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Antigenotoxic and antimutagenic activities of *Psittacanthus calyculatus* (Loranthaceae) leaves water extract

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ABSTRACT: Mistletoe (*Psittacanthus calyculatus*) is used for the prevention and treatment of numerous diseases. Samples of leaves from *P. calyculatus* were collected in April of 2019, and prepared an aqueous extract. The extract was lyophilized, and its polyphenols, flavonoids, and anthocyanins content were determined. Then, concentrations of lyophilized extract were prepared (5, 50 and 100 ppm) and assessed their antigenotoxic, antimutagenic and genotoxicity activities in human lymphocytes were evaluated using the comet assay system. The dry aqueous extract contained 73.54 mg of polyphenols AGE per g sample, 39.37 mg of flavonoid CE per g, and 0.1 mg of anthocyanins Cy-3-gluc E per g. No significant genotoxic activity was observed, with the exception of the concentration of 100 ppm at 10 hours of exposure ($p < 0.05$). There was also significant ($p < 0.05$) antigenotoxic and antimutagenic activity ($p < 0.05$). Clearly, low concentrations and short-duration exposures to lyophilized *P. calyculatus* do not induce genetic damage; however, high concentrations are genotoxic. The antigenotoxic and antimutagenic effects were due to a protective effect not only against induced DNA damage but also against basal genetic damage.

1. INTRODUCTION

Although practitioners of folk medicine used plants to treat various ailments, the efficacy, and safety including its genotoxic, mutagenic, and carcinogenic activities of these plants, are not well understood (Hussain et al., 2003; Klein & King, 2007; Sharma et al., 2010). Moreover, some compounds can enhance DNA repair or eliminate genotoxic damaged cells from tissue/cell populations are very important in the investigation of antimutagenic agents and compounds which can lower the frequency or rate of mutations (De-Flora, 1998). Therefore, it is essential to assess the genotoxic implications of traditionally used medicinal plants (Avila-Acevedo et al., 2012), ideally using different bioassays system (Zúñiga, 2013).

Psittacanthus calyculatus (Loranthaceae), known as "graft, injerto, or mistletoe," is a hemiparasite liveing in trees of the genus *Acacia*, *Propopis*, *Quercus*, and *Prunus*, among others (Pérez-López et al., 2010) and it is used in traditional Mexican medicine for treatment of different diseases (Avila-Acevedo et al., 2012; Rodriguez-Cruz et al., 2003). Its extract can modify immune-modulator and antitumor response, also to maintain blood glucose concentration within normal levels (Liu et al., 2012; B.G. Varela et al., 2001).

Mistletoe is a source of bioactive polyphenols, though only a few flavonoids (hesperetin, rutin, myricetin, luteolin, (+)-catechin, quercetin, apigenin, naringenin, kaempferol, and phenolic acids (protocatechuic, caffeic, p-coumaric, gallic and rosmarinic) as well as other compounds like trans 4-hydroxy-N-methylproline, isorhamnetin 3-O-β-Dxylopyranosyl (1→6)-β-D-glucopyranoside and quercetin-3-O-β-D-xylopyranosyl (1→6)-β-Dglucopyranoside have been identified (Ibarra-Alvarado et al., 2010; Moustapha et al., 2011). Gallic acid, myricetin and quercetin possess antimutagenic or antigenotoxic activities (Fedel-Miyasato et al., 2014; Hobbs et al., 2015; Patil et al., 2014; Sharma et al., 2010), suggesting that the aqueous extracts of this plant have both antimutagenic and antigenotoxic activities, but this has not been reported in the literature. Such properties are very important in neutralizing mutations resulting from exposure to xenobiotics that may be precursors of carcinogenesis (De-Flora, 1998; Hussain et al.,

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2003). In addition, it is known that high intake of antioxidants could lead to genetic instability (Klein & King, 2007). Due to the high consumption of *P. calyculatus* as aqueous extracts in México and to the high concentrations of bioactive compounds with antioxidant properties, it is also important to determine their antigenotoxic and antimutagenic activities in addition to genotoxicity.

