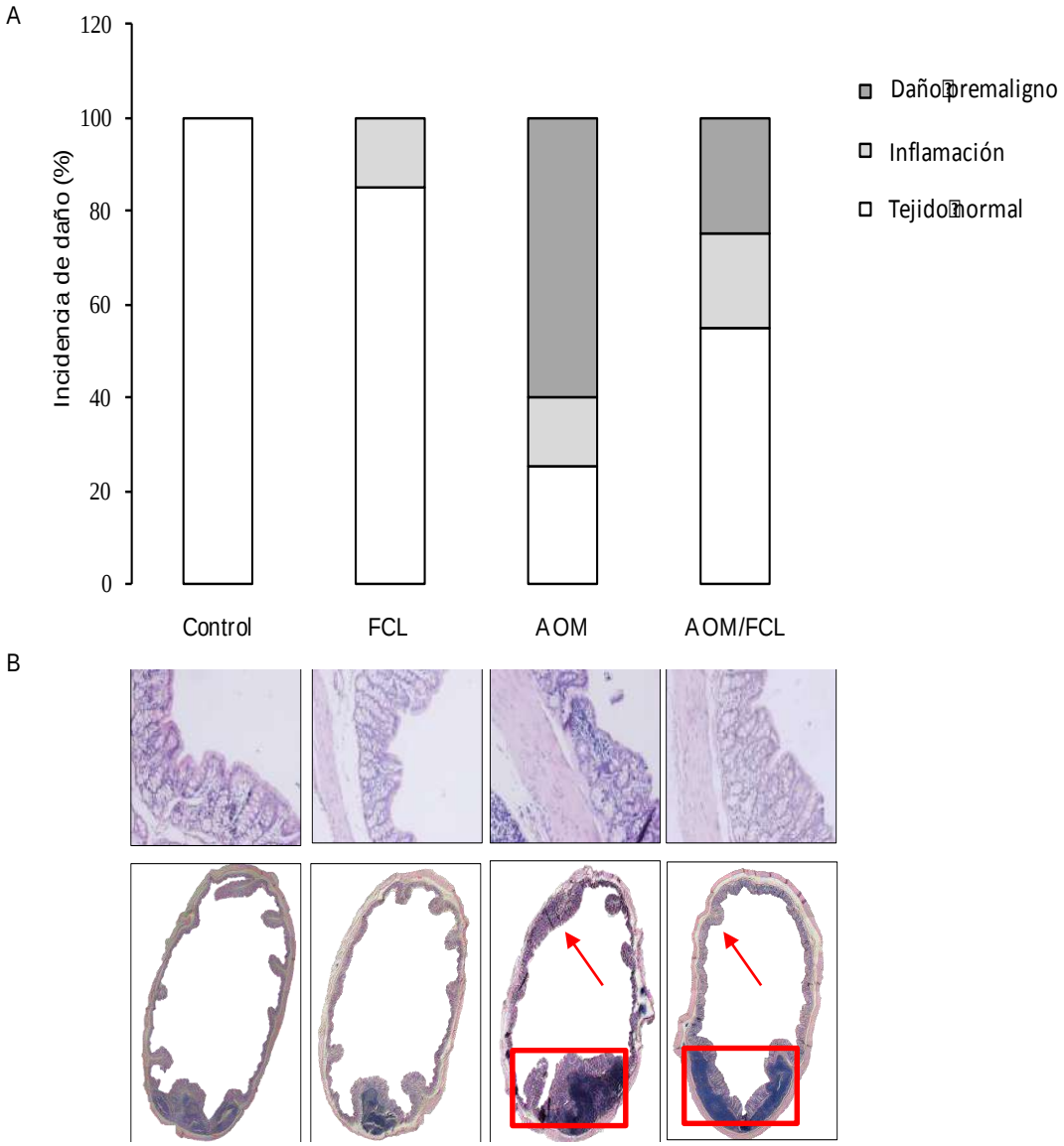

Figura 9. Análisis Histopatológicos.
Las ratas se administraron por vía intraperitoneal durante dos semanas con AOM y otras dos semanas con SSD en su agua potable diaria para desarrollar fosas crípticas aberrantes. A continuación, se les administró FCL (50 mg/kg) cada tercer día durante seis semanas mediante una cánula intragástrica. A) Incidencia de fosas crípticas aberrantes; B) Análisis histopatológico de hematoxilina-eosina de lesiones de colon (10X).

7.2.3 Evaluación Inmunohistoquímica

La proliferación es un proceso comúnmente desregulado en la carcinogénesis colónica, en especial diversos reportes indican que en los modelos animales en los cuales se induce el cáncer de colon con AOM se observa aumento de la proliferación celular evaluado por distintos marcadores como el BrdU, PCNA, Ki-67, entre otros (Tanaka y col., 2003; Thaker y col., 2012; Hong y col., 2015). Los resultados del presente trabajo muestran como la FCL produce una reducción de la presencia de PCNA en los animales a los que se les indujo fosas crípticas aberrantes con AOM (Figura 9). La vía de Akt se evaluó debido a su participación en la regulación de la proliferación y apoptosis, ya que comúnmente se desregula en el cáncer de colon. La vía de señalización de Akt aumenta la proliferación celular mediante la fosforilación de GSK-3, un agente bloqueador de Ciclina-D, y disminuye la apoptosis por fosforilación de caspasa 9 (Gorelik y col., 2001; Cheng y col., 2005). El presente trabajo muestra que no se observaron cambios entre los animales tratados con FCL y el control; sin embargo, el grupo AOM mostró un aumento en p-Akt como se informó en otros modelos de cáncer de colon (Roy y col., 2002). Así mismo, se observó un ligero incremento de p-caspasa 9; aunque, p-GSK-3 no mostró diferencias con respecto a las ratas de control (Figura 9).

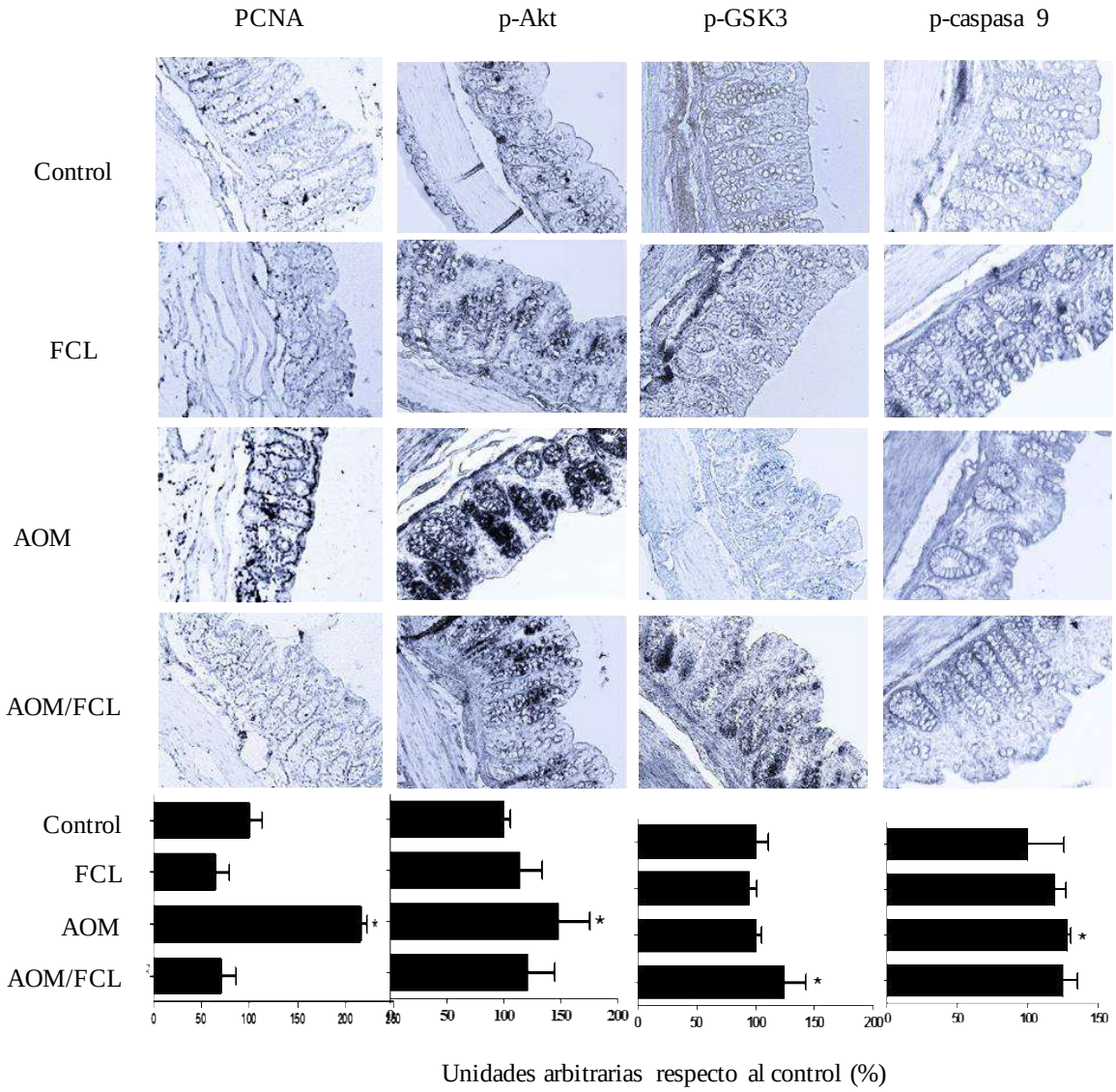

Figura 10. Evaluación de la vía de señalización Akt.
Las ratas se administraron por vía intraperitoneal durante dos semanas con AOM y otras dos semanas con sodio dextrán en su agua potable diaria para desarrollar fosas crípticas aberrantes. A continuación, se les administró FCL (50 mg/kg) cada tercer día durante seis semanas mediante una cánula intragástrica. A) Micrografías representativas de cada grupo (10X); B) Análisis de densitometría de tejidos de colon inmunoteñidos. (*) Diferencia significativa por ANOVA de una vía (Dunnett $p < 0.05$).

Cuando las ratas se trataron con AOM/FCL se observó una disminución de p-Akt con respecto a los animales tratados solo con AOM, lo que sugiere que FCL puede bloquear la ruta de Akt. Dados esos resultados, se esperaba un aumento en p-GSK-

3 y p-caspasa 9 sin embargo, la p-GSK-3 aumentó significativamente, tal vez debido a la participación de otras vías de señalización, como la proteína las activadas por proteín cinasa A (PKA) o proteína-quinasa C PKC).

En el mismo sentido y con la finalidad de evaluar si aparte del proceso de disminución de la proliferación se llevaba a cabo un proceso apoptótico, se evaluó la presencia de la caspasa 3 como marcadores de muerte celular por apoptosis. Se observó un incremento en la cantidad de caspasa 3 en el grupo que fue administrado con AOM y posteriormente con FCL; lo que indica un aumento de la actividad apoptótica. Lectinas de muérdago *Viscum album L. var. Coloratum,* (VCA) han mostrado efectos anticancerígenos induciendo apoptosis por vía de las caspasas en melanoma (Han y col., 2015). Aunque los resultados los describen principalmente en células de melanoma (B16BL6 y B16F10), proponen que el mecanismo se conserva en el modelo animal que manejan. Por otro lado, lectinas de *Eucheuma serra* (ESA) se probaron en células de adenocarcinoma de colon (Colon26) y en ratones con cáncer de colon BALB/c, donde nuevamente se observó un efecto apoptótico mediado por caspasas tanto *in vitro* como *in vivo* (Fukuda y col., 2006). De la misma forma, otros autores reportan efectos anticancerígenos de lectinas en lo que se involucra la apoptosis mediada por caspasas (Mukhopadhyay y col., 2014; Shi y col., 2014; Kabir y col., 2015). Por otro lado, se ha observado ausencia de mutación en el gen que codifica para p53 en los modelos de inducción de cáncer con AOM, pero si se da un aumento en la expresión de dicho gen promoviendo un incremento de la cantidad de proteína, aunque de la misma forma incrementa la cantidad de Mdm2 que es un regulador negativo de p53, no obstante aún no se tiene dato exacto de la interacción entre p53 y Mdm2 (Nambiar y col., 2002; Aizu y col., 2006). Los resultados del presente trabajo muestran de la misma forma que niveles de p53 se aumentaron con la presencia de AOM (Figura 10) lo cual se podría relacionar con el ritmo de crecimiento de las células transformadas, sin embargo se observó una disminución de los niveles de p53 en los animales que fueron tratados con FCL. Estos resultados difieren de los reportados por Hostanska

y col. (2003) de la lectina recombinante de muérdago (rML) en un modelo celular(Hostanska y col., 2003). De la misma forma, Bcl-2 es conocida por sus propiedades anti-apoptóticas. Los resultados obtenidos muestran niveles basales de Bcl-2 en el grupo control, así como en el grupo tratado únicamente con FCL, mientras que en el grupo tratado solo con AOM la presencia de Bcl-2 aumentó significativamente. Lo anterior sugiere cambios en los ritmos de proliferación inducidos por el AOM, así como alteraciones en las rutas de sobrevivencia. Sin embargo, los niveles de Bcl-2 disminuyeron a niveles basales cuando las ratas fueron tratadas con AOM y posteriormente con FCL, lo cual sugiere una importante participación de la FCL en el proceso apoptótico disminuyendo controladores negativos del proceso. De manera similar, mediante modelado *in silico* se propone que una lectina recombinante de muérdago, *Viscum álbum*, (ML-1) puede inducir apoptosis por medio del control de proteínas de la familia de Bcl-2 por la vía de señalización mediada por TNF-α (Khwaja y col., 2008).

Las mutaciones específicas del modelo de cáncer de colon con AOM son bien conocidas, especialmente en K-Ras (Jope y Johnson, 2004; Takahashi y col., 2004) que desregula la cascada de señalización correspondiente y permite la actividad proliferativa sostenida (Tong y col., 2011). Sin embargo, el tratamiento con AOM en modelos de cáncer de colon no se relacionan con mutaciones en el gen p53, pero se ha observado un aumento en la expresión de dicho gen (Aizu y col., 2006 y Nambiar y col., 2002). Los estudios en modelos de colitis y cáncer de colon que usan AOM como carcinógeno han mostrado un aumento tanto en la expresión como en la cantidad de p53; sin embargo, no se han observado mutaciones Rosenberg y col., 2009; Tong y col., 2011). Aunque la proteína p53 está típicamente involucrada en la reparación de daño o inducción de apoptosis en células dependiendo del daño del ADN, se ha descrito un mecanismo llamado proliferación inducida por apoptosis (AiP) (Mollereau y Ma, 2014; Valentin-Vega y col., 2008) donde un proceso apoptótico exacerbado conduce a la liberación de mitógenos inducir la proliferación celular. Este proceso ha sido implicado en el crecimiento de células intestinales en

ratones, que fue promovido por un aumento en la actividad de p53 (Mollereau y Ma, 2014). Del mismo modo, células moribundas de xenoinjertadas en ratones tratados con radioterapia mostraron un aumento en la actividad de la caspasa 3, que promovió la repoblación del tumor (Huang y col., 2011). Por lo tanto, en el desarrollo del cáncer el proceso AiP se activa como parte de su mecanismo. El grupo AOM/FCL no mostró diferencias estadísticamente significativas en p53 en comparación con el grupo control, lo que sugiere un efecto inhibidor de FCL en la inducción de cáncer con AOM (Figura 10).

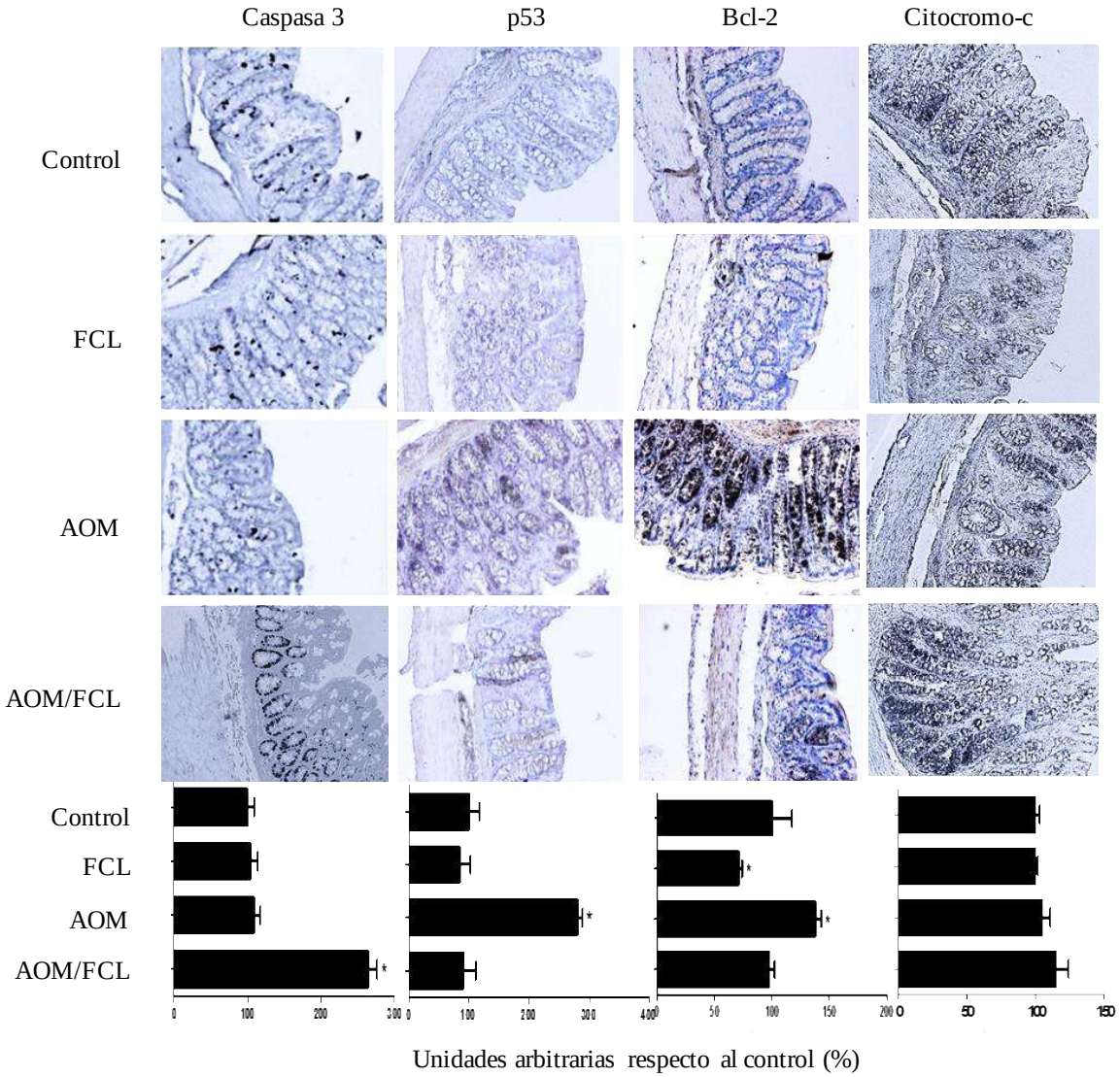

Figura 11. Evaluación de marcadores apoptóticos.

