Centro de Investigación en Alimentación y Desarrollo, A.C

POTENCIAL ANTIOXIDANTE DE LAUREL (*Litsea glaucescens*) Y SU APLICACIÓN EN UN MODELO CÁRNICO

Por:

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APROBACIÓN

Los miembros del comité designado para revisar la tesis de Julio César López Romero, la han encontrado satisfactoria y recomiendan sea aceptada como requisito para obtener el grado de Doctor en Ciencias.

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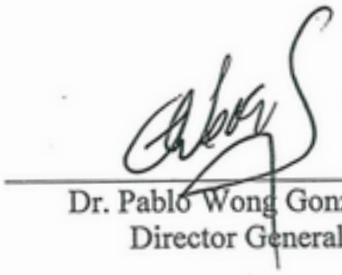

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CONTENIDO

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RESUMEN

Litsea glaucescens (LG) es una planta endémica de México, que es utilizada como especia y para el tratamiento de diversos padecimientos en medicina tradicional. Existen pocos reportes acerca de su potencial biológico y composición de compuestos fenólicos, y cómo ésta puede ser afectada por la temporalidad. Se desconoce también el potencial efecto de LG como aditivo alimentario. El objetivo de esta investigación fue purificar y caracterizar los compuestos bioactivos que determinan el potencial biológico de LG. Así como también, evaluar el efecto de la temporalidad sobre el potencial biológico y perfil de compuestos fenólicos de LG, y evaluar su efecto como aditivo en carne fresca de cerdo. Se elaboró un extracto metanólico de LG (EMLG),el cual fue sometido a cromatografía en columna (CC) logrando obtener dos fracciones (F-XI y F-XII) con elevada actividad antioxidante (AA) y contenido de compuestos fenólicos (CF). Las dos fracciones nuevamente fueron sometidas a CC, permitiendo obtener tres compuestos. Los resultados de RMN (^{1}H y ^{13}C), HPLC-ESI-MS y HPLC-DAD mostraron que los compuestos purificados correspondían a epicatequina, quercetrina y kaempferol. Posteriormente, se elaboraron EMLG de las diferentes épocas del año: otoño (ALGE) e invierno (WLGE) de 2015 y primavera (SLGE) y verano (SULGE) de 2016. Los resultados mostraron que los EMLG poseen alto contenido de compuestos fenólicos (CF) (92.9-138.2 mg EAG/g m.s.) y elevada actividad antioxidante (AA) a través de los distintos métodos evaluados (DPPH IC_{50}=14.7-27.2 µg/mL, FRAP=1466.4-2614.3 µM Fe (II)/g m.s. y ORAC=3413.3-3700 µM ET/g m.s.). A su vez, se observó que la temporalidad tiene un efecto sobre el contenido de CF y AA, donde ALGE y SULGE fueron los que presentaron mayor potencial ($P<0.05$). Posteriormente, se evaluó el efecto de ALGE, F-XI y F-XII como agentes antioxidantes en hamburguesas de cerdo almacenadas en refrigeración (11 días, 4 °C). Los resultados mostraron que los tratamientos evaluados disminuyeron la oxidación lipídica y proteica de las hamburguesas, logrando extender su vida de anaquel ($P<0.05$) (3 días con respecto al tratamiento control). Además, estos aditivos preservaron el color de las hamburguesas

durante un periodo más largo, comparado con el tratamiento control (sin aditivo). Los resultados obtenidos evidenciaron que la temporalidad afectó las actividades biológicas de los EMLG, y que su elevado potencial biológico está relacionado con el contenido de CF, lo que sugiere que los EMLG podrían representar una alternativa viable para la industria alimentaria y farmacéutica.

Palabras clave: *Litsea glaucescens*, potencial biológico, actividad antioxidante, compuestos fenólicos, aditivo alimentario.

ABSTRACT

Litsea glauscescens (LG) is a Mexican endemic plant commonly used as a food spice and for alternative treatment in folk medicine. However, there are only few studies about the biological potential and phenolic composition of this plant and how these features could be affected by year season. In spite of its wide use in local gastronomy, the effect of LG as food additive is still unkown. The objective of this study was purified and characterized the bioactive compounds that explained its biological potential of LG; as well as to evaluate the effect of season on LG biological potential and phenolic composition profile, and to evaluate the effect of LG as food additive in fresh pork meat. A methanolic extract of LG was prepared (EMLG) and eluted through a column chromatography (CC) separating two fractions (F-XI and F-XII) with high antioxidant activity (AA) and phenolic compounds content (PCC). Both fractions were eluted by CC, purifying three compounds. NMR (^{1}H and ^{13}C), HPLC-ESI-MS and HPLC-DAD results, determined that purified compounds were: epicatechin, quercitrin and kaempferol. Subsequently, methanolic extracts from LG (EMLG) collected from all 4 seasons were prepared: autumn (ALGE) and winter (WLGE) 2015, and spring (SLGE) and summer (SULGE) 2016. Results showed that EMLG presented high PCC (92.9-138.2 mg EAG/g d.w.) and AA by all tested methods (DPPH IC_{50}=14.7-27.2 µg/mL, FRAP=1466.4-2614.3 µM Fe (II)/g d.w. y ORAC=3413.3-3700 µM ET/g d.w.). Afterward, the effect of ALGE, F-XI and F-XII as antioxidant agents was evaluated in pork patties stored in preservation (11 days at 4 °C). Results showed that all evaluated treatments decreased lipid and protein oxidation in pork patties, extending their shelf life ($P<0.05$) (3 days related with control treatment). Thus, these compounds preserved color patties during a longer time, compared with no additive treatment. Results of this study, demonstrated the effect of season on biological activities and CF and biological activities of EMLG. EMLG´s high biological potential was related with it CF content, suggesting that EMLG could be considered as natural additive source for food and pharmaceutical industry.

Keywords: *Litsea glaucescens*, biological potential, antioxidant activity, phenolic compounds, food additive.

SINOPSIS

Inicialmente la presente disertación doctoral tenía como objetivo determinar el efecto antioxidante y antimicrobiano, y el modo de acción *in vitro* de extractos de *Agave*, y a su vez determinar el efecto como aditivo alimentario en hamburguesas de cerdo. Durante el desarrollo de las primeras etapas experimentales se observó que los extractos obtenidos de *Agave* presentaron un bajo potencial antioxidante, y se observó que dicho efecto era dependiente de la temporalidad en que las plantas fueron cosechadas las plantas. En función de estos resultados se realizaron ajustes en la temática de investigación considerando ahora la temporalidad como un factor importante en el potencial biológico del extracto. Por tal motivo, se decidió analizar bajo el mismo contexto otro material vegetal, siendo *Litsea glaucescens* el nuevo tema de estudio.

Litsea glaucescens (LG) es una planta nativa de México y América Central, que pertenece a la familia *Lauraceae* (Wang et al., 2016). En México, esta planta se distribuye principalmente en los estados de Veracruz, Tamaulipas, Chiapas y Nayarit, en donde es conocida regionalmente como laurel silvestre o laurel mexicano (Tucker et al., 1992). Las hojas (Figura 1) son utilizadas como sazonador de alimentos, debido a que proporciona características similares al laurel europeo en los alimentos elaborados (Guzmán-Gutiérrez et al., 2012). A su vez, las hojas de LG también han sido utilizadas en la medicina tradicional en forma de infusión, aceites esenciales, macerados alcohólicos o inhalaciones de vapor (López et al., 1995). Estas extracciones han sido empleadas para tratar diversos padecimientos como diarrea, vomito, cólicos, dolor de hueso, inflamación, problemas ginecológicos y enfermedades del sistema nervioso central (Jiménez-Pérez et al., 2011; Guzmán-Gutiérrez et al., 2012; Gamboa-Gómez et al., 2016). Sin embargo, existen pocos reportes científicos que confirmen el potencial biológico de esta planta, y a su vez cual es el perfil de compuestos bioactivos que pueden ejercer este potencial.

Basado en lo anterior, un estudio previo de nuestro grupo de trabajo evidenció que un extracto etanólico de hojas de LG presentó un elevado potencial antioxidante, basados en los métodos DPPH y FRAP. Además, se observó que el potencial antioxidante mostró

correlación positiva con el contenido de compuestos fenólicos. Este hecho sugiere que los compuestos fenólicos presentes en el extracto de LG están influyendo en gran medida su potencial antioxidante. Sin embargo, el perfil de compuestos fenólicos presentes es desconocido, así como los compuestos responsables de la actividad antioxidante de esta planta.

LG al igual que todas de plantas poseen metabolitos secundarios dentro de los cuales se encuentran los compuestos fenólicos, que se generan a través de las rutas pentosa fosfato, chiquimato y fenilpropanoide (Cartea et al., 2010; Heleno et al., 2015). Estos compuestos determinan en gran medida la adaptación de las plantas a factores bióticos y abióticos, así como en el crecimiento y reproducción de la planta (Akula y Ravishankar, 2011). A su vez, juegan un papel importante en el color y características sensoriales de frutas y vegetales (Pereira et al., 2009). Además, estos compuestos son asociados con diversas actividades biológicas tales como antimicrobianas, antivirales, antiinflamatorias, antialergénico, anticancer, antihipertensivo y antioxidante, las cuales están relacionadas directamente con la estructura de dichos compuestos (Cicerale et al., 2010). Los compuestos fenólicos están conformados por anillos aromáticos generalmente sustituidos por uno o más grupos hidroxilos, y estos se dividen en diferentes grupos tomando en cuenta la estructura del esqueleto fenólico básico, dentro de los cuales se encuentran los fenoles simples, ácidos benzoicos, fenilpropanoides y flavonoides (Michalak, 2006; Martins et al., 2011).

Los flavonoides son uno de los grupos de metabolitos secundarios ampliamente distribuidos en las plantas, y generalmente son responsables de la coloración de los frutos, hojas y flores (Pereira et al., 2009). Estos compuestos se pueden encontrar presentes en forma de agliconas, glucosidos y derivados metilados y se han identificado más de 4000 variedades de estos (Tapas et al., 2008). Los flavonoides están constituidos por una estructura base de 15 átomos de carbonos (C_6-C_3-C_6), conformados por dos anillos aromáticos A y B, unidos a un anillo heterocíclico C (Figura 2) (Balasundram et al., 2006). Basados en su estructura, los flavonoides se clasifican en flavanoles, flavonas, flavanonas, catequinas, antocianidinas, isoflavonas, dihidroflavonoles y chalconas (Figura 3) (Tripoli et al., 2007; Vermerris y Nicholson, 2008). Las actividades biológicas de los flavonoides estarán determinadas en gran medida por la estructura

Figura 1. Hojas de *Litsea glaucescens*

química, los sustituyentes y orientación relativa de sus componentes (Kumar and Pandey, 2013).

Unas de las actividades biológicas más destacadas de este tipo de compuestos es su actividad antioxidante (Rice-Evans et al., 1996; Tapas et al., 2008). La habilidad de estos compuestos para inhibir o estabilizar la formación de especies reactivas de oxígeno y quelar ó reducir metales dependerá de distintos factores. Uno de los factores importantes en el potencial antioxidante de un flavonoide es el grado de hidroxilacion (-OH) y la posición de estos grupos funcionales en la molécula (Bors et al., 1990; Balasundram et al., 2006). Por ejemplo, se ha observado que grupos hidroxilos presentes en la posición 3´, 4´- del anillo B en conjunto con grupo hidroxilo en la posición 3 del anillo C incrementan la actividad antioxidante, debido a que los protones o electrones en esta posición pueden ser donados con mayor facilidad, logrando estabilizar radicales libres con mayor eficiencia (Michalak, 2006; Farkas et al., 2004). A su vez, se ha observado que los flavonoides con esta distribución pueden incrementar hasta 10 veces más el potencial antioxidante con respecto a los que carecen de estas estructuras (Heim et al., 2002). Otra característica importante que favorece la capacidad antioxidante de los flavonoides es la presencia de doble enlace entre C-2 y C-3, conjugado con el grupo 4-ceto en el anillo, debido a que favorece la deslocalización de los electrones, permitiendo formar radicales fenólicos estables (Pietta, 2000; Amic et al., 2003). También se ha observado que el doble enlace entre C-2 y C-3 en combinación con 3-OH, incrementa el potencial de los flavonoides para estabilizar radicales (Heijnen et al., 2001; Seeram y Nair, 2002).

En relación a los extractos obtenidos de plantas, hay que considerar que su potencial antioxidante está influenciado por diversos factores, los cuales determinarán en gran medida el perfil y concentración de los compuestos fenólicos presentes (Khan et al., 2009; Verma y Shukla, 2015). Dentro de estos factores se encuentran el tipo de solvente utilizado y el método de extracción (Sultana et al., 2009). Por otra parte, factores bióticos tales como herbívoros, microorganismos y parásitos principalmente pueden afectar el perfil y concentración de los compuestos fenólicos (Esra et al., 2010). Otros factores que afectan fuertemente el perfil de estos compuestos son los factores abióticos,

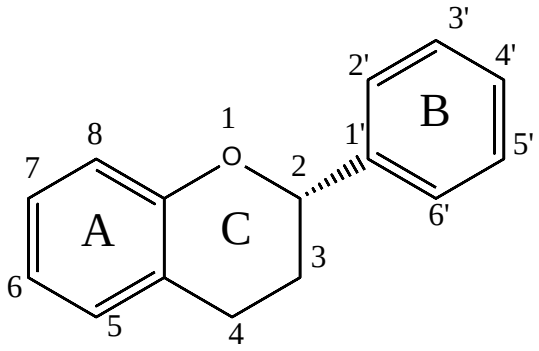

Figura 2. Estructura básica de los flavonoides

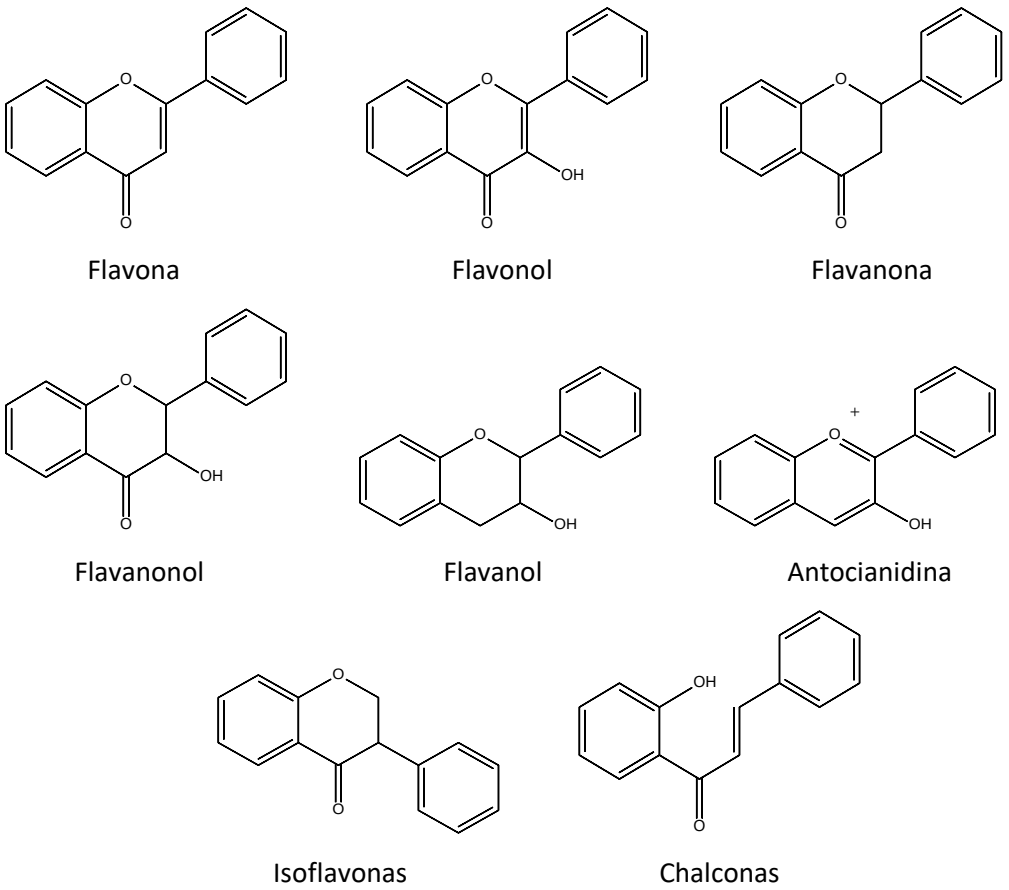

Figura 3. Clasificación de flavonoides

dentro de los cuales se encuentran los factores ambientales, en los cuales destacan la temperatura, la radiación ultravioleta (UV), la intensidad de la luz, humedad, disponibilidad de agua y minerales, por mencionar algunos (Zhi-lin et al., 2007; Akula y Ravishankar, 2011).

Se ha observado que el contenido de flavonoides en las plantas es afectado principalmente por la temporalidad, debido a que este tipo de compuestos se sintetizan en respuesta a las diferentes condiciones de temperatura, radiación UV y humedad en las distintas épocas del año, ya que normalmente estos compuestos se encuentran en mayor medida en las hojas de las plantas (Gouvea et al., 2012; Verma y Shukla, 2015). En este sentido, diferentes estudios han demostrado que el estrés producido por la temperatura y radiación UV induce la activación de la enzima fenilalanina amonio liasa (PAL), la cual es considerada como la enzima más importante en la síntesis de los fenil-propanoides (Cheynier et al., 2013). La activación de la enzima PAL produce una desaminacion de L-fenilalanina, teniendo como resultado la transformación a trans-ácido cinámico, el cual es el intermediario primario en la biosíntesis de compuestos fenólicos (Boudet, 2007). Por tal motivo, la PAL juega un papel primordial en la aclimatación celular contra el estrés térmico en plantas (Zhang y Liu, 2015). A su vez, también se ha observado que otros factores como el ataque por microorganismos incrementan la síntesis de flavonoides, debido a que estos compuestos tienen la capacidad de inhibir o matar a los microorganismos (Miranda et al., 2007; Cheynier et al., 2013). En este sentido, resulta de gran interés tratar de extraer fracciones ricas en este tipo de compuestos o compuestos purificados, los cuales podrían representar alternativas viables en diferentes áreas de estudio como la industria alimentaria y farmacéutica.

La obtención de extractos ricos en fenoles y compuestos puros se puede lograr mediante la aplicación de diferentes procedimientos, los cuales tienen como objetivo primordial lograr la separación de los compuestos deseados de la matriz vegetal (Azmir et al., 2013). Esto se puede lograr, mediante una extracción directa de los compuestos con solventes afines, debido a una alta solubilidad (Wijngaard et al., 2012). Sin embargo, hay que considerar que en ocasiones este tipo de compuestos bioactivos se encuentran unidos a la pared celular, por lo cual es necesario utilizar otros procedimientos que

logren separar a los compuestos bioactivos de los polímeros enlazados en la pared celular (Dai y Mumper, 2010). Esta separación, se puede lograr mediante hidrolisis, en la cual se utilizan agentes químicos, ácidos o bases y enzimáticos (Martins et al., 2011; Min et al., 2006). Una vez obtenidos los extractos crudos, se pueden realizar etapas físico-químicas con la finalidad de concentrar los compuestos de interés, que permite obtener extractos con mayor pureza y actividad.

Uno de los métodos más utilizados para obtener fracciones ricas en flavonoides es la extracción con disolventes, en la cual se utilizan solventes de mediana polaridad como cloroformo, diclorometano, éter etílico o acetato de etilo para lograr extraer flavonoides de polaridad intermedia (isoflavonas, flavanonas, flavonas y flavonoles), mientras que flavonoides con mayor polaridad como flavonoides glicosilados, chalconas, flavonoles y flavonas hidroxiladas son extraidos principalmente con alcoholes, acetona, agua, y sus mezclas (Ignat et al., 2011; Azmir et al., 2013). Por otra parte, otro método utilizado para la obtención de fracciones ricas en flavonoides y compuestos purificados es a través de métodos cromatográficos (Dai y Mumper, 2010). La cromatografía en columna es uno de los métodos más utilizados para la purificación de compuestos fenólicos debido a su simplicidad (Ignat et al., 2011). Este tipo de separación consiste en utilizar una fase estacionaria conocida como adsorbente (normalmente gel de silice), la cual es introducida dentro de una columna, que se impregna con los eluyentes o fase móvil (Çitoğlu y Acikara, 2012). Al gel de sílice se le incorpora previamente la muestra a separar, y se coloca en la parte superior de la fase estacionaria (Berrueta et al., 1995). Posteriormente, esta se eluye a través de adición sucesivas de solventes (eluyentes) de diferente polaridad, lo cual hace descender a las moléculas por la columna debido a la solubilidad de los compuestos con los solventes utilizados, permitiendo obtener compuestos purificados (Dai y Mumper, 2010).

