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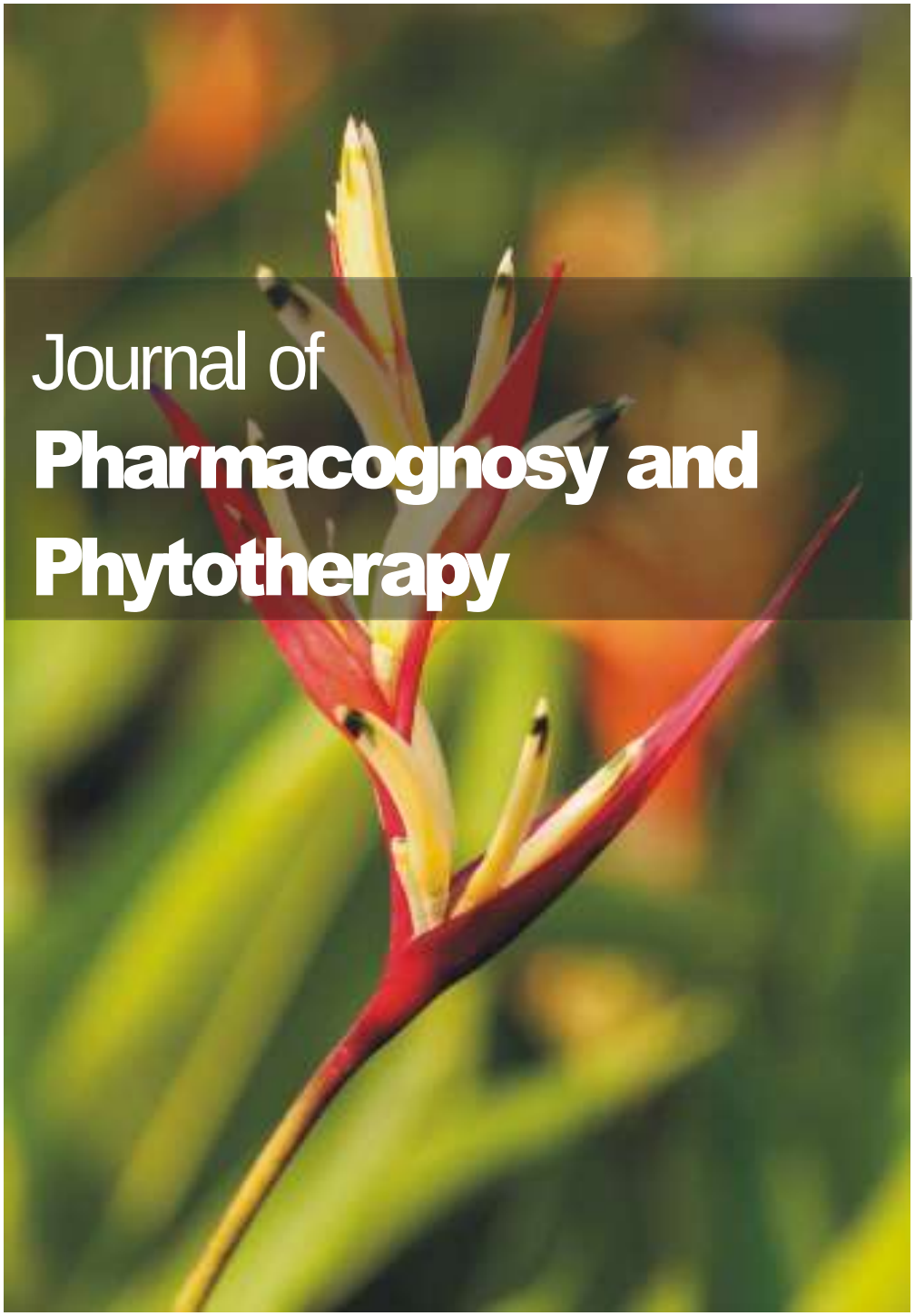

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Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

Clinopodium nubigenum (Kunth) Kuntze essential oil: Chemical composition, antioxidant activity, and antimicrobial test against respiratory pathogens

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The essential oil of leaves and flowers of *Clinopodium nubigenum* (Kunth.) Kuntze (Lamiaceae) collected in the province of Pichincha-Ecuador was steam distilled and analyzed by gas chromatography mass spectrometry (GC/MS) to determine its chemical composition. The majority of the compounds identified were carvacrol acetate (42.1%), carvacrol (20.6%), pulegone (6.3%) and thymol (5.5%). Antioxidant activity was assessed by the assays of diphenylpicrylhydrazyl (DPPH) (IC_{50}: 1.8 µl / ml), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (IC_{50}: 0.3 µl / ml) and β-carotene (IC_{50}: 0.031 µl / ml) compared to *Thymus vulgaris* and butylated hydroxyanisole (BHA) as referents. The specie also shows a promising medicinal potential exhibiting significant antibacterial activity at different concentrations against *Staphylococcus aureus* (2.5% v/v), *Streptococcus pyogenes* (0.6% v/v), *Streptococcus pneumoniae* (0.6% v/v) and *Streptococcus mutans* (0.6% v/v), suggesting an interesting natural alternative in the fight against bacteria that generate resistance to other antibiotics.

Key words: *Clinopodium nubigenum*, gas chromatography mass spectrometry (GC/MS), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), diphenylpicrylhydrazyl (DPPH), antimicrobial test.

INTRODUCTION

A minimal fraction of the known biodiversity has been sufficiently studied to know its properties and potentialities for the multiple benefits for humans (Estrella, 2005). The high biological and cultural diversity of the Ecuador have let it become one of the countries with great potential in terms of traditional therapeutics. Therefore, it is important to establish different aspects of importance, such as the methods of use of each plant and the curative benefits of traditional medicine to the different communities in the country (Zambrano et al., 2015). Moreover, if we consider Andean cultures to possess a wealth of knowledge about the use of plants and the quantity of species used, traditional medicinal practices could be more diverse than hitherto documented and published; Therefore, it is fundamental to continue with ethnobotanical studies that allow systematizing and disseminating this valuable knowledge (Ansaloni et al., 2010) in order to achieve greater use of both technical and economic resources, considering the traditional management them and the state of

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conservation (Ocampo, 1994).

According to Myers et al. (2000), the Tropical Andes is believed to contain at least 20,000 known plant endemics, and many more species, probably thousands, remain to be discovered there. However, it is important to mention that due to the increase in global climate change the native species that inhabit sensitive ecosystems such as those of the paramo are threatened because they will react by means of displacement, adaptation (either in terms of evolutionary changes or physiological adaptations) or local extinction of the species that form it, and locally these mechanisms could interact and lead to alterations of their compositions (Aguirre et al., 2014).

The *Clinopodium nubigenum* (Kunt) Kuntze may be found in this bio diverse zone. The genus *Clinopodium* belongs to the *Lamiaceae* family and comprises of 271 described species, with 142 being accepted as such. Within this group, *C. nubigenum* traditionally known as "sunfo" or "tipo de llano", is an aromatic medicinal plant native to Ecuador, which has been reported in the provinces of Carchi (Nudo del Boliche), Pichincha (Paso de Guamaní), Tungurahua (Páramo de Minza-Chica), Cañar (Páramo de Biblián), Azuay (Páramo de Tinajillas), El Oro (Páramo de Corredores) and Loja (Saraguro) (Epling and Jativa, 1964; Pulgar et al., 2010; Ansaloni et al., 2010; Missouri Botanical Garden, 2017).

It is an herbaceous plant that can reach approximately 15 cm in height and it is possible to identify it by its characteristic of being covered with small white hairs on its leaves, the stem is quadrangular and reddish brown color (Aguilar et al., 2009). This aromatic plant is also known with the synonyms of *Thymus nubigenus* Kunth, *Micromeria nubigena* (Kunth) Benth, and *Satureja nubigena* (Kunth) Briq (Gilardoni et al., 2011). According to Cantino and Wagstaff (1998), after making a generae reassessment, based on molecular data and some herbarium studies, they recommend that the genera *Satureja* and *Micromeria* should be considered in a narrow sense and restricted way for the Old World, while most of the specimens of the New World form a clade including the genera *Clinopodium* and *Calamintha*, all in order to group and facilitate further studies given their similarities.

Aiming at its medicinal usefulness, there are several reports of use as a hot infusion of flowers and leaves with anti-inflammatory, stomachal, anti-influenza and anti-infective activity against dysentery and attenuating menstrual syndromes (Gilardoni et al., 2011). They offer relief of general malaise and to counteract the cold (Ansaloni et al., 2010). Their use is also cited to prevent urinary incontinence in children (de la Torre et al., 2008). The study of Lituma and Molina (2008) is of the opinión that the “sunfo” has analgesic activity. Another study by Jerves-Andrade et al. (2014) details the ethnopharmaceutical uses for stomach conditions and gastritis. The species in question denotes a promising medicinal potential, and the aim of this study was to evaluate and elucidate the chemical composition, and its antioxidant and antimicrobial activities.

MATERIALS AND METHODS

Plant material and essential oil distillation

The plant material studied was collected from the paramos of the parish of Píntag (27.5 Km S.E. de Quito) in the province of Pichincha, Ecuador. The plant was identified as *C. nubigenum* (Kunth.) Kuntze by the National Herbarium of Ecuador. In order to obtain the essential oil, the vegetable sample of approximately 6 Kg was distilled by steam trapping in a distiller with a capacity of 40 L, the process took 5 h.

Essential oil characteristics

For characterization of the essential oil obtained, the percentage of yield and the different organoleptic (odour, colour, taste) and physicochemical parameters were gotten (density, refractive index, pH) at 20°C.

Gas chromatography mass spectrometry (GC/MS) analysis

The sample analyzed by GC/MS was prepared by dissolving 10 µl of essential oil in 1 ml of dichloromethane, the volume of injection was 2 µl. The analysis was carried out on a Varian 3900 chromatograph, a Factor Four® column (5% phenyl-95% dimethylpolysiloxane 30 mx 0.25 µm) and helium carrier gas was used at a flow rate of 1 ml/min with a Split at 1:50, oven programming is shown in Table 1. In the Varian Saturn 2100 mass spectrometer, the conditions were set to a current emission of 10 µAmp, ionization voltage 70 eV, mass range 35 to 400 Da, scanning speed 1 scan/min, trap temperature 220°C and temperature transfer line 260°C. The total GC-MS analysis time was 90 min. The chemical identification of the essential oil was done by comparing the mass spectra, using the commercial database of chemical compounds of the National Institute Standard and Technology NIST. In addition, experimental lineal retention indexes were determined in relation to the retention times of a series of C_8 to C_{20} alkanes, later compared to the theoretical retention indexes of Adams (2007).

DPPH and ABTS assays

The methods of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) developed by Brand-Williams et al. (1995) modified by Noriega et al. (2015) as well as the method with 2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) used by Kuskoski et al. (2004) were used. For both evaluations, the essential oil of *T. vulgaris* was a natural reference and as a positive control for Butylhydroxyanisole (BHA).

The DPPH reagent was prepared by dissolving 19.6 mg DPPH in 500 ml of 96% ethanol. For the positive control, BHA dilutions were performed to which 2.9 ml of DPPH reagent were added, the samples were prepared by taking 20 µl of the oils dissolved in 180 µl of dimethyl-sulfoxide (DMSO), then to prepare a range of concentrations to which 2.9 ml of reagent were added DPPH. Both the control dilutions and the oil samples were stirred for 30 min. Subsequently, the absorbances were measured at ʎ 517 nm, 96% ethanol was used as a blank.

For the preparation of the ABTS reagent, solution A (27 mg ABTS in 25 ml distillated H_2O) was prepared, to which 250 µl of

Table 1. GC-MS oven program.

Initial temperature (°C)	Increase (°C/min)	Time (min)	Final temperature (°C)
50	1	50	100
100	5	30	250
250	-	10	250

solution B (188.2 mg $K_2S_2O_8$ in 10 ml distillated H_2O) was added, subsequently after 24 h the reagent was adjusted with 96% ethanol to obtain an absorbance of 0.7±0.02 at ʎ 754 nm. A series of BHA dilutions were prepared to which 0.9 ml of ABTS reagent was added. Samples were prepared by taking 4 µl of the essential oils dissolved in 196 µl of DMSO. To prepare a series of concentrations, 0.9 ml of ABTS reagent was added. Absorbances were measured at ʎ 754 nm, 96% ethanol was used as a blank. For the calculation of the percentage of inhibition of the free radical DPPH and ABTS, the following formula was used:

$$Inhibition\ \% = \frac{A - B}{A} \times 100$$

Where:

A is equal to the absorbance of the blank, and B is the absorbance of the sample.

β-carotene assay

The β-carotene test was performed based on the method developed by Miller (1971) with certain modifications in the concentrations. The essential oil of *T. vulgaris* was used as a natural reference and BHA as a positive control. An emulsion of β-carotene was prepared as follows:

β-carotene 4 ml of 1000 ppm solution in chloroform to which 400 µl of a solution of linoleic acid was added in 8 ml Tween 20®.