Las ratas se administraron por vía intraperitoneal durante dos semanas con AOM y otras dos semanas con sodio dextrán en su agua potable diaria para desarrollar fosas crípticas aberrantes. A

continuación, se les administró FCL (50 mg/kg) cada tercer día durante seis semanas mediante una cánula intragástrica. A) Micrografías representativas de cada grupo (10X); B) Análisis de densitometría de tejidos de colon inmunoteñidos. (*) Diferencia significativa por ANOVA de una vía (Dunnett p <0.05).

Para corroborar los resultados inmunohistoquímicos, se determinó la expresión génica de TP53, Bcl-2, caspasa 9, GSK y Akt en tejidos de colon (Figura 11). Se observó un incremento significativo en la expresión de TP53, Bcl-2 y Akt en el grupo AOM, pero no se encontraron diferencias estadísticas significativas entre los grupos AOM/FCL y control. Por otro lado, la expresión del gen caspasa 9 se incrementó significativamente en el grupo del tratamiento con AOM/FCL, sugiriendo la inducción de apoptosis por efecto de la FCL. Los resultados concuerdan totalmente con los análisis inmunohistoquímicos descritos previamente; y tomados en conjunto, estos resultados sugieren la actividad apoptótica independiente de p53 inducida por FCL en tejido colónico precanceroso temprano.

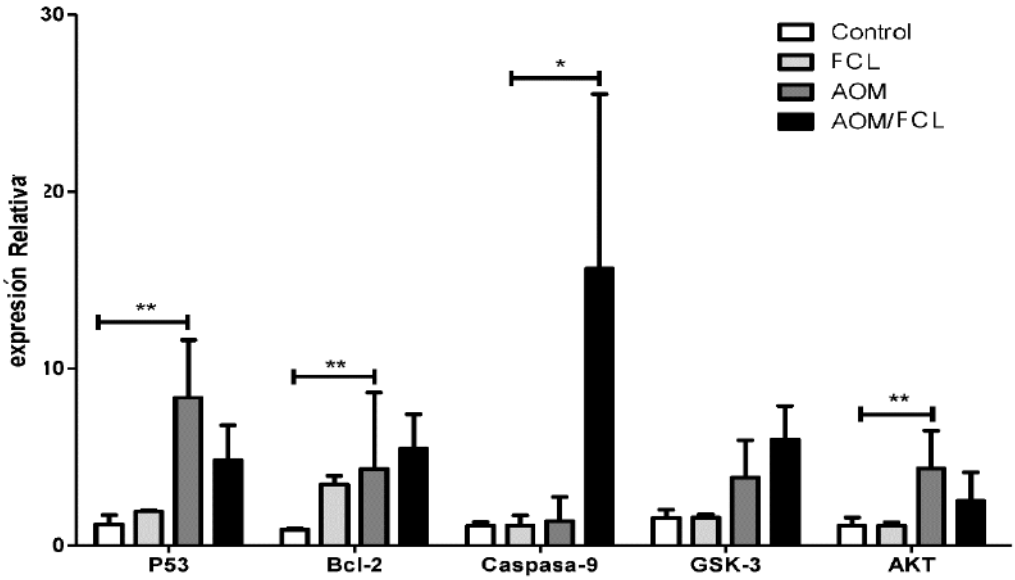

Figura 12. Evaluación de la expresión génica de TP53, Bcl-2, caspasa 9, GSK-3 y AKT en tejidos de colon de rata administrados con AOM/SSD y tratados con FCL. Las ratas se administraron por vía intraperitoneal durante dos semanas con OMA y otras dos semanas con SSD en su agua potable diaria para desarrollar ACF. A continuación, se les administró FCL (50 mg / kg) en días alternos durante seis semanas mediante una cánula intragástrica. Los resultados se muestran como una expresión relativa al gen constitutivo HPRT y como una proporción del control ± SD. (*) Indica una diferencia significativa p ≤ 0.05 y (**) indica una diferencia significativa p ≤ 0.01 por ANOVA de una vía (Dunnett).

8. Conclusiones

Con el presente trabajo se han logrado concluir diversos aspectos en relación a los efectos de la FCL en cáncer de colon, los cuales se enlistan a continuación:

- La FCL es capaz de inducir muerte celular de forma diferenciada en células de la misma estirpe patológica pero con distintas alteraciones, teniendo mejores efectos en aquellas que son menos malignas.
- La FCL induce muerte celular por apoptosis activando vías relacionadas con las caspasas, sin causar necrosis.
- Los principales genes alterados por el tratamiento con FCL son TP53 y Bcl-2, indicando la participación de la FCL en la activación de muerte por inducción de vías relacionadas a esas proteínas.
- La FCL no resulta tener efectos tóxicos de relevancia en animales tratados con la dosis segura de 50mg/kg de peso corporal.
- La FCL causa activación linfocitaria intestinal en las ratas.
- La FCL revierte el efecto cancerígeno causado con el AOM en ratas.
- La FCL induce apoptosis por medio de la desregularización de la vía de señalización mediada por Akt/Bcl-2/p53.

El presente trabajo permite proponer a la FCL como un posible tratamiento o coadyuvante para el cáncer de colon.

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Anexos

Publicación 1. Moreno-Celis, U., Lopez-Martinez, J., Blanco-Labra, A., Cervantes-Jimenez, R., Estrada-Martinez, L.E., Garcia-Pascalin, A.E., et al., 2017. **Phaseolus acutifolius Lectin Fractions Exhibit Apoptotic Effects on Colon Cancer: Preclinical Studies Using Dimethilhydrazine or Azoxi-Methane as Cancer Induction Agents**. *Molecules* (Basel, Switzerland) 22(10).

Publicación 2. Estrada-Martínez, L.E., Moreno-Celis, U., Cervantes-Jiménez, R., Ferriz-Martínez, R.A., Blanco-Labra, A., García-Gasca, T., 2017. **Plant Lectins as Medical Tools against Digestive System Cancers.** *International Journal of Molecular Sciences* 18(7), Doi: 10.3390/ijms18071403.

Publicación 3. Ferriz-Martínez, R., García-García, K., Torres-Arteaga, I., Rodriguez-Mendez, A.J., Guerrero-Carrillo, M. de J., Moreno-Celis, U., et al., 2015. **Tolerability assessment of a lectin fraction from Tepary bean seeds (Phaseolus acutifolius) orally administered to rats**. *Toxicology Reports* 2: 63–9.

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Phaseolus acutifolius Lectin Fractions Exhibit Apoptotic Effects on Colon Cancer: Preclinical Studies Using Dimethilhydrazine or Azoxi-Methane as Cancer Induction Agents

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Abstract: *Phaseolus acutifolius* (Tepary bean) lectins have been studied as cytotoxic molecules on colon cancer cells. The toxicological profile of a Tepary bean lectin fraction (TBLF) has shown low toxicity in experimental animals; exhibiting anti-nutritional effects such as a reduction in body weight gain and a decrease in food intake when using a dose of 50 mg/kg on alternate days for six weeks. Taking this information into account, the focus of this work was to evaluate the effect of the TBLF on colon cancer using 1,2-dimethylhydrazine (DMH) or azoxy-methane/dextran sodium sulfate (AOM/DSS) as colon cancer inductors. Rats were treated with DMH or AOM/DSS and then administered with TBFL (50 mg/kg) for six weeks. TBLF significantly decreased early tumorigenesis triggered by DMH by 70%, but without any evidence of an apoptotic effect. In an independent experiment, AOM/DSS was used to generate aberrant cryptic foci, which decreased by 50% after TBLF treatment. TBLF exhibited antiproliferative and proapoptotic effects related to a decrease of the signal transduction pathway protein Akt in its activated form and an increase of caspase 3 activity, but not to p53 activation. Further studies will deepen our knowledge of specific apoptosis pathways and cellular stress processes such as oxidative damage.

Keywords: colon cancer; apoptosis; lectins; Tepary bean; *Phaseolus acutifolius*

1. Introduction

Plant lectins are proteins that are able to bind to free or cell membrane carbohydrates, showing a high specificity for the recognition of surface glycosylations. Given these properties, they have been used for several disease diagnoses and as potential therapeutic agents, including against cancer [1–3]. Several studies have shown that traditional-use plant lectins exhibit biological effects such as cell agglutination, reactive oxygen species generation, and mitogenic or cytotoxic effects on different cells

as a function of concentration [3]. Lectins from edible and non-comestible plants, such as banana and ricin, have shown different effects that are related to different molecular mechanisms. The wide-range recognition ability of lectins is related to their specificity for membrane carbohydrates, therefore it is difficult to generalize the effects of these molecules [4,5].

Lectins can exhibit mitogenic effects, for example, peanut lectins have shown effects on cell proliferation [6,7], and others such as mistletoe (*Viscus album*) have mitogenic effects on immune cells [3]. On the other hand, soybean and other leguminous lectins have shown anticancer potential by promoting blockage of survival signaling pathways or inducing apoptosis by increasing reactive oxygen species (ROS), and/or caspase activity [8,9]. Changes in glycocalix are related to cancer progression due to their relationship with cell migration, invasion, evasion of the immune system, and metastasis [10–13], therefore, lectins have been widely studied for their anticancer effects [14], cell proliferation [15–18], and the increase in cell death by apoptosis and autophagy [12,13]. Some lectins can affect signal transduction pathways, such as mistletoe lectins that inhibit Akt phosphorylation [19] or *Polygonatum cyrtonema* lectins, which exhibit effects on the Ras-Raf and PI3K-Akt pathways [20] and induce apoptosis by deregulation of the ROS-p38-p53- signal pathway [21]. However, some lectins can induce apoptosis by independent p53 pathways [22].

Phaseolus acutifolius (Tepary bean) lectins have been studied recently because of their differential cytotoxic effects on cancer cell lines, particularly their specificity for colon cancer cells [17,23]. Toxic effects by intraperitoneal [24] and intra-gastric [25] routes have been tested and a Tepary bean lectin fraction (TBLF) showed good tolerability when administered in a dose of 50 mg/body weight kg, on alternate days for six weeks in Sprague Dawley rats by intra-gastric cannula. The TBLF was characterized, two lectins with no affinity for fetuin were sequenced and a differential biological activity was found [26]. Here, we demonstrate that the lectins present in TBLF are the only ones responsible for the cytotoxic effect and the evaluation of the intra-gastric administration of TBLF on chemical-induced colon cancer in Sprague Dawley rats using two cancer inductors, 1,2-dimetilhydrazine (DMH) and azoxi-methane/dextran sodium sulfate (AOM/DSS), is presented.

2. Results and Discussion

In previous work, we showed that protein inhibitors from Tepary beans did not exhibit cytotoxic effects but the TBLF is able to induce cell death in a differential manner on different cancer cells [11]. Prior to testing the effect of TBLF against colon cancer in rats, we confirmed that the non-lectin proteins (NLP) contained in the TBLF were not responsible for the cytotoxic effect. The ion exchange chromatogram obtained from the TBLF is shown in Figure 1. All the fractions with no agglutination activity were labelled as non-lectin protein (NLP); fractions with agglutination activity were pooled and labelled as lectin. The electrophoretic profile shows that the characteristic lectin band was separated from all the protein and the separation method showed good results in reproducibility. The lectin fraction was insufficient to perform the cytotoxic analysis but it was possible to test the effect of the NLP on cell proliferation. As cell harvesting was not achieved by using trypsin, we used an image analyzer to measure the cytotoxicity of the NLP. Image variables showed good correlation with cell proliferation by direct counting: cell circularity (-0.972, $p < 0.001$), Feret's diameter (0.854, $p = 0.015$) and cell perimeter (0.899, $p = 0.006$) but the cell area did not show correlation (0.578, $p = 0.174$). Taking this, we used only the significant image parameters to determine that lectins are the molecules mainly responsible for the cytotoxic effect since NLP exhibited a low cytotoxicity when compared with the TBLF (Figure 1C).

Two in vivo experiments were performed to study the effects of TBLF treatment on induced colon cancer. The first set of experiments consisted of inducing tumors in colonic tissue using DMH. Previous work showed that TBLF did not exhibit toxic effects in Sprague Dawley rats when orally administered in a dose of 50 mg/body weight kg; the only adverse effect observed until then was a 10% reduction of body weight gain [25]. Our results showed that TBLF caused a statistically significant decrease in body weight gain of about 10% and DMH administration caused a decrease of 5% ($p < 0.0001$, $F = 21.68$ and df = 71). The DMH/TBLF treated group showed a body weight gain reduction of up to 20%, with a recovery of 10% at the end of the treatment (Figure 2A). These indicated

a slight detrimental effect of DMH on body weight gain, but as TBLF naturally provokes a lessening in this parameter, it appeared that the co-administration of DMH/TBLF had a potentiated effect through the treatment that concluded with a partial recovery of body weight. In terms of food intake, there were variations in food consumption throughout the experiment; a decrease of 25% for TBLF and DMH/TBLF treatments even showed a complete recovery at the end of the experiment (Figure 2B) but there is no statistically significant difference (p = 0.4765, F = 0.8383 and df = 71). These results suggested that the detrimental effects on body weight gain and food intake were mainly related to the TBLF effect as an anti-nutritional factor. Besides anti-nutritional effects, TBLF could also exhibit effects on the immune system by mainly increasing granulocytes, and decreasing lymphocytes without effects on hepatic, renal or pancreatic markers [25], suggesting no toxic effects.

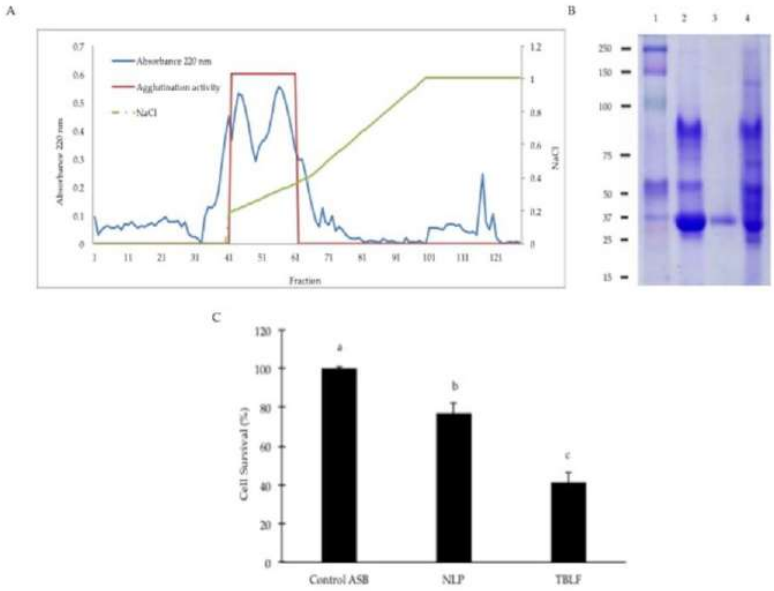

Figure 1. Cytotoxic effect of the non-lectin protein contained in the Tepary bean lectin fraction (TBLF). (**A**) TBLF was subjected to an ion exchange chromatography were non-lectin protein (NLP) and lectins (fractions with agglutination activity) were separated. (**B**) Electrophoretic profile of NLP and lectins. (1) Molecular weight marker, (2) TBLF, (3) Lectin pool, (4) Non-lectin protein pool. (**C**) Cytotoxic effect of NLP compared to TBLF. Small letters show significant difference by one-way ANOVA for the cytotoxic effect (Tukey, $p \leq 0.05$).