The alkaline comet assay, developed by Singh et al. (1988), is very efficient at detecting genotoxic agents (Belpaeme & Kirsch-Volders, 1998; Koppen & Verschaeve, 1996; Nacci et al., 1996), and is ideal for assessing the antigenotoxicity, and antimutagenicity activities (Gasiorowski & Brokos, 2001; Pesarini et al., 2013; Reynoso et al., 2014; Roy et al., 2003; Sondhi et al., 2010).

Although *P. calyculatus* is considered harmless in Mexico, few studies have examined its genotoxic, mutagenic, or antimutagenic effects. This study's goal was to assess the antimutagenic and antigenotoxic properties of *P. calyculatus* water extracts at levels similar to those seen in the infusion often eaten by Mexicans.

2. MATERIALS AND METHODS

2.1. Plants material

The leaves of *P. calyculatus* grown on host plant *Pithecellobium dulce* (guamúchil) were collected in Guadalajara, México, during the months of February to April 2019. The specimens were authenticated by Dr. Vazquez-Garcia, and deposited a voucher (reference number 198563) in the Herbarium IBUG, University of Guadalajara.

2.2. Preparation of water extract and analysis of polyphenols, flavonoid and anthocyanins

One hundred g of *P. calyculatus* leaves were boiled in 1 L of water for 30 min at room temperature. The solid particles filtered and the water was removed in a rotavapor at 50°C, then the suspension was freeze-dried (LABCONCO Corporation, USA) and stored the dry powder at 4°C until used.

For the determination of polyphenols, flavonoid and anthocyanins content, five g of dry powder with 50 mL of 80% methanol was mixed, sonicated it for 40 min, and collected the supernatant after centrifuging at 14,000 rpm for 15 min (Atanassova et al., 2011).

2.3. Determination of polyphenol

The polyphenolic content of the samples was evaluated using the Folin-Ciocalteu colorimetric test, and the results were represented as mg of gallic acid equivalents (GAE) per g of sample on a dry weight basis (dw) (Atanassova et al., 2011).

2.4. Determination of Flavonoids

The method aluminum chloride ($AlCl_3$) was used to quantify the extract flavonoids content and the results were represented as mg of catechin equivalents (EC)/g of dw (Atanassova et al., 2011).

2.5. Total anthocyanin

To quantify the extract anthocyanins content, it used the differential pH method described by Giusti et al. (2001) and the results were represented as mg of cyanidin-3-glucoside per g on a dry weight basis (dw).

2.6. Obtaining human lymphocytes

Blood samples were acquired from ten male volunteers between the ages of 18 and 20, who had never smoked or drank and had never been exposed to chemicals (information obtained from a previous questionnaire). The study was accepted by the Bioethics Committee of the CUCBA, Universidad de Guadalajara, who followed the Declaration of Helsinki and Tokyo principles for people and followed worldwide guidelines.

Five drops of blood from each person were dissolved in 10,000 IU heparin. Then 5 mL of cold phosphate-buffered solution (PBS) (160 mM NaCl, 8 mM Na2HPO4, 4 mM NaH2PO4, EDTA 50 mM, pH 7). A 10 minute centrifugation removed the supernatant and the pellet was re suspension in 500 mL PBS and stored at 4 °C until required. Each person's proportion of viable cells was determined by the trypan blue test on 1 ml of whole blood solution. Each group's mean percentage viability was over 90%.

2.7. Genotoxic study

For the genotoxic activity study, each sample was divided in 5 parts: two were used for positive control ethyl methanesulfonate (EMS) (5mM, 50 μL / suspension, 100 μL, 2 h) and negative control (PBS/suspension, 2 h). Another three parts of suspension were mixed v/v in independents tubes using 5, 50 and 100 ppm of lyophilized water extract of *P. calyculatus*. After of 5, 10 and 15 h at 4 °C, the suspensions were washed 3 times with PBS and centrifuged at 3,000 rpm for 10 min. Later, the pellet was re suspended in 100 μL of PBS and 5 μL were mixed on agarose gel (Alvarez-Moya et al., 2001).