The chloroform was evaporated at 40°C for 15 min and flushed at 1 L. For the positive control, a series of 5 ml BHA dilutions were prepared, samples were made by dissolving 50 µl of essential oils in 1 ml Tween 20® to subsequently perform a series of concentrations by adding 5 ml of the β-carotene emulsion. A blank solution (20 µl Ac. 400 µl linoleic + Tween 20 + 50 ml H2O + 0.1 M Tris-HCl pH 7.4) was used. An absorbance reading was performed at ʎ 470 nm followed by a new reading after 60 min at 50°C. For the calculation of the antioxidant activity, the following formula was used:

$$Antioxidant\ activity\ \% = \frac{DRC - DRS}{A} \times 100$$

Where:

DRC is the percent degradation of the control and DRS corresponds to the percent degradation of the sample.

To determine the respective percentages of degradation, the following formula was applied:

$$DR = \frac{\ln(\frac{a}{b})}{60} \times 100$$

Where:

a is the initial absorbance and b the absorbance after 60 min at 50°C.

Antimicrobial susceptibility test

For the test of antimicrobial resistance, four certified strains ATCC (American Type Culture Collection) of Gram-positive bacteria were acquired: *Staphylococcus aureus* ATCC®: 25923™, *Streptococcus pyogenes* ATCC®: 19615™, *Streptococcus pneumoniae* ATCC®: 49619™ and *Streptococcus mutans* ATCC®:25175™. Their subsequent inoculation was carried out on tryptic soy agar medium (TSA) except for *S. pneumoniae* requiring lamb's blood agar, the necessary environmental conditions were followed for each microorganism (24 H, 37 °C); *S. aureus* in aerobiosis and *Streptococcus* strains in anaerobiosis. The inoculum was obtained in tryptic soybean broth (TSB) (18 H, 35°C), followed by the measurement of the absorbance at ʎ 625 nm of 0.08 to 0.11 for standardization of the initial inoculum. To test for microbial susceptibility, the Well Diffusion assay was used for which 1 ml of inoculum in plaque (TSA) was dispersed for the case of *S. aureus* and for the *Streptococcus* strains brain heart infusion plaques (BHI). In each plate, 4 wells of 6 mm φ were made, in which 0.8 µl of the different experimental materials were placed: the dilutions of the essential oil (2,5-1,25-0,6-0,3-0,15 % v/v) in DMSO as well as the positive control Penicillin (106 IU), and the negative control (DMSO). The plates were incubated at 37°C 24 h under the above-described conditions to finally carry out measurement of the inhibition halos.

RESULTS AND DISCUSSION

Essential oil characteristics

The oil characteristics obtained by distillation (Table 2) allow to establish differences between other oils. Those properties are subject of change, attributed to the environmental conditions, soil factors, life cycle of the specie and the extraction method employed. This could be affirmed considering for example the research lead by Ruiz et al. (2010), plants collected in a province from Ecuadorian south sierra region showed major yield (1.42%) although the physical properties were similar. Other characteristic is the pungent odor present in the oil according to Jyoti (2016), due to the presence of carvacrol a monoterpenoid phenol and its derivatives as it occurs in the *Origanum sp.*

Chemical composition of the oil

In the essential oil of *C. nubigenum*, 25 compounds were found, representing 98.09% of the essential oil

Table 2. Characteristics of *Clinopodium nubigenum* essential oil.

Parameter	Characteristic / Value	
Yield	0.27 % (p/p)	
Odor	Citrus pungent	
Color	Amber	
Taste	Bitter	
Density	0.91 g/cm^3	
Refraction index	1.479	20°C
pH	5.6	

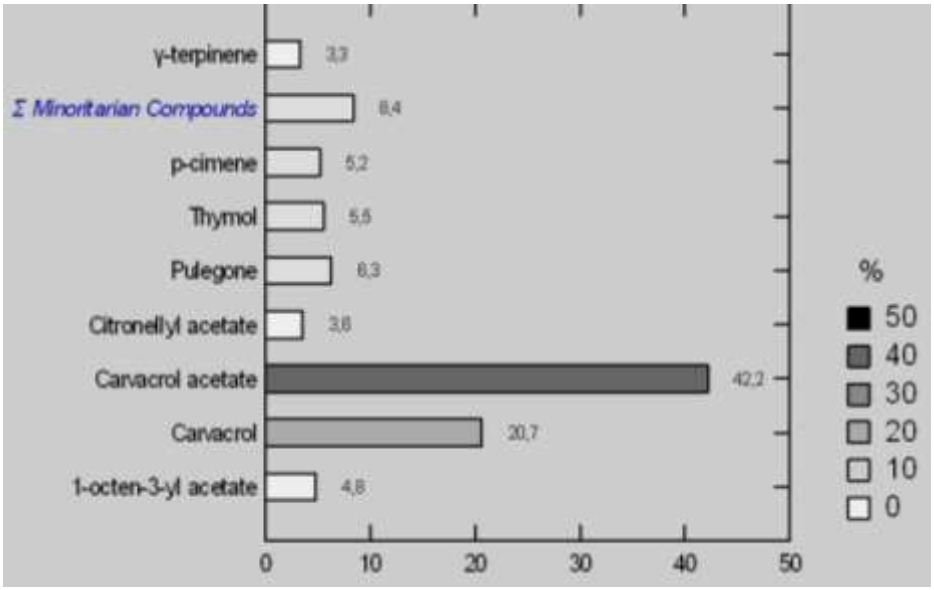


Figure 1. Amount of *C. nubigenum* (Kunth.) Kuntze essential oil main constituents.

composition (Figure 1). The preliminary GC/MS study (Table 3) reveals the presence of carvacrol acetate (40,95%), carvacrol (21,21%), pulegone (6,09%) and thymol (5,67%) as the main components. The chemical identification agrees with the research done by Ruiz et al. (2010), defining carvacrol acetate as the major component. Other studies have determined a significant difference in chemical composition, showing thymol and carvacrol as major components (El-Seedi et al., 2008), these changes in chemical composition could be attributed to climatic differences, soil composition, vegetative cycles, plant age and cultivation conditions (Gilardoni et al., 2011).

DPPH, ABTS and β-carotene assays

The capacity of the DPPH radical for the essential oil of *C. nubigenum* increased with the major concentration of essential oil in the prepared dilutions. The DPPH was scavenged by the antioxidant molecules forming the reduced form DPPH-H because of this reason, the color changes from purple to yellow in the reduction process quantified by the spectrophotometric method by decreasing the absorbance at 517 nm. The IC_{50} value for the DPPH radical assay (Figure 3) was 1,812±3,0 E-002 µl/ml, in contrast to the natural reference of *T. vulgaris* essential oil IC_{50} DPPH 0,759±1,0 E-002 µl/ml and BHA IC_{50} DPPH 5,2 E-003±1,3 E-005 µl/ml. However, the ABTS is radicalized in the presence of $K_2S_2O_8$ forming a blue-green compound which is decolorized by its reduction in the presence of the antioxidant molecules, a process quantified by spectrophotometry. In this way, the IC_{50} for ABTS was 0,3375±9.5 E-004 µl/ml in contrast to *T. vulgaris* IC_{50} ABTS 0,2107±3.3 E-004 µl/ml and BHA IC_{50} ABTS 1,22 E-003±4,0 E-005 µl/ml (Figure 3). In the third antioxidant test, the technique is based on oxidative discoloration of β-carotene in the presence of linoleic acid. Discoloration occurs when β-carotene reacts with the free radicals generated by linoleic acid. The presence

Table 3. Preliminary chemical composition of *Clinopodium nubigenum* essential oil analyzed by GC/MS and reported bioactivity of its phytochemical compounds.

S/N	Compound name	LRI[a]	LRI[b]	Preliminary identification	Relative composition (%)	Reported bioactivity	References
1	Tricyclene	916	926	RT, MS 70 eV, Adams and NIST databases	0.35	-	-
2	α-thujene	922	930	RT, MS 70 eV, Adams and NIST databases	0.59	-	-
3	α-terpinene	1014	1017	RT, MS 70 eV, Adams and NIST databases	0.33	-	-
4	p-cimene	1022	1024	RT, MS 70 eV, Adams and NIST Databases	3.80	Antifungal	Ksouri et al. (2017)
5	Limonene	1025	1029	RT, MS 70 eV, Adams and NIST databases	0.67	-	-
6	B-ocimene trans	1052	1050	RT, MS 70 eV, Adams and NIST databases	1.31	-	
7	γ-terpinene	1054	1059	RT, MS 70 eV, Adams and NIST databases	2.43	Anti-tumor and prooxidative, anti-inflammatory	Özkan and Erdoğan (2017); Leelarungrayub et al. (2017)
8	1-octen-3-yl acetate	1110	1112	RT, MS 70 eV, Adams and NIST databases	4,28	Repellent, insecticide, fungistatic, acaricide	Kihara et al. (2014); Re et al. (1999); Yang and Lee (2012)
9	Pulegone	1236	1237	RT, MS 70 eV, Adams and NIST databases	6.09	Antifungal	Ebadollahi et al. (2017)
10	Thymol	1298	1290	RT, MS 70 eV, Adams and NIST databases	5.67	*Antimicrobial*, anti-dermatophyte, anti-cancer, *antioxidant*	Ruiz-Rico et al. (2017); Mahboubi et al. (2017); Li et al. (2017); Llana-Ruiz-Cabello et al. (2015)
11	Carvacrol	1308	1299	RT, MS 70 eV, Adams and NIST databases	21.21	*Antibacterial*, neuroprotector, antifungal, herbicide	Engel et al. (2017); Dati et al. (2017); Hosseini et al. (2016); Hazrati et al. (2017)
12	δ-elemene	1333	1338	RT, MS 70 eV, Adams and NIST databases	0.96	-	-
13	Thymol acetate	1359	1352	RT, MS 70 eV, Adams and NIST databases	0.60	-	-
14	Citronellyl acetate	1364	1352	RT, MS 70 eV, Adams and NIST databases	3.54	-	-
15	Carvacrol acetate	1377	1372	RT, MS 70 eV, Adams and NIST databases	40.95	*Antibacterial*, anthelmintic, *antioxidant*, anti-inflammatory	Andre et al. (2016); Pires et al. (2014); Damasceno et al. (2014)
16	β-cubebene	1387	1388	RT, MS 70 eV, Adams and NIST databases	0.15	-	-
17	β-elemene	1388	1390	RT, MS 70 eV, Adams and NIST databases	0.42	-	-
18	β-caryophyllene	1411	1419	RT, MS 70 eV, Adams and NIST databases	0.35	-	-
19	β-Gurjunene	1423	1433	RT, MS 70 eV, Adams and NIST databases	0.21	-	-
20	α-humulene	1451	1454	RT, MS 70 eV, Adams and NIST databases	0,14	-	-
21	γ-muurolene	1477	1479	RT, MS 70 eV, Adams and NIST databases	0,31	-	-
22	Bicyclogermacrene	1491	1500	RT, MS 70 eV, Adams and NIST databases	1,85	Anti-inflammatory, anticancer	Morshedloo et al. (2017)
23	δ-amorphene	1504	1512	RT, MS 70 eV, Adams and NIST databases	0.13		
24	δ-cadinene	1513	1516	RT, MS 70 eV, Adams and NIST databases	1,32	Acaricide	Guo et al. (2017)
25	Spathulenol	1579	1578	RT, MS 70 eV, Adams and NIST databases	0.44	-	-
	Total				98.09	-	-

LRI[a] Lineal experimental retention index calculated in comparison to alkane series (C_8 to C_{20}) retention time. LRI[b] Theoretical Retention Index (Adams, 2007)..

of antioxidant substances prevents oxidative discoloration of the emulsion by the neutralization of free radicals. The antioxidant activity evaluated with the β-carotene bleaching assay obtained an IC_{50} de 0,031±3,0 E-003 µl/ml in contrast to the IC_{50} 0,022±1,0 E-003 µl/ml of *T. vulgaris* and IC_{50} 7,479 E-006±4,8 E-006 µl/ml of BHA. The results of this study show that the antioxidant activity is comparable to the natural reference *T. vulgaris*. If we review the data in Table 3, it can be deduced that within the preliminary chemical compounds of the essential oil we found limonene, δ-elemene, γ-murolene, carvacrol acetate y thymol as the possible antioxidant agents about which there is a reference to their activity with both thymol and

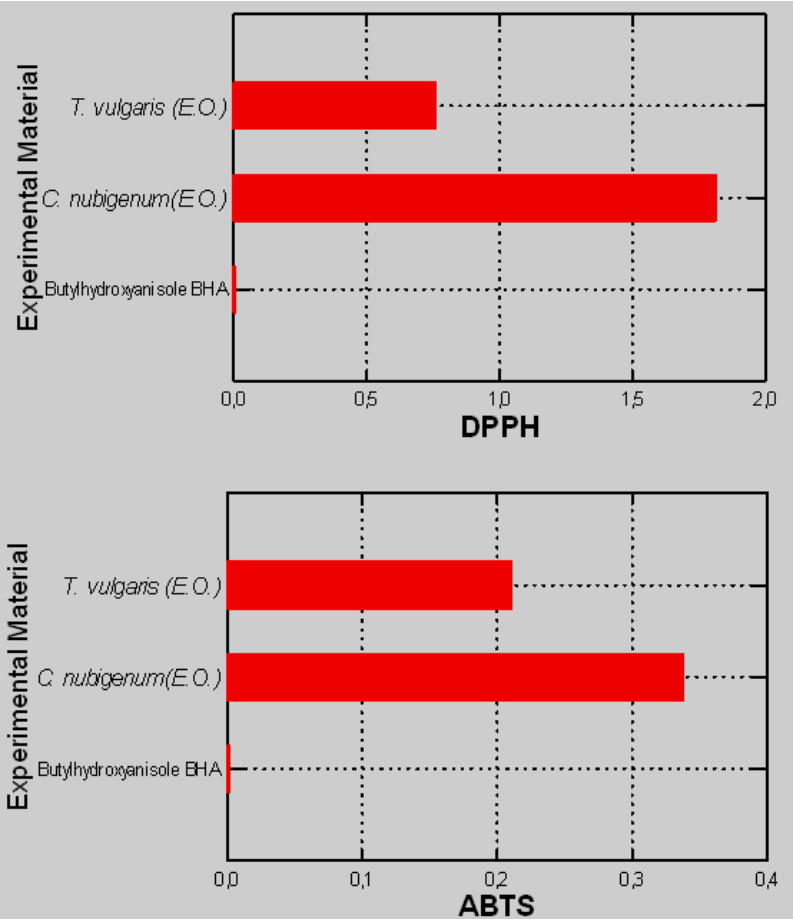


Figure 2. Free radical DPPH and ABTS scavenging activity. Comparison of IC_{50} mean between *Thymus vulgaris*, *Clinopodium nubigenum* and BHA control using both DPPH and ABTS methods.