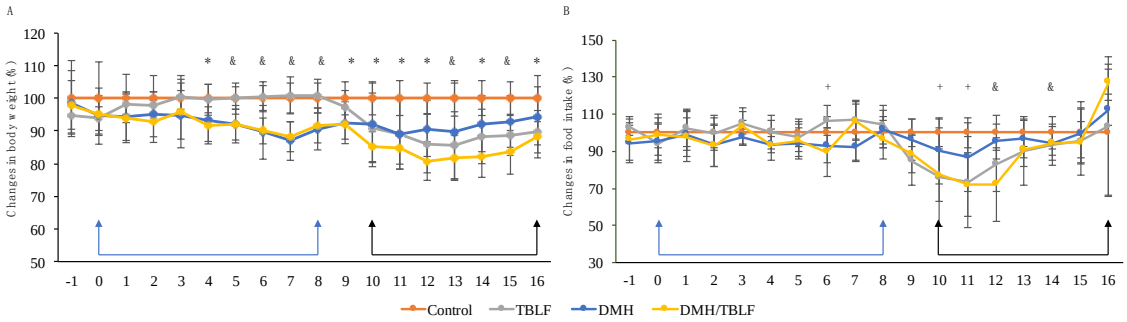

Figure 2. Body weight and food intake changes for the 1,2-dimethylhydrazine (DMH) experiment. Rats were administered DMH for eight weeks for the development of cancer lesions (blue arrows), followed by intra-gastric administration of TBLF (50 mg/kg) on alternate days for six weeks (black arrows). (**A**) Weekly body weight monitoring with respect to the control group. (**B**) Average of food intake changes per week with respect to the control group. A one-way ANOVA by Dunnett post hoc showed significant difference $p \leq 0.05$ (*), $p \leq 0.01$ (+) and $p \leq 0.001$ (&). TBL provoked a decrease in weight gain that affected also the combined DMH/TBLF treatment. Food intake decreased through TBLF administration but a final recovery was observed.

Tumor induction with DMH is shown in Figure 3. Histopathological findings showed different stages of tumor progression that were classified as inflammation, premalignant damage, low-grade damage and high-grade damage [27]. TBLF increased inflammation in colon tissues, lectins are considered anti-nutritional factors [2,28] because they can resist gut digestion for several hours, or even days [25]. They bind to intestinal membrane glycoproteins or glycolipids that affect nutrient absorption [7,29], and also activate the immune response [25,30], thereby provoking inflammation. Previous studies have shown that TBLF has exhibited a resistance to gut digestion, since agglutination activity was determined in faeces for at least 72 h with immune response activation [25], suggesting that they can interact with intestinal and colon epithelia, leading to an inflammation process.

DMH provokes different degrees of precancerous or cancerous injuries. Our results showed that DMH/TBLF treatment decreased 70% of premalignant lesions. Other lectins tested in different models have shown negative effects on tumorigenesis [31,32]. When proliferation and apoptotic markers were studied by immunohistochemical analyses, no differences were observed between the control rats and any of the treated groups (Figure 4). These results may be related to the tumor progression stage as the main activity of TBLF was observed in early tumorigenesis. Changes in glycocalyx are essential for lectin recognition, and it is well known that tumor progression is related to modifications in membrane glycosylation. Cancer cells display different aberrant membrane glycosylation patterns depending on the type of cancer and the tumor stage [21], therefore, TBLF was most likely unable to recognize low- and high-grade tumor cells.

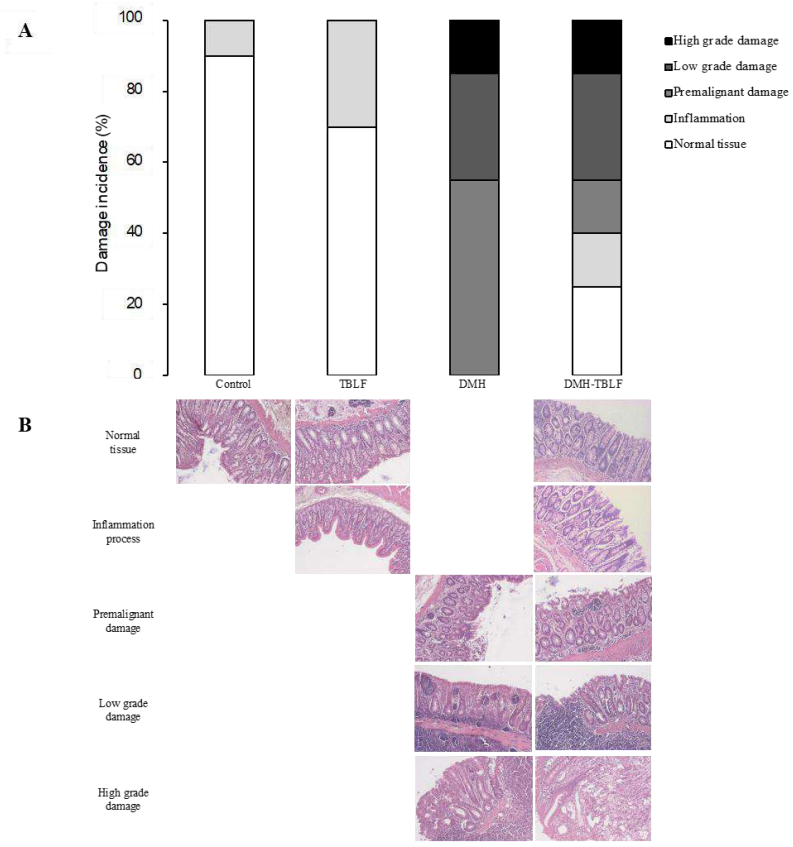

Figure 3. Histopathological analysis for colon lesions provoked by DMH and treated with TBLF. Rats were administered DMH for eight weeks for the development of cancer lesions followed by intra-gastric administration of TBLF (50 mg/kg) on alternate days for six weeks. (**A**) Incidence of colon tumors. (**B**) Hematoxylin-eosin histopathological analyses of colon lesions (10×). Microphotography shows the histological changes where it can be observed that tissue architecture was disrupted according to the malignancy.

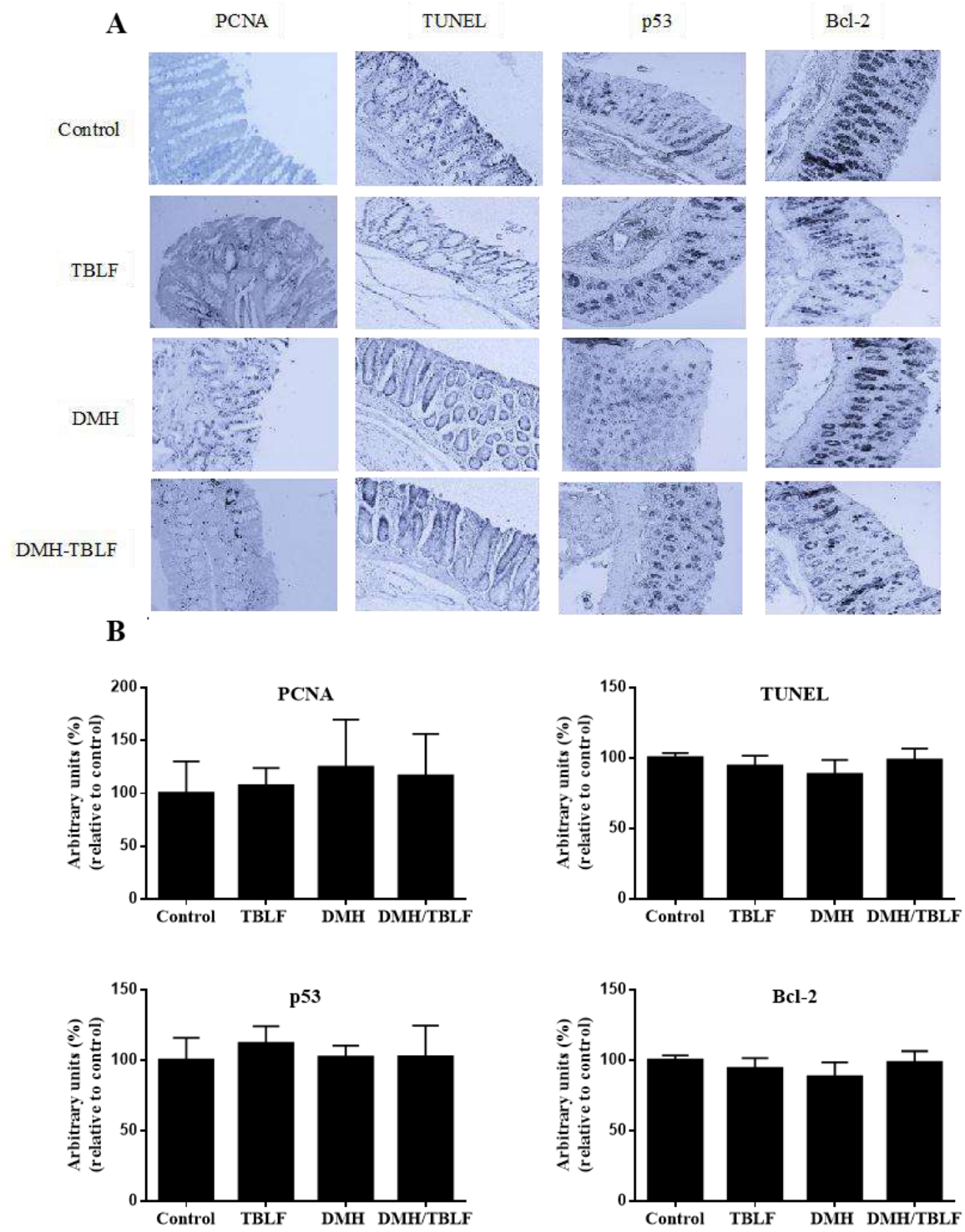

Figure 4. Immunohistochemical analyses of proliferation and apoptosis markers in colon lesions provoked by DMH and treated with TBLF. Rats were administered DMH for eight weeks for the development of cancer lesions followed by intra-gastric administration of TBLF (50 mg/kg) on alternate days for six weeks. (**A**) Representative micrographs of each group (10×); (**B**) Densitometry analyses of immuno-stained colon tissues. No significant difference by one-way ANOVA (Dunnett, $p < 0.05$) was found. No significant changes were observed between treatments.

As our results showed effects of TBLF on early tumorigenesis, a second set of experiments were performed using AOM/DSS in order to induce aberrant cryptic foci (ACF). AOM/DSS, alone or in combination with TBLF, showed a negative effect on body weight by up to 25%, which recovered by up to 5% at the end of the treatment (Figure 5A); the results were statistically significant ($p = 0.0004$, $F = 7.33$, df = 67). Food intake showed significant differences in AOM/DSS and AOM/DSS-TBLF treated groups (Figure 5B), and was statistically different ($p = 0.0001$, $F = 12.6$, df = 67). TBLF showed a similar effect on body weight gain as previously observed in this experiment, and a decrease in food intake had effects related to the anti-nutritional effects of different lectins.

Colon histopathology assays showed normal colonic tissue architecture in the control rats [27], while rats treated with the TBLF showed an atrophic effect on intestinal epithelia with decreased length and fusion of the colonic structure (Figure 6). These results were consistent with other studies, for example, Daprà et al. [23], observed that legume lectins provoked lymphocyte infiltration in the intestinal tissue as well as villi shortening and widening in trout (*Oncorhynchus mykiss*). As villi affectation by lectins can induce changes in organ architecture, this was related to the decrease in weight gain and in food consumption. The effect of AOM/DSS treatment induced inflammation in 15% and ACF in 60% of the rats' colon tissue and disrupted the entire architecture [27]. The co-administration AOM/DSS-TBLF reduced the ACF by 50% and partially restored the tissue architecture, but atrophy was still observed. This result showed the effect of TBLF in the early stages of colon tumorigenesis; and was consistent with studies reporting anticancer effects using lectins such as Concanavalina A lectin in other animal models [4,32], and *Euchema serra* agglutinin in mice [4]. Such results agree with the fact that glycosylation changed by tumor progression affected the TBLF affinity for cancer cells in different stages.

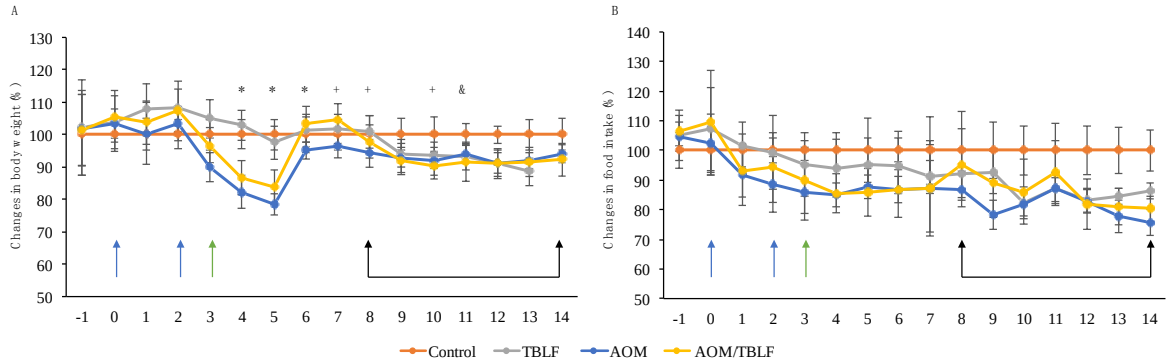

Figure 5. Body weight and food intake changes for the azoxy-methane/dextran sodium sulfate (AOM/DSS) experiment. Rats were intraperitoneally administered for two weeks with AOM (blue arrows) and a further week with dextran sodium sulfate (DSS) in their daily drinking water (green arrow) to develop aberrant cryptic foci (ACF). Next, they were administered TBLF (50 mg/kg) on alternate days for six weeks via intra-gastric cannula (black arrows). (**A**) Weekly body weight monitoring with respect to the control group; (**B**) Average of food intake changes per week with respect to the control group. A one-way ANOVA by Dunnett post hoc showed significant difference (*) $p \leq 0.05$, (&) $p \leq 0.01$ and (+) $p \leq 0.001$. A decrease in weight gain and food intake was observed for all treatments with no recovery at the end of the experiment.

Different studies have focused on elucidating the signaling pathways triggered by lectins, where it has been observed that lectins from different sources share some mechanisms of action on cell proliferation, survival, and apoptosis induction [33]. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) allowed us to observe that TBLF caused a significant reduction in cell proliferation when it was administered after the carcinogen (Figure 7). These results were consistent with in vitro and in vivo studies of different lectins on cancer [7,8,32,34,35].

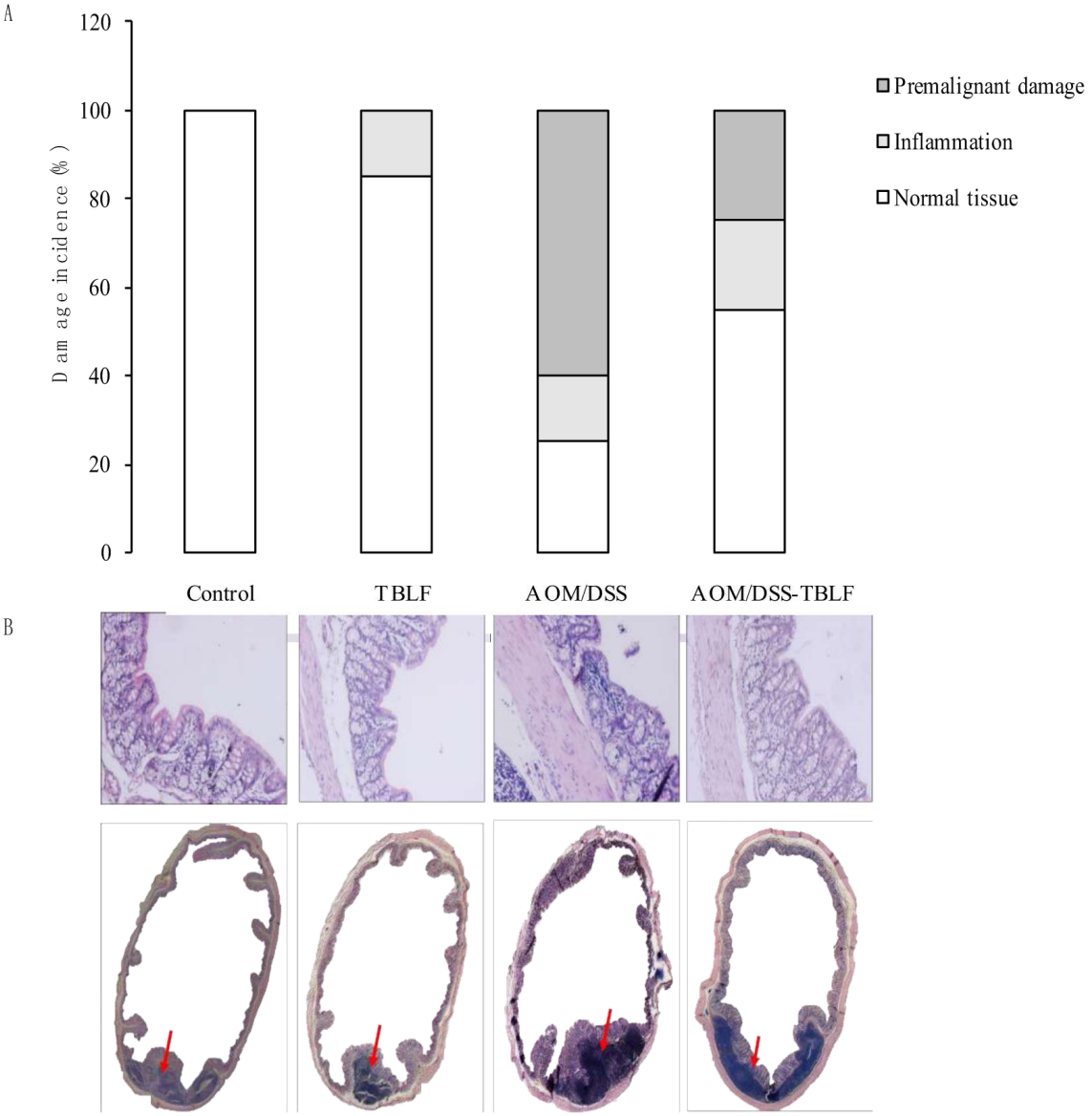

Figure 6. Histopathological analyses for colon lesions provoked by AOM/DSS and treated with TBLF. Rats were intraperitoneally administered for two weeks with AOM and a further two weeks with DSS in their daily drinking water to develop ACF. Next, they were administered with TBLF (50 mg/kg) on alternate days for six weeks via intra-gastric cannula. (**A**) Incidence of aberrant cryptic foci; (**B**) Hematoxylin-eosin histopathological analyses of colon lesions (10×). Red arrows show lymphocyte infiltration. Although no tumors were observed, AOM/DSS treatment showed a high disruption process that was diminished by TBLF.