Basado en lo anterior, actualmente no existen estudios en la literatura en los cuales se evalué el efecto de la temporalidad sobre el potencial biológico de LG, y como esta puede afectar la concentración de metabolitos secundarios presentes en la planta. A su vez, existen pocas investigaciones que evalúen su potencial antioxidante y se desconoce a detalle cuales son los compuestos responsables de dicho potencial biológico. Por tal motivo, resulta interesante estudiar a profundidad el potencial antioxidante de esta planta

y conocer a los compuestos responsables de dicha actividad, ya que estos (extractos y compuestos) podrían representar una excelente opción para diversas áreas, como podría ser la industria cárnica. En este sentido, es importante mencionar que el potencial antioxidante de LG sobre matrices cárnicas no ha sido evaluado con anterioridad, lo cual podría representar una buena opción para extender la vida de anaquel de carne y productos cárnicos manteniendo sus características de calidad, ya que es conocido que los procesos oxidativos (lípidos y proteínas) deterioran en gran medida la calidad de estos alimentos, teniendo como resultado una vida de anaquel corta y el rechazo por parte los consumidores.

Por lo anterior, la hipótesis del presente estudio fue la adición de un extracto y sus fracciones de *L. glaucescens* en hamburguesas de cerdo, permite retardar la formación de los productos primarios y secundarios de la oxidación lipídica y proteica, debido a la presencia de compuestos fenólicos con alto potencial antioxidante.

El presente estudio se dividió en tres etapas; La primera etapa (**Capítulo I)** consistió en purificar y caracterizar a los compuestos fenólicos que más influían en la capacidad antioxidante de un extracto de LG. La segunda de ellas (**Capítulo II)** consistió en evaluar el efecto de la temporalidad sobre el potencial biológico de extractos metanólicos obtenidos de hojas de *L. glaucescens* (LG). Así como también, determinar el efecto de la temporalidad sobre el perfil y la concentración de compuestos fenólicos en los extractos estudiados. Finalmente, en la tercera etapa (**Capítulo III**) se evaluó el efecto del extracto de LG y sus fracciones con mayor actividad antioxidante sobre la oxidación lipídica, oxidación proteica y vida de anaquel de un modelo cárnico almacenado en refrigeración.

En este contexto en el **Capítulo I,** se realizó un extracto metanólico de hojas de LG obtenidas de Xico, Veracruz. Posteriormente, se realizó una purificación y caracterización de los CF que influyen en mayor medida el potencial antioxidante del extracto de LG. El extracto de LG fue analizado por cromatografía en columna, utilizando sílica gel 60 como fase estacionaria, y hexano-acetato de etilo-metanol como fase móvil. Se obtuvieron 12 fracciones (I a XII), a las cuales se les evaluó el potencial antioxidante (DPPH y FRAP) y contenido de CF. Se obtuvieron dos fracciones (XI y

XII) con elevado potencial antioxidante y contenido CF, en comparación con las otras fracciones obtenidas.

Las fracciones XI y XII fueron analizadas mediante cromatografía en columna utilizando las condiciones antes mencionadas. De la fracción XI se logró purificar dos compuestos, los cuales fueron nombrados como compuestos 1 y 2, respectivamente. El análisis de la fracción XII permitió purificar un compuesto, el cual fue denominado como compuesto 3. Los compuestos purificados, fueron caracterizados mediante Resonancia Magnética Nuclear (^{1}H y ^{13}C en una y dos dimensiones), HPLC-ESI-MS y HPLC-DAD. Los resultados obtenidos mostraron que los compuestos 1, 2 y 3 correspondían a la estructura de epicatequina, quercitrina y kaempferol, respectivamente, de los cuales quercitrina no había sido reportado previamente en LG. Mediante este estudio se lograron purificar y caracterizar a los flavonoides que influían en mayor medida en el potencial antioxidante del extracto de LG. También, se logró identificar un nuevo flavonoide con potencial antioxidante en esta planta.

En el **Capítulo II** del presente estudio se evaluó el efecto de la temporalidad sobre el potencial biológico de extractos metanólicos obtenidos de hojas de LG y a su vez se analizó el impacto de la temporalidad sobre el contenido de compuestos fenólicos. Para cumplir con este objetivo, se utilizaron hojas de LG obtenidas de Xico, Veracruz, México durante otoño e invierno del 2015 y primavera y verano de 2016. Las hojas fueron maceradas en metanol durante cuatro días. Transcurrido este tiempo, el solvente fue eliminado bajo presión reducida en un evaporador rotatorio, y el solvente remanente se eliminó utilizando una bomba de alto vacío, permitiendo obtener los diferentes extractos. Los extractos fueron almacenados a -20 °C y se etiquetaron como extracto de otoño (ALGE), invierno (WLGE), primavera (SLGE) y verano (SULGE) de LG.

A los extractos obtenidos se les determinó el contenido de compuestos fenólicos (CF) y se les evaluó el potencial biológico (actividad antioxidante, antiproliferativa y antimicrobiana). El contenido de CF se determinó utilizando el método Folin-Ciocalteu y HPLC-DAD. La evalución de los CF mostró que hubo un efecto de la temporalidad sobre la concentración de estos compuestos bioactivos. De manera que los extractos ALGE y SULGE fueron los que presentaron un mayor ($P<0.05$) contenido, seguido de SLGE y WLGE. El análisis por HPLC-DAD permitió conocer el perfil de CF presentes

en los extractos, y la identificación de dos de los compuestos mayoritarios, los cuales fueron epicatequina y quercetrina. El primero de ellos se presentó a una mayor ($P<0.05$) concentración en ALGE, mientras que los otros extractos (WLGE, SLGE y SULGE) exhibieron concentraciones más bajas similares ($P>0.05$) de este compuesto. Por otra parte, el compuesto quercetrina mostró una mayor ($P<0.05$) concentración en SULGE.

La actividad antioxidante de los extractos fue evaluada a través de los ensayos de DPPH, FRAP y ORAC. Se observó que la actividad antioxidante fue afectada por la temporalidad, ya que los extractos ALGE y SULGE presentaron mayor potencial en todos los métodos analizados. A su vez, los métodos evaluados evidenciaron que todos los extractos de LG presentaron potencial antioxidante elevado tomando en cuenta las clasificaciones realizadas para extractos de plantas (Fidrianny et al., 2015; Wong et al., 2006) .Por otra parte, se realizó un análisis de correlación entre el contenido de CF y los métodos de actividad antioxidante. Los resultados obtenidos mostraron que se presentó una correlación positiva entre los CF y la actividad antioxidante de los extractos (CF:DPPH, r=0.92; CF:FRAP, r=0.93; CF:ORAC, r=0.80).

La actividad antimicrobiana de los extractos se determinó en base al método de microdilución utilizando como microorganismos de referencia a *Escherichia coli* y *Staphylococcus aureus*. La actividad antimicrobiana de los extractos analizados tuvo variación de acuerdo a la temporalidad, de manera que SULGE y SLGE presentaron un potente efecto antimicrobiano contra *S. aureus* a las concentraciones evaluadas (0-1000 µg/mL), logrando inhibir al menos el 50 % de este microorganismo. A su vez, se observó que SULGE logro inhibir entre 100 a 98 % de los cultivos de *S. aureus* a concentraciones de 1000 y 800 µg/mL, respectivamente, lo cual fue similar a la actividad exhibida por el control positivo utilizado (gentamicina, 12 µg/mL). Con respecto a *E. coli*, se presentó bajo potencial antimicrobiano, ya que ninguno de los extractos evaluados logró inhibir al menos en un 50 % a este patógeno a las concentraciones evaluadas (0-1000 µg/mL).

Por otra parte, el potencial antiproliferativo de los extractos de *L. glaucescens* se realizó a través del ensayo de MTT contra cuatro líneas celulares: HeLa (cáncer cervicouterino humano), LS-180 (cáncer de colon humano), M12.C3.F6. (linfoma de células B

murinas) y ARPE (epitelio pigmentario de retina humana). Los resultados obtenidos mostraron que los extractos de LG inhibieron la proliferación de las líneas celulares estudiadas. La línea celular más susceptible fue HELA, seguida de LS 180, M12.C3.F6 y ARPE. De manera general, se evidenció que la actividad antiproliferativa fue afectada por la temporalidad, ya que ALGE y SULGE fueron los tratamientos con mayor actividad.

En general, se observó un efecto de la temporalidad sobre el contenido de CF y las actividades biológicos de los extractos de LG, de manera que ALGE y SULGE fueron los que mostraron mayor concentración de CF. Dicho comportamiento podría ser asociado a un mayor estrés térmico de la planta durante estas temporadas del año, ya que normalmente durante este periodo se presentan temperaturas y radiaciones UV más elevadas. Este comportamiento, podría influenciar la síntesis de compuestos fenólicos para tratar de mitigar las condiciones ambientales adversas. A su vez, se observó que ALGE y SULGE fueron los extractos con mayor potencial en las actividades biológicas evaluadas, lo cual puede ser relacionado con el contenido de CF, ya que diferentes investigaciones evidencian que este tipo de compuestos presentan elevado potencial en las áreas de estudios evaluadas. Por lo tanto, los extractos de LG podrían representar una interesante alternativa como agentes antioxidantes y antimicrobianos, sin embargo es necesario realizar otros estudios para asegurar su efectividad y seguridad en humanos.

En el **Capítulo III** de esta tesis se evaluó el efecto antioxidante del extracto (ALGE) y sus fracciones (F-XI y F-XII) más activas sobre las características de calidad y vida de anaquel de hamburguesas de cerdo almacenadas en refrigeración. Para cumplir con este objetivo, se elaboraron hamburguesas de carne molida de cerdo, las cuales fueron asignadas a los siguientes seis tratamientos: Tratamiento 1: Control negativo (Sin aditivo); Tratamiento 2: BHT a 100 ppm; Tratamiento 3: Trolox a 100 ppm; Tratamiento 4: ALGE a 100 ppm; Tratamiento 5: F-XI a 100 ppm; Tratamiento 6: F-XII a 100 ppm. Las hamburguesas obtenidas fueron colocadas en platos de plástico y empacadas en empaque tradicional y mantenidas en refrigeración a 4 °C durante 11 días, y los análisis se realizaron al día 1, 4, 6, 8 y 11 de almacenamiento.

El potencial antioxidante de los aditivos utilizados fue evaluado sobre los parámetros de oxidación lipídica (dienos y TBARS) y proteica (grupos carbonilo) de las hamburguesas

de cerdo. Los resultados de oxidación lipídica mostraron que los aditivos incorporados en las hamburguesas disminuyeron ($P<0.05$) la formación de los productos primarios (dienos) y secundarios (malonaldehido) de la oxidación lipídica durante el almacenamiento. Los tratamientos con mayor efectividad fueron la fracción XI, Trolox y BHT, de manera que presentaron la menor formación de dienos y manolaldehido durante el almacenamiento. Es importante mencionar que los tratamientos ALGE y fracción XII también fueron efectivos al inhibir la oxidación lipídica, ya que mostraron menor oxidación que el control negativo (sin aditivo) durante la vida de anaquel. Con respecto a la evaluación de oxidación proteica, se presentó un comportamiento similar al de oxidación lipídica, ya que todos los aditivos fueron capaces de disminuir la formación de grupos carbonilo de las proteínas, los cuales se generan como resultado de la oxidación proteica. De manera general, se observó que el mayor contenido de grupos carbonilo se presentó en el tratamiento control negativo, lo cual evidenciaba el potencial antioxidante del extracto de LG y sus fracciones.

Por otra parte, el color superficial fue evaluado por el método CIE-LAB sobre la superficie de las hamburguesas. Los resultados más destacados mostraron que el extracto que ALGE, F-XI y F-XII presentaron una menor disminución ($P<0.05$) de los valores a* (índice rojo-verde), lo cual indicada que estos tratamientos fueron efectivos para disminuir la oxidación de la mioglobina. Otro de los parámetros evaluados fue la determinación de pH. Los valores obtenidos evidenciaron que se presentaron cambios al día 11 de almacenamiento, donde el tratamiento control presentó mayor valor de este parámetro en comparación con el resto de los tratamientos. Finalmente, se realizó una evaluación sensorial de las hamburguesas elaboradas mediante un panel sensorial entrenado. De manera general, los panelistas percibieron que la adición de ALGE y fracciones (F-XI y F-XII) afectaron de manera negativa la aceptabilidad de las hamburguesas, ya que tendieron a desarrollar sabores y olores poco aceptables. Sin embargo, los parámetros evaluados de perdida de olor y sabor a fresco no fueron afectados por la adición de estos tratamientos.

Los resultados obtenidos evidenciaron que el extracto de LG y sus fracciones presentaron efecto antioxidante al ser capaces disminuir los procesos oxidativos en las

hamburguesas de cerdo. Este comportamiento puede ser atribuido a la capacidad de los compuestos presentes en el extracto y fracciones, los cuales son capaces de disminuir la formación de radicales libres o la estabilización de los radicales generados al inicio de los procesos oxidativos, ya que como se demostró anteriormente los extractos de *L. glaucescens* presentaron una gran capacidad para estabilizar radicales libres y reducir metales, los cuales tienen un papel preponderante en los procesos oxidativos de lípidos y proteínas.

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Capítulo I

Isolation and characterization of antioxidant phenolic compounds from *Litsea glaucescens* Kunth extract

Artículo preparado para la revista Molecules

Isolation and characterization of antioxidant phenolic compounds from *Litsea glauscescens* Kunth extract

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Abstract: *Litsea glaucescens* is a native plant from Mexico known as laurel, and is commonly used as food seasoning and traditional medicine, however, there is little

information about its bioactive compound profile. Therefore, in this work, we studied the antioxidant activity of a methanolic extract of *L. glaucescens* (LGE) and isolated the bioactive compounds responsible for this activity. The antioxidant activity was evaluated by DPPH and FRAP assay and total phenolic content was evaluated by Folin-Ciocalteu method. In addition, we isolated antioxidant compounds by chromatographic methods and determine their structure by spectroscopic techniques (HPLC-DAD, HPLC-ESI-MS and NMR). We identified two fraction (F-XI and F-XII) with exceptional ($p \leq 0.05$) antioxidant activity by DPPH (100 µg/mL=93 and 91%, 50 µg/mL= 93 and 91%, 25 µg/mL =92 and 87% and 12.5 µg/mL=85 and 53% of radical inhibition, respectively) and FRAP methods (7729.8 and 4929 µmol Fe(II)/g, respectively), as well as phenolic content (462.9 and 347.5 mg GAE/g, respectively). Subsequently those fractions were selected to isolate their antioxidant compounds. Chromatographic separation of F-XI and F-XII yield three phenolic compounds identified as epicatechin, quercetin rhamnose and kaempferol, being quercetin rhamnose, first- reported for *L. glaucescens* plants. This is the first report that provides information about the responsible compounds for *L. glauscences* antioxidant, representing an alternative source of phytochemicals with bioactive potential for alimentary and pharmaceutical industries.

Keywords: isolated, antioxidant, phenolic compounds, *Litsea glaucescens*.

1. Introduction

Oxidative stress is produced as result of an unbalance between pro-oxidant and antioxidant compounds [1]. Pro-oxidant molecules are known as reactive oxygen species (ROS), and are produced under normal physiological conditions by human metabolism [2, 3]. In this regard, the human system has powerful enzymatic defense systems to neutralize the effect of pro-oxidant species. However, in several occasions an overproduction of ROS results in an oxidative stress process, affecting DNA, RNA, proteins, lipids and carbohydrates, producing cell death, damage and mutations, which are involved in the occurrence of chronic human diseases [4, 5].

Nowadays, chronic human diseases are considered a worldwide public health problem, and most of them are related to oxidative stress, which's play an important role in the

development of degenerative diseases such as cancer, atherosclerosis, neurodegenerative and cardiovascular damage, among others [6]. These diseases are the leading causes of mortality around the world [5, 7]. Therefore, it is required to find efficient alternatives to help the human enzymatic system to reduce the high incidence of chronic human diseases. In this regard, plants represent an interesting alternative source of bioactive compounds, because of their wide variety of secondary metabolites, which are associated to different health benefits, including antioxidant properties [8].

Litsea genus belongs to the Lauraceae family, with more than 400 species approximately and are mainly distributed throughout North America, South America and Asia [9]. Traditionally, *Litsea* plants have been used as medicinal plants and are widely used for the treatment of inflammation, stomach infection, vomit, colic and central nervous system illness, among others [10, 11]. Researches about chemical constituents of *Litsea* plants evidence the presence of a variety of bioactive compounds such as phenolic compounds, alkaloids, lactones, terpenes, terpenoids, butanolides, steroids and amides [10, 12]. Several of these compounds possess a wide spectrum of biological activities, such as protective effect on cardiovascular system, antioxidant, anticancer, antiproliferative, antimicrobial, anti-HIV and antidiabetic, among others [12]. One of *Litsea* species that have been little studied is *Litsea glaucescens*, which as a member of the *Litsea* genus, may present a good source of bioactive compounds with potential biological activities.

Litsea glaucescens is a native species from Mexico, principally distributed in the state of Veracruz, Chiapas and Nayarit, where it is commonly known as Laurel [13]. In Mexico, *L. glaucescens* plants have been conventionally used as food seasoning and traditional medicine [11]. However, there is limited investigation about the biological properties of *L. glaucescens* and its profile of bioactive compounds. In this regard, Tapia-Torres et al. [14] and our preliminary study (unpublished data) demonstrated that *L. glaucescens* extract exhibit a high content of phenolic compounds, as well as a good antioxidant activity. However, additional information is required to determine the phenolic compounds responsible of the antioxidant activity of *L. glaucescens*, with the aim to provide the phytochemical characterization of a potential nutraceutical plant for alimentary and pharmaceutical area.

Based on the above, the objective of present study was to isolate and identify the phenolic compounds responsible of antioxidant activity of *L. glaucescens*.

2. Materials and Methods

2.1. General information

NMR spectra were recorded at 400 MHz for 1H and 100.6 MHz for 13C on an Agilent 400 MHz NMR Magnet Fourier transform instrument in acetone-d6 at 25 °C. 1H-1H COSY, 1H-13C HSQC were acquired with a usual pulse sequence, and data were analyzed with an Agilent VmnrJ 3.2 Software. Preparative HPLC analysis was performed on a Varian ProStar 210 (Walnut Creek, Usa) equipped with a Hypersil ODS C18 column (250 x 4.6 mm, Ø 5 µm, Thermo Fisher Scientific, USA). Elution was with 5 % formic acid in water (solvent A) and methanol (solvent B). The elution was accomplished with a solvent flow rate of 1 mL/min, using a gradient elution of 30 % methanol (0-15 min), 40 % methanol (15-20 min), 45 % methanol (20-30 min), 60 % methanol (30-50 min), 80 % methanol (50-65 min) and 100 % methanol (65-75 min). Flavonoids were detected with diode array detector and monitored at 280 and 340 nm. Mass spectra was analyzed on HPLC-ESI-MS. Analytical TLC were performed on aluminum silica gel 60 F 254 plate (Merck). Solvents such as hexane and ethyl acetate were obtained from Reproquifin (Mexico). Methanol, ethanol and formic acid were purchased from Sigma-Aldrich (USA). Water (HPLC-grade) was filtered in a Milli-Q50 purification system (Millipore). Folin–Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$) , 2,4-dinitrophenylhydrazine (DNP), potassium hydroxide (KOH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri(2-pyridyl)-striazine (TPTZ), iron (III) chloride hexa-hydrate, sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), hydrochloric acid, gallic acid, quercetin and butylated hydroxytoluene (BHT) were obtained *from Sigma-Aldrich (USA).*

2.2. Litsea glaucescens and methanolic extracts elaboration (LGE)

L. glaucescens leaves were collected in Xico, Veracruz, Mexico during November 2015. Collected leaves were identified in the Herbarium of the Instituto de Investigaciones Biológicas of the Universidad Veracruzana, Mexico. The collected sample was washed

and dried at room temperature. Dried leaves were extracted with methanol (96%) at room temperature during 4 days with occasional stirring (2-3 time per day). After, the obtained methanolic extract was filtered through Whatman grade No. 4 filter paper and solvent was removed under vacuum at 40 °C. Finally, *L. glaucescens* extract (LGE) was stored at -8 °C until analysis.

2.3. Free-radical scavenging activity (DPPH assay)

The antioxidant activity by the DPPH radical scavenging activity assay was performed using the modified version of Usia et al. [15]. LGE was dissolved in ethanol, and 100 µL of extract was mixed with 100 µL of DPPH solution (300 µM). The sample was kept in the darkness for 30 min. After, the sample was reading at 517 nm in a microplate reader. LGE was evaluated at concentration of 12.5, 25, 50, and 100 µg/mL. Vit. C and Trolox (70 µM) were used as antioxidant standards.

2.4. Ferric reducing/antioxidant power (FRAP assay)

Antioxidant capacity assay was carry out using a modified FRAP assay as described by Benzie and Strain [16]. The working FRAP reagent was elaborated reacting 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 40 mM TPTZ (dissolved in 40 mM HCL) and 1 volume 20 mM of ferric chloride (dissolved in water). 280 µL of FRAP reagent was mixed with twenty µL of LGE and the absorbance was read at 630 nm after 30 min of storage in the dark. Results were reported as Fe (II)/g of dry weight (d.w.).

2.5. Total phenolic content

Total phenolic concentration was evaluated according to the method described by Popova et al. [17] with slight modifications. Ten µL of LGE was mixed with 80 µL of distillate water, 40 µL of Folin Ciocalteu reagent 0.25 N, 60 µL sodium carbonate (5 % in distillated water) and 80 µL of distillate water. The mixture was incubated at room temperature during 60 min. The absorbance was measured at 750 nm using a Microplate Reader. Results were expressed as milligrams of gallic acid equivalent (GAE)/ g d. w.