2.8. Antigenotoxicity and antimutagenicity assay

For the antigenotoxic activity study both simultaneous and post-treatment were used. Similar procedure described above was used; for simultaneous treatment, three parts of suspension were mixed (cellular suspension 200 μL + lyophilized extract 100 μL + EMS 5mM, 100 μL) in independents tubes using 5, 50 and 100 ppm of lyophilized extract. After of 5, 10 and 15 h at 4 °C, the suspensions were washed 3 times with PBS and centrifuged at 3,000 rpm for 10 min. Later, the pellet was re suspended in 100 μL of PBS and 5 μL were mixed on agarose.

Post-treatment (**antimutagenicity activity**): three parts of suspension were used, each mixed in independents tubes (cellular suspension 200 μL + EMS 5mM, 100 μL, at 2 h) washed 3 times with PBS and centrifuged at 3,000 rpm for 10 min. Later, the pellet was re suspended in 200 μL of PBS. Next 100 μL of 5, 50 and 100 ppm of lyophilized extract of P. calyculatus was aggregated in independents tubes using. After of 5, 10 and 15 h at 4 °C, the suspensions were washed 3 times

with PBS and centrifuged at 3,000 rpm for 10 min. Later, the pellet was re suspended in 100 μL of PBS and 5 μL were mixed on agarose (Alvarez et al. 2001)

Singh et alalkaline's comet test was employed (1988). The slides were covered with 1 percent agarose with a Normal Melting Point (NMP) melting point, which was used to determine the melting point. Agar was allowed to firm before being removed, resulting in an absolutely clean surface after the process was completed. Following the pouring of 300 μl of 0.6 percent Low Melting Point (LMP) agarose over the slide, the slide was dried. On top of the first layer, 95 μl of LMP agarose at a concentration of 0.5 percent was mixed with 5 mL of the cellular solution and put immediately on top of it. At the end of the procedure, a third layer consisting of 100 μl of 0.5 percent LMP agarose was placed on top of the previous layer.

Sodium lauroylsarcosinate 1%, Triton X-100 1% and dimethyl sulfoxide 10% were used to lyse all slides for 48 hours at 4°C. The slides were placed in an electrophoresis buffer (NaOH 300 mM, EDTA 1 mM, pH 13) for 45 minutes at 4°C to allow the DNA to unfurl. Electrophoresis was performed for 10 minutes at 1.0 V/cm and 300 mA. (Labconco, model 4333280). The slides were then cleaned with water and stained for 5 minutes with 0.1 ml ethidium bromide (20 μl / 1 ml). On the fluorescent microscope, the cover slip was placed over the gel after five washes (Zeiss, model Axioskop 40, excitation 515-560 nm). The evaluation was based on the tail length measurements (Comet assay system II). This was done twice.

2.9. Data analysis

The results were then subjected to one-way ANOVA in MINITAB 19. It was utilised to compare the data from both slides of human cells treated to various doses of *P. calyculatus* extracts. Each concentration used 100 lymphocytes. Statisticians use p0.05 as a cut-off.

3. RESULTS AND DISCUSSION

3.1. Polyphenol, flavonoids and anthocyanins content

On a dry basis, *P. calyculatus* aqueous extract shown acceptable amounts of polyphenols and flavonoids; however, the anthocyanin content was lowest (Table 1).

Table 1
Polyphenol, flavonoid, and anthocyanins content, on a dry weight basis, in *P. calyculatus* aqueous extract.