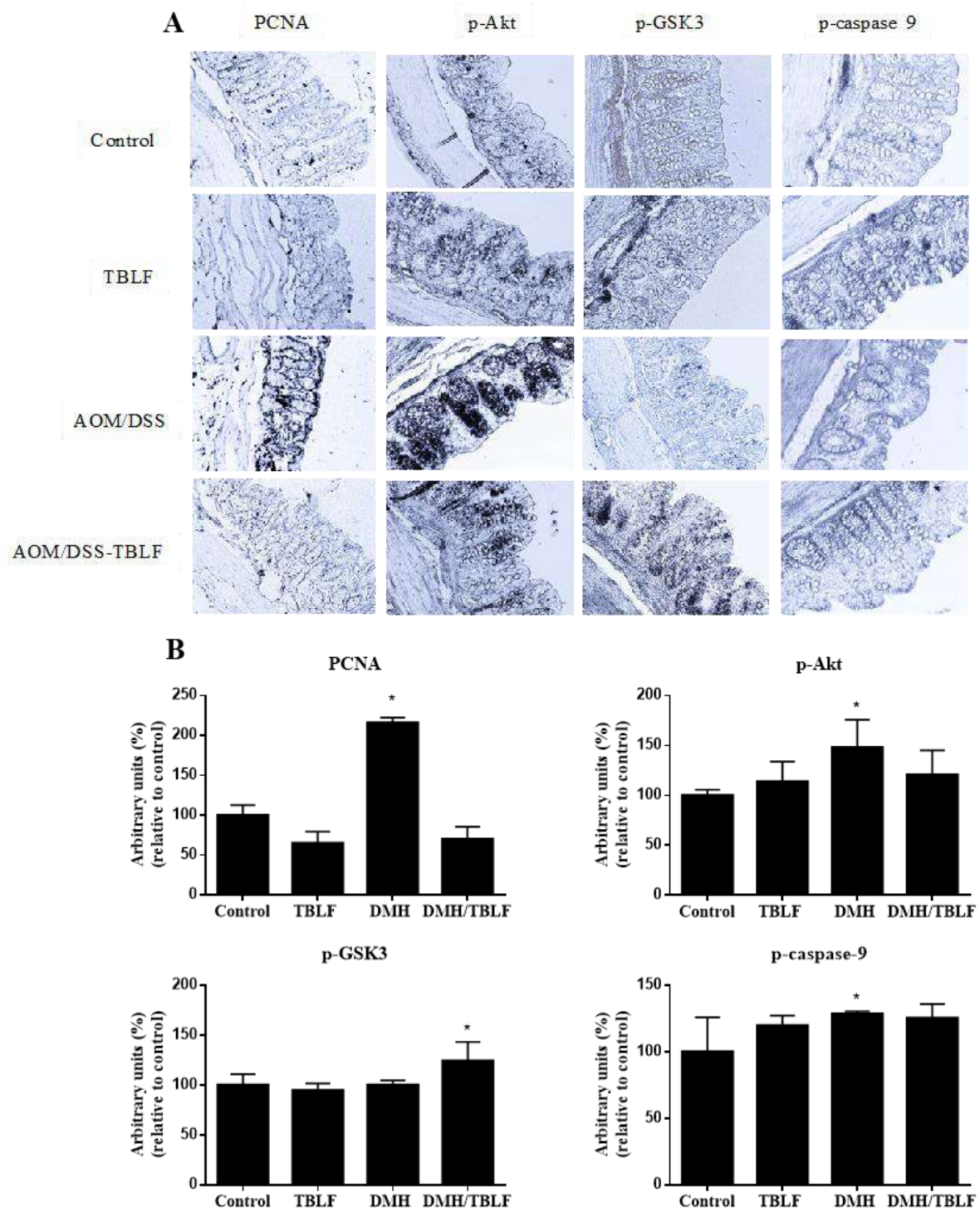

Figure 7. Immunohistochemical analyses of the proliferation and Akt-pathway targets in colon lesions provoked by AOM/DSS and treated with TBLF. Rats were intraperitoneally administered for two weeks with AOM and a further two weeks with DSS in their daily drinking water to develop ACF. Next, they were administered with TBLF (50 mg/kg) on alternate days for six weeks via intra-gastric cannula. (**A**) Representative micrographs of each group (10×); (**B**) Densitometry analyses of immuno-stained colon tissues. (*) Significant difference by one-way ANOVA (Dunnett $p < 0.05$).

The Akt pathway was evaluated due to its participation in proliferation-apoptosis regulation and as it is commonly deregulated in colon cancer [36,37]. The Akt pathway increases cell proliferation by phosphorylating GSK-3, a blocking agent of Cyclin-D, and decreases apoptosis by phosphorylation of caspase 9. No changes between the control and TBLF treated animals were observed; however, AOM/DSS exhibited an increase in p-Akt as reported in colon cancer models [38], and also a slight increment of p-caspase 9. Nevertheless, p-GSK-3 did not show differences with respect to the control rats. These results showed the participation of the Akt pathway by AOM/DSS in colon tissue. When rats were treated with AOM/DSS-TBLF, a decrease in p-Akt was observed with respect to the AOM/DSS-treated animals, suggesting that TBLF may block the Akt pathway. Given those results, no increase in p-GSK-3 and p-caspase 9 was expected; however, p-GSK-3 increased significantly, perhaps due to the participation of other routes such as the protein kinase A (PKA) or protein kinase C (PKC) pathways [39].

To assess whether cell death by apoptosis induction occurred, the presence of p53, Bcl-2, caspase 3, cytochrome-c was evaluated. Neither AOM/DSS, nor TBLF alone provoked an increment of caspase 3, but the co-treatment of AOM/DSS-TBLF showed a significant increase. Furthermore, the AOM/DSS-TBLF treatment showed an increase in cytochrome-c, and a decrease in the antiapoptotic protein Bcl-2. A significant increase of p53 was observed in the AOM/DSS treatment ($p < 0.05$) (Figure 8). The specific mutations of AOM/DSS are well known, especially in KRas with high frequency [40,41], which deregulates the corresponding signaling cascade and allows sustained proliferative activity [42]. However, AOM/DSS treatment in colon cancer models was not related to mutations on the p53 gene, but an increase has been observed in the expression of this protein [38]. Studies in colitis and colon cancer models using AOM/DSS as the carcinogen have shown an increase of both the expression and amount of p53; however, no mutations have been observed [43,44]. Although the p53 protein is typically involved in repairing damage or inducing apoptosis in cells depending on DNA damage, a mechanism called apoptosis-induced proliferation (AiP) has been described [38,45] where an exacerbated apoptotic process leads to a release of mitogens that induce cell proliferation. This process has been implicated in intestine cell growth in mice, which was promoted by an increase in p53 activity [45]. In the same way, dying xenograft cells in mice treated with radiotherapy showed an increase in caspase 3 activity, which promoted tumor repopulation [46]. Therefore, induces the AiP process as part of its action mechanism in the development of colon cancer. The AOM/DSS-TBLF group showed no statistically significant differences in p53 when compared with the control group, suggesting an inhibitory effect of TBLF on AOM/DSS cancer induction.

In order to corroborate the immunohistochemical results, gene expression of TP53, Bcl-2, caspase 9, GSK, and Akt was determined in colonic tissues (Figure 9). A significant increase in TP53, Bcl-2, and Akt expression were observed for AOM/DSS treatment, but no differences were found for AOM/DSS-TBLF-treated rats with respect to the control. Caspase 9 gene expression was significantly increased by AOM/DSS-TBLF treatment, suggesting apoptosis induction by TBLF. These results fully agree with the immunohistochemical analyses previously described and taken together, these results suggest the independent p53 apoptotic activity of TBLF on early precancerous colonic tissue.

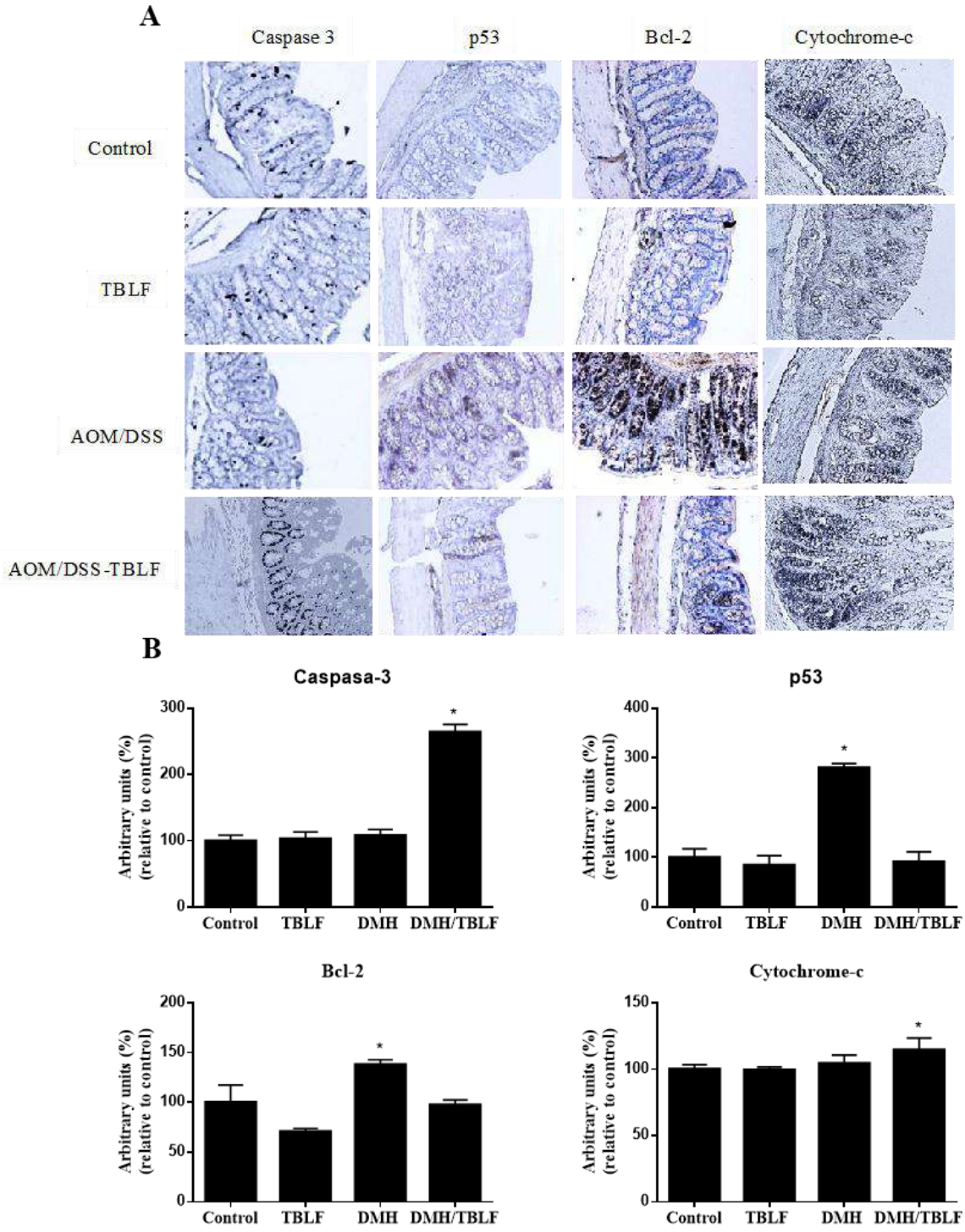

Figure 8. Immunohistochemical analyses of apoptosis markers in rats administered with AOM/DSS and treated with TBLF. Rats were intraperitoneally administered for two weeks with AOM and a further two weeks with DSS in their daily drinking water to develop ACF. Next, they were administered with TBLF (50 mg/kg) on alternate days for six weeks via intra-gastric cannula. (**A**) Representative micrographs of each group (10×); (**B**) Densitometry analyses of immuno-stained colon tissues. (*) Significant difference by one-way ANOVA (Dunnett $p < 0.05$).

It has been reported that several lectins induce apoptosis through caspase activity in different cancer cell lines [47] and in some animal models [32,44]. Mistletoe lectins (*Viscum album* L. var. Coloratum) have shown anticancer effects by inducing apoptosis by caspases in melanoma [44]. *Eucheuma serra* lectins have exhibited a caspase-mediated apoptotic effect in colon adenocarcinoma cells (Colon26) and in BALB/c mice with colon cancer [8]. The Akt-mediated signaling pathway has also been studied and the results showed that lectins such as *Canavalia brasiliensis* and *Viscum album coloratum* could induce cell death by deregulating this pathway [8,19,48]. The effect of Con-A was found to be dependent on the decrease in p-Akt, an increase in cytochrome-c, and an increase in caspase 9 activity [19,43]. In contrast, Korean mistletoe lectins (VAL) have the ability to induce p53-independent apoptosis, causing a decrease in Bcl-2 levels, and an increase in cytochrome-c and telomerase inhibitory activity on hepatic carcinoma [49]. Here, our study showed for the first time that Tepary lectins were able to induce apoptosis in colon precancerous lesions by a p53-independent way, suggesting Akt pathway blockade.

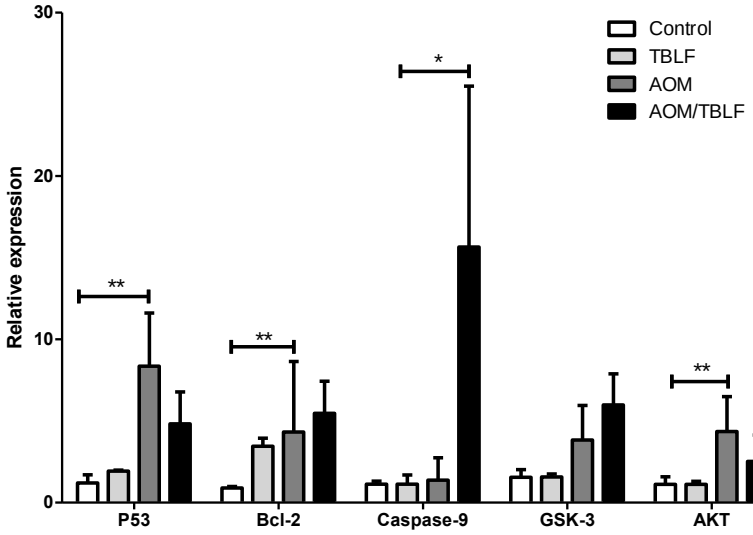

Figure 9. Evaluation of the gene expression of TP53, Bcl-2, caspase 9, GSK-3, and AKT in rat colon tissues administered with AOM/DSS and treated with TBLF. Rats were intraperitoneally administered for two weeks with AOM and a further two weeks with DSS in their daily drinking water to develop ACF. Next, they were administered with TBLF (50 mg/kg) on alternate days for six weeks via intra-gastric cannula. The results are shown as a relative expression to the constitutive gene HPRT and as a proportion of the control ± SD. (*) indicates significant difference $p \leq 0.05$ and (**) indicates significant difference $p \leq 0.01$ by one-way ANOVA with Dunnett post hoc.

3. Materials and Methods

3.1. Tepary Bean Lectin Fraction (TBLF) Extraction

Tepary bean (*Phaseolus acutifolius*) seeds were obtained from a local market in Hermosillo, Sonora, México. A sample of Tepary bean was deposited and identified in the herbarium of Dr. Jerzy Rzedowski of The Natural Sciences Faculty, Autonomous University of Querétaro, Santiago de Querétaro, Mexico. The TBLF was obtained as described previously [17,50]. In brief, ground bean seeds were degreased and an aqueous extract was obtained. A selective sequential precipitation (40–70% ammonium sulfate) was performed and the protein dialyzed and separated by molecular weight exclusion chromatography using a Sephadex G-75 column (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Absorbance at 280 nm was measured for protein profile determination using a Beckman DU-65 spectrophotometer (Beckman Coulter Inc., Brea, CA, USA), agglutinant activity was determined [51] and the samples were lyophilized and stored for further use at −20 °C.