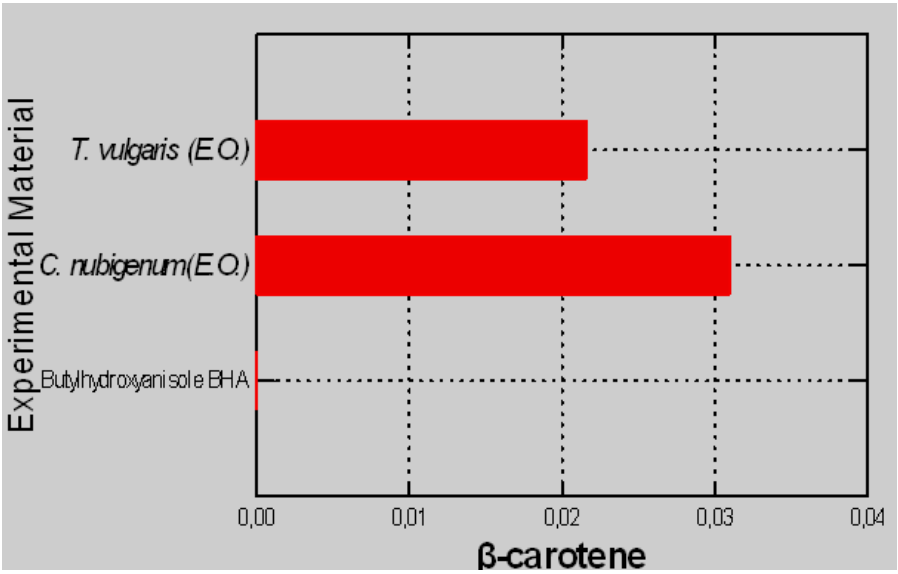
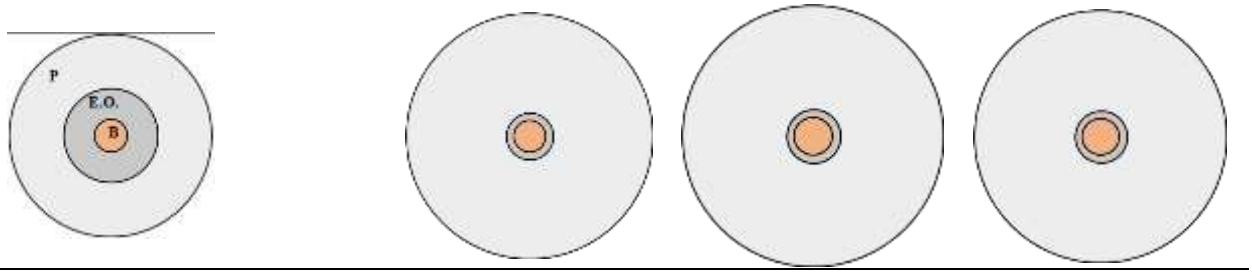


Figure 3. Inhibitory concentration (IC_{50}) results applying β-carotene antioxidant activity test.

Table 4. Comparative data between inhibition halos developed by Penicillin and *Clinopodium nubigenum* essential oil.

Variable	Microorganism			
	S. aureus	***S. mutans***	***S. pyogenes***	***S. pneumoniae***
Essential oil concentration to show m.o. inhibition	2.5%	0.3%	0.3%	0.3%
Essential oil halo diameter	12.68 mm	8.80 mm	8.46 mm	8.46 mm
Penicillium halo diameter	40.00 mm	43.58 mm	41.53 mm	40.51 mm
DMSO halo diameter			6.00 mm	
Well diameter			6.00 mm	
				

P: Penicillin halo (Positive control: 10^6 I.U.); E.O.: *Clinopodium nubigenum* essential oil halo; B: Initial well diameter equal to DMSO (Negative Control).

carvacrol acetate being two major compounds. However, all components can act synergistically, since from a chemical point of view essential oils are very complex matrixes and it is difficult to attribute their antioxidant activity to one or a few compounds (Shakeri et al., 2017), although generally the majority of those being responsible for the biological effects (Ksouri et al., 2017).

Antimicrobial susceptibility test

Statistical analysis showed that the antimicrobial results presented activity at different concentrations of essential oil (Table 4) 2.5% for *S. aureus* and 0.3% for *S. mutans, S. pyogenes and S. pneumoniae*. These results are in line with El-Seedi et al. (2008), presuming that this activity is mainly due to the presence of carvacrol. According to the study of Magi et al. (2015), this compound exerts a direct bactericidal activity causing damage to the bacterial cell membrane. In addition, it was shown that carvacrol is not prone to generate bacterial resistance in some species of *Streptococcus* and could exhibit a synergistic behavior in combination with other antibiotics such as erythromycin. However, the chemical composition also highlights thymol, carvacrol acetate and limonene that can accentuate this activity.

Conclusion

It was evidenced that the essential oil of *C. nubigenum* (Kunth.) Kuntze showed a significant antioxidant activity possibly attributed to the presence of one of its major components, carvacrol acetate, in addition to other compounds such as thymol, limonene, δ-elemene and γ-murolene, for this reason, it is interesting to know the relationship between compounds that potentiate this effect. Regarding microbial activity, the effect for all pathogens tested was observed, being more effective against *S. mutans*, *S. pyogenes*, *S. pneumoniae* and less so for *S. aureus* strains. The compounds carvacrol and carvacrol acetate are those of greater presence in the essential oil and bibliographically can be indicated as the main antimicrobial agents. These data suggest an interesting natural alternative in the fight against bacteria that generate resistance to other antibiotics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Anti-inflammatory property of the methanol leaf extract of *Parinari kerstingii* (ENGL) in rats

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The anti-inflammatory effect of the extract of *Parinari kerstingii* leaves was investigated using egg albumin-induced rat paw oedema, leukocyte mobilization, and acetic acid-induced vascular permeability assay methods. Heat and hypotonicity-induced heamolysis of human red blood cell membrane were also used to assess its membrane stabilizing effect and to determine its inhibitory property on phospholipase A_2 activity. Acute toxicity and phytochemical tests were also determined using standard methods. The methanol crude extract (MCE) of *P. kerstingii* reduced ($p<0.05$) the acetic acid-induced vascular permeability and increased agar-induced leukocyte mobilization in rats dose-dependently. Vascular permeability was inhibited by 24.85, 26.04 and 48.52% with 100, 200 and 400 mg/kg of the MCE, respectively. The total leukocyte count of the treated groups increased significantly ($p<0.05$) relative to the control group. The percentage membrane stability exhibited by the MCE was comparable with drug control, indomethacin. The MCE contains principles that protected the erythrocyte membranes effectively. More so, the extract inhibited ($p<0.05$) the activities of phospholipase A_2 and showed no significant difference in the phospholipase A_2 inhibitory effect as compared to the standard drug, prednisolone. The extract showed no toxicity at 5000 mg/kg. Phytochemical screenings revealed the presence of tannins, saponins, reducing sugars, phenols, soluble carbohydrates, alkaloids, terpenoids, steroids, hydrogen cyanide glycosides and flavonoids. This study indicated that the MCE of *P. kerstingii* leaf is relatively safe for consumption and has anti-inflammatory property. Also, it could prevent the haemolysis of human erythrocyte membrane.

Key words: Anti-inflammatory, *Parinari kerstingii,* acute toxicity, phytochemicals, membrane stability.

INTRODUCTION

Inflammation is a complex physiological response of vascular tissues to harmful stimuli and the body's protective mechanism to eradicate noxious stimuli and hence promote tissue repairs (Ferrero-Miliani et al., 2007). Activation of inflammation results in the generation of inflammatory mediators such as histamine, kinin,

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and prostaglandin leading to increased blood flow, leakage of plasma proteins and fluids, and migration of neutrophils from blood vessels into sicken tissues (Chaitanya et al., 2011). Chronic inflammation has become the major challenge in the world today and its implications in virtually all human diseases have motivated a global scientific research (Morales et al., 2014). For instance, a lot of inflammatory processes have been revealed to induce insulin resistance, reduce insulin secretion and dysfunction of β-cells (Akash et al., 2013; Uzayisenga et al., 2014). Several herbal drugs contain certain principles with the ability to stabilize biological membranes when exposed to induced lyses (Oyedapo et al., 1997, 2004).

Parinari kerstingii Engl. is an evergreen plant of the genus *Parinari* and family Rosaceae growing up to the height of 20 m with ovoid shaped fruit and is widely distributed in tropical West Africa (Burkill, 1997). It is variously called aramon (Ivory Coast), kakyiki (Ghana), ningelia (Togo), okpe (*Yoruba-Ife* of Togo), and ƙaiƙayi (Nigeria: Hausa). The plant is sometimes harvested from the wild for local medicinal use. It is used traditionally, for the treatment of bronchopneumonia and feverish pains and also serves as emetic and purgative agent (Burkill, 1997). Presently, there is no scientific report on the folkloric use of *P. kerstingii,* thus this research documented the observed anti-inflammatory effect of the MCE of the leaves of the plant in rats.

MATERIALS AND METHODS

All chemicals used in this study were acquired from Sigma Aldrich, Germany.

Plant

Fresh leaf samples of *P. kerstingii* were obtained from Otuku town of Nsukka Local Government Area, Enugu State, Nigeria. These were authenticated by Mr. A. Ozioko of the Bioresources and Development Centre Programme (BDCP), Nsukka. A voucher specimen (Interceded 0615) was deposited at the same centre. The leaves were air-dried and ground using a mechanical grinder. The resulting powdered crude drug (5000 g) was macerated in methanol for 24 h, filtered with a Whatmann No. 1 filter paper and then concentrated using a rotary evaporator (IKA, Germany) at an optimum temperature of 40 to 50°C.

Animals

Swiss albino mice (18 to 28 g) and Wistar rats (110 to 170 g) of both sexes were gotten from the animal house in the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. They were acclimatized to laboratory condition in a seven day period; water and growers mash (Niger Feeds, Nigeria) was given *ad libitum*. Ethical approval on the use of laboratory animals was obtained from the committee of University of Nigeria on the care and use of laboratory animals, in accordance to the revised National Institute of Health Guide for Care and Use of Laboratory Animal (Pub No.85-23, revised 1985).

Phytochemical analysis

Standard qualitative test (Trease and Evans, 2008) was carried out for the detection of alkaloids, glycosides, steroids, terpenoids, flavonoids, tannins, reducing sugars, soluble carbohydrates, resins and saponins.

Acute toxicity test

The method according to Lorke (1983) was used for the study, which was in two phases. Firstly, twelve mice of were grouped into four: Group 1 was not fed with any drug and used as vehicular control. Groups 2, 3, and 4 received 10, 100, and 1000 mg/kg extracts orally in that order. The mice were then observed under 24 h for irregularity in behavior and/or death. The second phase was similarly designed but group 2 mice were administered 1500 mg/kg of the extract while groups 3 and 4 were given the crude extract at 2900 and 5000 mg/kg, respectively. These were also similarly observed for signs of toxicity, and the lethality dose (LD_{50}) was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose or the geometric mean of consecutive doses for which 0 and 100% survival rates were recorded.

Egg albumin-induced paw oedema in rats

The procedure by Niemegeer et al. (1964) was adopted. The Wistar rats were grouped into 5, of 5 rats each. They were respectively administered via oral route, distilled water (5 ml/kg) and the crude extract (at 100, 200 and 400 mg/kg). The last group (5) was given 10 mg/kg indomethacin. After 30 min, 0.1 ml of freshly prepared egg albumin was injected into the sub plantar region of the left hind paw of each rat. The paw diameter was measured with the aid of vernier caliper at 0, 1, 2, 3, and 4 h after the injection of egg albumin.

Assay for membrane stabilization

P. kerstingii crude extract was screened for inhibition of haemolysis of HRBC induced by heat; hypotonic solution (distilled water) was evaluated (Shinde et al., 1999) with some modifications.