Polyphenols (mg GAE g^{-1})	Flavonoids (mg CE g^{-1})	Anthocyanins (mg Cy-3-glc E g^{-1})
73.54 ± 1.11	39.37 ± 2.15	0.10 ± 0.01

GAE = Gallic Acid Equivalents; CE = catechin equivalents; Cy-3-glc E = cyanidin-3-glucoside
Values represent the mean and standard deviation (±) of three determinations.

Ibarra-Alvarado et al. (2010) documented the use of *P. calyculatus* for the treatment of cardiovascular diseases as well as the polyphenols and flavonoid contents in a stem, leaves, and flowers water extract. The polyphenol content in their lyophilized extract was about three times higher than the levels in our leaves extract (253.1 ± 16.8 vs. 73.54 ± 1.11 mg GAE/ g). In contrast, the flavonoid content was slightly lower (33.4 ± 0.8 mg CE/g) than the concentration we found (39.37 ± 2.15 mg CE/ g). Besides, the authors reported an antioxidant activity (IC_{50}) with DPPH of 251.8 μg/ mL; although we did not quantify our extract antioxidant activity, it would be interesting to measure it in a later study. On the other hand, the polyphenols content in our study is lower than that of other medicinal plants used in Mexico, including *Ziziphus amolle* (Sessé and Moc.) MCJohnst., *Annona squamosa* L, *Gliricidia sepium* (Jacq.) Kunth ex Walp, *Bursera grandifolia* (Schltdl.) Engl., *Comocladia engleriana* Loesen, and *Licania arborea* Seem, (85 to 270 mg GAE/g of sample) (Ruiz-Terán et al., 2008). However, the flavonoid content in mistletoe leaves is higher (0.25 to 0.45 mg/CE g) that reported in commercial medicinal plants such as lemon balm (*Melissa officinalis*), sage (*Salvia officinalis*), and mint (*Menta piperita*) (Atanassova et al., 2011).

3.2. Genotoxic activity

The genotoxic activity of lyophilized extracts of *P. calyculatus* is depicted in Figure 1. There was no evidence of significant genotoxic action except at a dose of 100 ppm for ten hours of exposure ($p < 0.05$). Tail length was significantly decreased ($p < 0.05$) in lymphocytes exposed to 50 ppm for 5 hours and 5, 50, and 100 ppm for 15 hours, comparing to the negative control.

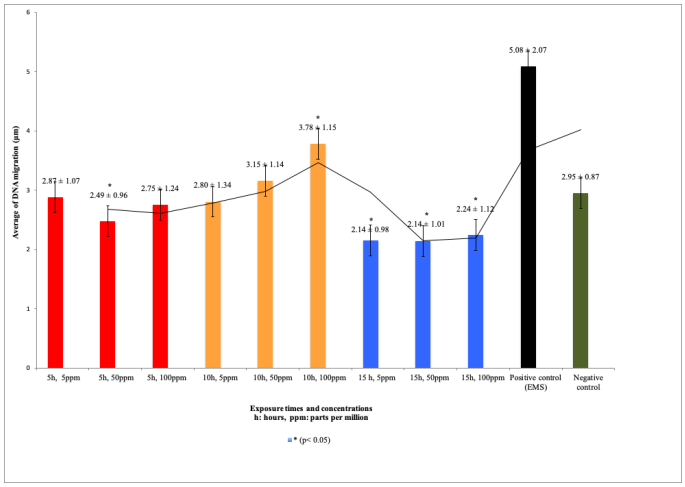

Figure 1. Genotoxic activity of extracts of *Psittacanthus calyculatus* in human lymphocytes exposed to different concentrations and times. * Indicates significant differences ($P < 0.05$).

Few scientific studies exist on the genotoxic activity and mutations resulting from exposure to medicinal plants consumed by the population; such mutations could lead to genetic instability (Klein & King, 2007) and are precursors to carcinogenesis (De-Flora & Ferguson, 2005; Hussain et al., 2003). As a result, it is critical to evaluate the genotoxic potential of historically used therapeutic herbs (Avila-Acevedo et al., 2012), ideally using different bioassay systems (Zúñiga, 2013).