3.2. Confirmation of the Cytotoxic Role of Lectins in the TBLF

TBLF was subjected to ion exchange chromatography using an Econo Pack High Q cartridge (ECONO, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a flow rate of 1 mL per min using a NaCl (0.1 to 1.0 M). A total of 120 fractions were collected and the presence of agglutination activity was determined. Two pools were obtained, one with all the fractions with no agglutination activity (non-lectin protein, NLP) and the other one containing the lectins. The two pools were dialyzed, lyophilized and stored at −20 °C. Cytotoxicity of NLP was determined using 3T3/v-mos cells [11]. Briefly, cells were plated in 24-well plates (1×10^4 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (FBS). After 24 h, the medium was changed to DMEM with 2% FBS for cell cycle synchronization for a further 24 h. Subsequently, the cytotoxic effect of the NLP was determined and compared to a TBLF positive control, using a protein concentration of 0.4 mg/mL for both treatments in DMEM with 0.5% of bovine serum albumin (BSA). Cells were incubated for 8 h and the cytotoxic effect was determined by image analysis (IMAGEJ 1.50b, National Institutes of Health, Bethesda, Rockville, MD, USA) using an inverted microscope (Carl Zeiss Axiovert A1, Carl Zeiss Inc., Oberkochen, Germany) at 10× resolution to measure the following morphometric parameters: area, perimeter, Feret's diameter and circularity. As a first step, images of the treated cells were processed converting the colored-image to an 8-bit format (grey scale image). Then, an automatic threshold by an algorithm default was applied to obtain a binary image [52]. Finally, cells were highlighted with the tool "fill" found in the same software. Correlation with cell proliferation by direct counting was determined for each morphometric parameter and for the cytotoxic evaluation only parameters with significant correlation were used. The criterion to discriminate between living cells and dead cells was based on the statistical evaluation of morphometric parameters using ANOVA, Tukey and Fisher tests ($p \leq 0.05$) using MINITAB 17 (Minitab Inc., Harrisburg, PA, USA) with respect to control cells where a confidence interval for each significant parameter was selected: perimeter (90.0 to 96.8 μm), Feret's diameter (32.0 to 34.0 μm) and circularity (0.31 to 0.35), cells whose parameter values did not belong to these intervals were classified as dead cells.

3.3. Experimental Animals and Cancer Induction

Five-week-old, male Sprague Dawley rats were obtained from the Neurobiology Institute-UNAM, Juriquilla, México. The animals were maintained with food and water ad libitum, under a circadian cycle of 12 h light and 12 h dark at 25 °C. All experiments started after a week of adaptation and were conducted observing the procedures of the Official Mexican Norm [29] for the use of laboratory animals and with the approval of the Bioethics Committee of the Neurobiology Institute, UNAM, México. Two independent experiments were performed (Figure 10). The first experiment (n = 10 per group) induced colon tumors by subcutaneous injection of 40 mg/body weight kg of 1,2-dimethylhydrazine (DMH, D161608-100G, Sigma Aldrich, St Louis, MO, USA), in 0.5 mL of saline solution (SS, 0.9% NaCl) twice a week for eight weeks, followed by two weeks without treatment and six weeks of TBLF (50 mg/kg each third day). The second experiment (n = 7 per group) was designed to provoke aberrant cryptic foci (ACF) using 10 mg/body weight kg of 2-azoximethane (AOM, A5486-100MG, Sigma Aldrich, St Louis, MO, USA) in 0.5 mL of 0.9% SS by a weekly intraperitoneal injection in weeks one and three, followed by one week of 2% dextran sodium sulfate (DSS) in their daily drinking water as a cancer promoter. After five weeks without treatment, TBLF was administered in a dose of 50 mg/kg in 0.5 mL SS via intra-gastric cannula [25] on alternate days for six weeks. Weekly body weight changes and average food intake were monitored in both experiments. Euthanasia was performed by decapitation a week after the last dose of TBLF. The organs were dissected under the supervision of a veterinarian and fixed in 10% formaldehyde, and the intestines were perfused with the same solution to achieve the best preservation. Tumor classification was performed by a veterinarian pathologist [27].

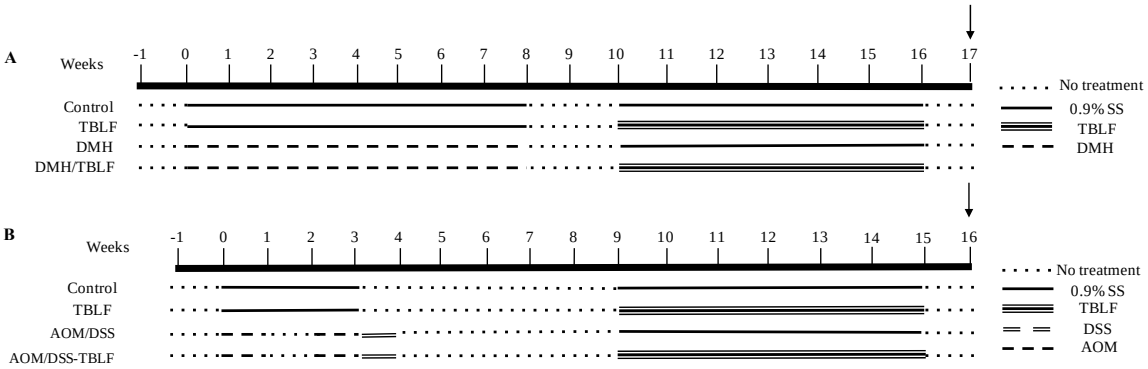

Figure 10. Experimental design. (**A**) DMH experiment (n = 10 per group). (**B**) AOM/DSS experiment (n = 7 per group). Two independent experiments were performed. Cancer induction with DMH was achieved after eight weeks of treatment to generate colon tumorigenesis while AOM induction took two weekly doses to generate aberrant cryptic foci. TBLF was administered for six weeks each third day and sacrificed a week later (black arrow).

3.4. Histopathology Analyses

The tissues were dehydrated and embedded in paraffin using a Histoquinete (Leica TP1020, Leica Camera AG, Wetzlar, Germany). Tissue cuts of 5 µm were obtained and mounted in gelatin-coated slides in hot water. Tissues were rehydrated and stained with hematoxylin and eosin, then dehydrated again using decreasing concentrations of alcohol and sealed with entellan and a coverslip. The analyses were performed under a microscope (Zeiss Axio Vert-A1, Carl Zeiss Inc., Oberkochen, Germany) using 5–10× magnification based on the morphology of normal colonic tissue. Lieberkühn crypts normally appear as simple tubular glands and can be classified according to morphological changes, inflammation process and structure loss for classifying damage as premalignant, low-grade damage, high-grade damage and neoplasia [27].

3.5. Immunohistochemical Analyses for Proliferation and Apoptosis

Dehydrated samples were embedded in paraffin blocks and cut into 5 µm thick positively charged Thermo Fisher slides (Thermo Fisher Scientific, Waltham, MA, USA) using a microtome and subsequently rehydrated. To unmask the epitopes, samples were placed in a water bath at 100 °C for 20 min in 0.1 M citric acid solution, pH 6.0. Endogenous peroxidases were quenched with 2% H_2O_2 for 30 min in phosphate buffer saline with 1% tween 20 (PBST). Blocking was performed with 1% bovine serum albumin (BSA) in PBST for 1 h and incubated with primary antibodies (PCNA, caspase 3, p53, Bcl-2, p-Akt, p-GSK3, p-caspase 9, and cytochrome-c overnight (16 h approx.) at 18 °C. Secondary antibodies were added and incubated for 2 h at 18 °C. Staining was achieved using diaminobenzidine 1:8000 w/v and H_2O_2 1:2500 in phosphate buffer saline (PBS). The reaction was stopped using ddH_2O. Hematoxylin was used as a contrast agent; samples were dehydrated and mounted for observation under a microscope. Photographs were obtained and an optical densitometry analysis was performed using Image J® software (v.1.5, National Institutes of Health, Bethesda, Rockville, MD, USA). Data were reported as arbitrary units ± SD.

3.6. Gene Expression Analysis

Formalin-fixed paraffin embedded tissue was extracted with a train of alcohols and rehydrated. RNA was extracted as per Reference Gouveia et al. (2014) [53]. cDNA was synthesized using a TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster city, CA, USA, Cat. No. N8080234), and a q-PCR was performed using the following primers:

TP53: fw AGTGGGAATCTTCTGGGACG; rv TCTTTTGCTGGGGAGAGGAG
Bcl-2: fw TTCTTTGAGTTCGGTGGGGT; rv CAGCCTCCGTTATCCTGGAT

Caspase 9: fw GATGCTGTCCCATACCAGGA; rv TCTCGATGTACCAGGAACCG
GSK-3: fw CTCAAGGCTCTCCCCACTAG; rv GTCTTGGCCAGTCTGAGTCT
AKT: fw CAAAGGATGAAGTCGCCCAC; rv TGCAAGTACTCCAGAGCTGA

Normalization was performed using the housekeeping gen hypoxanthine-guanine phosphoribosyl transferase (HPRT): fw AGGACCTCTCGAAGTGTTGG; rv CCACTTTCGCTGATGACACA. Data were reported with respect to the control group.

3.7. Statistical Analyses

A one-way ANOVA by Tukey or Dunnett post hoc ($p < 0.05$) was used to perform the data analyses. The results were presented as average ± SD. Analyses were performed using the IBM SPSS Statistics for Windows (Version 22.0, IBM Corp., Armonk, NY, USA).

4. Conclusions

TBLF affected DMH- and AOM/DSS-induced tumorigenesis in the colon where only premalignant lesions or ACF were affected. The DMH experiment showed that TBLF decreases the incidence of premalignant lesions but no evidence of apoptotic mechanisms was observed. On the other hand, our results suggested that AOM/DSS effects were related to the induction of the Akt pathway and AiP process since an increase in cellular proliferation was observed together with high levels of p53; however, more work is required to have a better understanding of such effects. The co-administration of AOM/DSS-TBLF reversed p53 and PCNA to basal levels, suggesting an anti-proliferative effect of TBLF, and apoptosis was confirmed by the increase of caspase 9 gene expression, a decrease of Bcl-2, and the increment of caspase 3 and cytochrome-c proteins. The decrease of p-Akt after the AOM/DSS-TBLF treatment appears to be related to a blocking effect of TBLF; however, it is necessary to explore this pathway further. These results allow us to propose that TBLF induces apoptosis in the early stages of colon malignancy in a p53-independent way. Further research will focus on the signaling pathways triggered and/or altered by TBLF in colon cancer, and will propose an evaluation of the ROS-mediated effects, the extrinsic and intrinsic pathways of apoptosis, as well as other possibly triggered cell death processes to unravel the anticancer potential of TBLF as a therapeutic agent against colon cancer.

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Author Contributions: Ulisses Moreno-Celis and Josué López-Martínez performed all the in vivo experiments and the experimental analyses; Ricardo Cervantes-Jiménez obtained the TBLF; Alejandro Eduardo García-Pascalin participated in the immunohistochemical analyses; Laura Elena Estrada-Martínez realized the in vitro studies; María De Jesús Guerrero-Carrillo performed the histopathological assays and their interpretation; Carmen Mejía, Alejandro Blanco-Labra, and Adriana Jheny Rodríguez-Méndez participated in the study design and interpretation; and Roberto Augusto Ferríz-Martínez and Teresa García-Gasca directed the entire work. All co-authors participated in general discussion and interpretation of the results.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Plant Lectins as Medical Tools against Digestive System Cancers

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Abstract: Digestive system cancers—those of the esophagus, stomach, small intestine, colon-rectum, liver, and pancreas—are highly related to genetics and lifestyle. Most are considered highly mortal due to the frequency of late diagnosis, usually in advanced stages, caused by the absence of symptoms or masked by other pathologies. Different tools are being investigated in the search of a more precise diagnosis and treatment. Plant lectins have been studied because of their ability to recognize and bind to carbohydrates, exerting a variety of biological activities on animal cells, including anticancer activities. The present report integrates existing information on the activity of plant lectins on various types of digestive system cancers, and surveys the current state of research into their properties for diagnosis and selective treatment.

Keywords: cancer; diagnosis tools; digestive system; plant lectins; therapeutic tools

1. Introduction

Cancer is a complex process in which genetic alterations modify the ability of cells to transduce signals, and allow them to acquire new functions, replicate beyond normal limits, evade apoptosis, and ultimately encroach other tissues [1,2]. Within this process, cell surface glycosylations play a key role on cell development, signalling, interaction, proliferation, differentiation and migration [3–5]. Digestive system cancers result from a combination of genetic and lifestyle factors that encompass a wide spectrum of diseases with different clinical characteristics, therapeutic specificities, and life expectancies [6,7]. They represent an important cause of mortality worldwide, generally related to late diagnosis due to the absence of symptoms or masking by other pathologies [8]. Inflammation is a physiological response that has been widely related to the presence of cancer in the digestive tract [9,10], and for which alterations in the glycosylation of proteins play an important role [11]. Although some epidemiological studies have found that from 10 to 15% of cancers were related to infections caused by viruses, fungi or bacteria, it has also been found that up to 25% of cancers are associated with chronic inflammation [12–14].

Most of the drugs currently employed in anticancer therapy seem to affect cell replication and therefore tumor growth, but they usually have nonselective mechanisms of action that affect vital macromolecules (such as DNA) or metabolic pathways that are important for both malignant and normal cells, causing undesirable and potentially toxic effects [15,16]. Efforts to treat cancer have led research to focus on the use of less toxic and more selective molecules. Membrane glycosylation, one of the most important facts of cell behaviour, has been pointed to as a valuable target for cancer diagnosis and treatment [17,18]. Cancer cells display aberrant membrane glycosylation patterns, which vary

depending on the type of cancer and the tumor stage. Among the major glycosylation changes are the blockage synthesis and the neo-synthesis of carbohydrates, altered branching, and the appearance of new structures. A greater occurrence of cell surface *N*-glycans, sialylations, and fucosylations, the abnormal production of mucin, the expression of Lewis X/A structures in glycosphingolipids (identified as a tumor antigen), and the increased expression of galectins constitute the main structural changes that mark the difference between cancer and normal cells. These changes are related with cell migration, invasion, evasion of immune system, and metastasis [5,19–23].

Lectins are proteins or glycoproteins of non-immune origin that display a ubiquitous distribution in living organisms, and are particularly abundant in plants. They have the ability to recognize and bind specifically and reversibly to either free carbohydrates or glycoconjugates, such as glycoproteins, glycolipids, or polysaccharides, without modifying their structure [24–27]. This type of proteins has the ability to agglutinate cells or precipitate glycoconjugates detonating a variety of important cellular processes [28–30]. Hundreds of plant lectins have been purified and characterized in order to investigate their biochemical properties, carbohydrate binding specificity, and biological functions [31,32], finding numerous applications in the agronomic and biomedical fields, including anticancer potential [31–34].

2. Potential of Plant Lectins against Cancer

The anticancer potential of lectins can be considered from two main angles: diagnostic and therapeutic. The first is due to their ability to recognize cancer cells, mainly by the presence of tumor glycosylations [35,36], which allows for a better diagnosis and prognosis of cancer tumors [37,38]. Their therapeutic potential is based on their antitumor activity and cytotoxic effects through the induction of programmed cell death, such as apoptosis and autophagy [27,39–44]; however, the mechanisms of cell death induction have not been fully unravelled [31].

In vitro studies have found a preferential attachment of some lectins to the membranes of cancer cells [27,39,45–47], a relevant aspect since selectivity is sought as a tool to improve the effectiveness of anticancer therapies. For example, mistletoe lectins (*Viscum album*) have been used on the European continent for years as alternative adjuvant agents in cancer therapy, lessening the adverse effects of chemo and radiotherapy, and improving patients' quality of life [47]. Further, some lectins have the ability to bind to the gastrointestinal epithelium cells, exhibiting high resistance to intestinal proteolysis and maintaining their biological activity and carbohydrate affinity intact [48,49]. Table 1 shows the growing diversity of plant lectins with cytotoxic, antiproliferative, apoptotic, or autophagic effects on cancer cell lines and on in vivo experiments.

Table 1. Antineoplastic activity of plant lectins.

Vegetal Source	Lectin	In Vitro Activity	In Vivo Activity	References
Abrus precatorius	AGG	Inhibition of protein synthesis, apoptosis induction.	Inhibition of tumor growth and angiogenesis, apoptosis induction.	[50–53]
Allium chinense	ACL	Antiproliferative effect, apoptosis induction.	Not reported.	[54]
Arachis hypogaea	PNA	Antiproliferative effect, apoptosis, and autophagy induction by oxidative stress.	Inhibition of tumor growth, apoptosis and autophagy induction.	[46]
Astragalus membranaceus	AML	Antiproliferative effect, apoptosis induction by caspases.	Not reported.	[55,56]
Canavalia ensiformis	Con A	Antiproliferative effect, autophagy, and apoptosis induction via caspase–mitochondrial pathway.	Inhibition of tumor growth, inhibition of tumor nodule formation.	[57–62]
Glycine max	SBL	Antiproliferative effect, apoptosis, and autophagy induction by oxidative stress and DNA damage.	Inhibition of tumor growth, apoptosis, and autophagy induction.	[63]
Momordica charantia	MCL	Differential antiproliferative effect, apoptosis induction by caspases.	Inhibition of tumor growth, apoptosis induction.	[64,65]
Morus alba	MLL	Apoptosis induction.	Not reported.	[66]
Phaseolus acutifolius	TBL	Differential antiproliferative effect, apoptosis induction.	Not reported.	[39,67]
Phaseolus vulgaris	PHA	Antiproliferative effect, apoptosis induction by death receptors.	Not reported.	[41,68,69]
Pinellia ternate	PTL	Antiproliferative effect, apoptosis induction.	Inhibition of tumor growth.	[70,71]
Polygonatum cyrtonema	PCL	Differential antiproliferative effect, autophagy, and apoptosis induction by caspases.	Not reported.	[72–74]
Polygonatum odoratum	POL	Differential antiproliferative effect, autophagy induction by oxidative stress, and apoptosis induction via caspase–mitochondrial pathway and death receptors.	Not reported.	[42,75,76]
Sophora flavescens	SFL	Antiproliferative effect, apoptosis induction by caspases.	Inhibition of tumor growth.	[62,77]
Triticum vulgaris	WGA	Differential antiproliferative effect, autophagy induction.	Not reported.	[78,79]
Urtica dioica	UDA	Antiproliferative effect, apoptosis induction.	Not reported.	[80]
Viscum album	ML	Antiproliferative effect, apoptosis induction.	Inhibition of tumor growth and metastasis, prolonged survival rate.	[81–86]

AGG, Abrus agglutinin; ACL, *Allium chinense* lectin; PNA, Peanut agglutinin; AML, *Astragalus membranaceus* lectin; Con A, Concanavalin A lectin; SBL, Soybean lectin; MCL, *Momordica charantia* lectin; MLL, Mulberry leaf lectin; TBL, Tepary bean lectin; PHA, *Phaseolus vulgaris* agglutinin; PTL, *Pinellia ternata* lectin, PCL *Polygonatum cyrtonema* lectin; POL, *Polygonatum odoratum* lectin, SFL, *Sophora flavescens* lectin; WGA, Wheat germ agglutinin; UDA, *Urtica dioica* agglutinin; and ML, Mistletoe lectin.