Heat induced haemolysis

The MCE of the study plant was dissolved in an isotonic phosphate buffer solution. Five centrifuge tubes were each loaded with 5 ml of the extracts in the respective doses of 100, 200, 400, 800, and 1000 µg/ml and arranged in sets of 4 per dose. Two sets of control tubes contained 5 ml of the vehicle and 5 ml of 200 µg/ml of indomethacin, respectively. A 0.1 ml HRBC suspension was gently mixed with the content of each tube. A regulated water bath at 54°C was used to incubate one pair of the tubes, while a second pair was maintained at -10°C in a freezer for 20 min. Next, the tubes were centrifuged at 1300 g for 3 min, followed by the estimation of the haemoglobin content of the supernatant using a Spectronic Spectrophotometer (21D Milton Roy) at 540 nm. Percent inhibition of haemolysis was calculated, viz.:

$$\% \text{ Inhibition of hemolysis} = 1 - \frac{OD2 - OD1}{OD3 - OD1} \times 100$$

where OD1 = absorbance of test sample unheated, OD2 = absorbance of test sample heated, and OD3 = absorbance of control sample heated.

Hypotonicity induced haemolysis

The MCE was reconstituted in distilled water to form a hypotonic solution. Similarly, 5 ml of the graded doses of the extracts (100, 200, 400, 600 and 800 µg/ml), in centrifuge tubes were grouped in pairs per dose. An isotonic solution containing 5 ml of the MCE in the dose of 100 to 1000 µg/ml was also similarly paired (per dose). A 5 ml of the distilled water and another 5 ml of indomethacin (200 µg/ml) were loaded into separate capillary tubes as vehicle and drug control, respectively. A 0.1 ml of erythrocyte suspension was added to each of the tubes and mixed gently. The tubes and contents were then incubated for 1 h at 37°C, and centrifuged for 3 min at 1300 g. Absorbance (OD) of the haemoglobin content of the supernatant was estimated at 540 nm using the Spectronic spectrophotometer. The percentage heamolysis was thus determined by the mathematical expression:

$$\% \text{ Inhibition of haemolysis} = 1 - \frac{OD2 - OD1}{OD3 - OD1} \text{ X } 100$$

where OD1 = absorbance of isotonic test solution, OD2 = absorbance of hypotonic test samples, and OD3 = absorbance of control hypotonic solution. Heamolysis produced in distilled water was taken to be 100%.

Phospholipase A_2 activity test

The phospholipase A_2 was prepared from *Aspergillus niger* and was used to evaluate the effect of the methanol crude extract (MCE) on its activity (Vane, 1971).

Preparation of enzyme

Nutrient agar plates were prepared by dissolving 28.0 g of nutrient agar in 1000 ml of distilled water. The nutrient agar solution was homogenized in a water bath, 100°C, for 10 min and dispensed into sterilized conical flasks. The agar was autoclaved at 121°C for 15 min and then distributed into sterilized petri dishes. *Aspergillus niger* was then inoculated on the plates and left to grow for 3 days. The nutrient broth was prepared by dissolving 15 g of Sabouraud dextrose agar in 1000 ml of distilled water, homogenized in a water bath for 10 min and dispensed into 250 ml conical flasks. The conical flasks were sealed with cotton wool and foil paper. The broth was then autoclaved at 121°C for 15 min. The broth was allowed to cool to room temperature and then the organism in the Petri dishes were aseptically inoculated into the broth and incubated for 72 h at room temperature. The culture was transferred into test tubes containing 3 ml of phosphate-buffered saline, and centrifuged at 3000 g for 10 min. The supernatant was used as the crude enzyme preparation. 5 ml of blood was drawn out from a healthy volunteer and dispensed into plastic tubes containing 0.01 ml of 1% EDTA as an anticoagulant. They were also similarly centrifuged and the supernatant (plasma) was discarded. The erythrocytes were re-suspended in a volume of normal saline and plasma (1:1) and centrifuged to discard the supernatant so formed. This solution of red cells was reformed to 40% (v/v) suspension with phosphate buffered saline and this served as the substrate for phospholipase. 0.2 ml of $CaCl_2$ (2 mM), 0.2 ml of human red blood cell (HRBC), 0.2 ml of the crude enzyme preparation and varying concentrations of normal saline and the test sample or reference drug were incubated in test-tubes for 1 h. The control contained the human red blood cell suspension, $CaCl_2$ and free enzyme. The blanks were treated with 0.2 ml of boiled enzyme separately. The incubation reaction mixtures were centrifuged at a speed of 3000 g for 10 min. Samples of the supernatant (1.5 ml) were diluted with 10 ml of normal saline and the absorbance of the solutions read at 418 nm. Prednisolone was used as the reference drug. The percentage of maximum enzyme activity and percentage inhibition was determined as follows:

$$\% \text{ Maximum enzyme activity} = \frac{OD \text{ of test}}{OD \text{ of control}} \times 100$$

$$\% \text{ Inhibition} = 100 - \% \text{ maximum activity of the enzyme}$$

Leukocyte mobilization test in rats

The method outlined by Ribeiro et al. (1991) was used to check the effect of the MCE *P. kerstingii* on *in vivo* leukocyte mobilization induced by an inflammatory stimulus. Twenty five adult Wistar rats of both sexes (110 to 150 g) divided into five groups of five rats each were used for the test. Groups 3, 4 and 5 were administered varied doses of the extract (100, 200 and 400 mg/kg), while groups 1 (vehicle control) and 2 (treatment control) received distilled water and indomethacin (10 mg/kg), respectively. Three hours after oral administration of the extracts, distilled water or reference drug, each animal in the respective groups received intraperitoneal injection (i.p) of 0.5 ml of 3% w/v agar suspension in normal saline. Four hours later, the animals were sacrificed and the peritoneal cavities washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS). The peritoneal fluid was recovered and both total and differential leukocyte counts (TLC and DLC) were performed on the perfusates using a manual cell counter after staining with Wright's stain. The percent inhibition of leukoctye migration was calculated using the formula:

$$\% \textit{ Leukocyte inhibition} = 1 - \frac{T}{C} \textit{ X } 100$$

where T represents the leukocyte count of the treated groups.

Vascular permeability test in rats

The effect of the extract on acetic acid induced vascular permeability was assessed by a modification of the method of Whittles (1964). Twenty five adult Wistar rats of both sexes (120 to 170 g) divided into five groups of five rats each were used. The animals were fasted for 10 h prior to the experiment and were then administered with varied doses of the extract and drug as stated earlier. Three hours later, each animal received 0.5 ml intravenous injection of 1% Evans blue solution. Vascular permeability was induced 30 min afterwards, by (i.p) injection of 1 ml of 0.6% acetic acid. The animals were sacrificed 20 min later, and their peritoneum washed with 10 ml of normal saline. The recovered peritoneal fluid was centrifuged and the absorbance of the supernatant measured at 610 nm using a spectrophotometer.

Statistical analysis

The data was expressed as Mean ± standard error of mean (SEM). Analysis of variance (ANOVA) followed by post hoc and Dunnet-t-test was used to statistically analyze the data. P values less than 0.05 ($P<0.05$) were considered as significant.

RESULTS

Acute toxicity

The LD50 > 5000 mg/kg body weight.

Qualitative phytochemical analysis

The paw volume of all the treated groups were significantly (P≤0.05) reduced from the first hour after oedema induction as compared to the control group (Table 2). The extent of oedema inhibition for the treated groups increased with time. *P. kerstingii* extracts at test doses of 100, 200 and 400 mg/kg reduced the egg albumin induced-oedema by 53, 46.7 and 47%, respectively at the 4th hour as compared to the standard drug (51%). In the control group, egg albumin-induced paw oedema was sustained for 2 h after which it reduces significantly (P≤0.05) when compared with the 4th hour.

Table 5 shows the effect of *P. kerstingii* extract on phospholipase activity. The result shows that phospholipase activity of the extract at different doses was non-significant (P≤0.05) as compared to the standard drug, prednisolone.

The extract of *P. kerstingii* leaves produced a significant increase (p≤0.05) in agar induced leukocyte mobilization into the peritoneal cavity. The proportion of neutrophils in the perfusate was higher than lymphocytes and other cells in all the groups. The total leukocyte count of the treated groups increased significantly (p≤0.05) when compared with that of the control.

Intraperitoneal injection of 0.6% acetic acid evoked an increased vasodilation and permeability of the blood vessel of the animals as indicated by the leakage of Evans blue across the epithelial walls of the blood vessel into the peritoneal cavity. Table 7 shows that the *P. kerstingii* extract elicited a significant and dose dependent reduction (p≤0.05) in vascular permeability.

DISCUSSION

Bioactive compounds present in plants exhibit varied biochemical and pharmacological actions in animals when ingested (Nwogu et al., 2008). The extract of *P. kerstingii* leaves revealed the presence of glycosides, reducing sugars, saponins, triterpenes, flavonoids, tannin, soluble carbohydrates, cyanide, steroids, phenols and alkaloids (Table 1). Some of the constituents of the extract have been documented to possess analgesic and anti-inflammatory activities (Park et al., 2001; Okoli et al, 2007).The presence of flavonoids in the leaf of *P. kerstingii* could account for its use as an antinflammatory agent as reported by Ekwueme et al. (2011), prevention of damage caused by free radicals in the body (Dweck and Mitchell, 2002), treatment of diarrhoea (Schuier et al., 2005), *P. kerstingii* leaves, due to its flavonoid content could also be used as antipyretic, analgesic and spasmolytic agents. Flavonoids exhibit dramatic effects on immune and inflammatory cells; these can be either immunosuppressant or immunostimulatory (Huang et al., 2010). The phytochemical analysis of the extract also revealed the presence of alkaloids and this indicates that the leaves could be used in hypertension treatment (Olaleye, 2007). Kumar and Subrahmanyam, (2013) reported that tannins possess immune-stimulating activities. Various plants that contain tannins are used for wound healing (Okwu and Josiah, 2006; Nayak et al., 2007), treatment of dysentery, diarrhoea and urinary tract infection (Okwu and Josiah, 2006; Fahey, 2005). This suggests the possible potential of *P. kerstingii* leaves in the treatment of dysentery, diarrhoea, urinary tract infection and in wound healing. Ekwueme et al. (2011), reported that the saponin content of *senna* might be responsible for its anti-inflammatory properties and for its immunomodulating effect and could as well be used to treat hyperglycaemia, to cleanse and purify blood, treat hypertension (Fahey, 2005), and might also have cholesterol binding properties, and haemolytic activities (Okwu, 2004). This also suggests the possible potential of *P. kerstingii* leaves in the treatment of inflammatory, hyperglycaemia, hypertension, cleansing and purification of blood and as an immunomodulating agent.

The egg albumin-induced inflammatory reactions have been shown to be due to the release of inflammatory mediators (Heller et al., 1998; Nunez-Guillen et al., 1997; Ndebia et al., 2007). Egg albumin causes inflammation of the rat paw similar to carrageenan and the extract inhibited the development of paw edema in the treated animals at 0.5 and 4.0 h post injection of irritant corresponding to the two phases of the inflammatory response. The extract of *P. kerstingii* produced significant acute anti-inflammatory effects on egg albumin-induced paw oedema, which is a model of acute inflammation used in the study of non-steroidal anti-inflammatory agents (Di Rosa et al., 1971). The effects of the extract was most pronounced at 3 h after induction of oedema, an action which was similar to that of the standard drug (indomethacin), suggesting its usefulness in the management of acute inflammation.

The inhibition of haemolysis was not dependent on the doses, increasing with decreased concentration of the extract in the medium and was comparable with that obtained for indomethacin. Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators (Chaitanya et al., 2011). It is well known that the vitality of cells depends on the integrity of their membranes (Ferrali et al., 1992). Exposure of red blood cell to injurious substances such as hypotonic medium, phenylhydrazine and heat results in lysis of its membrane and this is accompanied by haemolysis and oxidation of haemoglobin (Augusto et al., 1982; Ferrali et al., 1992). The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation (Augusto et al., 1982; Ferrali et al., 1992). This conception is

Table 1. Phytochemical content of methanol extract of *Parinari kerstingii* leaves.

Phytochemical	Bioavailability
Alkaloids	++
Saponins	++
Steroids	+
Terpenoids	++
Glycosides	++
Tannins	++
Flavonoids	+++
Reducing sugar	++
Phenols	+
Hydrogen cyanide	+
Soluble carbohydrate	+

+ Mild; ++ Moderate; +++ Abundance.

Table 2. Effect of extract on egg albumin-induced rat paw oedema.