2.6. Isolation of antioxidant phenolic compounds

Dried LGE (16.269 g) was fractioned through a silica gel 60 column (100 cm x 5 cm), and eluted with a stepwise gradient mixture of hexane-ethyl acetate-methanol (90:10:0, 80:20:0, 70:30:0, 50:50:0, 30:70:0, 0:100:0, 0:50:50, 0:0:100). All obtained fractions were analyzed by TLC, and combined in XII fractions according to its UV-Vis

absorption pattern. The antioxidant activity (DPPH and FRAP) and phenolic content were determine in all fractions (I-XII). Fraction XI and XII exhibited the highest antioxidant activity (DPPH and FRAP) and phenolic content in comparison with the other fractions. Fraction XI (0.15 g) was chromatographed on a silica gel 60 column (100 cm x 5 cm) eluted with hexane-ethyl acetate-methanol (10:90:0, 0:100:0, 0:90:10, 0:50:50, 0:0:100) to yield compound 1. Fraction XII (2 g) was separated using a silica gel 60 column (100 cm x 5 cm) eluted with hexane-ethyl acetate-methanol (10:90:0, 0:95:5, 0:80:20, 0:50:50, 0:25:75, 0:0:100) to afford compounds 2 and 3.

2.7. Statistical analysis

Data analysis was performed using the NCCS, 2007 statistical software. One way ANOVA was used, and mean comparisons were performed using the Tukey-Kramer test. Significance level in Type I error was $p \leq 0.05$.

3. Results and discussion

The high incidence of oxidative stress diseases are a worldwide concern, because cause a great number of deaths every year [18]. Therefore, it is required to find efficient alternatives to reduce the high incidence of chronic human diseases. In this way, traditionally used plants represent an excellent option, because these are perceived as safe and contain a wide variety of secondary metabolites, which exhibited interesting antioxidant potential [19]. *Litsea glaucescens* is a native plant from Mexico, which showed a good antioxidant potential and phenolic content, however, there is no information about the responsible compounds for this activity, which can represent a potential option for pharmaceutical industry. Therefore, the present study provides new information concerning to the responsible compounds of the antioxidant activity of *L. glaucescens.*

Analysis of antioxidant activity by DPPH assay of *Litsea glaucescens* extract (LGE) and their fraction are shown in Table 1. LGE presented antioxidant activity in a dose-response manner, because the DPPH inhibition increase ($p \leq 0.05$) in function to the evaluated concentrations of LGE (Table 1). In addition, was observed that LGE at 100 µg/mL and 50 µg/mL exhibited similar DPPH inhibition values ($p > 0.05$) to those

presented by the antioxidant standards Trolox and Vit. C at 70 µM. Regarding the antioxidant activity of LGE fractions, a dose-response effect was also observed. Results evidenced that F-XI presented the higher antioxidant potential, followed by F-XII. In addition, F-XI at 100, 50 and 25 µg/mL had a similar effect ($p>0.05$) than Trolox at 70µM. In this regard, Fidrianny et al. [20] suggested that natural extracts with IC_{50} values (concentration required to inhibit the 50 % of DPPH radical) lower than 50 µg/mL can be classified an extract with very strong antioxidant activity. Based on the above, our results showed that LGE, F-X, F-XI and F-XII can be considered as very strong antioxidants, because they presented IC_{50} values lower than 50 µg/mL.

Generally, the ability of LGE and its fraction to inhibitit DPPH were in the range of those observed in others studies on *Litsea* genus. In a study performed by Devib and Meera, [21], *L. glutinosa* exhibited DPPH antioxidant activity depending of the concentration, where the highest tested concentration (100 µg/mL) exhibited 85.46 % of DPPH inhibition and an IC_{50} value of 30.24 µg/mL. Hwang et al. [22], evidenced that *L. cubeba* extract and their fractions at 100 µg/mL exhibited between 60.25 to 90.57 % of DPPH inhibition values, obtained values using in fractionation were solvent-dependent, where methanolic extraction was the accurate due to the highest inhibition. Similarly, Yoon et al. [23] found that *L. japonica* extract and fraction exhibited IC_{50} values between 13.6 to 669.2 µg/mL, those values varied according with solvent type, due to medium (ethyl acetate) and highest (butanol) polarity fractions were the most active comparing with the lowest one (hexane and chloroform). Wong et al. [24] tested the antioxidant activity of different extracts from stem, root and inner bark of *L. elliptica* and *L. resinosa*. They observed that *L. resinosa* extracts (EC_{50} values ranged from 11.22 to >1000 µg/mL) exhibited higher antioxidant potential in comparison with *L. elliptica* extracts (EC_{50} values ranged from 23.99 to >1000 µg/mL). Overall, stem and root extracts presented better antioxidant activity than inner bark extract of mentioned plants. In addition, these authors observed that polar solvents (methanol) extraction reached extracts with stronger antioxidant activity, suggesting that these type of solvents were more effective to extract antioxidant compounds. This behavior is in agreement with our results, because fractions obtained with higher polarity solvents (F-XI and F-XII) were more actives antioxidants in comparison to the less polar fractions (F-I and F-II). This

can be attributed to the ability of polar solvents to mainly extract phenolic compounds, which are associated with potent antioxidant activity.

On the other hand, ferric reducing activity of LGE and their fractions was determine by the FRAP assay (Figure 1). LGE showed 4689.8 µmol Fe(II)/g, whereas the obtained values of the analyzed fraction ranged from 326.1 to 7729.8 µmol Fe(II)/g. Generally, it was observed that F-XII and F-XI provided the highest ($p \leq 0.05$) reducing activity compared with LGE and the other fractions (F-I to F-X). LGE exhibited higher activity that other reported *Litsea* extracts, however, antioxidant activity evaluted by this method was lower than positive control (Vit. C and Trolox). For example, methanolic and aqueous extracts from *L. garciae* showed values between 210-2050 µmol Fe(II)/g [25]. Similarly, Fu et al. [26] and Suksamerkun et al. [27] analyzed the reducing activity of *L. rotundifolia* and *L. elliptica* and observed antioxidant activity of 27.6 and 2020 µmol Fe(II)/g, respectively. Obtained results on FRAP evaluation are consistent with the DPPH assay, which evidenced that F-XII, F-XI and LGE extract were the most active, suggesting that these treatments are a good source of bioactive compounds with high antioxidant activity.

The present study confirms that *Litsea* genus possess strong antioxidant activity. This activity is mainly associate to the presence of phenolic compounds. In this regard, different authors have isolated and characterize different compound with exceptional antioxidant activity from *Litsea* plants, where phenolic compounds such as flavonol and flavanone were the most active [12, 28].

Total phenolic content of LGE and its fractions are presented in Figure 2. LGE presented 346.5 mg GAE/g d.w. and the obtained fractions showed significant variation ($p<0.05$), ranging from 45.5 to 462.9 mg GAE/g d.w., where F-I, F-XIII and F-IX exhibited the lowest values ($p<0.05$), while F-XI and F-XII had the highest phenolic content ($p<0.05$). Results demonstrate that phenolic compounds present in LGE were associated with the antioxidant potential of the analyzed extracts, since F-XI and F-XII showed the highest phenolic content and antioxidant activity. By the other hand, an opposite effect was observed for F-VI, due to its high phenolic content was not enough to improve the antioxidant potential. This premise suggest that F-VI phenolic compounds presented low

Table 1. DPPH free-radical scavenging activity of LGE and its fractions (F-I to F-XII).

Treatment	% of DPPH inhibition					
			Concentration µg/mL			
	Trolox (70 µM)	Vit. C (70 µM)	12.5	25	50	100
LGE	95.3±1.1^{cd}	96.7±1.1^{d}	44.6±0.9^{a}	79.9±0.4^{b}	93.3±2.6^{c}	94.6±0.2^{cd}
F-I	95.3±1.1^{c}	96.7±1.1^{c}	NA	NA	10.7±13.8^{a}	13.8±0.5^{b}
F-II	95.3±1.1^{c}	96.7±1.1^{d}	NA	NA	5±0.6^{a}	8.8±0.4^{b}
F-III	95.3±1.1^{b}	96.7±1.1^{c}	NA	NA	NA	6.5±0.3^{a}
F-IV	95.3±1.1^{e}	96.7±1.1^{e}	2.6±0.2^{a}	6.2±0.3^{b}	13±0.5^{c}	25.7±2.8^{d}
F-V	95.3±1.1^{e}	96.7±1.1^{e}	6.2±0.3^{a}	10.4±0.7^{b}	20.8±0.4^{c}	31.8±1.9^{d}
F-VI	95.3±1.1^{e}	96.7±1.1^{e}	13.3±1.5^{a}	25.6±1.6^{b}	37.8±0.9^{c}	52±2.6^{d}
F-VII	95.3±1.1^{e}	96.7±1.1^{e}	7±0.6^{a}	14.5±1.6^{b}	24.3±0.6^{c}	39.4±1.2^{d}
F-VIII	95.3±1.1^{e}	96.7±1.1^{e}	17.5±0.2^{a}	28±1^{b}	41.3±1.2^{c}	58.4±1.5^{d}
F-IX	95.3±1.1^{d}	96.7±1.1^{d}	2.8±1^{a}	4.6±0.3^{a}	7.3±0.1^{b}	10.6±1^{c}
F-X	95.3±1.1^{a}	96.7±1.1^{e}	30.4±1.9^{a}	43.8±1.1^{b}	60.3±3.1^{c}	79.9±1.3^{d}
F-XI	95.3±1.1^{c}	96.7±1.1^{bc}	85.3±2.4^{a}	92.3±0.4^{b}	93.2±0.3^{b}	93±0.6^{b}
F-XII	95.3±1.1^{d}	96.7±1.1^{d}	53.8±1.6^{a}	87.6±0.9^{b}	91±0.4^{c}	91±0.1^{c}

$^{a\text{-}e}$Different letter in each row indicate significant difference ($p<0.05$). Trolox (70 µM) and Vitamin C (70 µM) were used as antioxidant standards. LGE and its fractions were tested a different concentrations (12.5 to 100 µg/mL). Data are presented as the mean±standard deviation. NA: No antioxidant activity was observed.

antioxidant potential, contrary with the positive association of phenolic compounds and antioxidant potential. On the other hand, the obtained results in the present research are in agreement with other studies (5.85-753 mg EAG/g) [26, 29-31].

Since fractions, F-XI and F-XII showed the highest phenolic content and antioxidant activity. F-XI and F-XII were eluted by column chromatography (previously described in materials and methods section), obtaining compound 1 from F-XI fraction and compound 2 and 3 from F-XII. Subsequently, compounds structure were determined by HPLC-DAD, HPLC-ESI-MS and NMR (^{1}H and ^{13}C).

Results of HPLC-DAD and HPLC-MS in positive ionization mode of isolated compounds from F-XI and F-XII are shown in Table 2. Regarding HPLC-DAD analysis, band B, characteristic of flavanols group was identified with a maximum absorption at 280 nm. In the same way, compound 2 and 3 exhibited two maximum absorption at 362-366 and 256-267 nm, characteristic of band A and band B, respectively, structurally in reference to the structure of flavonols. HPLC-ESI-MS showed that compound 1 presented a parental ion with a m/z of 291.0861 [M+H]+. In addition, the fragmentation pattern of compound 1 yielded fragmented ions at m/z 273.0758, 249.0756, 123.0442, 139.0390 and 165.0547, which coincided with the initial dehydration, loss of CH_2CO group and fragmentations of the A- and B- rings, respectively, characteristic of a flavonol group. Results were consistent with previous studies and coincide with the presence of a flavonol structure named epicathechin (Figure 3) [32, 33]. By other hand, compound 2 showed a molecular ion $[M+Na]^+$ at m/z 471.0905. Dissociations of this ion produced a protonated molecular ion $[M+H]^+$ at m/z 449.1090 and 303.0502 as result of the loss of Na ion and rhamnosyl, respectively. Based on the above, compound 2 was identified as quercetin rhamnoside (Figure 3) [34]. Compound 3 exhibited a protonated molecular ion [M+H]+ at m/z 287.0565. In accordance to others works, compound 3 was identified as kaempferol (Figure 3) [32, 35].

To confirm the structures of isolated compounds analyzed by HPLC-ESI-MS, we carried out NMR spectroscopy analysis to determine the proposed structure. The ^{1}H NMR signal spectrum of compound 1 to compound 3 are presented in Table 3. The ^{1}H NMR spectrum of Compound 1 exhibited two doublets (δ 5.92 and δ 6.02 ppm, J=2.3 Hz),

which correspond to the protons H-6 and H-8 an aromatic A-ring. In addition, characteristic signals of catechol B-ring protons were observed by the ABX system, showing two doublets corresponding to protons H-2' and H-6' (δ 6.79 ppm, J=2 Hz) and protons H-5' and H-6' (δ 6.76 ppm, J=8.1 Hz), respectively. Also, a doublet of doublet signal (δ 6.82 ppm, J=1.9 and 8.1 Hz) was obtained, representing the coupling between H-6'-H-2' and H-6'-H-5'. Addtionally, signals for a characteristic C-ring was observed. One doublet of doublet was obtained by the coupling of H-4 (δ 2.70 ppm) and H-3 (δ 2.82 ppm), and then, a coupling between H-3-H2 and H-3-H-4 showed a multiplet signal (δ 4.21 ppm). Additionally, a 2,3-cis configuration in flavan structure was evident, due to the characteristic singlet signal (δ 4.87 ppm) from this functional group. Thus, this results are accordance with previous studies relating epicatechin as the estructure identified in compound 1 [36, 37].

The ^{1}H NMR spectrum of Compound 2 presented two doublets signals (δ 6.245 and δ 6.450 ppm, J=2.1 Hz) showing protons H-6 and H-8 associated with to the A-ring. The ABX-system aromatic signals at δ 6.96 ppm (J=2.1 Hz), δ 7.36 ppm (J=2.1 Hz) and δ 7.486 ppm (J=2.1 and 8.3 Hz) are typical for catechol B-ring. In addition, the two doublets obtained at δ 5.51 ppm (J=1.3 Hz) and δ 0.90 ppm (J=6.01 Hz) are characteristics to the rhamnose unit (H-1'' and H-6'', respectively). These results are in accordance with the HPLC-ESI-MS analysis and consistent with others studies [38] confirming that compounds 2 was quercetin rhamnose. The ^{1}H NMR spectrum of Compound 3 showed typical signals for aromatic A-ring with two doublets, representing protons H-6 (δ 6.18 ppm, J=2.06 Hz) and H-8 (δ 6.382 ppm, J=2.02 Hz). Additionally, two doublets signals characteristic with the protons H-2' (2H, δ 8.16 ppm, J=8.8 Hz) and H-3' (2H, δ 7.08 ppm, J=8.8 Hz) were obtained on the catechol B-ring. The NMR study of compound 3 confirm that structure of compound 3 was kaempferol, and are in agreement with the previously reported [39, 40].

The present study demistrated the presence of epicatechin, quercetin rhamnose and kaempferol in LGE, being this last one, first reported to *L. glaucescens*, representing a new contribution for its phenolic composition. Likewise, results of this research demonstrated that antioxidant potential of this plant is mainly attributed to previous phenolic compounds. This assumption is based in the antioxidant activity exhibited by

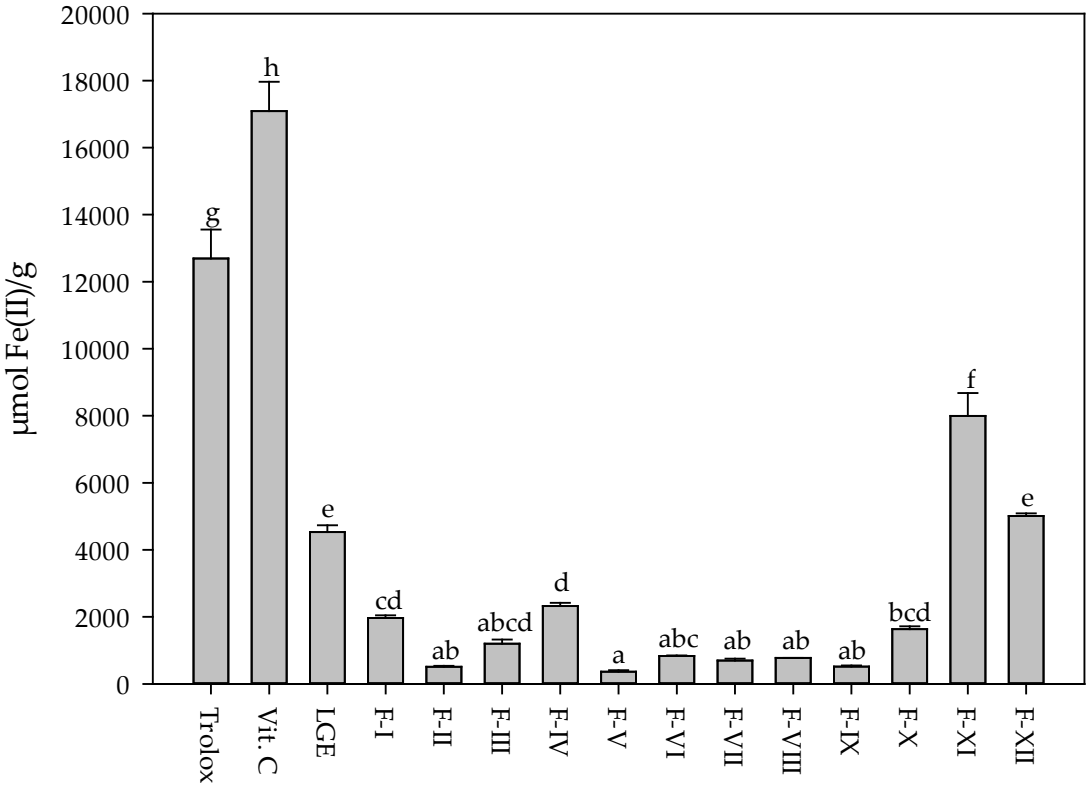


Figure 1. Ferric reducing activity of LGE and their fractions (F-I to F-XII). Trolox and Vitamin C were used as antioxidant standards. Data are presented as the mean±standard deviation. Different letter in each graphic indicate significant difference ($p<0.05$).

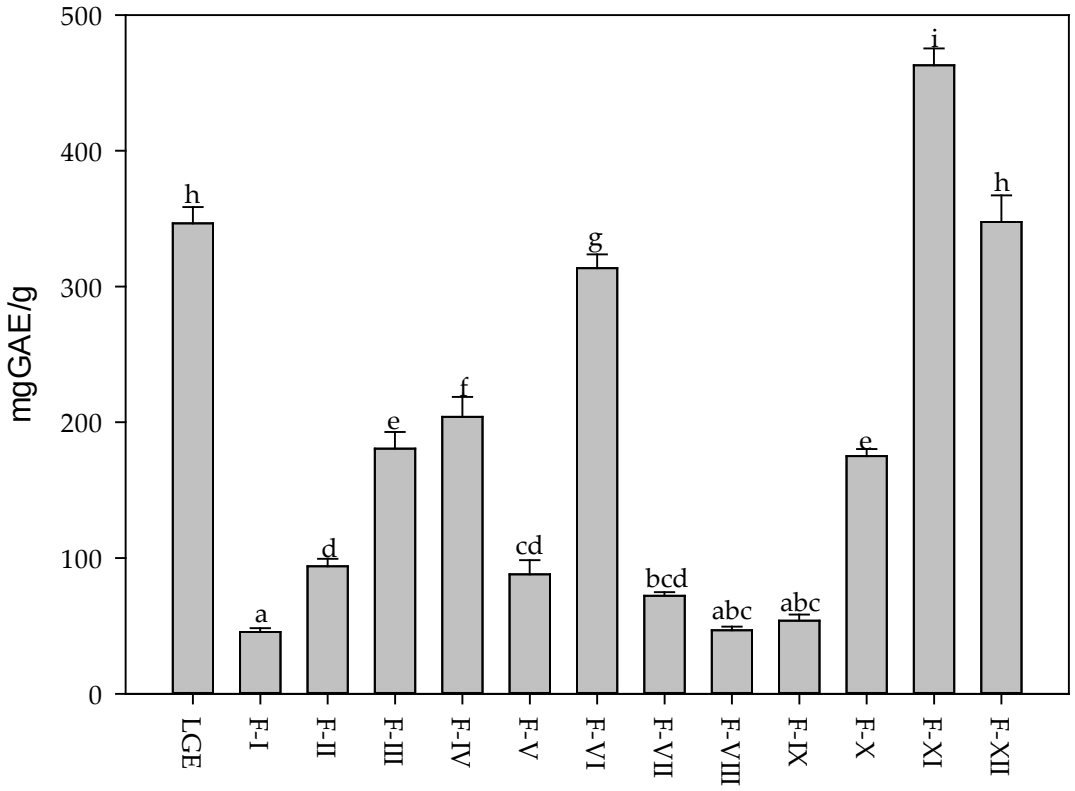


Figure 2. Phenolic content of *Litsea glaucescens* extract (LGE) and their fractions (F-I to F-XII). Data are presented as the mean±standard deviation. Different letter indicate significant difference ($p \leq 0.05$).

these compounds through different antioxidant assays, showing an excellent capacity to stabilize free radical and metals, inhibit peroxidation and DNA oxidation [41-45].