3. Plant Lectins against Esophageal Cancer

Esophageal cancer ranks eighth in prevalence and sixth in cancer mortality worldwide [87], and its incidence is expected to rise within the next few years [88] due to factors such as diet [89,90] and lifestyle that lead to pathologies such as obesity [91], gastresophageal reflux, and Barrett's esophagus [92,93]—themselves risk factors for changes at the cellular level.

Invasive esophageal cancer is a progressive multi-stage process that can be completed in two ways: by the conversion of normal epithelium to basal cell hyperplasia, dysplasia or carcinoma in situ that leads to squamous cell carcinoma; or by metaplasia caused by Barrett's esophagus, which represents a previous stage and leads to esophageal adenocarcinoma (EAC) [92,94]. However, the elasticity of the esophagus delays the presence of symptoms [95], so that cancer in this organ is habitually diagnosed in advanced stages and often in the presence of metastatic disease [96], decreasing the 5-year survival to less than 15% [97]. Diagnosis is usually invasive and exhibits limitations in the detection of cancer in its early stages [98]. According to this, and given the fact that esophageal cancer is mostly preceded by dysplastic and metaplastic changes in tissue, it is important to find biomarkers that allow identification of alterations at the cellular level, in the early stages of neoplastic formation [96].

At present, there is little evidence on the use of plant lectins as diagnostic agents or adjuvant treatment of esophageal cancer. However, in a recent study, the topical application of fluorescently labelled wheat germ lectins (WGA) on ex vivo esophagus tissues showed high affinity and specificity for sub-expressed glycans in neoplasia originated from Barrett's esophagus. Identification of dysplasia was better traced than by white light endoscopy, the technique commonly used for diagnosis [99], confirming that the use of lectins for optically detecting changes in glycan expression in dysplastic tissue represents a potential biomarker for the transformation towards EAC [100]. Additionally, new lectin-based biomarkers have been used for the detection of EAC in serum. A lectin-coated magnetic bead array, coupled with mass spectrometry and assembled with 20 lectins, mostly from plants, has distinguished between healthy, Barrett's esophagus, and EAC phenotypes and will be subject to further testing [98].

4. Plant Lectins against Gastric Cancer

Gastric cancer (GC) is one of the most aggressive malignancies, occupying the fourth place in morbidity and the second in mortality among cancers worldwide [101,102]. In spite of a downward trend in incidence and mortality shown in several countries [103,104], it continues to be a threat in developing countries [101]. This type of cancer tends to progress from chronic gastritis [105–107], developing over the course of years and even decades, remaining clinically undetectable in the absence of specific symptoms [108–110]. Since it is fatal in about 80% of cases [111] due to diagnosis in advanced stages or even metastasis [110], surgical and chemotherapeutic treatments no longer have the desired effect [112]. Therefore, it is necessary to find more precise markers that allow more efficient diagnosis in the early stages [113].

Gastric cancer shows an outstanding aspect within its multifactorial aetiology, which is the presence of *Helicobacter pylori* bacteria in up to 95% of cases [114]. This bacterium, classified as a class I carcinogen since 1994 [115], has the ability to adhere to epithelial cells and the gastric mucosa by adhesins, extra-membrane proteins that bind to glycosylated receptors from the host, modifying the glycophenotype and promoting infection and chronic inflammation. The resulting alterations in the glycosylations affect the activity of cadherins and integrins, proteins that regulate cell–cell and cell–extracellular matrix interactions, respectively. Proliferation, migration and invasion processes are affected, facilitating carcinogenesis, and therefore representing important targets in anti-cancer therapy [116].

The studies that have been carried out using lectins as tools for GC have been focused on diagnosis due to the differential assessment of healthy and neoplastic tissues, metastasis, or by evaluating recurrence through the analysis of glycans present in different tissues. Lectin microarrays have been used to differentiate between gastric ulcer (GU) and GC. For instance, 40 human GU and GC tissue

samples previously diagnosed by pathologists were analysed by a microarray made up by 37 lectins, mostly from plants. Differences between the two diseases were found in the glycopatterns, as well as a higher presence of glycosylations in GC than in GU, which showed higher binding affinity for MPL (*Maclura pomifera*) and VVA (*Vicia villosa*) lectins [117]. Another diagnostically oriented study evaluated the ability of a microarray of 17 lectins integrated in a microfluidic "lab-on-a-chip" platform to identify alterations in the glycan structure of biopsies and serum from 39 patients, either healthy gastric epithelium, type B chronic gastritis associated with *H. pylori*, type C chronic gastritis, or gastric adenocarcinoma. The microarray was able to discriminate between the four clinical stages from tissue samples. For the serum samples, it was only possible to distinguish between normality and disease. Additionally, it was possible to determine the glycoprofiles of the three disease stages [113]. In another study, the expression of glycans was analyzed through a microarray made up of 45 lectins in 60 healthy tissues as well as in 60 tissues resected from gastric cancer patients. Twenty-four out of the 45 lectins tested showed significant differences at binding to cancer tissues in comparison to healthy tissues; in particular, the BPL lectin (*Bauhinia purpurea*) showed promise as predictor of gastric cancer recurrence [118]. Moreover, a microarray constituted of 41 lectins, mostly from plants, was successfully used to differentiate cancer phenotypes through glycan profiling of 242 advanced GC tissue samples, and was found to be a more accurate quantitative assessment than immunostaining. Lymph-node-metastasis-associated lectins were also discerned [119].

On the other hand, recent studies have shifted to analysing the cytotoxic effect of lectins in gastric cancer as a therapeutic possibility. *Pseudomonas fluorescens* lectins (PFL) showed cytotoxicity against human gastric cancer cells (MKN28). A dose-dependent effect on cell viability was shown in doses of 0.5 μM and higher; however, at lower doses a slight increase in cell viability was observed [120]. The cytotoxic and apoptotic effect of *Urtica dioica* (UDA) lectins on human gastric cancer cells (AGS) was likewise tested. The cells were exposed to different concentrations of the lectin for 24 h and a decrease in cell proliferation and apoptosis induction was observed [80].

5. Plant Lectins against Small Intestine Cancer

Although the small intestine makes up 75% of the gastrointestinal tract and 90% of the total mucosal surface, the presence of tumors in this organ is rare [121,122], representing only 3% of malignancies [123]. This situation can be explained by factors such as rapid transit, the content of circulating fluid, the presence of the enzyme benzopyrene hydroxylase and low bacterial load, which means less exposure to carcinogens and irritants and less formation of carcinogenic metabolites [124]. However, small intestine cancer prevalence is increasing [122], and like other neoplasias of the gastrointestinal tract lacks specific symptoms until later stages [125], hindering diagnosis and appropriate treatment and reducing lifespan.

Food lectins affect intestinal function since they interact with the small intestine epithelial cells [126] and can remain active for several hours, as they can resist the digestive process. Some of them show tolerance to variables such as elevated temperatures, acid pH, and digestive enzymes [19,127,128]. The administration of raw leguminous beans or their lectins to rats can provoke effects ranging from weight loss to death. Chronic exposure to lectins can cause small intestine hyperplasia [129–131]. However, a recent study found that after the administration of *Phaseolus vulgaris* L. var. Beldia to rats, some marked structural changes in the small intestine villi were observed, but not weight loss [132]. The evidence described suggests that the feasibility of lectins for the diagnosis or treatment of small bowel cancer, although no studies have been found on that matter.

6. Plant Lectins against Colorectal Cancer

Colorectal cancer (CRC) ranks third in incidence among all types of cancer and has a mortality rate of about 50% [133], with a high prevalence in developed countries [134]. Glycosylation alterations are important changes in the inflammation process, ulcerative colitis, Crohn's disease, precancerous adenomatous polyps, hyperplastic polyps, and colon cancer [135]. They usually occur in *O*-linked

mucin-type glycans that start with *N*-acetylgalactosamine (GalNAc), causing the shortening of *O*-glycans. Plant lectins can recognize these changes and interact with various colon cell types. Taking into account that changes in glycosylations have been associated with the presence of CRC [136,137], and that some lectins remain intact through the intestinal tract [19], they have the potential to be used as diagnosis or therapeutic tools.

Diagnosis of CRC can take advantage of lectin's recognition properties. A lectin glycoarray was used to detect biomarkers that could discriminate between normal, adenoma, and CRC in human plasma, identified marked differences in CRC and adenomas compared with normal tissues. Changes consisted of a notable elevation of sialylations and fucosylations in complement C3, histidine-rich glycoprotein, and kininogen-1, which were identified as useful biomarkers for this disease [138]. In tissue samples, lectin microarrays have been used for the identification of glycan differences between colon cancer and normal tissues from patients, where *Solanum tuberosum* lectin (STL) recognized with high affinity GlcNAcylation, enabling distinction between both types of tissues [139].

Regarding the cytotoxic effects of plant lectins on CRC, in vitro studies have shown that Korean mistletoe lectins (VCA) exhibit a dose-dependent effect on a cell line of colon cancer (COLO). Approximately 65% of the treated cells showed apoptosis mediated by the activation of caspases-2, -3, -8, and -9 and the inhibition of antiapoptotic proteins. COLO cells were inoculated into naked CD1 nu/nu mice and VCA lectins were injected around the tumor mass for 5 weeks. Complete tumor regression was observed [140]. Lectins from leaves of *Morus alba* (MLL) exhibited cytotoxic effect on HCT-15 cells from human colorectal adenocarcinoma and an antiproliferative effect by apoptosis induction [66]. Additionally, a lectin obtained from *Lotus corniculatus* (LCL) showed a dose-dependent antiproliferative effect on HCT116 cells from human colonic carcinoma by apoptosis induction [141].

Lectins from Tepary beans (*Phaseolus acutifolius*, TBL) exerted a dose-dependent antiproliferative effect on different cancer cell lines [39,67], particularly on human colorectal adenocarcinoma CaCo-2 [39]. In vivo studies showed low TBL toxicity in short-term experiments, depending on the administration route [48,142]. In a study to determine the effect of TBL on colon cancer in rats, a 6-week intragastric administration demonstrated good tolerability with no toxic effects however; a 10% decrement of body weight gain was observed [48]. Similarly, an aqueous lectin extract of *Moringa oleifera* seeds caused moderate cytotoxicity on HT-29 colon cancer cells. When administered to mice in a dose of 2000 mg/kg, no signs of acute or systemic toxicity were observed [143].

However, some lectins exhibit contrary effects on cell proliferation. Peanut agglutinin lectin (PNA) showed a mitogenic effect on HT-29 and SW-1222 cells [144]. This lectin binds to the Thomsen–Friedenreich (TF) oncofetal carbohydrate antigen that is abundant in colon cancer, adenomas, and inflammatory bowel disease, and has shown a mitogenic effect on colon epithelial cells, both in vitro and in vivo. This effect has been related to the mitogen-activated protein kinase (MAPK) pathway [145]. Colon cancer metastasis is also related to signalling pathways such as MAPK [146], ribosomal s6 kinase (RSK) via the extracellular signal-regulated kinase (ERK) [147], proto-oncogene tyrosine-protein kinase (Src) [148], and protein kinase B (Akt) [149,150]. To date, the T-LAK-cell-originated protein kinase (TOPK) pathway has been described as a regulator of the metastasis process in colon cancer cells [150]. Therefore, efforts must focus on molecular targets of signalling pathways to understand the specific effects of lectins on colon cancer.

In vivo studies have shown that some lectins can affect several relevant cellular processes in colon cancer. Chinese mistletoe lectins (ACML-55) induced antitumor immunity in mice inoculated with CT26 colon cancer cells, delaying tumor development [151].

7. Plant Lectins against Liver Cancer

Liver cancer is one of the most aggressive cancers, with a high mortality rate worldwide [152]. Its prevalence is higher in Asia and Africa [153], where China accounts for slightly more than half of all deaths worldwide [154]. Infections by hepatitis B and C viruses are the main risk factor for liver cancer, accounting for up to 77% of cases [155]. Other important risk factors are alcoholism, smoking, diabetes

mellitus, metabolic syndrome, and exposure to aflatoxins [154,156], all of which are preventable to some extent. Up to 90% of primary liver cancer cases are hepatocellular carcinomas [156].

Of interest for diagnosis, *Lens culinaris* agglutinin (LCA) has been used as a tool for hepatocellular carcinoma identification, taking advantage of its specific binding to α1-6 fucose [157,158]. Research concerning the effect of plant lectins on liver cancer cells has determined that wheat germ lectin (WGA) promotes a high cytotoxic effect [78]. Korean mistletoe lectins (VCA) were tested on the SK-Hep-1 human hepatoma cell line, which expresses p53, and on Hep3B, which does not express p53. A dose- and time-dependent cytotoxic effect was observed on both cell lines, which were similarly affected by both lectins. The study therefore concluded that the mechanism of cell death was independent of p53. Apoptosis induction and inhibition of telomerase were found [83]. In another work, when Hep3B cells were exposed to VCA an apoptotic effect related to the increase of reactive oxygen species and the decreased of mitochondrial membrane potential was reported. The phosphorylation of JNK appeared to be responsible for triggering a modification in the ratio of Bax/Bcl-2, Bax translocation, the consequent release of cytochrome c, and ultimately activation of caspase 3 [159].

Concanavalin A (ConA) lectins inhibit growth and elicit autophagy in ML-14a, Huh-7 and HepG2 hepatoma cell lines. In addition, an in vivo assay injected the spleens of mice with severe combined immunodeficiency with human hepatoma cells, which migrated to the liver to form tumor nodules. One week after inoculation, treatment with intravenous ConA lectin was initiated. The results showed that ConA lectin treatment significantly inhibited the formation of tumor nodules at doses of 20 mg/kg, presumably through lymphocyte activation [60]. *Phaseolus vulgaris* var. blue tiger king (BTKL) lectins were tested on HepG2 cells from human hepatocellular carcinoma and WRL 68 from human embryonic liver tissue. The results suggested a selective cytotoxic effect, affecting HepG2 cells more than their non-carcinogenic counterparts, whose proliferation was not significantly affected. It was also determined that the most prevalent type of cell death was apoptosis, with the presence of DNA fragmentation, apoptotic bodies, chromatin condensation and membrane depolarization; however, necrosis was also found [68]. Lectins of *Phaseolus coccineus* L. var. Albonanus Bailey (CHL) were also tested on HepG2 cells, showing an antiproliferative effect [160].

Recently, the effect of *Momordica Charantia* lectins (MCL) was studied on five human hepatoma cell lines, including HepG2 and PLC/PRF/5. The results showed a dose- and time-dependent cytotoxicity induction that inhibited cell proliferation. The presence of apoptosis and autophagy was specifically detected by G2/M arrest, as well as the activation of MAPK pathways and caspases-3, -8, and -9 in the apoptotic processes. Additionally, in an in vivo xenotransplant-type assay, human hepatoma cells were injected into nude mice, which were then administered with MCL and/or the antineoplastic drug Sorafenib. A dramatic decrease in tumor size by apoptosis was observed in rats treated with the lectin–drug combination. Based on the results, the authors suggest MCL as a promising chemotherapeutic agent [161]. *Allium chinense* lectins (ACL) were studied on Hep-3B human hepatoma cells, where a cytotoxic effect was observed in a dose-dependent manner. Apoptosis by mitochondrial route was determined [54]. Additionally, Broccolini lectin (BL) from *Brassica oleracea* Italica showed a selective dose-dependent cytotoxic effect on HepG2 cells [162].

8. Plant Lectins against Pancreatic Cancer

Pancreatic cancer (PC) is one of the most lethal cancers, with a 5-year survival prognosis below 5%, independent of surgical resection of the neoplasm [163,164]. This is because, like most cancers of the digestive tract, is usually diagnosed at advanced stages, commonly when metastasis is already present. This situation is largely due to the inaccessibility of the organ for diagnostic testing, its late clinical presentation, and a lack of biomarkers that identify early pancreatic cancer stages [38,164,165]. Due to its rapid clinical progression, PC remains a real challenge for early detection.