Treatment	Dose (mg/kg)	0 h	½ h	1 h	2 h	3 h	4 h
Control	-	0.27±0.03	0.68±0.06	0.69±0.05	0.74±0.10	0.69±0.05	0.66±0.035
Indo	10	0.27±0.06	0.66±0.11 ⌂ 82.9 ₳ 17	0.57±0.10 ⌂ 62.5 ₳ 37.5	0.55±0.11 ⌂ 52.8 ₳ 47	0.49±0.06 ⌂ 41.7 ₳ 58	0.49±0.07 ⌂ 48.9 ₳ 51
Extract	100	0.30±0.02	0.61±0.07 ⌂ 65.9 ₳ 34	0.57±0.04 ⌂ 56.2 ₳ 43.7	0.56±0.06 ⌂ 49 ₳ 50.9	0.51±0.07 ⌂ 43.8 ₳ 56	0.51±0.05 ⌂ 47.6 ₳ 53
Extract	200	0.24±0.03	0.57±0.03 ⌂ 78 ₳ 28	0.57±0.02 ⌂ 68.75 ₳ 31	0.53±0.03 ⌂ 54.7 ₳ 45	0.55±0.057 ⌂ 62.5 ₳ 37.5	0.47±0.04 ⌂ 53 ₳ 46.7
Extract	400	0.25±0.04	0.62±0.08 ⌂ 78.7 ₳ 21	0.61±0.02 ⌂ 75 ₳ 25	0.58±0.05 ⌂ 62 ₳ 37.7	0.49±0.05 ⌂ 50 ₳ 50	0.49±0.04 ⌂ 53 ₳ 47

⌂ = % Inflammation; ₳ = % Inhibition of inflammation; Indo = indomethacin.

consistent with the observation that the breakdown of bio-membranes leads to the formation of free radicals which in turn enhance cellular damage (Halliwell et al., 1988; Maxwell, 1995). The human erythrocyte membrane was protected by *P. kerstingii* extract against lysis induced by hypotonic solution and heat. Lyses of lysosomes occur during inflammation, thus releasing their component enzymes that result to various disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes (Mounnissamy et al., 2008). Since human red blood cell (HRBC) membranes are like lysosomal membrane components (Mounnissamy et al., 2008), the inhibition of hypotonicity and heat induced red blood cell membrane lysis was employed as a measure of the mechanism of anti-inflammatory activity of *P. kerstingii* extract (tables 3 and 4). Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators (Aitadafoun et al., 1996) and according to Anosike et al. (2012), anti-inflammatory drugs usually offer protection of erythrocyte membrane against lysis induced by heat and water. These results indicate that the observed anti-inflammatory activities of this plant are related to their membrane stabilization activity and contained principles that protected the erythrocyte

Table 3. Effect of *P. kerstingii* extract on heat induced haemolysis of HRBCs.

Treatment	Conc. (µg/ml)	Mean absorbance±SD at 540 nm		% Inhibition of analysis
		Heated solution	Unheated solution	
Control	-	0.707±0.002		-
Extract	100	0.034±0.060^{b}	0.705±0.013^{ef}	0.3
	200	0.094±0.050^{d}	0.701±0.090^{def}	1.0
	400	0.084±0.408^{c}	0.575±0.002^{b}	21.1
	600	0.076±0.003^{a}	0.591±0.008^{bc}	18.4
	800	0.091±0.001^{a}	0.472±0.052^{a}	38
	1000	0.107±0.005^{a}	0.445±0.044^{a}	44
Indomethacin	200	0.017±0.004^{a}	0.621±0.0014^{bcde}	13.1

Values represent mean ± standard deviation of triplicate sample. Percent inhibition of of haemolysis was calculated relative to control. Mean values, down the column, with different letters as superscripts are considered significant at $p < 0.05$.

Table 4. Effect of *P. kerstingii* extract on hypotonicity induced haemolysis of HRBCs.

Treatment	Conc. (µg/ml)	Mean absorbance±SD at 540 nm		% Inhibition of analysis
		Isotonic solution	Hypotonic solution	
Control	-	0.390±0.04		-
Extract	100	0.236±0.13^{ab}	0.150±0.02^{abc}	69.22
	200	0.147±0.01^{a}	0.167±0.02^{ab}	62.79
	400	0.279±0.07^{b}	0.146±0.01^{ab}	99.30
	800	0.243±0.06^{ab}	0.216±0.02^{c}	69.28
	1000	0.238±0.03^{ab}	0.178±0.03^{abc}	63.04
Indomethacin	1000	0.195± 0.01^{ab}	0.160± 0.02^{ab}	84.48

Values represent mean ± standard deviation of triplicate sample. Percent inhibition of of haemolysis was calculated relative to control. Mean values, down the column, with different letters as superscripts are considered significant at $p < 0.05$.

Table 5. Effect of *P. kerstingii* extract on phospholipase A_2 activity.

Treatment	Conc. (µg/ml)	Mean absorbance ± SD	% Inhibition
Control	-	1.6±0.31^{b}	-
Extract	100	0.81±0.05^{a}	49.1
	200	0.78±0.03^{a}	50.9
	400	0.80±0.04^{a}	49.6
	600	0.86±0.02^{a}	46
	800	0.85±0.06^{a}	46.5
Prednisolone	200	0.76±0.01^{a}	51.8

membranes effectively. The mode of action of the extract and standard anti-inflammatory drug could relate to binding to the erythrocyte membranes followed by alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the haemolysis of red blood cells. It has been reported that flavonoids,

Table 6. Effect of methanol extract of *P.kerstingii* on leukocyte mobilization.

Treatment (%)	Dose (mg/kg)	TLC (×10^4)	Differential leukocyte mobilization (%)				
			Neutrophils	Lymphocytes	Monocytes	Basophils	Eosinophils
Control		7800±25.4^a	59.8±2.6^b	35.6±2.6^a	2.4±0.5^a	-	1.4±0.5^a
Indomethacin	10	12400±3.8^b	61.2±3.1^b	35.2±2.3^a	1.8±0.8^b	-	1.6±0.5^a
	100	11680±9.6^d	62.6±3.7^b	33.6±2.5^a	1.8±0.5^b	-	1.8±0.4^a
Extract	200	15880±1.3^c	64±3.1^{ab}	33.2±2.2^{ab}	1.4±0.5^b	-	1.0±0.0^a
	400	12400±7.9^b	66.8±3.8^a	30.6±2.6^b	1.4±0.5^b	-	1.2±0.8^a

Table 7. Effect of methanol extract of *P. kerstingii* on acetic acid-induced vascular permeability test in rats.

Treatment (%)	Dose (mg/kg)	Absorbance	% Inhibition
Control	-	0.033±0.002	-
Indomethacin	10	0.027±0.001	18.34
	100	0.025±0.003	24.85
Extract	200	0.025±0.001	26.04
	400	0.017±0.001	48.52

triterpenoids and other phenolic compounds exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro* and possess ability to bind cations, thereby stabilizing erythrocyte membranes and other biological macromolecules (Middleton, 1996; El-Shabrany et al., 1997; Awe et al., 2009; Oyedapo et al., 2004).

Inflammation, pain and fever are all associated with enhanced production of prostaglandins (Rang et al., 2003). Phospholipase A_2 cleaves free fatty acids from membrane phospholipids, for instance, from erythrocyte phospholipids. The phospholipase A_2 enzyme activity was carried out using its action on erythrocyte membrane which created a leakage that resulted in extravasation of hemoglobin into the medium. The enzyme activity is thus directly related to the amount of hemoglobin in the medium, hence the increase in absorbance since hemoglobin absorbs maximally at 418 nm. Inhibition of phospholipase A_2 suggests that the extract may suppress the synthesis of free fatty acids from membrane phospholipids and hence prostaglandin synthase is depriving of substrates for the production of prostaglandins (Iwueke et al., 2006). Anti-inflammatory and immunosuppressive steroids inhibit arachidonic acid and its metabolites (prostaglandins) by induction of lipocortin which inhibits phospholipase A_2. The sequential inhibition of these two enzymes leads to potent suppression of prostaglandins synthesis and possible amplification of the anti-inflammatory activity of the extract.

Lymphocyte and neutrophils were mobilized than the other leukocytes. Neutrophil mobilization of the groups treated with different concentrations of extract increased in a concentration dependent manner. The increased neutrophil mobilization in our study is in agreement with the report of Vega and Corbi (2006) and Ekwueme et al. (2015) which state that, the number of phagocytic cell increases in the blood stream during injury and are responsible for increase in white blood cell count during infection. The extract triggered an increase mobilization of neutrophil which then fight and eradicate the harmful agent using myeloperoxidase present in the primary granules, lactoferrin and gelatinase present in the secondary granules, to degrade extracellular matrix, digest phagocytosed material, exert antimicrobial activity and initiate inflammation (Dale et al., 2008). The increase in neutrophil by the extract shows its ability to recruiting dendritic cells and monocyte that complete innate clearance of invading microbes and also initiate more specific adaptive immune responses (Vega and Corbi, 2006).

Again, the mobilization of neutrophil by the extract(table 6) indicates that the extract stimulates the generation of respiratory burst, hence generates ROS used in killing microbes. This is consistent with the report of Puga et al. (2012) which states that the mobilization of neutrophil stimulates the generation of respiratory burst by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which generates ROS used in killing microbes. In addition, the extract can also mop up this ROS when they are produced in excess due to its

antioxidant effect. The mobilization of lymphocyte decreased gradually as the concentration of the extract increased.

The inhibitory effect of *P. kerstingii* extract at various doses was comparable to that of indomethacin. Results demonstrated the effectiveness of the methanol extract of *P. kerstingii* against induced vascular permeability and infiltration of inflammatory cells to an injured area as was carried out using acetic acid induced vascular permeability test and agar induced leukocyte migration. Two major components of the inflammatory response mechanism are vascular changes leading to increased vascular permeability and emigration of leukocytes from the circulation to the site of inflammation (Ezike et al., 2015). Inhibition or suppression of any of these reactions could ultimately alleviate the extent and magnitude of the inflammatory response. In this study, intraperitoneal injection of 0.6% acetic acid resulted in an increased dilation and permeability of the blood vessels of the animals which was indicated by the increased leakage of fluids, including Evans blue across the blood vessel epithelial walls. Administration of the extract of *P. kerstingii* caused a significant and dose dependent reduction in vascular permeability of the extract treated animals.

Conclusion

The study revealed that *P. kerstingii* is rich in flavonoids and other phenolic compounds have been associated with decreased risk of developing inflammatory and other related diseases and also has inhibitory effect of vascular changes that occurs during inflammation. It was reported that stimulation of leukocyte by the extract plays a significant role in immune response and that the anti-inflammatory property of the extract is not at the level of leukocyte mobilization (Ekwueme et al., 2015). This is in line with our result which showed stimulatory effect on leukocyte mobilization. It can therefore be deduced from this study that the extract has anti-inflammatory and immunostimulatory effect and could be used in boosting immune response as revealed in the models studied. This study has membrane stabilizing and phospholipase A_2 inhibitory effects, and may offer some beneficial effects on haemolytic diseases and in the management of inflammatory conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genotoxic effects of *Peganum harmala* L. in relation to traditional use

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***Peganum harmala* L. is a well-known medicinal plant, widely distributed and used in folk medicine. Recently, numerous reports have been published regarding the pharmacological and therapeutic effects of *P. harmala* and its major alkaloids components. However, mammalian toxicity due to *P. harmala* alkaloids extract has been rarely investigated. The present research aimed to study the genotoxic effects of *harmala* plant extract in relation to traditional use. *Harmala* crude alkaloid was extracted and the toxicity of the extract was examined on albino mice by intraperitoneal injection. The genotoxic effects of the alkaloid extract was tested on bone marrow cells isolated from sacrificed mice. The total seeds alkaloids showed moderate toxic effects on male albino mice with LD_{50} 350 mg/ Kg body weight. *P. harmala* alkaloids extract showed significant reduction in the mitotic index (MI) and increased depression of mammalian cells division possibly through chromosomal aberrations. *P. harmala* alkaloids induced different types of chromosome aberrations including rings, breaks, polyploidy, sticky, laggards and bridges with the sticky form as the most abundant type. Furthermore, *P. harmala* alkaloids induced a significant increase in sister chromatid exchange (SCE) compared to untreated controls. The frequency of micronuclei was increased with increasing the concentration but was not affected by increasing the exposure time. The medicinal use of *harmala* should be under control since higher doses and/ or longer exposure is genotoxic. An amount of plant that contains ≥ 12 mg alkaloids cannot be safe for traditional use.**

Key words: *Peganum harmala*, genotoxicity, chromosome aberration, sister chromatid exchange.

INTRODUCTION

Peganum harmala L. is a perennial herbaceous and glabrous plant that belongs to the family *Zygophyllaceae* and can grow up to 30-100 cm in semi-arid condition. The plant is widely distributed in Asia, North Africa and Middle East (Moshiri et al., 2013). All parts of the plant including leaves, seeds, fruits, roots and barks have been used in

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folk medicine (Moloudizargari et al., 2013). The plant is well-known for its medicinal values including hypotensive and cardiovascular activities (Tahraoui et al., 2007), antimicrobial effects (Prashanth and John, 1999), anti-fungal (Saadabi, 2006), antitumor activities (Li et al., 2007), anti-diabetics (Waki et al., 2007), anti-inflammatory effects (Hamsa and Kuttan, 2010), anti-leishmanial effects (Mirzaie et al., 2007) and in some nervous system disorders such as Parkinson's disease, psychiatric nervousness, and in rigorous pain (Leporatti and Ghedira, 2009)

P. harmala is known as a major source of alkaloids (Rafie et al., 2017) including β-carbolines such as harmine, harmaline, harman and harmalol and the quinazoline derivatives such as vasicine and vasicinone (Di Giorgio et al., 2004). Harmala alkaloids showed a variety of biochemical, psychopharmacological, and behavioral effects on animals and humans (Farzin and Salimi, 2009). For example, harmine is considered as an important antitumor and anti-HIV agent (Begum et al., 2008). Similarly, harmaline, can cause 96.6% inhibition rate of human retinoblastoma (Rb) cell line within 72 h (Jin, 1990). On the other hand, γ -harmine is considered as the first alkaloid with relatively good radio-protective effect (Li et al., 1995). Furthermore, gastro-protective mechanism by peganine (Singh et al., 2013) and treatment of psychiatric disorders by deoxyperganine (Tursunkhodjaeva et al., 2015) were reported.