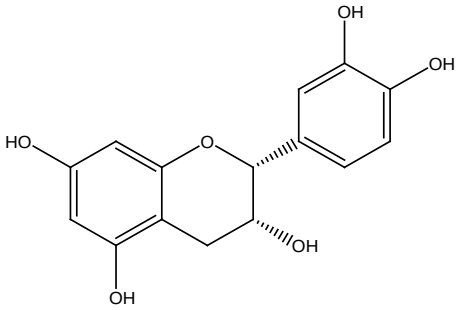
The antioxidant activity of these compounds is largely associated to the number and position of hydroxyl groups in the molecule and the 2-3 double bond and 4-oxo function [43, 46]. Phenolic compounds with the presence of hydroxyl group in the position 3', 4' catechol and 3-OH have a notable antioxidant activity, being 10 times greater than compounds with no hydroxyl group substitution [46]. This feature in combination with the double bonds allows the molecule efficiently stabilize the produced free radical (Resonance effect). In addition, different researches demonstrated that these compounds can act synergistically, causing a substantially increase in the antioxidant activity [47, 48]. Isolated compounds (epicatechin, quercetin rhamnose y kaempferol) from LGE fractions presented these characteristics, explaining the high antioxidant potential of LGE.

Also, fraction F-XI was the most antioxidant potential fraction, due to its association with epicatechin, which is recognized to be a powerful antioxidant by in vitro tests. By the other hand, fraction F-XII presented lower activity than fraction F-XI, possibly for an antagonist or indifference effect by the presence of phenolic compounds.

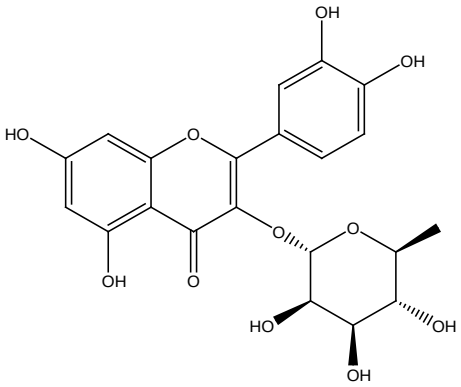
In conclusion, this research provides original information about phenolic profile of *L. glaucescens* which has an important transcendence to understand antioxidant activity behavior by two different assays. In this sense, further studies about *L. glaucescens* antioxidant properties are needed, in order to lead its application for alimentary and pharmaceutical industries thought the extraction of natural and efficient bioactive compounds.

Table 2. Retention times, UV and ESI-MS data of compound 1 to compound 3 of LGE.

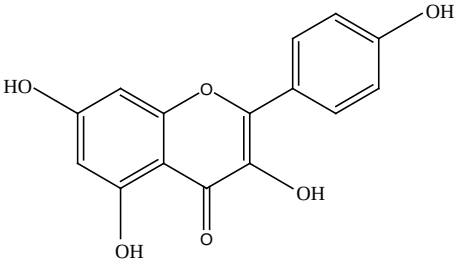
N°	Tr (min)	λmax (nm)		[M+Na]+ (m/z)	[M+H]+ (m/z)
		Band A	Band B		
1	24.2	-	279		291.0861, 273.0758, 249.0756, 123.0442, 139.0390, 165.0547
2	49.2	355	254	471.0905	449.1090, 303.0502
3		366	267		287.0555



Compound 1: Epicatechin



Compound 2: Quercetin rhamnoside



Compound 3: Kaempferol

Figure 3. Isolated compounds from LGE

Table 3. ^{1}H NMR data (400 MHz, acetone-d6) of compounds 1-2 and (400 MHz, DMSO-d6) compound 3.

Position	1 δh (J in Hz)	2 δh (J in Hz)	3 δh (J in Hz)
2	1H, 4.87		
3	1H, 4.21		
4a	1H, 2.82, dd, (4.4, 16.4)		
4b	1H, 2.70, dd (3.1, 13.5)		
5			
6	1H, 5.92, d (2.3)	1H, 6.24, d (2.1)	1H, 6.18, d (2.06)
7			
8	1H, 6.02, d (2.3)	1H, 6.45, d (2.1)	1H, 6.38, d (2.02)
9			
10			
1’			
2’	1H, 6.79, d (2)	1H, 7.48, d (2.1)	2 H, 8.1, d (8.8)
3’			2H, 7.0, d (8.8)
4’			
5’	1H, 6.76, d (8.130)	1H, 6.96, d (8.3)	
6’	1H, 6.82, dd (1.9, 8.1)	1H, 7.36, dd (2.1, 8.3)	
S 1´´		5.51 d (1.3)	
S CH_3		3 H, 0.90 d (6.01)	

Conflicts of Interest

The authors declare no conflict of interest.

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Capítulo II

Seasonal effects on the biological activities of *Litsea glaucescens* Kunth extracts

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Seasonal effect on the biological activities of *Litsea glaucescens* Kunth extracts

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Abstract

This study demonstrate the seasonal effect on the antioxidant, antiproliferative and antimicrobial activities of *L. glaucescens kunth* (LG) leaves extracts, a native species from Mexico used as remedy in folk medicine. Antioxidant activity of LG extracts was evaluated by DPPH, FRAP and ORAC assays and phenolic content (PC) was determined by using Folin-Ciocalteu method and HPLC-DAD analysis. Antiproliferative activity was determined by MTT assay against HeLa, LS 180, M12.C3.F6 and ARPE cell lines. Antimicrobial potential was evaluated against *Staphylococcus aureus* and *Escherichia coli* using microdilution method. LG extracts presented high PC (92.9±4.4 to 138.2±6.7 mg EGA/g d.w.), being quercitrin and epicatechin the main flavonoids presented. A notable antioxidant activity was obtained by all tested methods (IC_{50DPPH}=14.73 to 27.34 µg/mL; FRAP=1466.4±147.6 to 2614.3±183.1 µM Fe (II)/g of d.w.; ORAC=3413.3±46.1 to 3700±52.9 µM TE/g of d.w.). Antioxidant activity and PC were affected by the season, where autumn (ALGE) and summer (SULGE) extracts exhibited the highest potential ($p<0.05$). All extracts presented activity against the cell lines evaluated, being HeLa the most susceptible one. Antiproliferative activity was affected by the season, being ALGE and SULGE the most actives. About antimicrobial activity, SULGE (MIC_{90}<800 µg/mL; MIC_{50}<400 µg/mL) and SLGE (MIC_{50}<1000 µg/mL) showed inhibitory effect against *S. aureus*. Findings provide a vanguard-information about LG seasonal effect on PC regarding with biological potential effect, representing an alternative source against oxidative stress and microbial diseases.

***Keywords*:** *L. glaucescens*, seasonal effect, phenolic compounds, antioxidant, antiproliferative, antimicrobial.

1. Introduction

Nowadays, diseases related to oxidative stress and to antimicrobial resistance are considered the main public health concern, leading the highest mortality rates worldwide [1, 2]. Oxidative stress has been explained in terms of the overproduction of intracellular reactive oxygen species, which may produce damage to biomolecules such as DNA, RNA, lipids and proteins [3]. As a consequence, the cellular damage would eventually results in the development of chronic diseases such as cancer, atherosclerosis, rheumatoid arthritis, diabetes, chronic inflammation and cardiovascular ills, among others [4].

On the other hand, antimicrobial resistance is the result of antibiotic misuse, which conduces to stronger infections with a complicated clinical treatments such as respiratory tract infections, rhinosinusitis, otitis media, cystic fibrosis lung infection, dental caries and chronic wounds, among others [5, 6]. These complications reduce the conventional antibiotics efficacy, length the hospitalization stays and increase the medical treatment costs associated to the research and application of broad spectrum antibiotics [7]. Each year in the United States, around 2 million people was infected by antibiotic resistant bacteria in USA, and at least 23000 deaths were confirmed due to infections with clinical complications [8]. In this context, natural agents emerge as a safe alternative to reduce the problem of the oxidative stress and antimicrobial diseases.

Plants are traditionally used in folk medicine to treat different illnesses and nearly 80% of worldwide population had used them with this purpose, especially for being a natural source easily available for the communities [9, 10]. Their positive health benefits are associated with the presence of chemical compounds derived from secondary metabolism, such as phenolic compounds, essential oils, terpenes, saponins, alkaloids, polypeptides, which are used by plants as part of their defense mechanism [11, 12]. In addition, these compounds had shown a broad spectrum of biological activities, demonstrating the potential of plants as alternative drugs [13, 14]. However, the content of bioactive compounds depends on biotic and abiotic factors such as the presence of microorganisms and competitor species around the plant, temperature, light intensity, UV radiation, humidity, water, minerals and environmental contamination [15, 16]. These factors regulate the production of secondary metabolites and subsequently the

potential use of medicinal plants [17]. In this sense, the study of the effect that the different seasons has on the chemical composition and biological properties of plants, can contribute to its optimal use in the folk medicine [18, 19].

Litsea glaucescens Kunth is a native plant from Central America and Mexico, mainly distributed in the state of Veracruz, Chiapas and Nayarit, which is known as "laurel" in this geographical region [20]. Its leaves have been traditionally used as food seasoning, as well as remedy in folk medicine against central nervous system illness, depression, colic, pain, vomit and diarrhea [21]. These activities are mainly related to the presence of different compounds such as terpenes and phenolic compounds [22, 23]. The goal of the present study was to evaluate the seasonal effect on the antioxidant, antimicrobial and the antiproliferative activities of *L. glaucescens kunth* leaves extracts, as well on its content and profile of phenolic compounds, since in the best of our knowledge, this is the first effort to describe at this level, the biological properties and chemical composition of "laurel", commonly used as a remedy by the communities from the mountainous region of Veracruz, México.

2. Materials and Methods

2.1. Reagents. Folin–Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), gallic acid, epicatechin, quercitrin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri(2-pyridyl)-striazine (TPTZ), iron (III) chloride hexa-hydrate, sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), hydrochloric acid, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), gentamicin, sodium chloride (NaCl), dimethyl sulfoxide (DMSO), isopropyl alcohol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were obtained from Becton Dickinson (USA). HPLC-grade water (18 mΩ) was performed by a Milli-Q50 purified system (Millipore Corp., Bedford, MA).

2.2. Plant material and extracts preparation. L. glaucescens leaves were collected during autumn (November 2015), winter (February 2016), spring (May 2016) and summer (September 2016) from Xico, Veracruz, México. *L. glaucescens* leaves were identified in the Herbarium of the Instituto de Investigaciones Biológicas of the Universidad Veracruzana, México. Collected leaves were washed and dried. Dried leaves were extracted with methanol (96 %) during 4 days with occasional stirring (2-3 time per day). The extracts were filtered using filter paper (Whatman grade No. 4) and the solvent was evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator. The obtained extracts were stored at -20 °C and identified as *L. glaucescens* autumn, winter, spring and summer extracts (ALGE, WLGE, SLGE and SULGE, respectively).

2.3. Total phenolic content. Total phenolic concentration was determined with Folin-Ciocalteu reagent, according to the method described by Velazquez et al. [24]. Briefly, 10 µL of extracts were mixed with 80 µL of distilled water, 40 µL of Folin-Ciocalteu reagent 0.25 N, 60 µL sodium carbonate (5 % in distilled water) and 80 µL of distilled water. The mixtures were incubated at room temperature (1 h). The absorbance of the samples was measured at 750 nm on a Fluostar Omega microplate reader (BMG Labtech), and the results were expressed as milligrams of gallic acid equivalent (GAE)/gram of dry weight (d. w.).

2.4. HPLC-DAD analysis. Analytical HPLC-DAD analysis was carried out on an Agilent 1220 Infinity DAD LC (Waldbronn, Germany) equipped with a Zorbax SB-C18 column (250 x 4.6 mm, Ø 3.5 µm, Agilent, USA). The mobile phase consisted of 5 % formic acid in water (solvent A) and methanol (solvent B). The elution was accomplished with a solvent flow rate of 1 mL/min, using a gradient program as follow: 5 % B (0-5 min), 10 % B (5-10 min), 15 % B (10-18 min), 25 % B (18-28 min), 30 % B (28-40 min), 40 % B (40-45 min), 45 % B (45-55 min), 60 % B (55-60 min), 80 % B (60-65 min), 100 % B (65-76 min) and 30 % B (76-86 min). Flavonoids were monitored at 280 and 340 nm. The chromatographic peaks were assigned using authentic standards

of epicatechin and quercitrin. Quantification of both compounds was performed trough calibration curves. Results were expressed as mg of each compound/100 mg of d.w.

2.5. DPPH assay. Free-radical scavenging activity was measured following the modified method reported by Usia et al. [25]. *L. glaucescens* extracts (100 µL) were mixed with a 300 µM DPPH solution (100 µL). Samples were kept in the dark for 30 min. Afterward, absorbance at 517 nm was measured on a microplate reader (Fluostar Omega microplate reader, BMG Labtech). Results were expressed as mg of trolox equivalents (TE)/g of d.w.

2.6. FRAP assay. Ferric reducing ability was performed according to the methodology described by Benzie and Strain, [26]. Working FRAP reagent was elaborated reacting 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 40 mM TPTZ (dissolved in 40 mM HCl) and 1 volume of 20 mM ferric chloride (dissolved in water). Subsequently, 280 µL of FRAP reagent were mixed with 20 µL of *L. glaucescens* extracts, and the absorbance was read at 630 nm after 30 min of storage in the dark (Fluostar Omega microplate reader, BMG Labtech). Results were reported as mg of Fe (II)/g of d.w.

2.7. ORAC assay. Oxygen radical absorbance capacity assays was carry out using a modified method described by Ou et al. [27]. AAPH reagent was used as peroxyl radical generator, fluorescein as the fluorescent indicator. Reaction mixture contained 150 µL of fluorescein (10 nM), 25 µL of phosphate buffer (75 mM, pH 7.4) as blank, and 25 µL of extracts. Reaction was started by the addition of AAPH (240 mM). Samples were preincubated at 37 °C (15 min) and the fluorescence was monitored every 90 s for 1.5 h at 485-520 nm (Fluostar Omega microplate reader, BMG Labtech). Results were expressed as µM TE/g d.w.

2.8. Bacterial strains and growth conditions. Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used in the experiments. These strains were

maintained at -70 °C in cryovials containing glycerol (10%) broth and subculture in Mueller-Hinton broth, at 37 °C during 24 h before testing.

2.9. Antibacterial assay. Antibacterial activity of extracts was evaluated by the modified microdilution broth method [24]. Briefly, after overnight growth at 37 °C in Mueller-Hinton agar, 15 µL (1.5 x 10^6 CFU) of a suspension of a logarithmic phase bacterial culture [10^8 CFU ml^{-1}, the turbidity of this bacterial suspension matching the turbidity of a 0.5 McFarland standard] were inoculated into each well of a flat 96-well microplate (Costar, Corning, NY), containing 200 µL of different concentrations of extracts (100-1000 µg/mL). *L. glaucescens* extracts were dissolved previously in DMSO and subsequently diluted in sterile MHB. The percentage of DMSO did not exceed 2 % (v/v) of the total volume per well (215 µL). Gentamicin (12 µg/mL) was used as positive control of bacterial growth inhibition. Plates were incubated for 48 h at 37 °C, and read later at 620 nm, on a microplate reader (Benchmark Microplate Reader Bio-Rad, Hercules, CA), at 6, 12, 24 and 48 h. The minimal inhibitory concentration was defined as the lowest extracts concentration that inhibited at least 50 % (MIC_{50}) or 90 % (MIC_{90}) of the bacterial growth after incubation (37 °C x 24 h). MICs values were calculated from the Optical Density (OD_{620nm}) data using the following equation:

MIC_{50}: (OD_{620nm} untreated bacteria-OD_{620nm} test concentration)/(OD_{620nm} untreated bacteria)x100≥50%

MIC_{90}: (OD_{620nm} untreated bacteria-OD_{620nm} test concentration)/(OD_{620nm} untreated bacteria)x100≥90%.

2.10. Cell lines. Cell lines LS 180 (human colonic adenocarcinoma), HeLa (human cervix carcinoma) and ARPE-19 (human retinal pigmented epithelium) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cell line M12.C3.F6 (murine B-cell lymphoma) was provided by Dr. Emil R. Unanue (Department of Pathology and Immunology, Washington University in St. Louis, MO, USA).

2.11. Antiproliferative assay. Cell proliferation was evaluated through the MTT assay [28] modified by Hernandez et al. [29]. Briefly, 50 µL ($1x10^4$ cells) were placed in each well of a flat 96 well plate and incubated for 24 h (37 °C, 5 % of CO_2 atmosphere). Then, 50 µL of medium were added containing different concentrations of extracts and the cell cultures were incubated for 48 h. Extracts were previously dissolved in DMSO. DMSO did not exceed 0.5 % of the total volume per well (Preliminary studies showed that DMSO at this concentration does not cause damage cell). CAPE was used as a positive control in the antiproliferative assay. In the last 4 h of the LS 180, HeLa and ARPE cell line cultures, each well was washed with PBS and refilled with new fresh culture medium. Subsequently, 10 µL of a MTT solution (5mg/mL) were added to each well (in the case of the M12.C3.F6 cell line culture, only MTT solution (5mg/mL) was added). Metabolically active cells reduced tetrazolium salt to colored formazan crystals, which were dissolved with acidic isopropyl alcohol. Microplates were read at 570 and 650 nm (Multiskan EX, ThermoLabSystem). Results were expressed as IC_{50} values (IC_{50} is defined as the required concentration to inhibit 50 % the cell proliferation).

2.12. Statistical analysis. Data analysis was performed using the NCCS, 2007 statistical software. One way ANOVA was used, and mean comparisons were performed using the Tukey-Kramer test. Significance level in Type I error was $p \leq 0.05$. Pearson correlation between phenolic content and DPPH, FRAP and ORAC values were estimated too.

3. Results and discussion

3.1. Phenolic compounds. The phenolic content of the *L. glaucescens* extracts ranged from 92.9±4.4 to 138.2±6.7 mg EGA/g d.w. The highest concentrations ($p<0.05$) were found in ALGE and SULGE followed by SLGE and WLGE (Figure 1). These data are in agreement with those reported by Iqbal and Bhanger, [30], Brahmi et al. [31] and Sivaci and Duman, [32] who evaluated the seasonal effect of phenolic content of moringa, olive and almond leaves extracts, respectively. In the three studies, they found

that autumn extracts presented the highest phenolic concentrations, in comparison with the samples of the other seasons.

In order to identify the main phenolic compounds of the extracts of *L. glaucescens*, a HPLC-DAD analysis was performed. The chromatographic profiles of the four seasonal extracts are shown in Figure 2. As can be observed the evident difference among them, is the height of the chromatographic peaks (related to the concentration of the phenolics), which seem greater in the chromatograms corresponding to the extracts ALGE, SULGE and SLGE respect to the labeled as WLGE. In addition, the HPLC-DAD analysis allowed us to identify two of the main phenolic compound present in *L. glaucescens* extracts: epicatechin and quercitrin, which present quantitative variation in *L. glaucescens* throughout the year (Figure 2). Quercitrin was the most abundant phenolic compound in the four extracts, according with Lopez-Romero et al. [Unpublished data's] who previously reported this compound in *L. glaucescens* extract. SULGE presented the highest amount ($p<0.05$) of quercitrin, followed by SLGE, ALGE and WLGE, respectively (Table 1). Epicatechin, the second-major flavonoid found in the extracts, has been reported before by Lopez-Romero et al. [Unpublished data's] and Gamboa-Gomez et al. [33] as a secondary metabolite of *L. glaucescens*. In this work, the ALGE extract presented the highest ($p<0.05$) epicatechin content, while a similar amount ($p>0.05$) of this compound was observed in the others extracts.

Phenolic composition of plants is mainly affected by biotic and abiotic factors. In normal conditions, abiotic factors such as thermal stress play an important role in the biosynthesis of phenolic compounds in plants, because induce the phenylalanine ammonia-lyase (PAL) activation, which is the main enzyme involved in the biosynthesis of phenyl propanoid [34, 35]. In addition, the increase in the enzymatic activity of PAL is related to an adaptation to stress [36]. Therefore, it is possible to hypothesize that *L. glaucescens* plants were subjected to a higher thermal stress during summer and autumn, compared with spring and winter, resulting in an increasing of phenolic compounds during these seasons. On the other hand, phenolic compounds are associated with a wide range of biological activities. In order to contribute to the biological characterization of this plant, we evaluated its potential as antioxidant, antimicrobial and antiproliferative agent.

3.2. Antioxidant activity. Nowadays, different assays have been performed to evaluate the antioxidant activity of plant extracts. Most of them are based on scavenging specific radicals such as DPPH and peroxyl radicals, or metal reducing potential such as the FRAP assay. In the present study, we evaluated the antioxidant activity of *L. glaucescens* extracts measured throughout three chemicals assays DPPH, FRAP and ORAC (Table 2). Results obtained through the DPPH method, shown variations among the seasonal extracts (IC_{50}: from 14.73 to 27.34 µg/mL), however ALGE and SULGE were the most active ($p<0.05$) against the DPPH radical, in comparison with WLGE and SLGE. In addition, based on the Blois, [37] and Fidrianny et al. [38] classifications, the antioxidant capacity of the four extracts must be categorized as very strong antioxidant, since all of them had IC_{50} values lower than 50 µg/mL. In addition, our results are in agreement with previous studies related with plants from *Litsea* genus such as *L. glaucescens* (Lopez-Romero et al. [unpublished data's]), *Litsea glutinosa, Litsea floribunda* and *Litsea japonica* which IC_{50} values ranged from 9.68 to 669.2 µg/mL [39-41].