The ability of plant lectins to differentiate between healthy pancreatic tissue and neoplastic tissue, based on their differential affinity to cell glycosylation patterns, has been tested though the use of lectin microarrays. A study was performed on serum samples from 24 patients, both healthy

and with confirmed diagnosis of chronic pancreatitis or pancreatic cancer. The samples were processed and subjected to a microarray composed of MAL (*Maackia amurensis*); SNA (*Sambucus nigra*); PNA (*Arachis hypogaea*); ConA (*Canavalia ensiformis*); and a mushroom lectin, AAL (*Aleuria aurentia*), in order to detect differences in glycans. Bioinformatic analyses found that samples from healthy and pancreatitis-affected patients showed greater similarity between them than to samples of pancreatic cancer. The most prominent alterations in the expression of glycosylations during the progression of PC were sialylations and fucosylations in different proteins [165]. In a larger study, a trial was conducted to diagnose structural differences in serum glycans from 183 healthy patients with chronic pancreatitis, type II diabetes mellitus, or pancreatic cancer using an antibody/glycoprotein/lectin sandwich assay with lectins from *Aleuria aurentia* (AAL), *Sambucus nigra* (SNA), *Lens culinaris* (LCA), and *Canavalia ensiformis* (ConA). The results showed that the microarray was able to discriminate between cancer samples, the other pathologies, and the healthy control group with a high sensitivity and specificity, particularly by SNA lectin [166].

In an in vitro study, the effects of wheat germ lectins (WGA), concanavalin A (ConA), and Phytohemagglutinin-L (PHA-L) were tested on membrane binding and proliferation of 9 pancreatic cancer cell lines (BxPC, MIA, Panc-1, CFPAC, ASPC, HS-766T, HTB-147, CaPan-1 and CaPan-2), using a lectin-blot assay and the incorporation of thymidine. A marked dose-dependent cytotoxic effect of WGA lectin was observed on all cancer cell lines, being higher than the effects of the other two lectins, even at a lower concentration. WGA lectin was able to bind to sialic acid residues in membrane glycoproteins, causing chromatin condensation, nucleus fragmentation, and DNA release, and internalization and localization in the cell nucleus were determined [167]. Recently, the activities of *Benincasa hispida* (BhL) and *Datura innoxia* (DiL9) on pancreatic cancer cells lines have been studied. A considerable antiproliferative, dose-dependent effect triggered by a mitochondrial apoptotic pathway, along with anti-angiogenic features, were reported [168].

In vivo experiments showed the effects of mistletoe extracts and lectins from *Viscum album* and were compared to those of Gemcitabine, an antitumor drug used in the treatment of pancreatic cancer. Xenotransplants in athymic nude mice (NMRI nu/nu) were performed using human pancreatic adenocarcinoma cells PAXF 736 and treated with mistletoe extract, mistletoe lectins or Gemcitabine in equivalent doses. Mistletoe extracts showed more antitumor activity than Gemcitabine, presenting partial regressions and total remission of the tumors. Mistletoe lectins showed similar but lower activity than the extract, also with partial regressions [169]. Tröger and colleagues [170] also evaluated the effect of a mistletoe extract on 220 patients with localized advanced cancer or metastatic pancreatic cancer receiving palliative care only (best supportive care-BSC) without chemotherapeutic treatment at the time of the study. The results were favorable, with a clear increase in survival in the 110 patients who received mistletoe extract treatment over the same number of control patients without treatment. In addition, the patients who were given the extract reported a lower presence of adverse events. The authors suggest the administration of such extract as a second line therapy for patients with advanced or metastatic pancreatic cancer. In this phase III study, the effects of mistletoe extract were attributed mostly to the presence of lectins and viscotoxins.

9. Lectin-Based Analytical Techniques with Biomedical Applications

Although lectins of the same family are highly conserved in the binding site amino acid residues, the specificity of binding is related with amino acids of different regions of the carbohydrate-binding site. Lectin's specificity for glycans include mannose and glucose (Man/Glc) for concanavalin A (Con A), *N*-acetylglucosamine (GlcNAc) and Nacetylneuraminic acid (Neu5Ac) for wheat germ agglutinin (WGA), galactose (Gal) and *N*-acetylgalactosamine (GalNAc) for soybean agglutinin (SBA), and Gal for ricin agglutinin (RCA) [171]. The ability of lectins to recognize glycans allows for the use of several analytical methods that take advantage of two important features: specificity and reversible binding. Table 2 shows some of the traditional and modern techniques used for glycans recognition by lectins in biomedicine.

Table 2. Lectin-based analytical techniques for glycan detection (modified from [171]).

Technique	Fundament	References
Cell agglutination	Specific recognition of cell membrane carbohydrates or glycoconjugates.	[28,29,172]
Cytochemical and histochemical assays	Recognition of cell surface carbohydrates or glycoconjugates by labelled lectins or immuno-recognition of lectins.	[157,158,173,174]
Enzyme-linked lectin assay (ELLA)	Marked lectins used for binding to immobilized glycoconjugates.	[175–177]
Lectin affinity chromatography (LAC)	Affinity chromatography using immobilized lectins.	[178]
Lectin blotting	Qualitative method for detecting carbohydrates moieties in a western blot-like method.	[179,180]
Crossed affinity immunoelectrophoresis	Based in migration patterns changes of glycosylated proteins in an agarose gel which contain an embedded lectin. A second dimension is needed for detecting of the protein with embedded specific antibody in the gel and a final staining of proteins is required.	[175]
Flow cytometry	Lectins labelled with a fluorophore are used in order to detect cell surface glycoconjugates.	[181,182]
Surface plasmon resonance (SPR)	Immobilized lectins to a glass surface (optical biosensor) and binding to carbohydrates in solution is determined as changes in the refractive index.	[183,184]
Lectin microarrays	A panel of immobilized lectins in a chip is used for glycans recognition.	[4,37,139,166,177]
Antibody-Lectin Sandwich Array (ALSA)	Biomarker glycoprofiling by lectins and glycan-binding antibodies.	[158,185,186]
Electrochemical Impedance Spectroscopy biosensors (EIS)	A label-free biosensor based on the lectin–glycan interaction.	[187,188]

Taking advantage of lectin's recognition properties, they have also been used for drug delivery. Oral administration is the most conventional method for drug delivery; however, its passage through the gastrointestinal tract entails a series of obstacles such as pH variation, low stability, solubility, bioavailability, and absorption [189]. Lectins' ability to prevail in aggressive environments (e.g., pH, heat, and enzymes) and interact with cell membrane glycans allows for their use as vehicles for targeted drug delivery [190,191]. Several plant lectins have been used for this purpose and have been aimed toward specific cells and tissues (direct lectin targeting) [192]. An increase in the cellular uptake of lectin-conjugated particles has been reported [193]. Another modality of targeted drug delivery consists of glycans coupled to nanoparticles to target endogenous lectins within specific tissues (reverse lectin targeting) [194,195]. Its use in cancer therapeutics has also been explored, for example, by associating wheat germ agglutinin (WGA) to a paclitaxel-loaded particle, an effective chemotherapeutic for colon cancer. The conjugated molecule was able to exert anti-proliferative activity against colon cancer cell lines Caco-2 and HT-29, showing greater cellular uptake and retention compared to non-conjugated particles [196].

10. Final Remarks and Conclusions

Plant lectins as bioactive molecules are characterized by their ability to recognize animal cell carbohydrates. This property enables them to generate cellular responses depending on cell linage, from immune system activation to cancer cell death. Lectins exhibit a vast potential for diagnostic and therapeutic use against cancer due to the cytotoxic, apoptotic, autophagic, and antitumor effects triggered after exposure to these proteins in cells, tissues, and even patients with cancerous processes of the digestive system. The reported information regarding the activity of plant lectins on digestive cancer cell lines indicates the presence of dose- and time-dependent cytotoxicity, generally affected by induction to apoptosis. In vivo experiments have shown inhibition of tumor growth and in some cases even complete remission of tumors. Phase III studies of the effect of plant lectins in cancer patients have shown favorable effects. The ability to induce cell death in a selective manner is a desirable

attribute in anticancer therapy and, paradoxically, a trait most of the current chemotherapeutics lack but which lectins have shown. Hence, the growing interest in the study of the activity of plant lectins is due to the biological effects they exert on cancer cells, from identification of tumors to antitumor activity and, additionally, decreased side effects caused by chemotherapeutics. The diagnostic potential of plant lectins has been exposed using microarrays however; despite their multiple beneficial effects, it is important to acknowledge their possible toxicity that depends on the lectin source, the dose, and the administration route. There is a need to increase the study about the biological effects of lectins, and deepen into their molecular mechanisms in order to take advantage of the biomedical potential of these amazing proteins.

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Tolerability assessment of a lectin fraction from Tepary bean seeds (*Phaseolus acutifolius*) orally administered to rats

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ABSTRACT

Our previous studies have shown that a lectin rich fraction (TBLF) extracted from Tepary bean seeds differentially inhibits cancer cells proliferation *in vitro*. Before testing the *in vivo* anticancer effect, the acute and subchronic toxicological assays in rats were conducted, where an oral dose of 50 mg/body weight kg was determined as the NOAEL. This study evaluated the resistance to digestion and complete blood count (CBC) after 24 h of the orally administered 50 mg/kg TBLF. The digestion resistance test showed lectins activity retention after 72 h and the CBC study showed a high level of eosinophils, suggesting an allergic-like response. Tolerability was assayed after 6 weeks of treatment by dosing with an intragastric cannula every third day per week. It was observed a transient reduction in food intake and body weight in the first weeks, resulting in body weight gain reduction of 10% respect to the control group at the end of the study. Additionally, organs weight, histopathological analysis and blood markers for nutritional status and for liver, pancreas and renal function were not affected. Our results suggest that 50 mg/kg TBLF administered by oral route, exhibit no toxicity in rats and it was well tolerated. Further studies will focus on long-term studies.

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1. Introduction

Lectins include a group of proteins from non-immune origin that share the property of binding specifically and reversibly to carbohydrates [1]. Many plant lectins have attracted the attention due to their effects on proliferation and differentiation of animal cells, including lymphocytes and cancer cells. The *in vitro* and the *in vivo* antitumor effects of plant lectins are apparently associated with their ability to modulate growth, differentiation, proliferation and apoptosis [2–4].

Toxicity of lectins must be considered before used as medical tools, mainly because they are considered antinutritional factors. It has been shown that binding lectins to intestinal epithelium can interfere with nutrient absorption, reduction of nitrogen retention, increased urine nitrogen excretion and reduction of insulin production in rats [5–8]. Antinutritional and negative effects on digestion and absorption have been described for lectins from different sources [9–12]. Studies with common bean (*Phaseolus vulgaris* L.) lectins show that they can interfere with bowel function, causing changes in systemic metabolism and affecting the growth in rats, decrease in glucose, lipids, vitamin B12 and nitrogen uptake [13,14]. Adverse effects in organs are produced by some diet lectins, which included *Phaseolus vulgaris*. Rats fed with navy beans showed morphological changes that include increased weight of kidney and heart, pancreatic acinar atrophy, fatty liver and multiple histological lesions as thymus atrophy respect to control healthy rats. Death by lectin administration can be produced by different mechanisms including the reduced availability of nitrogen in the body, the induction of bacterial overgrowth in the small intestine, the effects on blood cells or acting as ribosome inactivating proteins [15,16].

Some studies showed that intraperitoneal administration of Tepary bean (*Phaseolus acutifolius*) crude extract presented toxic effects as weight loss, negative efficiency on protein ratio, negative net protein utilization, poor digestion of proteins and death of rats and mice after 10 days treatment, however, after autoclaving the crude extract, the toxic effects were lost [17]. Studies on the toxicity of semipure lectins from Tepary bean intraperitoneally administrated in CD-1 mice, found a lethal dose (LD_{50}) of 1100 and 1120 mg/kg body weight for males and females, respectively [18].

A semipure lectin fraction from Tepary bean seeds (TBLF) obtained by a molecular weight exclusion chromatography protocol exhibits *in vitro* antiproliferative differential effect on cancer and normal cells [19]. Before testing the *in vivo* anticancer effect, we studied the acute toxicity of TBLF using intragastric doses from 5 to 2000 mg/body weight kg suggesting a secure dose of 50 mg/kg. The intragastric 50 mg/kg TBLF dose was assayed for subchronic toxicity (daily dosing for 28 days) where no toxic or adverse effects were observed, therefore 50 mg/kg TBLF was determined as the NOAEL [20]. Here we present a short-term assay in order to know the digestion resistance of lectins and the effect on complete blood count (CBC) after 24 h of 50 mg/kg TBLF single-dose administration. The anti-nutritional effects and toxic parameters of a 6-week schedule study (intragastric administration every third day) were studied; where food intake, body weight, biochemical blood markers and histopathological analysis were included.

2. Materials and methods

2.1. Experimental animals

Sprague Dawley (SD) rats were purchased from Institute of Neurobiology, Universidad Nacional Autonoma de Mexico (INB-UNAM) and placed in individual cages with *ad libitum* water and rodent chow food (Rodent Laboratory Chow 5001, Saint Louis, MO, USA). The animals remained 1 week for acclimatization where the circadian cycle was adjusted to 12 h light/12 h darkness, at 22 °C and a relative humidity of 30%. The animals were sacrificed by decapitation at the end of the experiments. The experimental protocol was based on the Mexican official standard [21] and approved by the INB-UNAM ethics committee.

2.2. Tepary bean lectin fraction (TBLF)

We have performed a standardized method for TBLF obtaining [19]. Some modifications were done in order to improve the lectin enrichment. Briefly, Tepary bean seeds were grinded (A-10 Analytical Tekmar mill) and degreased with chloroform-methanol 2:1 in a 4:1 w/v proportion, stirring for 15 min and then vacuum filter; this process was repeated 2 more times and flour was dried at room temperature in a fume hood. In order to obtain the crude extract, 100 g of degreased flour were dissolved in 500 mL of 50 mM Tris–HCl pH 8 with stirring for 12 h at 4 °C and centrifuging at 39,200 × *g* for 60 min (Bekman JA-20 centrifuge). A sequential precipitation was done using 40% ammonium sulphate saturation with slow stirring for 30 min, equilibrating for 30 min at 4 °C and centrifuging at 39,200 × *g* for 45 min. The supernatant was precipitated with 60% ammonium sulfate saturation and treated as previously described, but in this case the precipitate was recovered and the supernatant was discarded. The fraction was resuspended in a minimum volume of deionizer water, dialyzed through a 3-kDa pore size membrane and centrifuged, loading 4 mL aliquots on a Sephadex G-75 gel filtration 167 cm × 1.7 cm column (Pharmacia Biotech, Uppsala, Switzerland). The column was equilibrated with 0.01 M ammonium bicarbonate buffer pH 7.8. The experiment was performed at 4 °C collecting 0.3 mL/min fractions with the same buffer. Protein was monitored at 280 nm in a Beckman DU-65 spectrophotometer. Agglutination activity [22] was determined by microscopic counting using glutaraldehyde-fixed type A^+ human erythrocytes [23]. Specific activity was determined using protein concentration [24]. Electrophoretic profile was obtained by 10% polyacrylamide SDS-PAGE [25]. Glycoproteins were confirmed by periodic acid-Schiff staining (PASS) [26]. Additionally, lectins were observed by western blot using an anti-phytohemagglutinin antibody from *Phaseolus vulgaris* (Vector Laboratories Inc. Burlingame, CA, USA. cat.

No. AS-2300). The fraction was dialyzed against deionized water, lyophilized and stored at −20 °C until use.

2.3. Digestion-resistance assay and complete blood count (CBC) determination after a single dose of TBLF

Five-week old male SD rats were divided into 2 groups ($n = 8$ per group). After fasting for 24 h, the treated group received a single dose of the lyophilized 50 mg/kg TBLF dissolved in standard saline solution (0.9% NaCl in deionized water) using an intragastric cannula [20], while the control group received saline solution. Autoclaving (121 °C for 15 min) was necessary for denature food lectins. Feeding was restarted with *ad libitum* water and autoclaved chow food (Rodent Laboratory Chow 5001. Saint Louis, MO, USA). Feces form 4 rats per group were collected at 0, 24, 48, 72, 96 and 120 h, fecal protein was extracted in PBS, filtered through a 0.22 mm membrane and agglutination specific activity was determined by microscopic counting [22]. Other 4 rats per group were sacrificed at 24 h in order to recover blood for CBC (CellDyn® 1600) and a commercial kit for differential blood cells staining was used for cell counting from blood smear (Hycel, Mexico; cat. number 548). Erythrocytes, neutrophils, eosinophils, basophils, lymphocytes, monocytes and platelets were counted using a 100× microscope objective. Results are expressed as absolute blood counts or percentage respect to control animals.

2.4. Tolerability study

Fifteen-week old male SD rats were randomly selected in 2 groups ($n = 12$ per group). Treated rats were dosed with 50 mg/kg TBLF dissolved in saline solution and control group was administered with saline solution by using an intragastric cannula. For CBC determination, 4 rats per group were daily administered for 30 days and sacrificed. For the tolerability assessment, treated group was administered with the 50 mg/kg TBLF in saline solution every third day for 6 weeks and control group administered with saline solution. This administration schedule was defined from the digestion resistance data and it will be used in further studies, *i.e.* against colon cancer. Food intake was determined twice a week and body weight weekly. After the 6-week administration schedule, rats were sacrificed by decapitation. Blood was collected in vacutainer tubes without anticoagulant and serum was recovered by centrifugation at 5000 × *g* for 5 min and stored at −80 °C until use for clinical chemistry parameters determination as described below. Liver, kidney, stomach, pancreas, small intestine, colon, thymus and spleen were dissected, weighted and fixed in 10% buffered formalin. A veterinary pathologist conducted the histopathological analyses for liver, kidney, small intestine and colon using Hematoxylin-Eosin staining and analyzed by microscopy (Olympus, model BX51, Evolution MP).