With all of the aforementioned pharmacological effects of *P. harmala* and its known folk use medicine, its cytotoxicity in particular genotoxicity on mammalian cells was rarely investigated. Here, in this study, the genotoxicity of *P. harmala* alkaloids on mammalian cells was investigated using different genotoxic parameters including mitotic index, chromosome aberration, sister chromatid exchange and micronucleus assay.

MATERIALS AND METHODS

Chemicals and reagents

Mitomycin C (MMC) was obtained from Kyowa Hakko Co. (Tokyo, Japan) and used as positive control. Fifty mg of 5-BrdU tablets were obtained from Boehringer-Mannheim (Mannheim, Germany). DMSO and Hoechst 33258 were purchased from Sigma Chemical Co. (St Louis, MO). Giemsa stain was procured from Glaxo India Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Preparation of *P. harmala* alkaloid extract

P. harmala L. seeds were collected from marginal and desert lands from various locations in Jordan including Irbid, Salt, Dead Sea, Zarqa, Mafraq and Ma'an, at the end of summer, 2015. A voucher specimen was preserved in the herbarium of the Hashemite University, Department of Biology and Biotechnology, Zarqa, Jordan.

P. harmala seeds were extracted according to Tharib and El-Migirab (1984) (Brkljača et al., 2015) with some modifications. Briefly, 500 g air dried and finely ground seeds were refluxed with ethanol in a Soxhlet extraction apparatus. The ethanol was evaporated using rotatory evaporator and the residual extract was mixed with10 % HCl and re-extracted with chloroform. The aqueous acidic layer was collected and its pH was adjusted to 9.5 by ammonium hydroxide (NH_4OH) solution. The aqueous basic solution was re-extracted 3 times with chloroform. The presence of alkaloids in the chloroform layer was confirmed by Mayer's precipitation test. The chloroform layer was evaporated under vacuum by a rotary evaporator at 45-50ºC to afford 0.5 g alkaloid residue (Memelink et al., 2001). A stock solution of the alkaloid residue was prepared by dissolving 500 mg of the alkaloidal fraction in 5 mL dimethyl sulphoxide (DMSO) and aliquoted in different concentrations and kept at 4ºC for further analysis.

Determination of the median lethal dose LD_{50}

Animal study performed in this report was approved by institutional review board (IRB) at the Hashemite University. Crude alkaloid from *P. harmala* were demonstrated using Swiss albino male mice (*Mus musculus*, 2n = 40); JVC 1, 10–12 weeks old and 20–25 g weight, were obtained from the animal house (Biology Dep., Hashemite University, Jordan) and maintained under conventional laboratory conditions at room temperature on a 12 h dark/ 12 h light cycle. LD_{50} was determined using behavioral and pharmacological studies of the extract (Benbott et al., 2013) with some modifications. The extract of *P. harmala* was administered at different doses intraperitoneally. Crude alkaloid extract with 200 mg/Kg b.w. was chosen as primary dose. The mice were divided into 8 groups each of 5 animals including a control group.

Preparation of mouse bone marrow cells

Mice were sacrificed by cervical dislocation, after a single intra-peritoneal injection with alkaloids. Femora were cleaned from adherent tissues and the tip of each bone was removed followed by harvesting the bone marrow using sterile saline. Metaphase bone marrow cells were prepared for mitotic chromosomal aberrations by classical methods (Omari et al., 1996). The prepared cells were stained with Giemsa solution and the slides were coded and scored for the presence of dividing cells. Different alkaloid concentrations including 12.5, 25, 50, 75 and 100 mg/ Kg b.w were applied to investigate their genotoxic effects on bone marrow cells at different time periods: 8, 24, 48 and 72 h. At least, 3000 cells from four male mice were used for each experimental and control group. Control groups received equivalent volumes of saline solution. Frequency of normal and aberrant chromosomes was examined, and chromosomal aberration was calculated according to the following formula:

Chromosomal aberration (CA) (%) = (Total number of chromosomal aberrations / Total number of cells examined) x 100

The mitotic index and mito-depression were calculated according to the following equations (Omari et al., 1996):

Mitotic index= (Number of divided cells/Total number of cells) x 100
And the mito-depression index was calculated using the following equation:

MI (control) - MI (treatment) x 100/ MI (control).

Sister chromatid exchange (SCE)

The SCE assay was applied as described by Goto et al. (1978)

with some modifications. The bromodeoxyuridine 5-BrdU tablets (50 mg) were prepared and implanted. The treated animals were sacrificed by cervical dislocation after 24 and 48 h treatments and the femora were dissected. The animals were treated intraperitoneally with colchicine (4 mg/ Kg) for 90 min. Different concentrations of alkaloids extract including 12.5, 25, 50, 75 and 100 mg /Kg b.w, were applied and 4 male mice were used for each injected alkaloid dose.

Bisbenzimide (Hoechst 33258) dye was applied and metaphase bone marrow cells were prepared by the classical methods. The preparations were stained in Giemsa solution and at least 200 well-spread second division metaphases were analyzed for SCE. Mitomycin C was chosen as a positive control, and DMSO was used as negative control.

Micronucleus assay (MN)

Different alkaloids concentrations (12.5, 25, 50 and 100 mg / Kg b.w) were injected intra peritoneal for 24, 47 and 72 h. Four male mice were used per each concentration. The micronucleus test was performed according to Schmid (1975) with minor modifications. The preparations were stained with Giemsa solution and a total of at least 4000 cells were scored for each animal at a magnification 1000 x using the oil immersion lens. Mitomycin C and DMSO were chosen as a positive and a negative control respectively.

Statistics

Statistical analysis was performed using SPSS version 17. Significant differences between the results of different groups using one way analysis of variance (ANOVA) was applied. The differences were considered statistically significant when $P \leq 0.05$ and highly significant at $P \leq 0.001$.

RESULTS

P. harmala is generally toxic

To establish a base line of extract cytotoxicity, LD_{50} of *P. harmala* alkaloids extract in mice was measured. The LD_{50} of mice treated intraperitoneally with *P. harmala* alkaloids were calculated using the second phase and was found to be ≥350 mg / Kg b.w.

P. harmala is genotoxic

P. harmala reduced mitotic index (MI) and increased mito-depression of mammalian cells

The cytological effect of the alkaloids was estimated on the basis of the mitotic index in bone marrow cells of Swiss albino mice following a single intraperitoneal dose of 12.5, 25, 50, and 100 mg / Kg b.w. The mitotic index was detected from 8 to 72 h following injection. A significant reduction in the mitotic index from ~4.45 to ~3.31 was evident in all treated mice cells compared to positive and negative controls (Table 1). The depression index was increased with increasing the exposure time in almost all treatments (Table 1).

P. harmala caused significant cchromosome aberrations

Cytogenetic studies showed that *P. harmala* seeds alkaloids extract induced significant increase in the % of chromosome aberrations in almost all treatments when compared to control. Chromosome aberrations including rings, breaks, polyploidy, sticky, laggards and bridges were shown. The sticky chromosomes were the most abundant, while rings, broken and poly ploidy were produced in considerable frequencies (Table 2). Other types of aberrations (gaps, dicentric and fragments) were also produced in very low frequencies (Table 2). Furthermore, *P. harmala* alkaloids induced sister chromatid exchange (SCE) at all tested concentrations (12.5, 25, 50 and 100 mg / Kg b.w) compared to untreated controls. The potency of SCEs due to alkaloid treatment was significantly lower than those produced by the positive control (MMC) by ~ 60% and the frequency of SCE was not increased by increasing exposure time (Table 3). Additionally, the frequency of micronuclei was significantly increased with increasing the alkaloid concentrations compared to control, while the increase in exposure time did not alter this frequency. However, there were no significant differences between treated groups (Table 4).

DISCUSSION

P. harmala alkaloid extract showed LD_{50} of ≥ 350 mg / Kg b.w which is consistent with that obtained previously (Benbott et al., 2013). These identical values may be attributed to similar conditions used in this respect. To assess the lethal effect of the extract, several genotoxic effects of the extract were tested including mitotic index (MI), chromosome aberration (CA), sister chromatid exchange (SCE) and micronucleus (MN) assays. The extract showed marked reduction in the mitotic index (MI) of the injected mice compared to controls; similar to results obtained with *Catha edulis* (khat) alkaloid (Kabarity and Malallah 1980) and *Rubia cordifolia* alkaloid extracts (Abderrahman 2004).

In reference to Kabarity and Malallah (1980), the marked reduction in MI suggested that the alkaloid extract could affect the onset of mitosis and the spindle formation. Thus, possible chromosomal aberrations (CA) due to the alkaloid extract were tested. Cytogenetic analyses showed significant increase in the % of chromosome aberrations due to alkaloid extract including rings, breaks, polyploidy, sticky, laggards and bridges with sticky chromosomes being the most abundant type. Other types of aberrations including gaps, dysenteric and fragments were also observed but in very low

Table 1. Mitotic index in bone marrow cells treated with different concentrations of *Peganum harmala* alkaloid extract.

Exposure Time (h)	Dose (mg/kg)	Number of cells	Mitosis	Mitotic index (MI)	Mitodepressive index
8	100	3100	122	3.93*	13.81
	75	3001	117	3.90*	14.47
	50	3675	143	3.89*	14.69
	25	3120	133	4.26	6.58
	12.5	3000	133	4.43	2.85
	Cont.	4300	196	4.56	-
24	100	3195	116	3.63*	13.37
	75	3020	112	3.71*	11.46
	50	3515	136	3.87*	7.64
	25	3110	120	3.86*	7.88
	12.5	3121	138	4.42	5.49
	Cont.	3009	126	4.19	-
48	100	3002	110	3.66 *	15.86
	75	3005	112	3.73*	14.25
	50	3111	117	3.76*	13.56
	25	3035	125	4.12	5.29
	12.5	3105	132	4.25	2.30
	Cont.	3440	150	4.35	-
72	100	4125	-	-	-
	75	4012	-	-	-
	50	3177	105	3.31*	24.77
	25	3940	161	4.09	7.05
	12.5	3935	175	4.45	1.14
	Cont.	3126	138	4.40	-

*Significant value.

frequencies. The results are consistent with previously reported results of extract using *Vicia faba* plants (Mekki et al., 2015). On the other hand, chromosomal aberration (CA) is considered to be very sensitive end points recognizing the genotoxicity induced by chemicals (Majak et al., 2010). It is evident that sticky chromosomes caused spindle deformation (Tawab et al., 2004) and it is attributed to an increase in the viscosity of cytoplasm (Abderrahman, 1997). *P. harmala* alkaloids showed significant genetic toxicity measured by sister chromatid exchange (SCE) test. SCE is considered as sensitive indicator of genetic toxicity and biomarker of genotoxic substances (Jeyapradha et al., 2011). *P. harmala* alkaloids showed significant increase in the frequency of SCEs and the increase was dose-dependent, but not time-dependent, consistence with results obtained on other genotoxic agents (Das et al., 2004; Abderrahman and Modallal, 2008).

Furthermore, assay of micronuclei (MN) serves as an indicator of genetic damage (Jois et al., 2010). MN are very small extra-nuclear bodies which arise from acentric chromatid / chromosome fragments caused by unrepaired or misrepaired DNA breaks during anaphase (Fenech et al., 2011). Thus, the quantitative estimation of MN may serve as an indicator of genetic damage that has taken place (Jois et al., 2010). MN assay was chosen to assess alkaloids possible genotoxic and cytotoxic effects on mice cells. MN assay has become one of the most commonly used methods for assessing chromosome breakage and loss in mammalian lymphocytes (Jiménez et al., 2008). The increase in the frequency of MN in exfoliated cells revealed a statistically significant effect on mice cells treated with *P. harmala* alkaloids when compared to control group. This result is similar to data reported on the genotoxic induction by the Brazilian medicinal plant, *Strychnos pseudoquina* (Santos et al., 2006).

Conclusion

In summary, our results showed clearly that alkaloids

Table 2. Frequency of chromosome aberrations in mice cells treated with different concentrations of alkaloids from *Peganum harmala.*

Exposure Time (h)	Conc. (mg/kg)	Cells Examined	Types of chromosome aberrations						% of CA
			Rings	Breaks	Polyploidy	Sticky	Lagg.	Bridges	
8	100	3820	25	26	15	49	2	1	3.09**
	75	3200	20	20	12	40	1	2	2.97**
	50	3711	22	23	14	40	-	1	2.69**
	25	3686	14	14	8	26	1	-	1.71**
	12.5	3502	9	8	4	18	-	1	1.14*
	Cont.	3005	2	2	2	4	-	-	0.33
24	100	3522	28	28	18	61	2	1	3.92**
	75	3010	22	22	12	50	1	2	3.62**
	50	3711	25	26	17	56	2	2	3.45**
	25	3900	18	21	14	50	1	1	2.69**
	12.5	3615	14	12	8	30	-	1	1.80*
	Cont.	3623	2	2	1	3	1	1	0.28
48	100	3520	36	34	24	68	4	3	4.80**
	75	3800	29	30	20	54	4	4	3.71**
	50	3908	27	28	18	48	3	2	3.22**
	25	3912	18	20	10	30	2	-	2.06**
	12.5	3844	12	13	9	21	1	1	1.48*
	Cont.	3998	4	111	1	3	1	1	0.25
72	100	3622	38	37	23	72	5	3	4.91**
	75	3400	30	30	20	45	5	4	3.94**
	50	3952	31	30	18	60	4	3	3.69**
	25	3945	30	30	20	55	3	2	3.55**
	12.5	3800	18	20	11	24	1	1	1.97*
	Cont.	3678	3	3	23	33	1	1	0.27

*Significant value, ** The result shows exceptionally a significant value.