On the other hand, ferric reducing power of the *L. glaucescens* extracts was evaluated through their ability to reduce the ferric complex Fe3+-tripyridyltriazine to Fe2+-tripyridyltriazine. The FRAP values of *L. glaucescens* extracts were in the range: 1466.4±147.6 to 2614.3±183.1 µM Fe (II)/g of d.w. (Table 2), and significant differences were observed ($p<0.05$) among them. Particularly, ALGE and SULGE exhibited the stronger power, whereas WLGE had the lowest activity. These values are higher than those reported before for other *Litsea* species (1.4-638 µM Fe (II)/g of d.w.) [40, 42, 43]. In addition, according to the classification performed by Wong et al. [44] for medicinal plants, the *L. glaucescens* extracts had an extremely high ferric reduction power, since the obtained values were higher than 500 µM Fe (II)/g of d.w.

The capacity to scavenging peroxyl radical generated by AAPH of *L. glaucescens* extracts was evaluated using the ORAC assay. ORAC values obtained for the different extracts varied from 3413.3±46.1 to 3700±52.9 µM TE/g of d.w. (Table 2). The *L. glauscences* extracts that showed the highest ($p<0.05$) scavenging peroxyl radical ability

were SULGE, SLGE and ALGE, while WLGE presented the lowest ($p<0.05$) antioxidant capacity. These results by ORAC assay evidenced that season had a significant effect on antioxidant activity, even when all extracts presented strong antioxidant activity.

The three type of tests performed in this study, provided evidences about the high ability of the four extracts to transfer electron and hydrogen atoms to stabilize free radicals and reduce metals, which is related to their strong antioxidant activity. In addition, it is notorious the significant effect that the seasons had on the antioxidant capacity of *L. glaucescens* extracts. Particularly, ALGE and SULGE shown a higher activity, respect to SLGE and WLG. Additionally, the four extracts exhibited an interesting ability to act as preventive and chain-breaking antioxidants with activity against biological and synthetic radicals. These facts suggest that they have the potential to stabilize biological radicals and to inhibit the generation of reactive oxygen species, which could contribute to reduce the oxidative stress caused by them and, as a consequence, to avoid the DNA damage .

It is well known that the antioxidant activity of natural products is strongly related with the content of phenolic compounds that they have, and the results obtained in this work are in agreement with that fact. In order to demonstrate the correlation between both parameters, a series of plots of the data obtained through the DPPH, FRAP and ORAC assays against the concentration of phenolic compound (CPC) in the four *L. glaucescens* extracts were performed (Figure 3). The regression coefficients (R) of the linear correlations for each series are presented in the Figure 3. As can be observed, positive slopes were obtained in the three cases, and the regression values were 0.93 and 0.92 for the correlations between the data obtained from the FRAP and DPPH assays *vs* CPC, respectively. On the other hand, the lowest regression coefficient was obtained for the correlation between the data of the ORAC test *vs* CPC (R=0.80), however the value is still into an acceptable range. As we hypothesized, it seems that phenolic compounds were the main responsible of the antioxidant activity (evaluated by three different methods) of the four *L. glaucescens* extracts. In this sense, the high antioxidant potential of ALGE and SULGE could be associated with their content of epicatechin and quercitrin. Previously, different studies have demonstrated that both phenolics are

considered among the most antioxidant phenolic compounds [45, 46], and that capacity has been attributed to the catechol and chromane moieties present at them (Figure 4). Particularly, the presence of hydroxyl groups in 3'-and 4´-position of ring B, the hydroxyl group 3- of ring C and double bond between C2-C3 enhance the antioxidant activity of these phenolic compounds, since they are able to transfer electrons and protons to stabilize free radicals, or to reduce and chelate metals. These structural features confer to both compounds a greater stability, compared with those that lack them [13, 47]. In addition, these facts determine also the redox potential and therefore the antioxidant activity of phenolic compounds [48].

3.3. Antiproliferative activity. The results of the Antiproliferative activity evaluation of *L. glaucescens* extracts against Hela, LS 180, M12.C3.F6 and ARPE cells are shown in Table 3. All the extracts inhibited cell proliferation of human and murine cells lines, and clearly there was an effect of the season on their capacity to do it in three of the four cases. As can be observed in Table 3, HeLa resulted the more sensitive cell line to the *L. glaucescens* extracts, particularly to the SULGE and ALGE ones, which exhibited the highest activity [IC_{50} 45.8±1.6 µg/mL and 48.7±1.8 µg/mL, respectively ($p<0.05$)] against its proliferation. Regarding with LS 180, ALGE and SULGE showed the stronger activity too [53.1±1.2 µg/mL and 55.6±1.5 µg/mL, respectively ($p<0.05$)], whereas in the case of the cancerous murine cell line (M12.C3.F6), the four extracts had a similar antiproliferative effect ($p>0.05$) with ranged values from 68.1±1.3 to 73.2±2.5 µg/mL. In addition, ALGE showed the lower IC_{50} value [62.1 ±3.6 µg/mL, ($p<0.05$)] to inhibit the proliferation of the non-cancerous ARPE cell line, however much higher concentrations of SLGE, SULGE and WLGE were required [(101.9 ±5.6 µg/mL), (102.2 ±1.9 µg/mL) and (166.1 4.9 µg/mL), respectively]. Even more, these last three values are the higher of the Table 3, and constitute an evidence of the selectiveness of the *L. glaucescens* extracts to inhibit the proliferation of cancerous cell lines respect to those non-cancerous. On the other hand, in all the cases the positive control CAPE resulted several times more active than the *L. glaucescens* extracts, (see Table 3). However, it should keep in mind that the purity of the CAPE used in the assays (above 95%), is

much higher than those of the active compounds present in the extracts, and this fact could contribute to the differences observed in the IC_{50} values. These findings are consistent with previous studies from *Litsea* plants. For example, in a study performed by Herrera-Carrera et al. [49], it was demonstrated that an herbal infusion obtained from *L. glaucescens* was able to inhibit the proliferation of human colon cancer cell line (HT-29). In the same way, Ndi et al. [50] observed inhibition on HT-29 (IC_{50}=37.9 µg/mL) and melanoma (SK-MEL-28) (IC_{50}=>100 µg/mL) cells treated with of *L. glutinosa* extract. Subarnas et al. [51] evaluated the antiproliferative activity of *L. mappaceae* extracts against human breast cancer (MCF-7), and reported that 200 µg/mL of plant extract were required to inhibit the 50 % of cell proliferation.

The obtained results in this work are an evidence of the antiproliferative effect of *L. glaucescens* against cancerous cell lines, in comparison with those non-cancerous. In addition, it was demonstrated that antiproliferative activity of analyzed extracts is affected by the season. This fact could be related to the total phenolic content and explain why ALGE and SULGE presented the highest activity respect to SLGE and WLGE. However, the structural features of these phenolics are important too, and they are the same described above as enhancer of the antioxidant activity of the *L. mappaceae* extracts [52]. In this sense, Kinjo et al. [53] and Nagarajan et al. [54] proposed that epicatechins, one of the most abundant phenolics of the four extracts reported here, possess potent antiproliferative activity against cancerous cells lines, which was related to an arrest in G2 phase of the cell cycle. On the other hand, previous studies have reported that phenolic compounds exhibit different mode of actions against cancerous cell lines. One of the most reported is the induction of apoptosis, as well as cell cycle arrest and preventing carcinogen metabolic activation, among others, with the subsequently cell death [55, 56].

3.4. Antimicrobial activity. Results of antibacterial activity of *L. glaucescens* extracts are summarized in Table 4 and Figure 5. As can be observed from Table 4, SULGE and SLGE showed the strongest activity against *S. aureus*, since the MIC_{50} values obtained for both of them are below the maximum concentration evaluated (1000 µg/mL), which constitutes an evidence of the important seasonal effect on the biological properties of *L.*

glaucescens. In contrast, no antimicrobial activity of any of the four extracts was observed against *E. coli*. The fact that *S. aureus* (Gram-positive) was less resistant than *E. coli* (Gram-negative) to *L. glaucescens* can be attributed to the cell structure and composition of both type of microorganisms. In this regard, Gram negative bacteria have an outer membrane constituted by phospholipid and lipopolysacharides, which provide resistance to antimicrobial treatments [57]. In addition, the presence of porins in outer membrane regulates the penetration of hydrophilic substances and reduce the fluidity of lipopolysaccharides layer, decreasing the rate of transmembrane diffusion [58]. Moreover, Gram positive bacteria lack of these characteristics, which result in a lesser resistance to antimicrobial treatments.

In addition, the dose-depend relationships of the active extracts (SULGE and SLGE) against *S. aureus* were explored, and the corresponding plots are shown in Figure 5. As can be observed, there is a clear effect of the concentration of both of them on their antimicrobial activity. SULGE, the most potent extract, was able to inhibit the 100 % of bacteria growth at the highest tested concentration (1000 µg/mL), the 98 % at 800 µg/mL, the 70 % at 600 µg/mL, and until 63 % at 400 µg/mL. In addition, higher concentrations, such as 1000 and 800 µg/mL had a similar activity respect to gentamicin (>98 % inhibition), which is an evidence of the strong antimicrobial activity of this extract. Although in a minor proportion than SULGE, SLGE also exhibited antimicrobial effect depending on concentration against *S. aureus*. As can be observed from Figure 5, at a concentration of 1000 µg/mL inhibited the 51 % of bacteria growth, while the others concentration (800-400µg/mL) provoked an inhibition lower than the 50 %. Phytochemicals are classified as antimicrobials on the basis of susceptibility test that achieve inhibitory concentrations in the range of 100 to 1000 µg/mL [59]. In this sense, ALGE and SLGE could be include into this classification, and represent a viable alternative for the treatments of diseases caused by *S. aureus*.

On the other hand, the antibacterial activity of *L. glaucescens* extracts evaluated in the present research, is consistent with previous works about the *Litsea* genus. For example, in a study performed by Ahmmad et al. [60] *L. monopetala* extracts presented inhibitory effect against *S. aureus* and *E. coli* at concentrations of 62.5 and 250 µg/mL,

respectively. Similarly, Areekul et al. [61] evaluated the antimicrobial activity of *L. glutinosa* extracts against *S. aureus* and *E. coli*. They observed that *L. glutinosa* extract at 6.97 %(w/v) showed low antimicrobial activity against *S. aureus* (Inhibition, 8.78 mm) in comparison to the positive control (chloramphenicol, 21.34 mm), while for *E. coli* no antimicrobial activity was observed. In addition, Pradeepa et al. [62] report a behavior close to the results reported here, since they observed that the *L. glutinosa* extracts presented greater antimicrobial activity against *S. aureus* (MIC=2.5 mg/mL) compared with *E. coli* (MIC=5 mg/mL).

Antimicrobial effect of *L. glaucescens* extracts could also be related to the high content of phenolic compounds. In this regard, Borges et al. [63] and Andrade et al. [64] demonstrated that phenolic compounds induced alteration of membrane properties, producing changes in the hydrophobicity, surface charge and membrane integrity with the subsequent leakage of essential intracellular constituents of Gram positive and Gram negative bacteria. On the other hand, Cushnie and Lamb [65, 66] have concluded that phenolic compounds have different mechanism of action as antimicrobial, such as inhibitors of nucleic acid synthesis, of the energy metabolism or of the cytoplasmic membrane function of the microorganisms. Likewise, they mentioned the antimicrobial potential activity of phenolic compounds is strongly related to functional groups present at them, their hydrophobicity, and also to the conformations that they adopt. In this study, the antimicrobial of the activity of the *L. glaucescens* extracts seems related to the higher concentrations of quercitrin present in SULGE and SLGE, the most active against *S. aureus.* However a synergism effect with epicatechin and other phenolics present in low concentrations should not be discarded.

4. Conclusions

This research provides a vanguard-information about seasonal effect on the composition and the concentration of phenolics compounds present in *L. glaucescens* extracts, as well on a series of biological activities of it, which could be a promising alternative to combat the oxidative stress and microbial diseases. From the results reported here, it is clear that the antioxidant and antiproliferative activities of *L. glaucescens* are enhanced during the

autumn and summer, which is related to the major concentration of phenolic compounds produced by the plant as response to the environmental conditions. In addition, the extract of leaves collected during summer and spring represent a viable alternative for the treatments of diseases caused by *S. aureus*. The antimicrobial effect exhibited for *L. glaucescens*, seems to be related, at least in part, to the higher concentrations of quercitrin that the plant produces during those seasons. Nevertheless the interesting properties that this Mexican plant has, subsequent studies are required to support its effectiveness and safety doses for human applications.

Conflict of interest

The authors declare there is no conflict of interest.

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Tables and Figures

Table 1. Concentration of major phenolic compounds identified in *L. glaucescens* extracts

	L. glaucescens extracts (mg/100 mg d.w.)			
Compound	ALGE	WLGE	SLGE	SULGE
Epicatechin	1.56±0.19[b]	0.88±0.009[a]	0.73±0.02[a]	0.68±0.01[a]
Quercitrin	2.11±0.05[b]	1.39±0.17[a]	3.01±0.16[c]	3.89±0.32[d]

[a-c]Means with different superscript within the same row, indicate statistical differences ($p<0.05$). All values represent mean±standard deviation. ALGE, autumn *L. glaucescens* extract.WLGE, winter *L. glaucescens* extract. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract.

Table 2. Antioxidant activity of *L. glaucescens* extracts

Extract	Antioxidant assay			
	DPPH (μM ET/g of d.w.)	DPPH (IC_{50}, μg/mL)	FRAP (μM Fe (II)/g of d.w.)	ORAC (μM ET/g of d.w.)
ALGE	1264.5±18.5[c]	14.7±0.07[c]	2614.3±183.1[c]	3673.3±61.1[b]
WLGE	668.1±19.9[a]	27.2±0.8[a]	1466.4±147.6[a]	3413.3±46.1[a]
SLGE	841.1±25.9[b]	24.31±0.9[b]	1999.7±42.4[b]	3693.3±46.1[b]
SULGE	1221.9±32.6[c]	15.2±0.3[c]	2573.4±138.9[c]	3700.3±52.9[b]

[a-c]Means with different superscript within the same column, indicate statistical differences ($p<0.05$). All values represent mean±standard deviation. ALGE, autumn *L. glaucescens* extract.WLGE, winter *L. glaucescens* extract. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract.

Tabla 3. Antiproliferative activity of *L. glaucescens* extracts

L. glaucescens extracts IC_{50} (µg/mL)					
Cell line	ALGE	WLGE	SLGE	SULGE	CAPE (µg/mL / mM)
HeLa	48.7±1.8[b]	53.9±2.6[bc]	59±7.8[c]	45.8±1.6[b]	9.7±0.07[a] / 34.1±0.2
LS 180	53.1±1.2[a]	85.2±3.5[c]	67.5±3.9[b]	55.6±1.5[a]	17.8±0.2[a] / 62.6±0.7
M12.C3.F6.	71.9±6.2[b]	70.6±2.1[b]	73.2±2.5[b]	68.1±1.3[b]	0.58±0.04[a] / 2.04±0.1
ARPE	62.1±3.6[b]	166.1±4.9[d]	101.9±5.6[c]	102.2±1.9[c]	10.2±0.18[a] / 35.9±0.6

[a-c]Means with different superscript within the same row, indicate statistical differences ($p<0.05$). All values represent mean±standard deviation. ALGE, autumn *L. glaucescens* extract.WLGE, winter *L. glaucescens* extract. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract.

Table 4. Growth-inhibitory activity of *L. glaucescens* extracts against *S. aureus* and *E. coli*

	L. glaucescens extracts (μg/mL)							
	ALGE		WLGE		SLGE		SULGE	
Strain	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
S. aureus	>1000	>1000	>1000	>1000	<1000	>1000	<400	<800
E. coli	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000

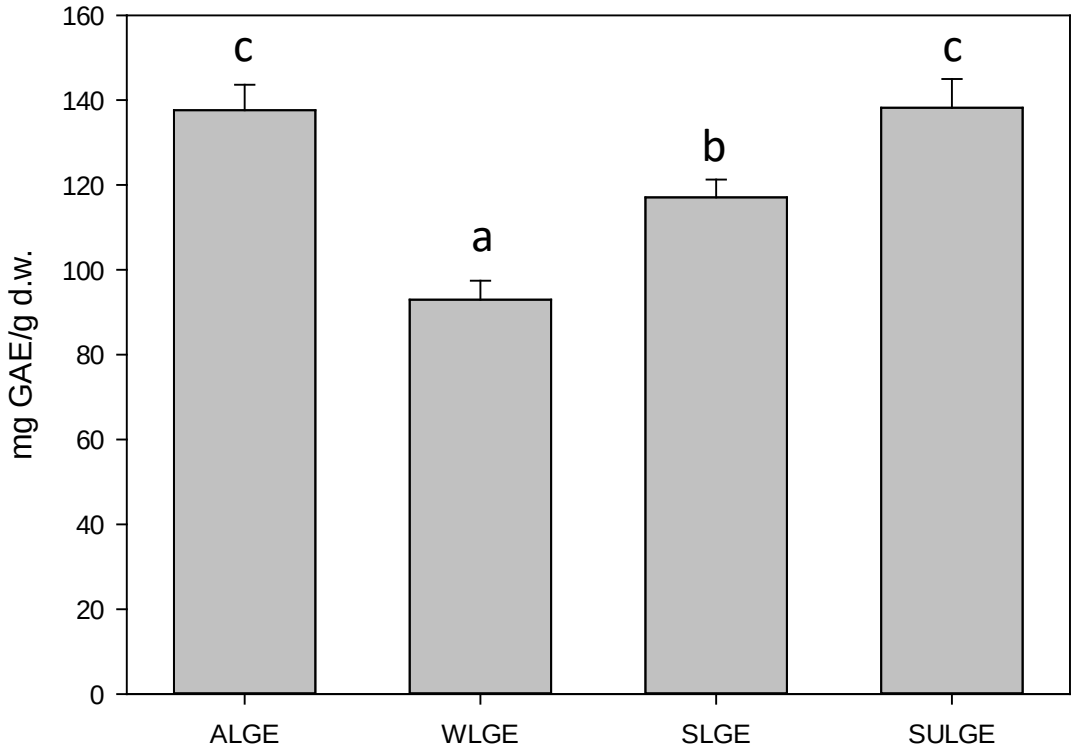

Figure 1. Phenolic content of *L. glaucescens* extracts. [a-c]Bars with different superscript, indicate statistical differences ($p<0.05$). All values represent mean±standard deviation. ALGE, autumn *L. glaucescens* extract. WLGE, winter *L. glaucescens* extract. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract.

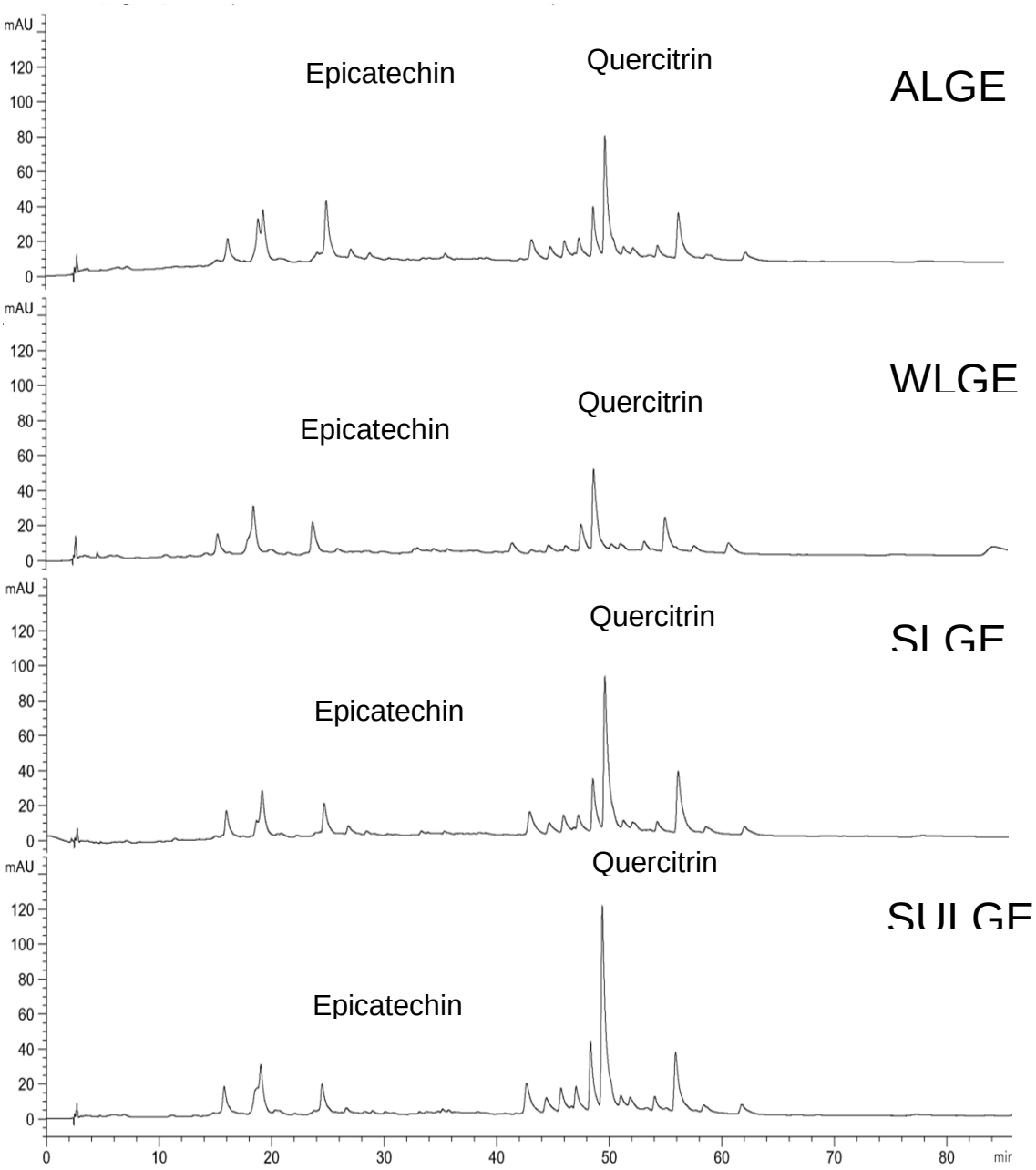

Figure 2. HPLC chromatogram of *L. glaucescens* extracts (recorded at 280 nm). ALGE, autumn *L. glaucescens* extract. WLGE, winter *L. glaucescens* extract. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract.