Commercial kits (Diagnostic Chemicals Limited, Canada) were used for determination of liver function using aspartate aminotransferase (AST) (Catalog No. 319-10), alanine aminotransferase (ALT) (Catalog No. 318-10) and total bilirubin (Catalog No. 243-17) kits. Urea (Catalog No. 283-17) and α-amylase (Catalog No. 341-10) were measured as renal and pancreas function markers, respectively. Serum creatinine (Catalog No. 221-30), total protein (Catalog No. 200-55), glucose (SL ELITech, Clinical Systems, France. Catalog No. B01-4509-01), and albumin (SL ELITech, Clinical Systems, France. Catalog No. ALBU-0600) were determined as nutritional status markers.

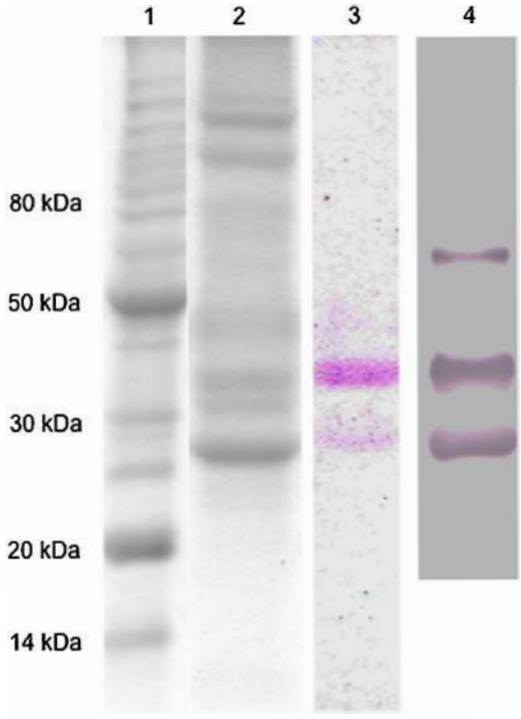

Fig. 1. TBLF electrophoretic profile and lectins identification. TBLF was obtained by molecular weight exclusion chromatography. Monodimensional SDS-PAGE was performed. (1) Molecular weight markers, (2) TBLF, (3) glycoprotein PASS, (4) western blot using anti-phytohemagglutinin from *Phaseolus vulgaris*.

2.5. Statistical analysis

Differences between TBLF treated rats against control rats were calculated by the *t*-student test ($p \leq 0.05$) using the SPSS 17 software.

3. Results and discussion

3.1. TBLF profile

The molecular weight exclusion chromatography protocol shows a reproducible profile for TBLF obtainment. The method allows observing the two main lectins (Fig. 1), similar than the observed profile previously obtained [19]. The presence of lectins was confirmed by PASS and western blot. The specific agglutination activity for the TBLF was 5566 AU/protein mg.

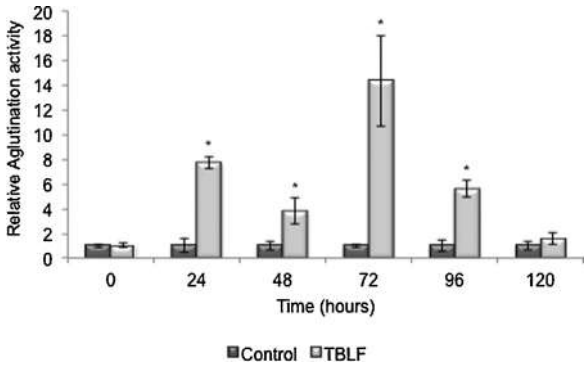

Fig. 2. Agglutination activity from rat feces administrated with TBLF. Rats were administrated with a single dose of TBLF (50 mg/kg) and feces were recollected every 24 h for 5 days. Agglutination activity was determined using A^+ human erythrocytes. Asterisks show statistical significant difference (*t* test $p \leq 0.05$).

3.2. Lectins from TBLF resist digestion up to 72 h

Some lectins exhibit high resistance to digestion by proteolytic enzymes in mammals, allowing them to effectively bind to intestinal epithelial cells. Lectins can also resist bacterial degradation and can remain in their biological and immunological intact forms [5–7]. It has been reported that this kind of proteins can be recovered with their intact biological activity after passing through the digestive tract of mice over a period of 24 h as *Pisum sativum* and Kintoki bean lectins [27–29]. In order to establish the resistance to gastric digestion of TBLF, agglutination activity was monitored through 120 h in feces after a 50 mg/kg TBLF single dose (Fig. 2). Agglutination activity in TBLF-treated rats was 8 and 4 times higher than in control rats after 24 and 48 h, respectively. However, instead of diminishing, it increased 15 times after 72 h and then gradually diminished until basal levels at 120 h. This result suggests that no retained lectin was excreted within the first 48 h and retained lectin releases after 72 h maintaining its biological activity.

3.3. TBLF exhibits an allergic-like response after 24 h

CBC is shown in Table 1 where only granulocytes count showed difference ($p = 0.001$) with an increase of 3.86 times in TBLF-treated animals respect to control rats. The proportion of granulocytes and lymphocytes was different respect to control animals, mainly due to an increment of granulocytes (Fig. 3A). Blood smears were used to differential counting of cells (Fig. 3B). Lymphocytes decreased 20% while neutrophils and eosinophils increased 2.4 and 20 times, respectively. Basophils, monocytes, erythrocytes, and platelets did not show significant changes (data not shown). This result suggests an allergic-like response, mainly indicated by the eosinophils increase.

3.4. Six weeks TBLF administration provoked partial reduction of food intake and decrease in body weight gain

Fifty mg/kg TBLF dose was administrated *via* intragastric cannula every third day for 6 weeks. Significant decreased in food consumption was observed from the first week of administration until the fourth week respect to control group ($p \leq 0.05$). However, on the fifth week, food consumption was the same than the control group (Fig. 4A), maybe as the result of compensatory mechanisms where the treated animals overcame the negative effects of the lectins administration. Rats body weight also showed significant changes ($p \leq 0.05$) between the two groups (Fig. 4B). Treated animals presented a transient decrease of body weight in the first weeks (5.25% respect to the start of dosing) however; at the end of the study, a recovery of weight was observed resulting in a reduction in body weight gain of 10% respect to the control group. It is known that lectins can provoke nonspecific interference with nutrient absorption, causing changes in animal nutrition status. Our results show that TBLF administration causes antinutritional effects at the beginning of the experiment with a final recovery, which resulted in a reduction in body weight gain.

3.5. Six weeks TBLF administration did not cause organs or blood markers alterations

The effect of TBLF on organs and blood markers is shown in Table 2. No significant differences were observed in spleen, heart, liver, kidney, stomach, thymus, pancreas, small intestine and colon weight. Small intestine and colon length were also determined and no significant differences were found with respect to the control group. No histopathological alterations were observed in colon, small intestine, liver and kidney (Fig. 5). A strong association between changes in the morphology and structure of the intestine and the ingestion of lectins have been observed,

Table 1
CBC after 24 h of a single-dose TBLF oral administration.

Blood parameter	Control	TBLF
White blood cells ($10^3/\mu L$)	5.16 ± 1.35	6.78 ± 1.56
Lymphocytes ($10^3/\mu L$)	4.70 ± 1.27	5.00 ± 1.44
Granulocytes ($10^3/\mu L$)	0.46 ± 0.21	1.78 ± 0.53*
Red blood cells ($10^6/\mu L$)	6.73 ± 0.20	6.55 ± 1.59
Platelets ($10^3/\mu L$)	874.40 ± 84.97	737.75 ± 216.71
Hemoglobin (g/dL)	12.50 ± 0.37	12.24 ± 2.88
Hematocrit (%)	42.20 ± 1.06	40.76 ± 9.52
Mean corpuscular volume (fL)	62.80 ± 2.77	62.40 ± 1.14
Mean corpuscular hemoglobin (pg)	18.60 ± 0.99	18.74 ± 0.33
Mean corpuscular hemoglobin concentration (g/dL)	29.62 ± 0.77	30.00 ± 0.48

* Statistically significant difference (*t* test, $p = 0.001$).

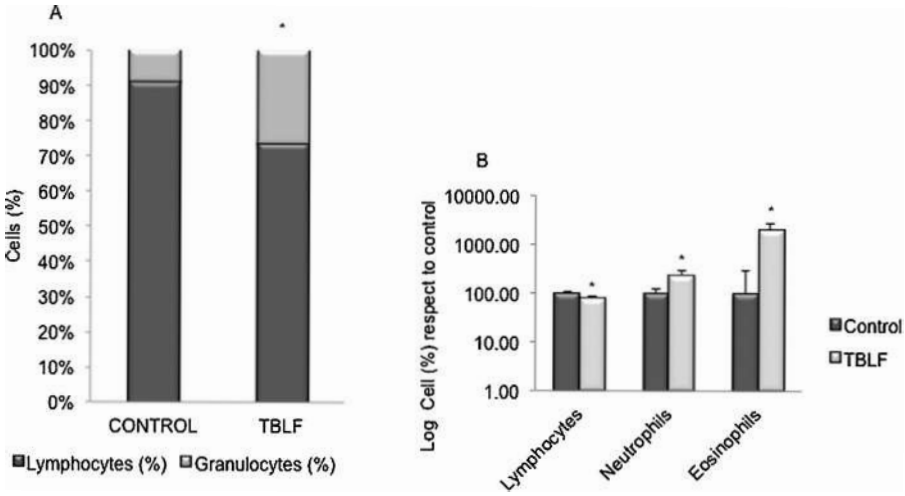

Fig. 3. White blood cells counting after 24 h of a single dose of TBLF. CBC was determined and blood cells were counted from blood smears by differential staining. (A) Percentage of granulocytes and lymphocytes were determined by automated counting. (B) White cells count was determined by microscopic counting. Results are expressed as percentage respect to control animals in a logarithmic scale. Asterisks show statistical significant difference (*t* test $p \leq 0.05$).

such changes may result from the decrease in intestinal permeability as shown with Con A, wheat agglutinin and navy bean lectin. The intestinal growth is maybe the result of compensatory process in order to maintain nutrient uptake, since some lectins directly stimulate intestinal cells causing hyperplasia and hypertrophy [9]. However, TBLF at the tested dose din not provoked macroscopic effects or histological changes on the intestinal tract.

Different blood markers were determined in order to study hepatotoxicity (AST and ALT), renal injury (urea, creatinine), pancreatic damage (α-amylase), and nutritional status (albumin, total protein, creatinine and glucose). No differences between groups were found, suggesting that the oral TBLF administration exhibit no toxicity at the end of the treatment (Table 3). Urea levels were found out of range, according to reference values for SD rats [30] but the high values applied to both, control and treated animals. To find out if blood parameters could change during the treatment, in a separated experiment total protein, albumin, creatinine and ALT were measured every 2 weeks and CBC was determined after 4 weeks. No significant differences between groups were found, suggesting

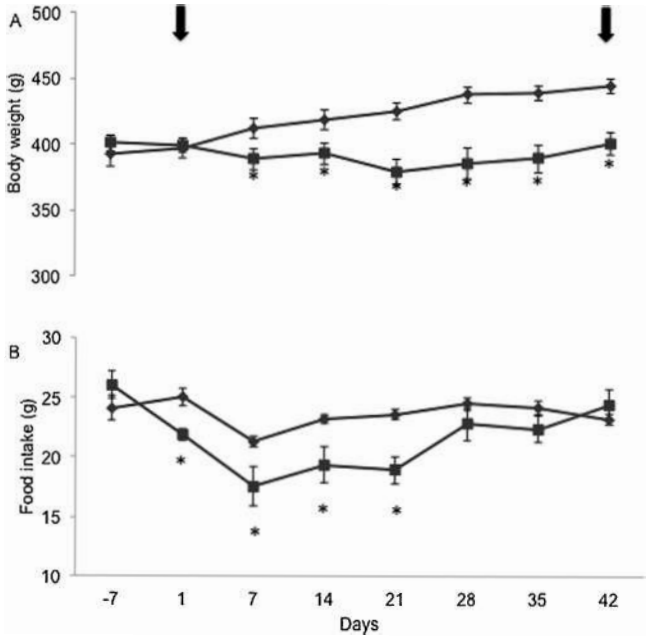

Fig. 4. Food intake and body weight changes after TBLF oral administration. Rats were orally administrated with TBLF (50 mg/kg) every third day for 6 weeks after an adaptation period (7 days). Dosing was done to complete 6 weeks, from day 1 to day 42 (arrows indicate the start and end of treatment). (A) Net food intake, (B) Net body weight changes. Asterisks show statistical significant difference (*t* test $p \leq 0.05$).

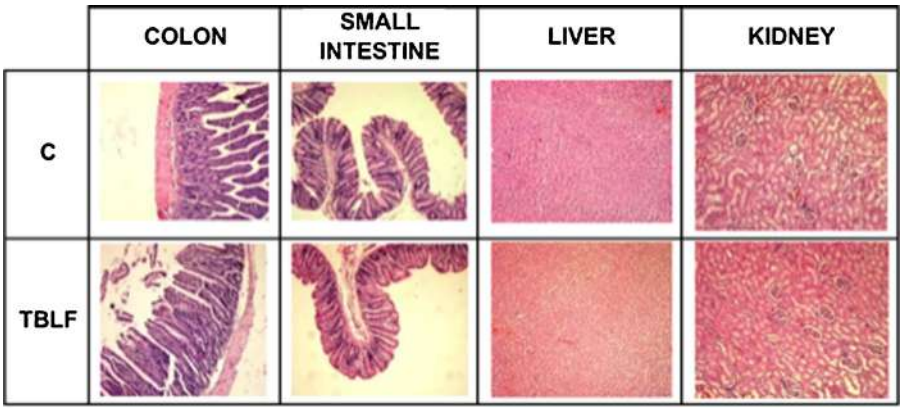

Fig. 5. Histopatological analysis after TBLF oral administration. Rats were orally administrated with TBLF (50 mg/kg) every third day for 6 weeks. Colon, small intestine, liver and kidney were collected after sacrifice, stained and observed (10×).

no adverse effects after long-term treatment (data not shown).

Other studies have observed that intragastric administration of saline extract of *P. acutifolius* to rats caused intestinal cells microvilli destruction, as well as breaking of endoplasmic reticulum outline [31]. These authors attributed the toxicity and poor nutritional value of *P. acutifolius* to high concentrations of phytohemagglutinin, however, it is known that Tepary bean protease inhibitor is also present in crude protein extracts [17]. TBLF does not contain the protease inhibitor form Tepary bean as a result of the chromatographic procedure [19]. Therefore, the results obtained in the present work could be attributed to the lectins contained in TBLF.

Table 2
Organs weight or length after TBLF subchronic oral administration.

Organs	Control	TBLF
Colon weight (g)	2.94 ± 0.40	2.68 ± 0.40
Colon length (cm)	19.38 ± 1.14	19.60 ± 0.81
SI weight (g)	8.06 ± 0.23	8.28 ± 0.52
SI length (cm)	125.96 ± 2.40	122.02 ± 5.08
Spleen (g)	0.84 ± 0.03	0.83 ± 0.05
Heart (g)	1.65 ± 0.06	1.52 ± 0.06
Liver (g)	11.98 ± 0.42	10.57 ± 0.37
Kidneys (g)	3.31 ± 0.12	3.18 ± 0.15
Stomach (g)	2.21 ± 0.08	2.14 ± 0.07
Thymus (g)	0.45 ± 0.04	0.48 ± 0.05
Pancreas (g)	1.46 ± 0.27	1.73 ± 0.12

No statistically significant difference was founded (t test, $p \geq 0.05$).

Table 3
Blood markers after TBLF subchronic oral administration.

Blood markers	Control	TBLF
Glucose (mg/dL, RV: 50–160)	93.5 ± 7.10	96.9 ± 6.69
TP (mg/dL, RV: 5.9–7.9)	8.3 ± 0.30	8.4 ± 0.44
ALB (mg/dL, RV: 3.8–4.8)	3.2 ± 0.02	2.9 ± 0.13
AST (U/L, RV: 39–262)	186.7 ± 21.50	160.1 ± 10.90
ALT (U/L, RV: 35–80)	65.5 ± 3.10	66.8 ± 4.93
Urea (mmol/L, RV: 9.28–22.13)	29.5 ± 4.03	30.8 ± 5.07
Serum creatine (mg/dL, RV: 0.5–1.5)	0.9 ± 0.02	0.9 ± 0.02
α-Amylase (U/L, RV: NA)	2.0 ± 0.04	1.9 ± 0.07

No statistically significant difference was founded (t test, $p \geq 0.05$).
TP: Total protein, ALB: Albumin, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, RV: Reference values (16), NA: Data not available.

4. Conclusions

Here we report that after subchronic oral administration, TBLF provoked antinutritional effects in rats resulting in a transient decrease of food intake and body weight in he first weeks. The final result was observed as a reduction in body weight gain respect to the control group. The digestion assay suggests that lectins from Tepary bean can remain intact into the digestive tract up to 72 h. CBC at 24 h post-administration showed an allergic-like response that disappeared after 4 weeks of treatment. Blood markers suggest no toxic effects and no alterations in the evaluated organs. Taking together, our results showed that TBLF provoke a reduction in body weight gain with no other remaining effects, suggesting compensatory mechanisms and good tolerability. More studies are needed to determine effects on nutrient availability and intestinal integrity after TBLF administration, especially in long-term assays and in different development stages.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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