Table 3. Frequency of sister chromatid exchange in mice cells treated with different concentrations of alkaloids from *Peganum harmala.*

Parameter	Dose (mg/ Kg b.w)				
	12.5	25	50	75	100
24 h					
MMC[a]2.0	12.85±0.20				
DMSO[b]	3.85±0.28				
SCE/cell (mean±S.D)	4.35±0.32*	5.44±0.34*	6.68±0.22**	7.85±0.28**	8.25±0.42**
48 h					
MMC[a]2.0	12.54±0.36				
DMSO[b]	3.52±0.26				
SCE/cell (mean±S.D)	4.48±0.38*	5.58±0.40*	6.74±0.42*	8.02±0.24**	8.44±0.38**

[a]MCC, mitomycin C (positive control); [b]DMSO, dimethyl sulfoxide (negative control). *Significant value; **The result shows an exceptionally significant value.

isolated from *P. harmala* exhibited genotoxic effects by inducing significant mitodepression, chromosomal aberration, sister chromatid exchange and micronuclei. These effects were more prominent at higher doses, and

Table 4. Effect of alkaloids extracted from *Peganum harmala* on micronucleus (MN) formation in mouse bone marrow cells.

Exposure Time (h)	Dose (mg/kg b.w)	Number of cells examined	Number of micronuclei	Percentage of micronuclei
24 h	**12.5**	**4005**	**42**	**1.04**
	25	4100	59	1.44*
	50	4002	61	1. 52*
	100	4021	95	2.36**
	DMSO	4000	25	0.86
	Mytomycin C 2.0	4012	174	4.34
48 h	12.5	4001	43	1.07
	25	4010	57	1.42*
	50	4015	61	1.52*
	100	4116	95	2.31**
	DMSO	4008	29	0.72
	Mytomycin C 2.0	4015	179	4.46
72 h	12.5	4105	44	1.09
	25	4211	56	1.33*
	50	4005	58	1.45*
	100	4211	97	2.30**
	DMSO	4019	29	0.90
	Mytomycin C 2.0	4113	185	4.50

*Significant value; ** the result shows an exceptionally significant value.

hence it is important to direct the traditional use of the medicinal plant to avoid any genotoxic effects. However, further investigation is still needed to identify the active compounds from the crude alkaloid extracted that is responsible for these activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In vitro anticancer screening of Colombian plants from *Piper* genus (Piperaceae)

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Historically, knowledge of ethnobotany, which has revealed different phytochemical and pharmacological compounds from traditional plants, has formed the basis for new anticancer drug discovery. The use of some *Piper* species in traditional medicine against cancer, suggests that genus *Piper* is a promising source of new compounds with anticancer activity. A total of 28 ethanolic extracts were obtained from 16 different *Piper* spp., then *in vitro* cytotoxicity activity was performed with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay in three certified human cancer cell lines (A549 lung, PC-3 prostate and MDAMB-231 breast). Seven ethanolic extracts obtained from different parts of *Piper eriopodon*, *Piper cumanense* and *Piper bogotense* showed promising anticancer effect with IC_{50} values below to 30 µg/mL. The most potent cytotoxic effect was found in the leaves ethanolic extract of *P. eriopodon* with an IC_{50} of 17.7 µg/mL for A549, 11.8 µg/mL for PC-3 and 20.7 µg/mL for MDAMB-231. Bioassay guided fractionation was performed for the most active extract and a highly cytotoxic compound was isolated and identified by spectroscopic means, mainly 1D and 2D RMN spectroscopy. The isolated compound identified as gibbilimbol B was shown to be a strong cytotoxic effect against cancer cell lines with IC_{50} values in the range of 11.4 and 41.9 µg/mL.

Key words: *Piper*, Piperaceae, alkenylphenols, cytotoxicity, anticancer, gibbilimbol B.

INTRODUCTION

According to the World Health Organization, cancer disease is a public health problem. By 2030, the number of people with cancer will reach 26.4 million in the whole world and now is considered one of the leading causes of death worldwide (WHO, 2014). Most of the drugs used in the pharmacological treatment of cancer disease are highly toxic and show low specificity to tumor cells, considerably affecting the survival prognosis of patients. Historically, natural product and ethnobotanical knowledge have been traditionally the main source of the discovery of new active principles that provide new horizons for cancer treatment (Cragg et al., 2014).

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However, many developing countries are still using traditional medicine due to their low cost and limited access to pharmacological treatments. The *Piper* genus is the most important genus of the Piperaceae family and recently was considered as potential source of new compounds with anticancer activity based on the use of some *Piper* species in traditional medicine (Sanubol et al., 2017; Calderón et al., 2006; Durant-Archibold et al., 2018; Mgbeahuruike et al., 2017). The vast majority of the global distribution for *Piper* spp. reside in the tropical zone of America (700 species) and Asia (300 species) (Jaramillo and Manos, 2001). In Colombia, the *Piper* genus is known as "cordoncillo" and is widely distributed in the tropical and humid forest, especially in the Chocó, Antioquia, Valle del Cauca, Santander and Cundinamarca regions.

Some of the *Piper* spp. reported in traditional medicine to treat cancer are *Piper aduncum, Piper boehmeriifolium* Wall*, Piper capense L.f., Piper* cubeba L., *Piper gibbilimbum* C.D.C., *Piper guineense* Schum and Thonn*, Piper longum* L., *Piper nigrum* L., *Piper sylvaticum* Rox*b* and *Piper barbatum* (Wang et al., 2014). Phytochemical reports on the genus *Piper* have shown that it contains a high amount of cytotoxic compounds, especially amide alkaloids (Bezerra et al., 2013; Meegan et al., 2017; Greenshields et al., 2015). Piperlongumine is a promising anticancer alkamide present in different plants of the *Piper* genus and it was shown to have selectively cytotoxic effect against cancer cells. The cytotoxic effect of piperlongumine in cancer cells, include induction of apoptotic cell death by cell cycle arrest in G1 or G2/M phase, increase of oxidative stress, inhibition of angiogenesis in xenograft-tumour mice model and destabilizing microtubules (Raj et al., 2011; Meegan et al., 2017). Other cytotoxic chemical constituents isolated from *Piper* plants are phenolic compounds such as phenylpropanoids (Ferreira et al., 2014; Hematpoor et al., 2018), flavonoids (Rossette et al., 2017; Niu et al., 2016; Freitas et al., 2014), lignans (Rajalekshmi et al., 2016; Sriwiriyajan et al., 2017) and alkenylphenols (Orjala et al., 1998; Lopes et al., 2013).

The present study investigated the *in vitro* cytotoxic effect of 28 ethanolic extracts, from 16 different Colombian *Piper* spp. against a panel of three human cancer cell lines. In order to explore the cytotoxic compounds, the chromatographic ultra-high performance liquid chromatography (UHPLC) profile of leaves, flowers and wood for the most active specie are presented. The majority component of the extracts was isolated, identified as an alkenylphenol and showed highly cytotoxicity activity.

MATERIALS AND METHODS

General experimental procedures

Flash chromatography was carried out with silica gel (230-400 mesh, Merck), analytical chromatography was performed using silica gel 60 PF254 (0.25 mm, Merck) and Shepadex® LH20 (Sigma). ^{1}H and ^{13}C NMR 1D and 2D were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using the solvent peaks as internal references, the spectra were in $CDCl_3$ (δ_H 7.26 in ^{1}H and δ_C 77.0 in ^{13}C). High-resolution mass data were collected on an Accurate-Mass quadrupole Time-of-Flight (q-TOF) (Agilent Technologies) mass spectrometer, ESI positive mode, Nebulizer 50 (psi), Gas Flow 10 L/min, Gas Temp 350°C, Fragmentor 175 V, Skimmer 75 V, Vpp 750 V. UHPLC was performed on a Thermo Dionex UltiMate 3000 equipment, coupled with photodiode array (PDA) and evaporative light scattering detector (ELSD), using a Phenomenex Sinergy RP-C8 column (5 µm, 4.5 × 250 mm).

Plant

A total of 16 plants (Table 1) were collected mainly from the humid forest in Cundinamarca, Boyaca and Santander departments. The voucher specimens were deposited and identified at the Herbario Nacional Colombiano, Instituto de Ciencias Naturales, Universidad Nacional de Colombia by biologist Adolfo Jara Muñoz.

Preparation of the extracts

The plants were dried at room temperature protected from light. Then, the dried parts (e.g. leaves, wood or inflorescences) of the different plants were powdered and submitted to exhaustive extraction with ethanol 96% three times for 72 h at room temperature through a maceration process (Table 1). The ethanolic extracts were obtained after filtration through Wathman No. 1 filter and the evaporation of solvent with a rotatory vacuum evaporator at 40°C (Mesquita et al., 2009).

Cell culture

The human ATCC cell lines were cultured according to the ATCC protocols. Briefly, human cancer cells lines A549 lung cancer, PC-3 prostate cancer, MDAMB 231 and MCF7 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (Lonza) in a humidified atmosphere at 37°C in 5% CO_2.

In vitro cytotoxicity test

The cytotoxic effect of ethanolic extracts and isolated compound was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Riss et al., 2004). Briefly, cells were seeded in 96-well plates (10^4 cell/well) in 100 µL of medium supplemented with 10% FBS and 1% penicillin-streptomycin and allowed to settle for 24 h in a humidified atmosphere at 37°C in 5% CO_2. Then, the medium was removed and cells were treated with 100 µL of different concentrations (100, 30, 10, 1 and 0.1 µg/mL) of extracts or isolated compound prepared in cell culture medium. After 24 h, the treated medium of each well was removed and replaced by 100 µL of fresh medium containing 0.5 mg/mL of MTT and the plates were incubated for 4 h. Finally, the supernatant was removed and 100 µL of saline lysis buffer was added and measured in a Tecan Sunrise Eliza-Reader (Hombrechtikon, Switzerland) at λ = 595 nm. Untreated cells were used as negative control and the IC_{50} values (concentration that inhibits 50% of cell growth) of tested extracts and gibbilimbol B were calculated using GraphPad Prism software. The data is expressed as mean ± standard error of mean (SEM; n = 4).

Table 1. Plants and parts of plants used for the *in vitro* cytotoxic assay against the cancer cell lines: A549, PC-3 and MDA-MB-231.

Species	Voucher number	Plant part	IC_{50} (µg/mL)*		
			Lung A549	Prostate PC-3	Breast MDA-MB-231
Piper aduncum	COL595171	Leaves	>100	>100	>100
		Inflorescences	78.16 ± 10.7[a]	>100	>100
Piper amalago	COL 510519	Leaves	>100	>100	>100
Piper arboreum	COL 519815	Leaves	>100	>100	>100
		Wood	60.29 ± 7.22[b]	27.25 ± 2.08[a]	61.29 ± 3.65[a]
Piper arthante	COL 515965	Wood	>100	>100	>100
Piper cf. asperiusculum	COL 579924	Leaves	89.45 ± 10.3[c]	>100	>100
		Inflorescences	>100	>100	>100
Piper bogotense	COL 517696	Leaves	38.83 ± 3.89[de]	27.89 ± 2.44[b]	49.21 ± 5.22[b]
		Inflorescences	>100	>100	>100
Piper cumanense	COL 518183	Leaves	43.78 ± 3.23[e]	30.08 ± 1.59[b]	44.75 ± 1.85[c]
		Inflorescences	31.10 ± 2.56[f]	18.03 ± 1.35[c]	55.78 ± 4.07[d]
Piper el bancoanum	COL 518182	Leaves	>100	>100	>100
Piper eriocladum	COL 517694	Wood	>100	>100	>100
		Wood	>100	>100	>100
Piper cf. eriopodon	COL 516757	Leaves	17.84 ± 2.24[g]	11.88 ± 0.69[d]	20.75 ± 1.12[e]
		Inflorescences	33.74 ± 3.83[df]	16.90 ± 1.85[c]	53.22 ± 4.22[d]
		Wood	26.60 ± 1.85[f]	44.70 ± 3.70[e]	39.56 ± 2.51[f]
Piper hispidum	COL 510518	Leaves	>100	>100	>100
		Roots	>100	>100	>100
Piper holtonii	COL 517184	Wood	>100	>100	>100
		Leaves	>100	>100	>100
Piper marginatum	COL 591820	Wood	>100	>100	>100
		Leaves	>100	>100	>100
Piper peltatum	COL 512098	Leaves	>100	>100	>100
Piper pertomentellum	COL 579920	Leaves	>100	>100	>100
		Inflorescences	>100	>100	>100
Piper pesaresanum	COL 553307	Leaves	77.41 ± 4.92[g]	82.51 ± 4.75[f]	83.63 ± 7.23[g]

*****Values labeled with different letters are significant (Tuckey's HSD, $p < 0.05$).

UHPLC-DAD-ELSD analysis

The three most active extracts (leaves, inflorescences and wood of *Piper eriopodon*) were analyzed by liquid chromatography in order to determinate their complexity and similarities. 10 mg of each ethanolic extract were solubilized in 1 mL MeOH HPLC-grade. These solutions were filtered through 0.22 µm polytetrafluoroethylene (PTFE) membrane and placed in chromatography vials. The samples were analyzed by liquid chromatography (UHPLC) coupled with photodiode array (PDA) and evaporative light scattering detector (ELSD), in a Thermo Dionex UltiMate 3000 equipment. The separations were performed on a Phenomenex Sinergy RP-C8 column (5 µm, 4.5 × 250 mm). The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in gradient mode. The gradient started at 5% B for the first 5 min and increased linearly to 100% B over 25 min. It was kept constant

for 3 min and then returned to 5% B (30 min) and remained constant for 5 min. The flow rate was kept at 0.3 mL/min. The UV spectral was acquired between 200 and 400 nm. The retention times, peak areas and UV spectra of the major peaks were analyzed. Finally, the compound isolated from the leaves of *P. eriopodon* was examined under the same chromatographic conditions to dereplicate its presence in the other organs.