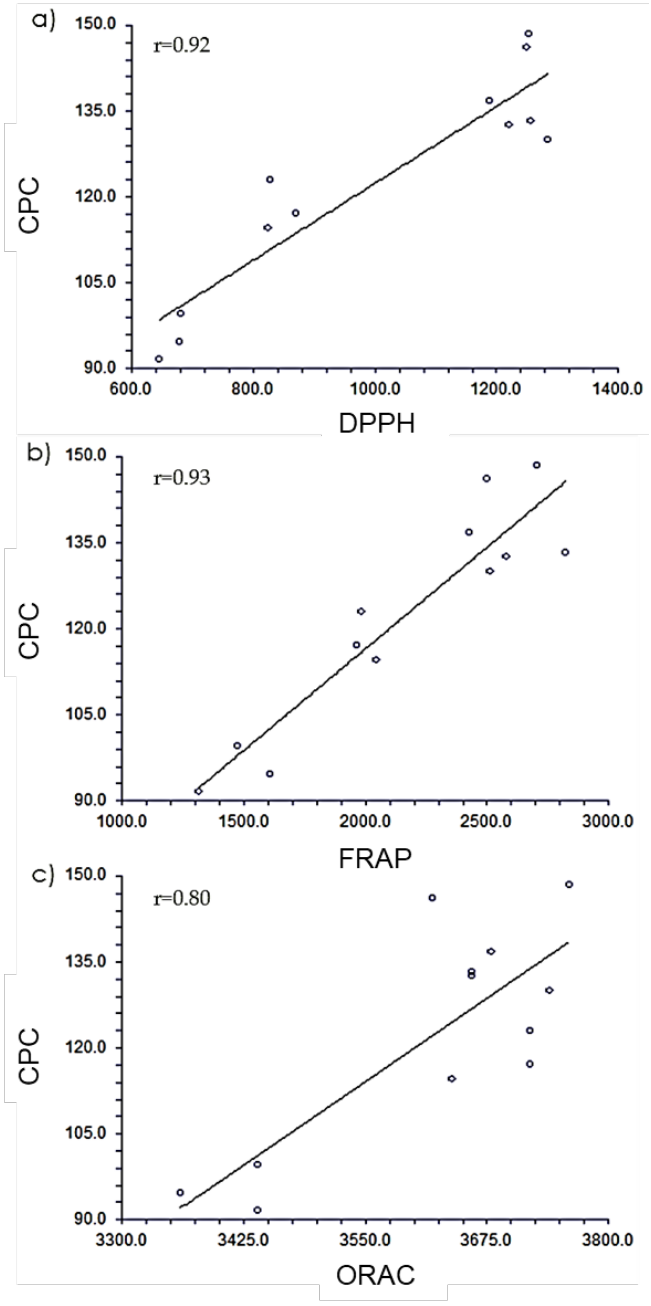

Figure 3. Correlation analysis. a) Correlation between content of phenolic content (CPC) and DPPH assay, correlation coefficient r=0.92; b) Correlation between content of phenolic content (CPC) and FRAP assay, correlation coefficient r=0.93; c) Correlation between content of phenolic content (CPC) and ORAC assay, correlation coefficient r=0.80.

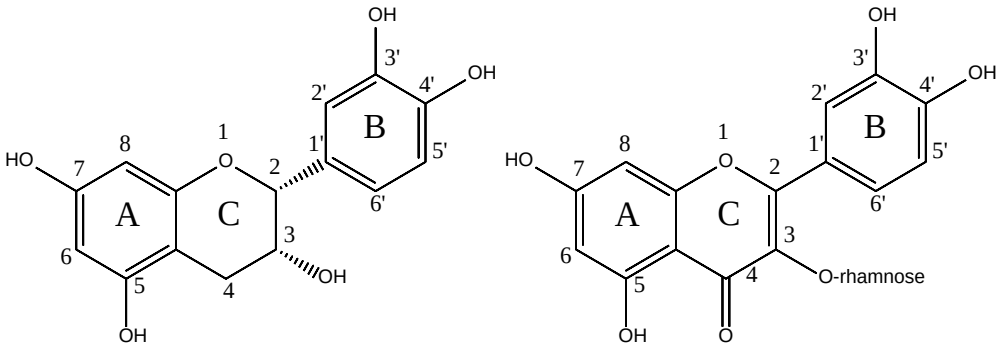

Figure 4. Structure of identified compounds in *L. glaucescens* extracts

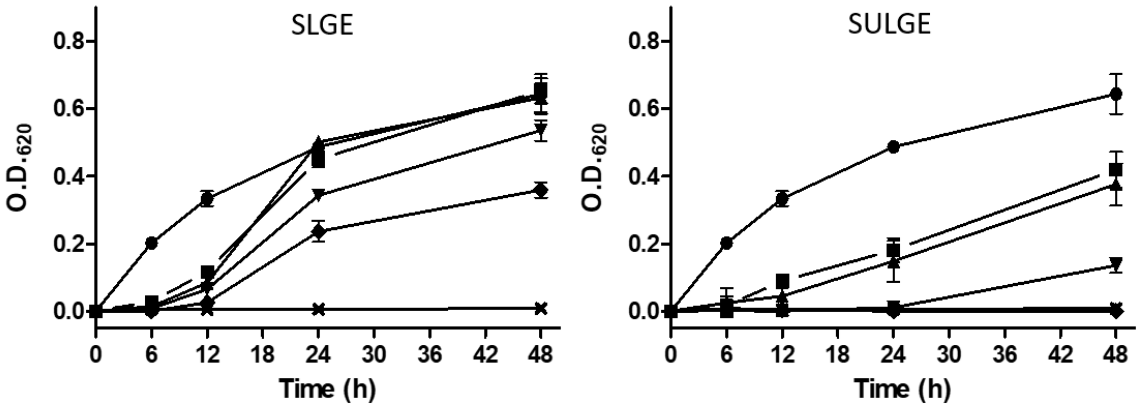

Figure 5. Antibacterial activity of *L. glaucescens* extracts against *Staphylococcus aureus*. SLGE, spring *L. glaucescens* extract. SULGE, summer *L. glaucescens* extract. Bacterial cell cultures were treated with different concentrations of *L. glaucescens* extracts during 48 h. ♦ 1000 µg/mL; ▼800 µg/mL; ▲600 µg/mL; ■ 400 µg/mL; ● 0 µg/mL; **x** gentamicin. Control bacterial cultures were incubated with DMSO (0.8-2 %). Gentamicin (12 µg/mL) was used as positive control. All values represent mean±standard deviation.

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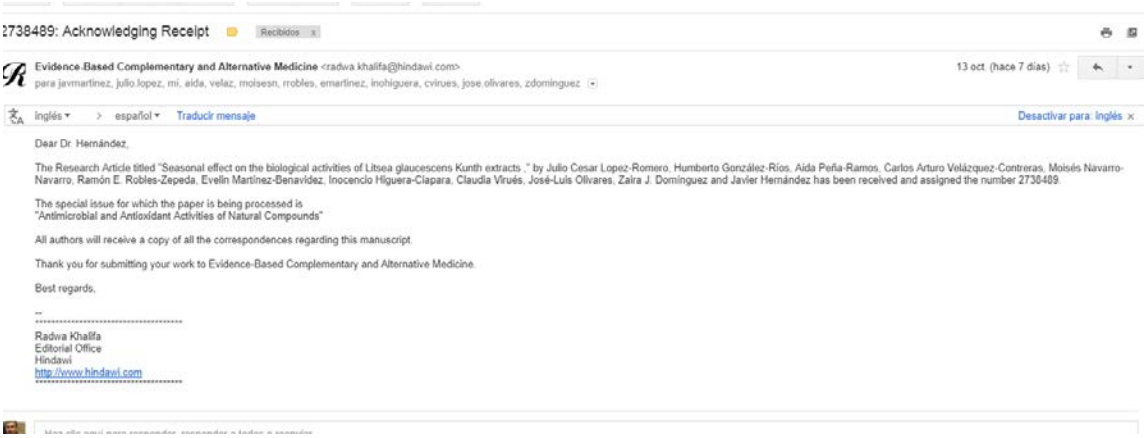
Seasonal effects on the biological activities of *Litsea glaucescens* Kunth extracts



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Dear Dr. Hernández,

The Research Article titled "Seasonal effect on the biological activities of Litsea glaucescens Kunth extracts ." by Julio Cesar Lopez-Romero, Humberto González-Ríos, Aida Peña-Ramos, Carlos Arturo Velázquez-Contreras, Moisés Navarro-Navarro, Ramón E. Robles-Zepeda, Evelin Martínez-Benavídez, Inocencio Higuera-Ciapara, Claudia Virués, José-Luis Olivares, Zaira J. Domínguez and Javier Hernández has been received and assigned the number 2738489.

The special issue for which the paper is being processed is
"Antimicrobial and Antioxidant Activities of Natural Compounds"

All authors will receive a copy of all the correspondences regarding this manuscript.

Thank you for submitting your work to Evidence-Based Complementary and Alternative Medicine.

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Capítulo III

Antioxidant potential of *Litsea glaucescens* extract and their fractions on refrigerated pork patties against lipid and protein oxidation

Artículo preparado para la revista Journal of the Science of Food and Agriculture

Antioxidant potential of *Litsea glaucescens* extract and their fractions on refrigerated pork patties against lipid and protein oxidation

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ABSTRACT

BACKGROUND: *Litsea glaucescens* (LG) is a native plant from Mexico commonly used in traditional medicine and as food seasoning; however, its effect as food preservative has not been evaluated. The objective of this study was to evaluate the antioxidant effect of LG extract (ALGE) and its fractions (F-XI and F-XII) on lipid and protein oxidation of pork patties stored at 4 °C.

RESULTS: HPLC analysis of ALGE (Epicatechin, EP: 1.56 mg 100 mg^{-1} d.w.; Quercitrin, QR: 2.11 mg 100 mg^{-1} d.w.), F-XI (EP: 24.541.56 mg 100 mg^{-1} d.w.) and F-XII (QR: 6.491.56 mg 100 mg^{-1} d.w.) allowed the identification of two majority compounds. The antioxidant evaluation by ORAC assay showed that ALGE (3673.3 µmol TE g^{-1}) and F-XII (3593.3 µmol TE g^{-1}) exhibited higher activity ($P<0.05$) than F-XI (3553.3 µmol TE g^{-1}). In patties shelf life study, ALGE, F-XI and F-XII showed to be effectives to decrease ($P<0.05$) primary (dienes) and secondary (TBARS) products of lipid oxidation, as well as protein oxidation (carbonyl content) of pork patties, comparing with the control treatment (without additive). No differences were observed in color evaluation, but ALGE, F-XI and F-XII tended to show high a* values during the storage of patties. Sensory evaluation evidenced that addition of ALGE, F-XI and F-XII did not affect organoleptic characteristics evaluated.

CONCLUSION: ALGE, F-XI and F-XII were effectives to decrease the oxidative process in the evaluated pork patties and subsequently extend their shelf life. This findings suggest these treatments represent an interesting alternative for meat and meat products industry.

Keywords: *Litsea glaucescens*, antioxidant, lipid oxidation, protein oxidation, pork patties.

INTRODUCTION

Lipid and protein oxidation plays an important role decreasing the quality and shelf life of meat products[1,2]. These oxidative processes induce alterations of lipid and proteins resulting in a deterioration of flavor, odor, color, nutritional value, texture, tenderness, juiciness and water-holding capacity[3,4]. Oxidation process development is determined by lipid profile, protein content (amino acid profile), oxygen presence, light, heat and heme pigments[5,6]. Most of these factors trigger a chain radical reaction consisting in three known stages: initiation, propagation and termination; same that could be reduced it by using antioxidant compounds[7].

Synthetic antioxidants such as butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tertiary butyl hydroxyl quinone (TBHQ) and propyl gallate are traditionally used to delay oxidative processes and subsequently extend the shelf life of the foods[8]. However, the usage of synthetic antioxidants have been restricted for their association with negative health effects and toxicity[9]. In this sense, antioxidant agents from natural sources highlight over traditional ones as a new alternative to provide safety meat products for consumers, due to the current consumer trend towards natural and low-processing products and their association with health and wellness.

Nowadays, natural sources represent an interesting alternative to enhance functional properties in meat products, especially spices extracts which represent a natural seasoning used in meat product elaboration worldwide. Previous studies have demonstrated that spices extracts can delay meat and meat products oxidative processes, extending their shelf life and quality[10-12]. The presence of phenolic compounds have been related with the high effectiveness of mentioned extracts, showing a strong relation between their presence and some biological properties, especially antioxidant activity[13,14]. Based on the above, natural sources represent an available alternative to acquire antioxidant compounds with a potential application in meat products industry.

Litsea glaucescens Kunth (LG) is a native plant from Central America and Mexico, mainly distributed in Veracruz, Chiapas and Nayarit, regionally known as laurel[15]. LG leaves have been traditionally used as food seasoning, as well as remedy in folk medicine against central nervous system illness, depression, colic, pain, vomit and diarrhea[16]. In addition, several biological properties as antimicrobial, antiproliferative

and antioxidant activity have been identified on its leaves. Lopez-Romero *et al.*[17,18] (Unpublished data's) demonstrated that LG extracts possess a high antioxidant potential, strongly correlated (r=0.93) with its high content of phenolic compounds. LG extract fractionation showed two powerful antioxidant potential fractions (F-XI and F-XII) with high phenolic compound content, even with higher activity than LG extract. This behavior is attributing to main phenolic compounds such as epicatechin and quercitrin. Therefore, LG and its fractions (F-XI and F-XII) may represent an excellent option as a natural antioxidant agent in different food products such as meat, since its quality and stability is strongly affected by oxidative processes.

Based on the above, the objective of the present study was to evaluate the effectiveness of LG extract and their fractions (F-XI and F-XII) to inhibit lipid and protein oxidation in pork patties stored under refrigeration at 4 °C.

MATERIALS AND METHODS

Reagents

2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein, butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), 2-thiobarbituric acid (2-TBA), 1,1,3,3-tetramethoxypropane, 2,4-Dinitrophenylhydrazine (DNPH), bovine serum albumin (BSA), epicatechin and quercitrin where purchased from Sigma-Aldrich (USA).

Preparation of LG extracts

Extraction of LG was achieved followed the method described by Lopez-Romero *et al.*[17,18]. Leaves of LG were obtained from Xico, Veracruz, Mexico (November, 2015). LG leaves were washed and dried. Extraction were performed with methanol (96 %) for 4 days with occasional stirring (2-3 time per day). The obtained extract was filtered through filter paper Whatman grade No. 4 and evaporated with a rotary evaporator below 40 °C. Afterward, the obtained extract (ALGE) was stored at -20 °C.

Fractionation of *L. glaucescens* extract (LGE)

ALGE fractionation was carried out by following the previous method described by Lopez-Romero *et al.*[18]. ALGE extract was fractioned using a silica gel 60 column (100 cm x 5 cm), and eluted with a gradient of hexane-ethyl acetate-metanol (90:10:0,

80:20:0, 70:30:0, 50:50:0, 30:70:0, 0:100:0, 0:50:50, 0:0:100). All fractions were analyzed by TLC, obtaining XII fractions. Antioxidant (DPPH and FRAP) and phenolic compounds determinations were evaluated for all fractions (I-XII). Fraction XI and XII exhibited the higher antioxidant activity (DPPH and FRAP) and phenolic compounds values compared to the other fractions. Based on the above, F-XI and F-XII were selected to evaluate their antioxidant potential in ground meat.

HPLC analysis for LGE and active fractions

Samples were analyzed in an Agilent 1220 Infinity DAD LC (Waldbronn, Germany) equipped with a Zorbax SB-C18 column (250 x 4.6 mm, Ø 3.5 µm, Agilent, USA). The solvent used were: 5 % formic acid in water (solvent A) and methanol (solvent B) stablishing the following gradient: 5 % methanol (0-5 min), 10 % methanol (5-10 min), 15 % methanol (10-18 min), 25 % methanol (18-28 min), 30 % methanol (28-40 min), 40 % methanol (40-45 min), 45 % methanol (45-55 min), 60 % methanol (55-60 min), 80 % methanol (60-65 min), 100 % methanol (65-76 min) and 30 % methanol (76-86 min), with a flow rate of 1 mL/min. Flavonoids were detected with diode array detector and monitored at 280 and 340 nm. The assignment of peaks were determinate using authentic standards: epicatechin and quercitrin. Quantification of these compounds was performed using calibration curves established with the authentic standards. Results were expressed as mg of each compound 100 mg^{-1} of d.w.

ORAC assay

ORAC assay was measured following the method reported by Ou *et al.*[19]. AAPH reagent was used as peroxyl radical generator, fluorescein as the fluorescent indicator. 100 µL of samples were mixture with 1.65 µL of phosphate buffer, 150 µL of AAPH (0.8 M), and 100 µL of fluorescein (10 mM). Phosphate buffer was used as the blank. Reaction was started by the addition of AAPH (240 mM). Samples were preincubated at 37 °C (15 min) and the fluorescence was measured every 90 s for 1.5 h at 485-520 nm (Fluostar Omega microplate reader, BMG Labtech). Results were reported as µmol TE g^{-1} d.w.

Preparation of pork patties and treatments

Pork meat was purchased from a local meat market with at 1-2 days post-slaughter. Pork meat was ground through a 4.5 mm plate (Hobart Dayton 4152, Troy, Ohio, USA). After mincing, ground meat was divided into six portions, and each portion was randomly

assigned to one of the following six treatments: Treatment 1: Control (without preservative); treatment 2: BHT at 100 ppm; treatment 3: Trolox (TR) at 100 ppm; treatment 4: *L. glaucescens extract* (LGE) at 100 ppm; treatment 5: Fraction XI (F-XI) at 100 ppm and treatment 6: Fraction XII (F-XII) at 100 ppm. All treatments were dissolved in 2 mL of ethanol-water (1:1, v/v) and mixed in patties preparation, excepting control treatment which was prepared only with based-solved solution. Each treatment was manually mixed. A total of, 25 pork patties of 70 g each, were formed for each treatment. Pork patties were placed in polypropylene trays, wrapped with PVC film and stored for 11 days at 4 °C. Samples were analyzed at 1, 4, 6, 8 and 11 days of storage. All analyzed samples were performed by duplicate.

Analysis of pork patties

Conjugated dienes

Total lipids of pork patties were extracted followed the methodology described by Bligh and Dyer,[20].

Lipids were weight into 50 mL centrifuge tubes and filled up to volume with ciclohexane. A 1 % solution was made, and if it was necessary, an extra dilution was performed to obtain an absorbance between 0.1 and 0.8. Absorbance was read at 232 nm against the blank of ciclohexane. Concentration of conjugated dienes was calculated using the molar extinction coefficient of 25,000 M^{-1} cm^{-1}. Results were expressed as µM mg^{-1} pattie.

Thiobarbituric acid reacting substances (TBARS)

Lipid oxidation of pork patties was determined by the 2-thiobarbituric acid (2-TBA) method[21.] Pork patties (10 g) were mixed with 15 mL of trichloroacetic acid (10 %, w/v). Samples were centrifuged (2300 x g for 20 min at 4 °C). After centrifugation, the supernatants were filtered and 2 mL of filtered was mixed with 2 mL of 2-TBA (20 mM). Samples were placed in a water bath (97 °C) for 20 min. Samples were cooled in cold water for 15 min, and the absorbance was read at 531 nm. Results were expressed as mg malondialdehyde (MDA) kg^{-1} per patty.