In our study, the partial absence of genotoxic activity of the extract in human lymphocytes agrees with that reported

in mouse micronuclei (Avila-Acevedo et al., 2012). However, increasing the dose and exposure time cause genetic damage. Previously, N. Varela et al. (2007) demonstrated that flavonoids such as quercetin, which are present in *P. calyculatus* water extracts, acted as pro-oxidant flavonoids and genotoxic agent at high concentrations. These findings may be related to previous results, which indicated that a high ingestion of phytochemicals could lead to genotoxicity (Klein & King, 2007).

Another change was observed at 15 h of exposure for all concentrations tested: genetic damage significantly decreased ($p \leq 0.05$) compared to the negative control indicating a protective effect, even against of basal genetic damage.

3.3. Antigenotoxicity and antimutagenic activities

In the study of antigenotoxicity using simultaneous treatment, significant decrease ($p < 0.05$) in tail length means relative to both controls showed a strong antigenotoxic effect (Figure 2) regardless of the concentration of the extract. The average migration observed between concentrations 5, 50 and 100 ppm no showed significant differences.

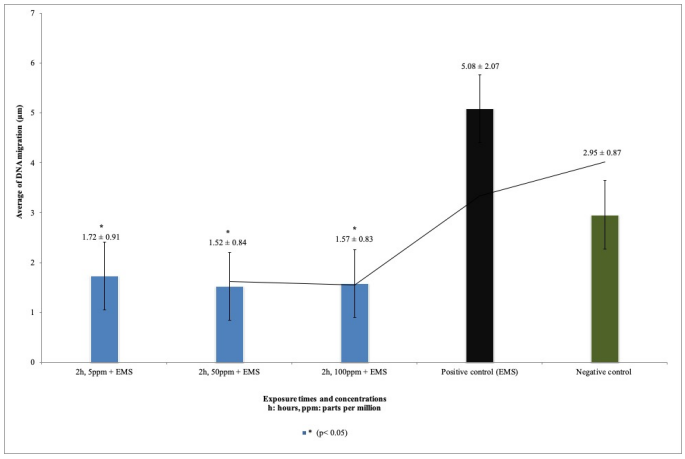

Figure 2. Antigenotoxic activity of *Psittacanthus calyculatus*. Human lymphocytes exposed simultaneously to different concentrations of lyophilized extracts and EMS during 2 hours (EMS). * Indicates significant differences ($P < 0.05$).

Post-treatment study. The tail length means at 3 h were very similar to observed in genotoxicity study at 15 h independently of concentration; tail length mean is reduced respect to negative control but doesn't do it in same magnitude than at 3 h. A light relationship doses-response was observed in the treatment for 2 h (Figure 3).

Moreover, compounds that are able to enhance either DNA repair or the elimination of genotoxicity damaged cells from tissue/cell populations are very important in the investigation of antimutagenic agents and compounds that are able to lower the frequency or rate of mutations (Azqueta & Collins, 2016; De-Flora, 1998).

Apparently, little time and low concentrations do not induce genetic damage. Longer exposure times decrease genetic damage, suggesting that the time increment prevents DNA