Carbonyl content

Carbonyl content was determined by derivatization with DNPH based on the modified method of Oliver *et al*.[22]. One g of pork patties was blended with 10 mL of 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) on an Ultraturrax homogenizator for 30 s. Two equal aliquots of 0.2 mL of the mixture were dispensed in 2 mL eppendorf tubes. Proteins were precipitated with 1 mL of TCA (10 %) and centrifuged (5000 rpm for 5 min). One pellet was treated with 1 mL 2M HCl (protein concentration measurement) and the other with 1 mL DNPH (0.2 %, w/v) in 2 M HCl (carbonyl concentration measurement) for 1 h at room temperature in the dark. Samples were precipitated with 1 mL TCA (10 %), washed two times with 1 mL ethanol:ethyl acetate (1:1, v/v), dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5), stirred and centrifuged (5000 rpm for 2 min). Protein concentration was read at 280 nm using BSA as standard. Carbonyls were read at 370 nm and were expressed as nM of carbonyl/mg of protein using an absorption coefficient of 21.0 nM^{-1} cm^{-1}.

CIE-Lab color

Colorimetric analysis of pork patties after 30 min blooming was evaluated using a colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc. Japan) with D65 illuminant, with 10° and 8 mm of aperture in the observer. L* (lightness), a* (redness) and b* (yellowness) were evaluated on the outer surface of pork patties. At least 5 different determinations were carry out per sample.

Sensory evaluation

Pork patties were cooked in a classic grill until and internal temperature of 71 °C was sustained for 3 min. After, sensory evaluation was performed by a trained 8 member panel using controlled condition in a room partitioned into booths (21±1 °C and 55±5% relative humidity). A 5-point hedonic scale was used to evaluate the loss of odor and flavor freshness in pork patties, where the minimum value (1) means no loss of fresh odor and flavor, while the highest one (5) indicated extreme loss of these attributes.

Statistical analysis

Data was analyzed by analysis of variance in a 5 x 6 factorial arrangement (storage time x treatments) with the NCSS statistical software (vers. 2007). The model included

treatments, storage time, and its interaction as fixed effects. Means were compared using Tukey-Kramer test. Statistical significance was considered at $P<0.05$.

RESULTS AND DISCUSSIONS

Phenolic compounds and antioxidant evaluation

Analysis of phenolic compounds by HPLC-DAD of ALGE, F-XI and F-XII are shown in Figure 1 and Table 1. HPLC-DAD analysis of ALGE showed several phenolic compounds at different concentrations. Epicatechin and quercitrin were two of the majority compounds observed in the extract with a concentration of 1.56 and 2.11 mg 100 mg^{-1} d.w., respectively. Chromatogram for F-XI, exhibited a high purity fraction, highlighting the presence of epicatechin, which was 15.7 times higher ($P<0.05$) than the found in ALGE. Analysis of F-XII showed quercitrin as majority compound in this fraction, found in a higher concentration ($P<0.05$) than ALGE. These results were consistent with previous studies, which reported the presence of epicatechin and quercitrin in *L. glaucencens* extracts[17,18,23].

Phenolic compounds are largely associated with antioxidant potential. Different methods have been described to evaluate antioxidant potential from plant extracts, some of them based on free radicals stabilization by protons or electrons transferring. ORAC is one of the most popular method, based on the antioxidant agent capacity to decreased peroxil radicals. Additionally, there is a significant correlation between ORAC method and lipid hydroperoxides[24]. Therefore, this method could support to determine the effect of antioxidant agents in matrices or systems where lipid oxidation plays and important role in product spoilage. Antioxidant activity of ALGE, F-XI and F-XII by ORAC assay is shown in Figure 2. The ORAC values for the evaluated treatments ranged from 3553.3 to 3673.3 µmol TE g^{-1} d.w. ALGE and F-XI presented the highest activity ($P<0.05$), followed by F-XII. This result demonstrated a powerful antioxidant activity by ALGE, F-XI and F-XII, in agreement with previous study by Lopez-Romero *et al*.[18], where evaluated the compounds antioxidant activity to stabilize synthetic and biological radicals and reducing metals, all of them, involved in particular oxidative stage. Due to properties described above, ALGE, F-XI and F-XII represent an available and efficient

source of antioxidant agents with a wide application on foods, especially in those where oxidative processes have a negative effect on food quality and stability. This antioxidant effect is related with phenolic compounds content such as epicatechin and quercitrin, which have shown to have potent antioxidant activity[18,25]. This effect is related with their structural composition such as the presence of a 3', 4'-dihidroxy group in the B ring, 3-hidroxy group in the C ring and double bond (C2-C3) in combination with a 4-keto group. Previous characteristics make flavonoids a great free radical stabilizer and, in consequence, a stable phenolic radical for resonance effect [14, 26].

Analysis of pork patties

Conjugated dienes (CD) values of pork patties during refrigerated storage

The initial stage of meat lipid oxidation is associated with conjugated dienes production, because double bonds of unsaturated fatty acids are transformed to conjugated systems[27]. The effect of ALGE, F-XI and F-XII on CD of raw pork patties storage under refrigeration are shown in Figure 3. The level of CD ranged from 0.12 to 0.41 μmol mg^{-1} of meat. It was observed that CD increased in all evaluated treatments until day 6 of storage, and subsequently decreased until day 11. This behavior can be attributed to CD hydroperoxydes decomposition to secondary lipid oxidation products[28]. Generally, it was observed that F-XI presented the lowest formation ($P<0.05$) of CD during the storage period. Additionally, F-XI presented a similar activity ($P>0.05$) than positive controls (BHT and Trolox) at the end of storage, demonstrating the powerful potential to prevent the CD formation. ALGE and F-XII, were also effectives ($P<0.05$) in reducing CD formation comparing with control (with no additive). This results evidenced that evaluated treatments were effectives against primary lipid oxidation products formation. Different studies demonstrated that the incorporation of naturals extracts in raw pork meat (patties and ground meat) stored under refrigeration significantly decreased CD formation during shelf life[29,30]. These same authors suggested that CD diminution was related to the presence of phenolic compound in the extracts, which are responsible to scavenge free radicals and reduce or chelate metals, playing an important role in the beginning of oxidative process. In the present study, we identified the presence of epicatechin and quercitrin. Epicatechin demonstrated an effect as free radical terminator in lipid system, delaying CD formation[31].

TBARS values of pork patties during refrigerated storage

TBARS assay is performed in meat and meat products to evaluate lipid oxidation process, specifically the production of secondary lipid oxidation products such as aldehydes, related to the development of unacceptable flavors and aroma in meat and meat products, affecting their quality and stability[32]. The effect of ALGE, F-XI and F-XII on TBARS values of pork patties stored under refrigeration are shown in Figure 4.

TBARS score ranged from 0.15 to 1.91 mg MDA kg^{-1} of meat during 11 days of storage (4 °C). At 8 day of storage, it was observed a significant difference ($P<0.05$) in evaluated treatments where control treatments (with no additive) exhibited the highest value (1.40 mg MDA kg^{-1}), in comparison with treated samples. F-XI was the most effective treatment to decrease TBARS values followed by ALGE and F-XII, presenting 50.7, 31.2 and 29.2 % (respectively) less oxidation vs control treatment (with no additive). Similarly, a significant difference ($P<0.05$) was observed at day 11 of storage, with a significant oxidation reduction vs control by all treatments: 52.3 % (F-XII), 28.2 % (ALGE) and 11.5 % (F-XII) vs 1.91 mg MDA kg^{-1} (control treatment). Additionally, F-XI was the most effective treatment to reduce the production of secondary lipid oxidation products, presenting a similar behavior that BHT and Trolox during the storage period. In addition, ALGE and F-XII also were effective to delay lipid oxidation process in the pork patties. Campo *et al.*[33] suggested that TBARS concentrations higher than 1 mg MDA kg^{-1} meat in pork meat are considered the rancidity detection limit by consumers. Thus, F-XI was evaluated with lower rancidity value than reference during 11 days of storage, while ALGE and F-XII were considered acceptable (until 8 day of storage) in this parameter due to their low values comparing with reference.

The obtained results evidenced that ALGE, F-XI and F-XI were an effective alternative to decreased lipid oxidation in pork patties, increasing their shelf life and maintaining their quality and stability. The protective effect of evaluated treatment against lipid oxidation have demonstrated a powerful effect to stabilize free radicals by *in vitro* tests, which confirm that these compounds can stabilize free radicals and avoiding oxidative process, besides reducing and chelating transition metals which also have an important role in the beginning of oxidative process[34,35].

Based on the above, different studies have demonstrated that natural extract rich in phenolic compounds incorporated in meat and meat products reduced secondary lipid oxidation products production. In a study performed by Lee *et al.*[29], addition of mustard leaf extracts in ground pork were effectives to reduce lipid oxidation (TBARS values) compared to the control. Choe *et al.*[36] observed that ground pork treated with pumpkin leaf extracts exhibited protective effect against lipid oxidation, allowing to extend their shelf life. On the other hand, the effectiveness to reduce the lipid oxidation of ALGE, F-XI and F-XII also could be related with the identified compounds. For example, incorporation of epicatechin on fish meat, demostrated a reduction on malondialdehyde content, achieving a reduction of 34 to 40 % of the TBARS values during the storage period (7 days at 4 °C) [37]. Similarly, Tang *et al.*[38] observed that epicatechin reduced at least 50 % of oxidation of a liposomal system during the evaluation period. In a study performed by Alves *et al.*[39] evaluated the antioxidant effect of tomato products added in chicken meat. Tomato treatments significantly reduced lipid oxidation in chicken meat, because showed lower values than control. The antioxidant effect was attributed to the presented extract compounds, where quercitrin was the third major compound identified in the tomato extract.

Protein carbonyls values of pork patties during refrigerated storage

Protein oxidation in meat and meat products can be measured by the formation of protein carbonyls, which indicates the oxidative reactions that affect muscle proteins. Carbonyls content of pork patties throughout storage are presented in Figure 5. Carbonyls values ranged from 0.28 to 1.04 nM carbonyls/mg protein during shelf life. The obtained results evidenced that carbonyls content significantly increased ($P<0.05$) during shelf life in all evaluated treatments. This suggested that meat proteins were affected by oxidative stress, associating with oxidative degradation of some amino acid such as lysine, proline, arginine and histidine[40]. Evaluated treatments were effective to reduce carbonyl content during test evaluation period. For example, at day 6 of storage F-XI significantly reduced ($P<0.05$) protein oxidation, compared with control (with no preservative). Similar results were observed at day 8 and 11, where ALGE, F-XI and F-XII reduced between 25.49 to 49.3 % the carbonyl content in the pork patties ($P<0.05$), respect to the control treatment. In addition, the most active treatment was F-XI,

followed by ALGE and F-XII. Additionately, F-XI exhibited similar activity than positive controls (Trolox and Vit. C) during pork patties storage. At the same time, ALGE and F-XII reduced patties protein oxidation comparing with control (with no additive), however, presented less potential than F-XI, Trolox and Vit. C. These results proved that evaluated treatments were suitable to reduce oxidation process, suggesting their effectiveness to prevent or delay degradation, aggregation and fragmentation of proteins, allowing to preserve their characteristics and functionality for a long time.

By the other hand, aldehydes (lipid oxidation product) induced protein oxidation by their interaction with side chains from aminoacids, this issue demonstrate a positive correlation between meat products lipid and protein oxidation [4,41,42]. Our results were consistent with this fact, because during storage pork patties' TBARS values increased as well as carbonyl values. This issue may suggest that the lipid oxidation products induced protein oxidation of pork patties. Also, metal transition have a primordial role in protein oxidation processes [4]. In this sense, ALGE, F-XI and F-XII showed that they were effective to delay lipid oxidation in the pork patties and also decreased protein oxidation with the same affectation. These results supported the usage of tested treatments as preservatives, due to their ability to reduced primary and secondary lipid oxidation products production, reducing protein oxidation. Previous behavior may be associated with phenolic compounds, molecules generally known as great antioxidants with powerful stabilizing capacity to reduce free radicals and metals, both last ones involved, at first stage lipid and protein oxidation in meat and meat products. In addition, this effect may be to compounds presents in the extract and each fractions. For example, epicatechin have demonstrate a high inhibition of carbonyl group formation in myofibrilar proteins and BSA, which was attributed to the ability of epicatechin to decrease lipid oxidation, stabilize free radical and reduce or chelate metals [44]. Also, Martinez *et al.*[45] observed that epicatechin acts as protein protector against oxidation induced by AAPH, a peroxyl radical initiator which produce cell membrane damage through free radicals attack. The protective effect of this compound is attributed to the ability to interact with the polar head groups of phospholipid membrane, maintaining their integrity, acting as a barrier against toxic agents such as reactive oxygen species

and metals that induce the initiation of lipid and protein oxidation. Moreover, other compounds with similar structure such as catechin and rutin (flavonol glycoside) have shown to be effective in reducing myofibrilar proteins oxidation, and also specific products derived from lysine protein oxidation such as α-aminoadipic and γ-glutamic acid semialdehydes [46]. Accordingly, our results support the usage of LG as a potential source of compounds with high antioxidant activity, which could reduce protein oxidation processes in meat and meat products.

On the other hand, our results were consistent with previous studies that reported a protein oxidation reduction in meat and meat products by the incorporation of antioxidant plant extracts with high amount of phenolic compounds. Similarly, Rodríguez-Carpena *et al.*[47] studied avocado extracts addition on protein oxidation of pork patties (15 days at 4 °C). Avocado extract incorporation reduced protein oxidation of pork patties during storage comparing with control. Notable changes were identified at 10 and 15 days of storage, where most effective treatments presented between 20 and 30 % less protein oxidation at the same days of storage. These results are in agreement with the present study; due to a significant reduction of pork patties protein carbonyls production at final storage days. Similarly, Jia *et al.*[35] studied the effect of black currant extract in pork patties during your storage (9 days at 4 °C). They observed that evaluated extract reduced the carbonyl content and sulfhydryl loss of pork patties in a dose-depend relation during storage, once an increasing of extract concentration presented less protein oxidation in pork patties, being changes greater at the end of storage days. In a study performed by Turgut *et al.*[48] observed that the pomegranate extract added in meatballs stored under refrigeration significantly reduced protein carbonyls and sulfidryl content compared to the control during the storage (8 days at 4 °C). Additionally, pomegranate treatments presented similar behavior comparing with positive control (BHT) and significantly lower than control negative values during 8 days of storage.

Color of pork patties during refrigerated storage

The evaluation of L* (lightness), a* (redness) and b* (yellowness) is presented in Figure 6. Results obtained for L* ranged from 54.3 to 57.9, however not significantly difference was observed, indicating that addition of all evaluated treatments did not affected L* values of pork patties during storage. Yellowness evaluation showed a decreased trend

of pork patties b* values closely the end of storage, however, significant differences was only observed at day 0 (starting evaluation).

On the other hand, a* values varied between 7.5 to 14.9 during refrigerated storage. Generally, it was observed that control treatment tended to present low a* values during the storage, however no differences were observed ($P>0.05$). Moreover, it is important to mention that at day 8 (a*=8) and 11 (a*=7.5) of storage, control treatment presented the lowest a* values, compared to the other treatments, where at 8 day the other evaluated treatments exhibited a* values between 9.1 to 10.4, whereas at day 11 a* values ranged from 8.9 to 9.7. This suggests that evaluated treatments presented a protective effect on the redness parameter of pork patties. Diminution in a* values is associated with the formation of metmyoglobin, which produced a brown color in meat and meat products, caused by the myoglobin oxidation associated with lipid oxidation and exposing to reduced system, triggering for primary (hydroperoxides) and secondary (aldehydes) products exposed to reactive oxygen species [49]. This interaction induced the conversion of ferrous to ferric iron, metal that constitute myoglobin heme group [6,50].

Our results were consistent with previous studies, due to lipid oxidation increased in the pork patties while a* values tended to decrease, suggesting that oxidative processes interfered with red color lost in the pork patties. Moreover, ALGE, F-XI and F-XII showed high a* values during shelf life, compared to the control. This behavior can be related to the antioxidant potential of these treatments, which presented a lower formation of primary and secondary lipid oxidation products. These results suggest that the evaluated treatments were a suitable alternative to preserve red color of meat and meat products, being able to inhibited metmyoglobin formation.

Sensory evaluation of pork patties during refrigerated storage

The mean score of loss of fresh odor and loss of fresh flavor of pork patties during shelf life are summarized in Figure 7. Generally, it was observed that sensory evaluation score increased during shelf life in all evaluated treatments. Related with loss of fresh odor, the evaluated treatments showed differences ($P<0.05$) at 8 day of storage, where control (without additive) exhibited highest losses of this parameter (2.85), followed by BHT (2.18), ALGE (1.30), F-XII (1.29) and F-XI (1.23) treatments. Similarly, at 10 day

of sampling the control (4.40) and BHT (4.32) treatments ($P<0.05$) exhibited moderately losses of fresh odor, while the other treatments (2.57 to 2.95) presented slightly loss of fresh odor.

On the other hand, evaluation of loss of fresh flavor showed that in the fists 6 days of storage the evaluated treatments did not show differences ($P>0.05$). However, at 8 day of storage, control (without additive) showed the highest losses (3.88) of fresh flavor ($P<0.05$), compared with the other treatments (1.36 to 2.66). Additionally, at 10 day of sampling BHT, Trolox and control treatments exhibited ($P<0.05$) moderate to extreme loss of fresh flavor, while ALGE and fractions presented slightly loss of fresh flavor.

The obtained results in the present study showed that ALGE, F-XI and F-XII did not have negative effect on this sensory parameter, because showed slight losses in fresh odor and flavor during 11 days of storage. In addition, this results are in accordance with TBARS evaluation, because the trainer panel detected differences at day 8 of storage, where the evaluated treatment showed values near or higher than 1 mg MDA/kg patties, considered the threshold detection for pork patties undesirable odors and flavors.

Several studies also demonstrated that plant extract incorporation did not affect meat products sensory properties. Muthukumar *et al.*[51] observed that a trained panel did not perceive sensory changes by moringa extract (300 to 600 ppm) incorporation to pork patties. Similarly, a study performed by Carpenter *et al.*[52] evidenced no significant effect on sensory properties of pork patties storage at 4 °C/ 4 days by grape seed (400 and 1000 µg/g) and bearberry (80 and 1000 µg/g) extracts. Same effect was reported to pomegranate peel extract (0.5-0.1%) incorporated to fresh meatballs where no negative changes in sensory parameters were observed after 8 days of storage [48].

By the other hand, it is important to highlight that ALGE, F-XI and F-XII imparted herbal odor to pork patties samples affecting their general acceptance, although all treatments did not change odor lost and freshness of pork patties. Due to this, subsequent research is needed, to study doses-effect of these extracts on sensory and physic-chemical characteristics of these products, keeping their acceptance and an extended shelf life.

CONCLUSIONS

In the present study, two of the main compounds present in ALGE, FXI and F-XII were quantified and also exhibited a great antioxidant potential. The addition of these treatments in pork patties stored under refrigeration at 4 °C for 11 days, demonstrated to be effective in reducing lipid and protein oxidation, and preserving color, which reflected an extended shelf life of the pork patties (3 days greater than control treatments). Results suggest that the evaluated treatments represented an excellent source of compounds with high antioxidant activity, identifying them as an effective potential alternative for meat industry to improve quality characteristics in meat products for a long time.

Conflict of interest

The authors declare there is no conflict of interest.

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Tables and figures

Table 1. Concentration of major phenolic compounds identified in *L. glaucescens* extract and their fractions

L. glaucescens extracts and fractions (mg 100 mg^{-1} d.w.)			
Compound	ALGE	F-XI	F-XII
Epicatechin	1.56 ± 0.19^{a}	24.54 ± 0.82^{b}	ND
Quercitrin	2.11 ± 0.05^{a}	ND	6.49 ± 0.10^{b}

$^{a-b}$Means with different superscript within the same row, indicate statistical differences ($p<0.05$). All values represent mean±standard deviation. ND: No detected.

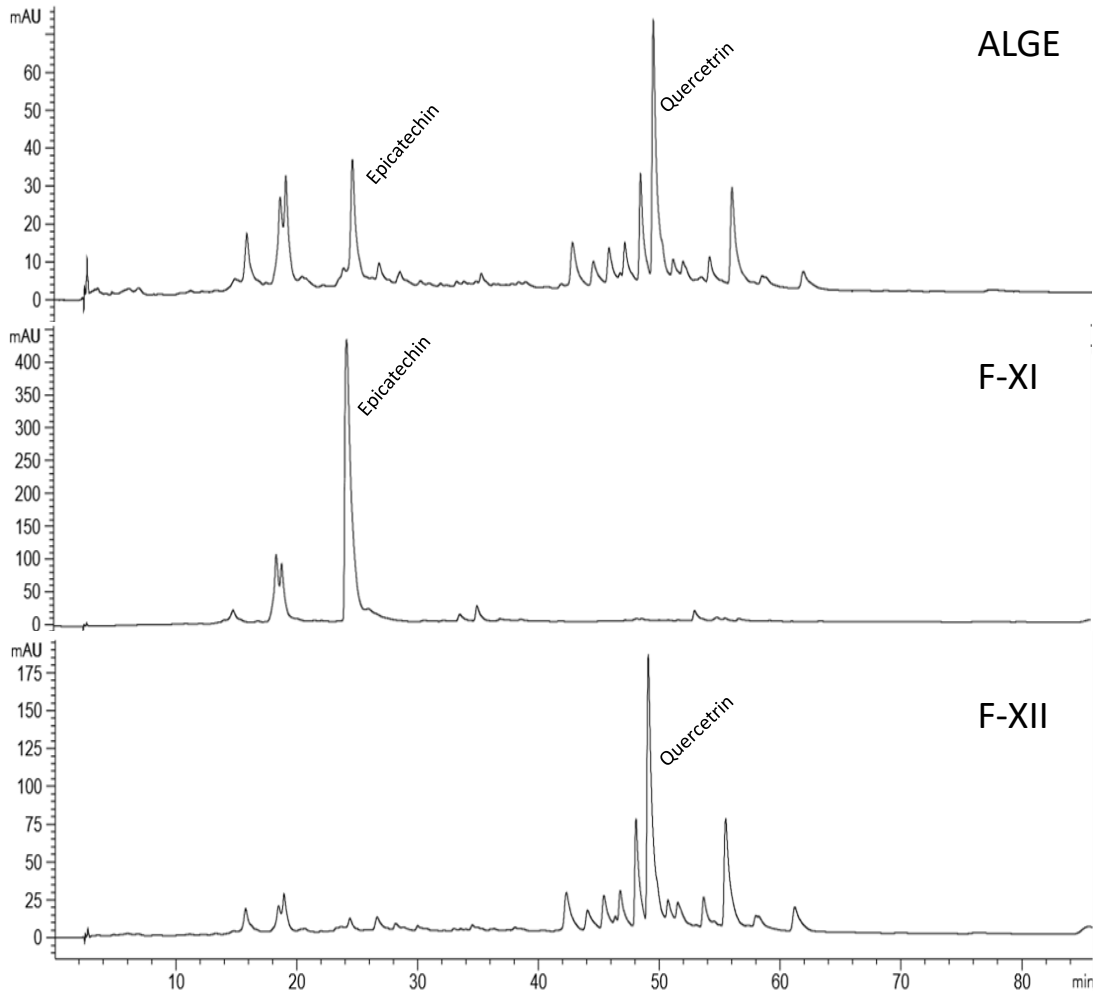

Figure 1. HPLC chromatogram of ALGE, F-XI and F-XII (recorded at 280 nm).

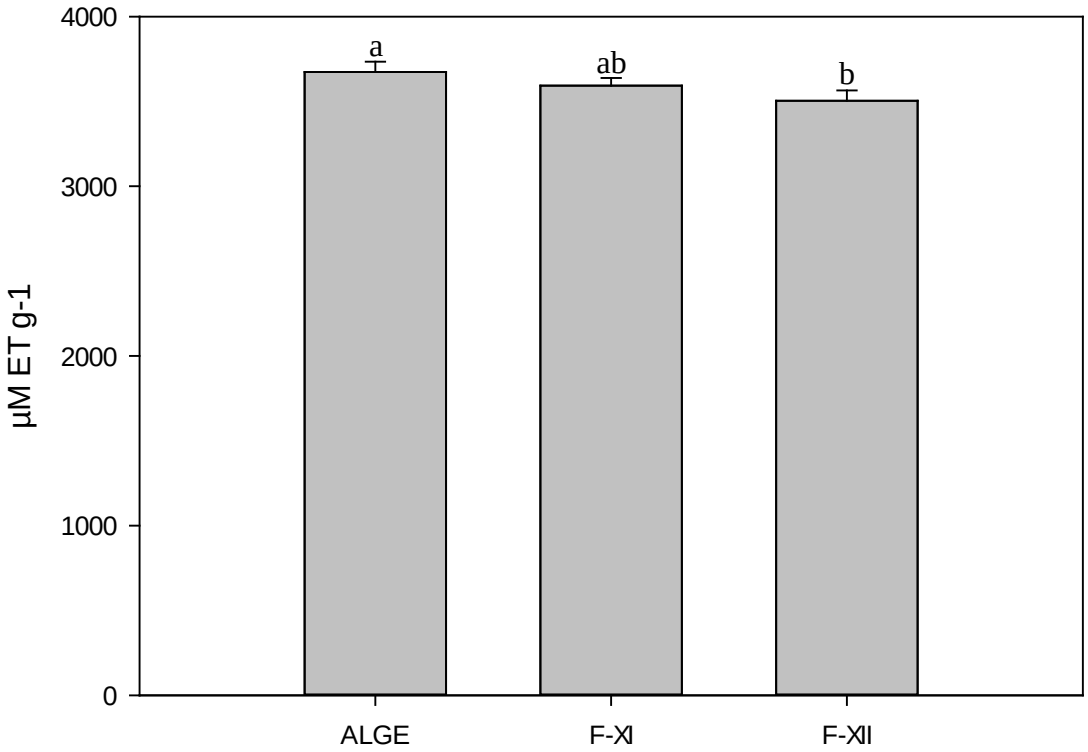

Figure 2. ORAC values of ALGE, F-XI and F-XII. [a-b]Different superscript indicate statistical differences (P<0.05). All values represent mean±standard deviation.

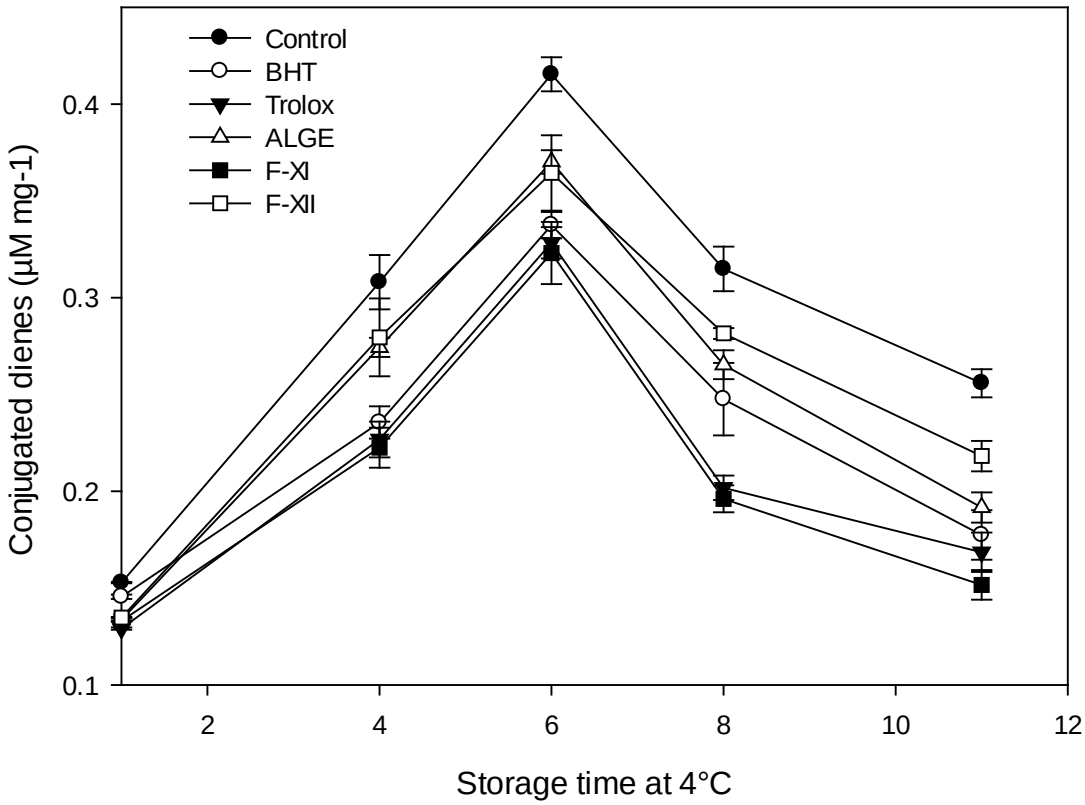

Figure 3. Conjugated dienes (CD) values of pork patties during refrigerated storage. Axis X: days at 4 °C, Axis Y: Conjugated dienes values (µM mg^{-1}).

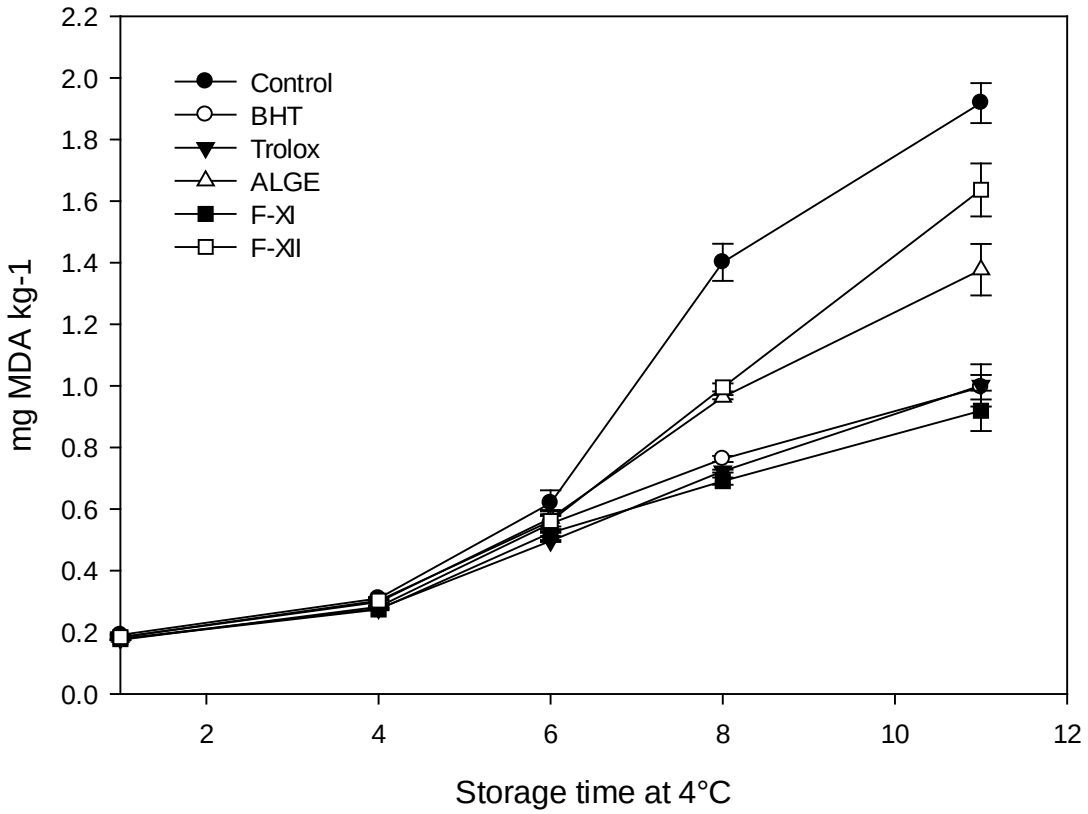

Figure 4. TBARS values of pork patties during refrigerated storage. Axis X: days at 4 °C, Axis Y: TBARS values (mg MDA kg^{-1}).

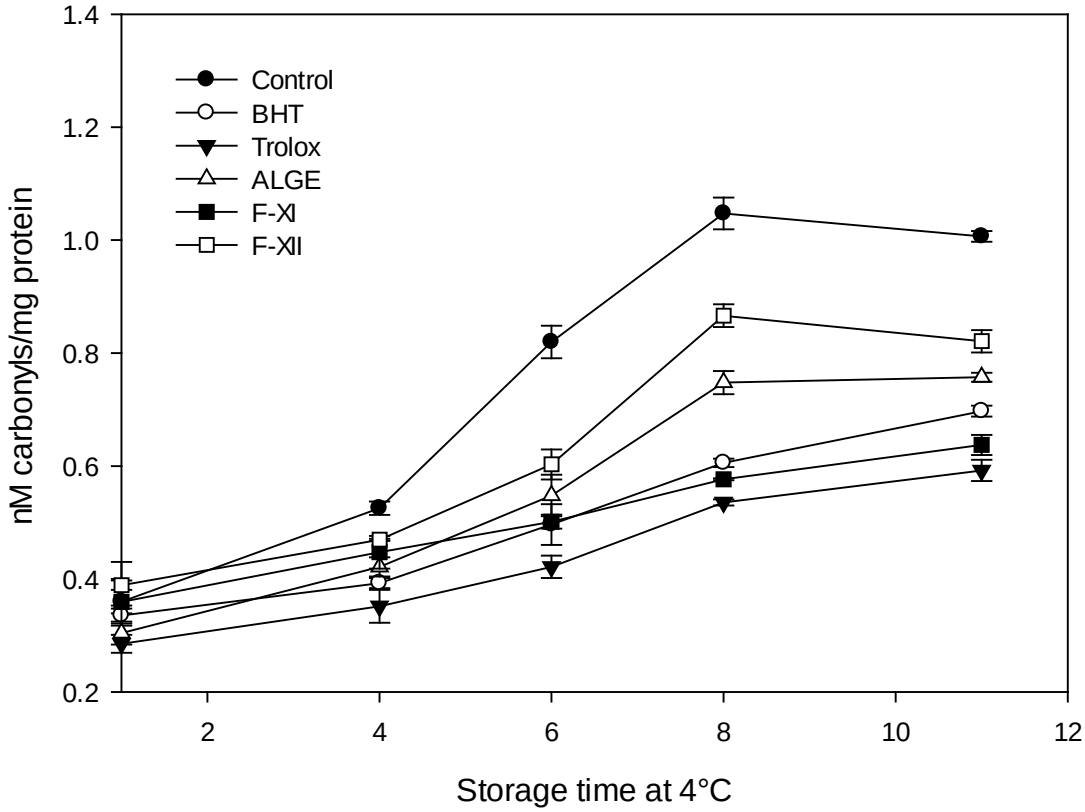

Figure 5. Carbonyl values of pork patties during refrigerated storage. Axis X: days at 4 °C, Axis Y: Carbonyl values (nM carbonyls mg^{-1} protein).

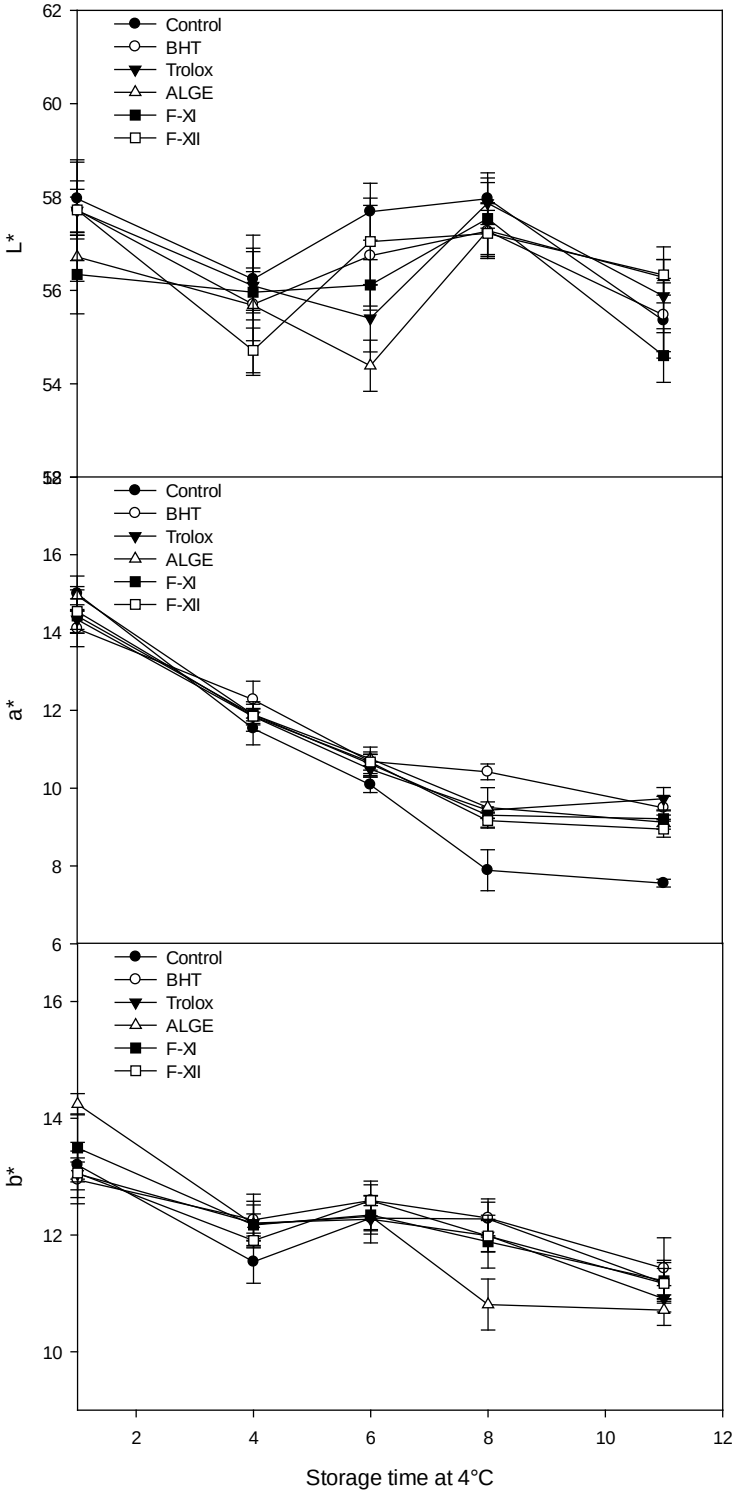

Figure 6. Color values of pork patties during refrigerated storage. Axis X: days at 4 °C, Axis Y: a) L* values, b) a* values and c) b* values.

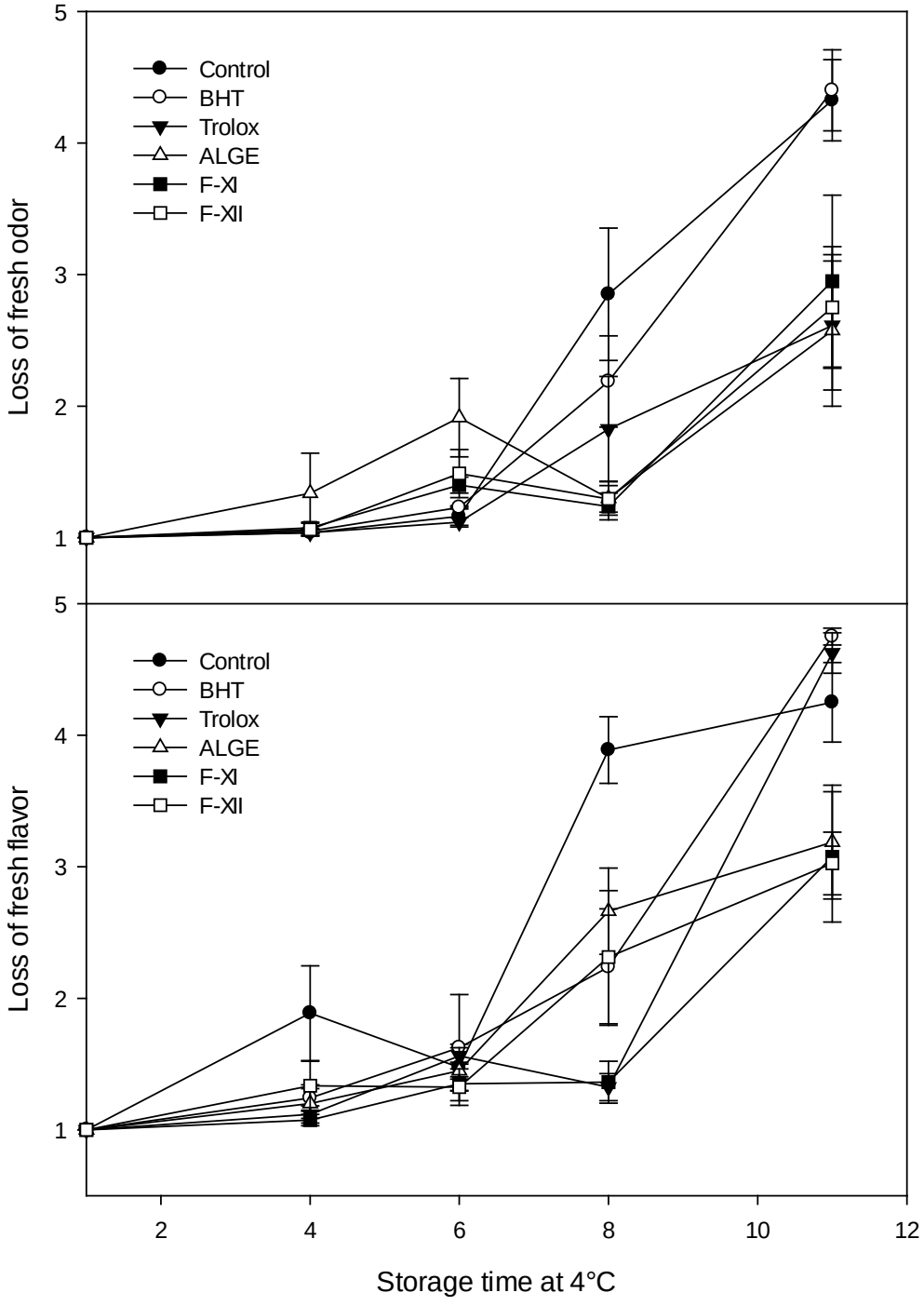

Figure 7. Sensory evaluation of pork patties during refrigerated storage. Axis X: days at 4 °C, Axis Y: a) Loss of fresh odor, b) Loss of fresh flavor