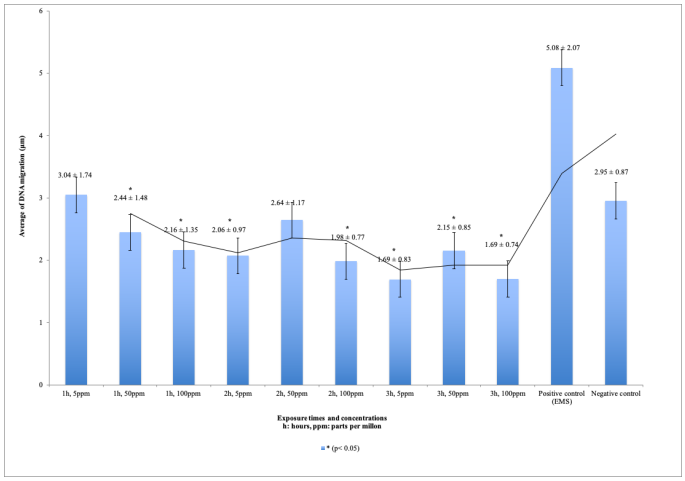

Figure 3. Antimutagenic activity of *Psittacanthus calyculatus* extracts. Human lymphocytes were first exposed to EMS for 2 hours and then (after washing) at different times and concentrations of the extracts. * Indicates significant differences ($P < 0.05$).

damage or promotes DNA repair (Fedel-Miyasato et al., 2014; Pérez, 2003). The behaviour suggests that increment of both concentration of extract and time exposition affect to tail length mean following a normal distribution. Possibly times exposition increases the DNA protection in lymphocytes probably due to the optimization of the enzimatic antioxidant defence: glutathione and glutathione S-transferase (Pérez, 2003). The optimization of the functions of the antioxidant defence system could also be related to the modification of the biological antitumor and immunomodulatory responses of mistletoe extracts earlier reported (Liu et al., 2012; B.G. Varela et al., 2001).

The decrease in basal genetic damage observed at 15 h (was also observed in the antigenotoxicity assay) in both, simultaneous and post-treatment. In simultaneous treatment, all concentrations of P. calyculatus extract neutralized chemically the mutagenic effect induced by the EMS regardless of dose, although, the maximum exposition time was 2 h. This time it no possible enhancement of the DNA protection system in lymphocytes (Pérez, 2003). Using post-treatment and 15 h exposition all the extract concentrations reversed the genetic damage caused by EMS and with a slight dose-response relationship it was observed, but only at 15 h the genetic damage was below the baseline level (negative control). The above indicates the importance of time for lymphocytes to increase their protection system and possibility increased efficiency of DNA repair (Fedel-Miyasato et al., 2014; Kada & Shimoi, 1987; Pesarini et al., 2013).

Mistletoe extract are important sources of bioactive compounds (Rodriguez-Cruz et al., 2003). Presence of gallic acid and catechins (Fedel-Miyasato et al., 2014; Roy et al., 2003) and quercetina (Ceruks et al., 2007), bioactive compounds with antigenotoxic activity (Ibarra-Alvarado et al., 2010; Moustapha et al., 2011) has been reported. These compounds are probably responsible for the protective effect on DNA (Azqueta & Collins, 2016).

Although the resistance to DNA damage caused by genotoxic agents in cells previously exposed to epigallo catechin gallate (one of the bioactive compounds of P. calyculatus) is attributed to a chemopreventive effect (Roy et al., 2003), the mechanism of DNA protection is not clear; it may be due to chemical neutralization of genotoxic agents or due to an enhancement of the DNA protection system. In all cases, our data suggest that an increase in DNA protection in longer exposure times to lyophilized *P. calyculatus* clearly exists.

4. CONCLUSION

P. calyculatus leaf water extract is poor in anthocyanins, but rich in flavonoids as quercetin. This bioactive compound have been shown protects DNA against the effects of EMS; additionally, it is able to decrease basal levels of genetic damage and do so in two ways: a) chemical neutralization of the mutagen and b) enhancement of the DNA protection system and/or repairing genetic damage, these are clearly associated with the time of exposure to the extract of P. calyculatus. Mutagenic activity of *P. calyculatus* indicates interaction with DNA. Further studies should be performed on human health associated with the consumption of this plant.

CONFLICTS OF INTEREST

The authors state that they have no commercial or financial relationships that could create a conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. CAM: Material preparation and prepared the first manuscript draft, MARL: analysis and final writing, JFLG: collection and formal analysis, MRS: Data analysis and interpretation, and PMGL: data collection and critical revision of the article, all authors commented on and improved it. All authors read and approved the final version of the manuscript.

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