Isolation of cytotoxic compound

The leaves (1145 g) of *P. eriopodon* were extracted according to the procedure described earlier and solvent was evaporated to dryness resulting in 103.6 g of ethanolic extract. 100 g of extract were fractionated by flash chromatography on silica gel, eluted with a toluene/EtOAc in gradient mode (0 to 100% EtOAc) to obtain 8 fractions. The activity was retained in fractions 1 (34.2 g) and 2 (unpublished data). Fraction 1 (34.2 g) was submitted to silica gel chromatography eluted with a mixture of dichlorometane/ hexane/EtOAc (70:25:30) to obtain 10 fractions (P1-P10). The major component of fraction P5 (10 g) was submitted to a further purification by Sephadex LH-20 with a mixture (2:2:1) of hexane-chloroform-methanol to obtain the phenolic compound gibbilimbol B (7.93 g). The same process was used in air-dried wood (1490 g), which was obtained at 50.3 g of ethanolic extract and 768 mg of the same compound. The structure of isolated compound was identified by interpretation of the spectral data IR, HREIMS, ^{1}H and ^{13}C NMR (including DEPT, COSY, HMQC and HMBC experiments), as well as by comparison of the spectral data with those reported in the literature.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). The IC_{50} values were obtained by non-linear regression curve analysis of the concentration effect responses, using the GraphPad Prism program (Graph Software, San Diego, CA). Data for each cancer cell line were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD test. All differences with $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

A total of 28 ethanolic extracts were tested in the cytotoxicity assay. The species and the parts of plants used in the experiments, as well as the voucher number are shown in Table 1. Of the 28 extracts tested, it was found that 7 extracts showed strong cytotoxic activity against all tested cancer cell lines. These extracts were able to inhibit the growth of cancer cells in a dose-dependent relation. The IC_{50} values were calculated in a complete dose-response curve against three cancer cell lines A549, PC-3 and MDA-MB-231, the values are shown in Table 1. Among the 28 ethanolic extracts from Colombian *Piper* spp., 10 showed IC_{50} values lower than 100 µg/mL, 7 showed IC_{50} values lower than 50 µg/mL, and 3 values were lower than 20 µg/mL against the cancer cell lines tested.

The results showed that there are significant differences in the IC_{50} values for the cytotoxic extracts. Analysis of the data showed that the ethanolic extract from leaves of *P. eriopodon* has the most potent cytotoxic effect against the three human cancer cell lines tested, whereas the lowest cytotoxic effect was found in the ethanolic extract from wood of *Piper asperiusculum* (Table 1). The ethanolic extracts from leaves of *P. aduncum* and *P. asperiusculum* showed moderate anticancer activity only against lung cancer cells (A549). This cell line showed the most sensitive response, 10 ethanolic extracts inhibited the cell growth of lung cancer cells (A-549) with IC_{50} values lower than 100 µg/mL, in contrast to prostate (PC-3) and breast (MDA-MB-231) cancer cells, which eight of them showed cytotoxic activity lower than 100 µg/mL. The ethanolic extract of *P. aduncum* previously reported the presence of dillapiole, a phenolic compound isolated from many species of the *Piper* genus that induce apoptosis in cancer cells through the activation of caspases by the intrinsic pathway through the mitochondria (Ferreira et al., 2014). Other studies report that the ethanolic extract of leaves from *P. aduncum* has cytotoxic activities against HeLa cells (IC_{50}=3.91 µg/mL) and the dichloromethane extract against KB (IC_{50}=12 µg/mL) (Orjala et al., 1994), MCF7 (IC_{50}=27 µg/mL), H-460 (IC_{50}=25 µg/mL) and SF-268 (IC_{50}=23 µg/mL) cancer cells (Calderón et al., 2006), whereas *P. asperiusculum* have no previous reports.

According to the U.S. National Cancer Institute, an active extract is one with an IC_{50}<30 µg/mL (Suffness and Pezzuto, 1990). The present results showed that the ethanolic extracts from *P. eriopodon, P.cumanense and p.bogotense* have values of IC_{50} around or below to 30 µg/mL against human cancer cells, suggesting the presence of promising cytotoxic compounds in these *Piper* spp.

The cytotoxic activities of *P. eriopodon, P. cumanense, pesaresanum, P. bogotense* and *P. asperiusculum* against human cancer cell lines are reported for the first time in this study. The present data indicate that this ethanolic extracts are cytotoxic against human cancer cells and deserves further investigation in other cancer cell lines, as well as might be a potential source for the isolation of anticancer and chemotherapeutic agents.

The results showed that *P. eriopodon* has the most potent cytotoxic effect, specially the ethanolic extract from leaves with IC_{50} values of 11.88 µg/mL against prostate (PC-3), 17.84 µg/mL for breast (MDA-MB-231) and 20.75 µg/mL for lung (A549) cancer cells. In order to establish the presence of cytotoxic compounds, decision was made to study the three ethanolic extracts of *P. eriopodon* (leaves, wood and inflorescences) by liquid chromatography UHPLC to understand the complexity of the different extracts. The chromatographic profiles for the three ethanolic extracts were obtained in two detection systems, a first PDA profile measure at 254 nm and a second profile measure with an Evaporative Light Scattering Detector (ELSD) (Figure 1).

The UV chromatographic profiles showed higher complexity with many minority peaks at all retention times, mainly between 18 and 30 min which indicates a

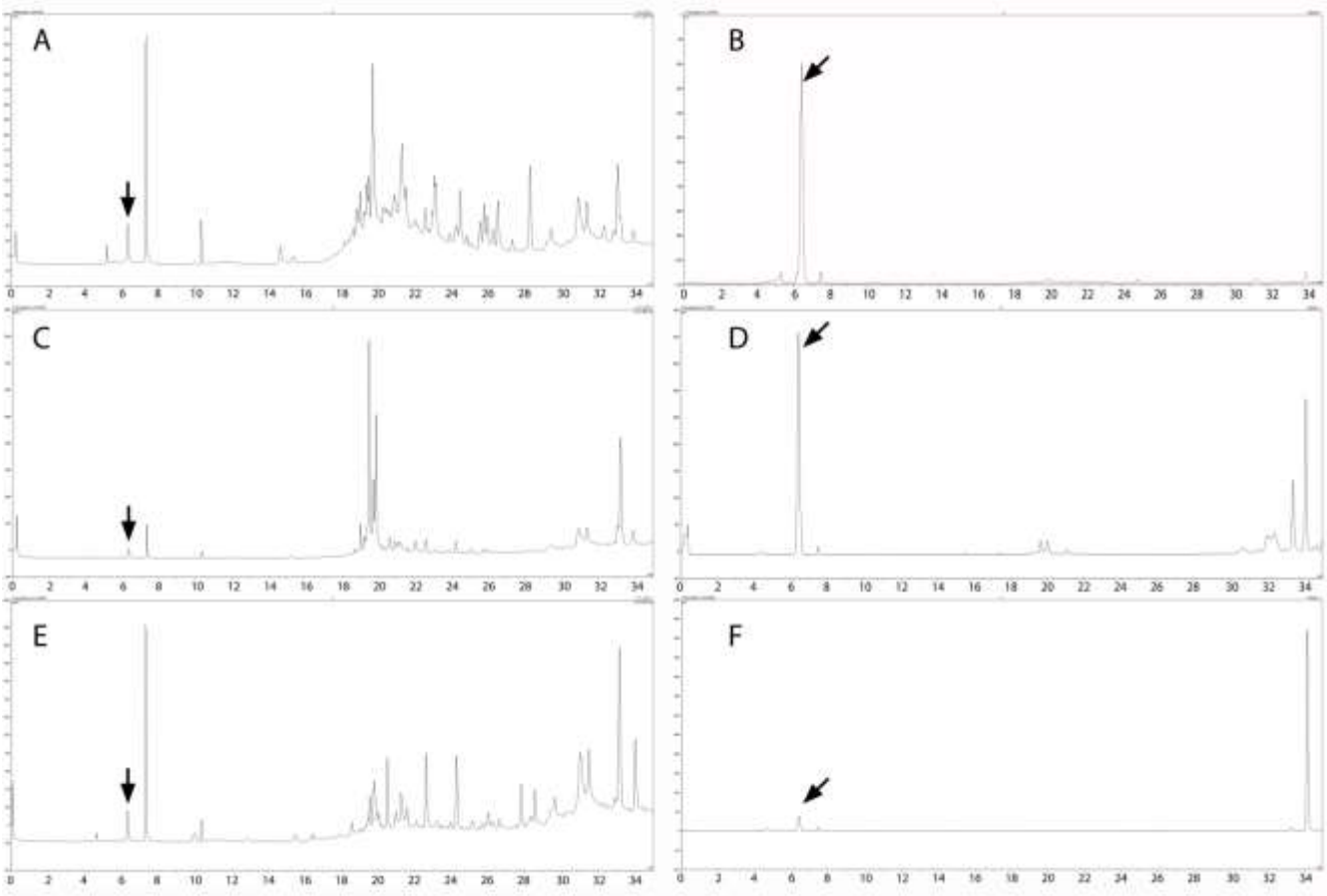

Figure 1. Chromatographic profiles at 254 nm for leaves (A), wood (C), inflorescences (E) and profiles with ELSD detector system for leaves (B), wood (D) and inflorescences (F) of the ethanolic extracts from *P. eriopodon*. The *X* axis corresponds to retention time in minutes and black arrow in all chromatograms corresponds to isolated compound gibbilimbol B.

massive presence of nonpolar metabolites. In the wood extract, the presence of two high peaks close to 20 min was observed, which was not current in the other extracts. In contrast, the chromatographic ELSD profiles for all extracts (Figure 1) showed low complexity, especially in leaves and wood, where almost a single peak is noted at 6.50 min. This observation can be considered indicative of large amounts of one compound in the ethanolic extracts. The ELSD detector response is related to the absolute quantity of analyte present, significantly different to UV detector, in which the analyte must have a chromophore, resulting in a signal completely dependent of the compound spectral properties (Swartz, 2010). The results, clearly showed the large presence of one compound in the ethanolic extracts of leaves and wood, followed by a smaller amount in inflorescences from *P. eriopodon*.

To establish the relation between this compound and the cytotoxic effect of *P. eriopodon*, the ethanolic extracts were subjected to a further purification, as described in the experimental section. The isolated compound was obtained from leaves in large amounts (7.93 g) and identified as an alkenylphenol named gibbilimbol B. The chemical structure of gibbilimbol B was elucidated completely by the interpretation of the spectral data and the comparison with those reported in the literature (Figure 2) (Orjala et al., 1998). The presence of gibbilimbol B was verified through comparison of chromatograms in all extracts, mainly in leaves, wood and in a smaller proportion in the inflorescences; which might be related to the greater cytotoxic activity of the ethanolic extract of leaves from *P. eriopodon* against cancer cells. Previously, this alkenylphenol had shown cytotoxic activity in KB carcinoma cells (ED_{50} = 3.9 ug/mL) (Orjala et al., 1998). Thus, was decided to evaluate the cytotoxic effect of gibbilimbol B against A549, PC-3, MDAMB-231 and MCF7 cells. The present results confirm that gibbilimbol B is effective in suppressing cancer cells growth in a dose-dependent manner, with IC_{50} values of 39.7, 32.1, 11.1 and 11.9 µg/mL for A549, PC-3, MDAMB-231 and MCF7 cells, respectively (Figure 3).

Gibbilimbol B was isolated previously in small quantities from the medicinal plant *P. gibbilimbum*, a

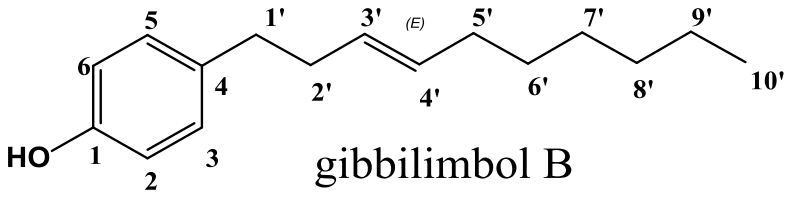

Figure 2. Chemical structure of isolated compound.

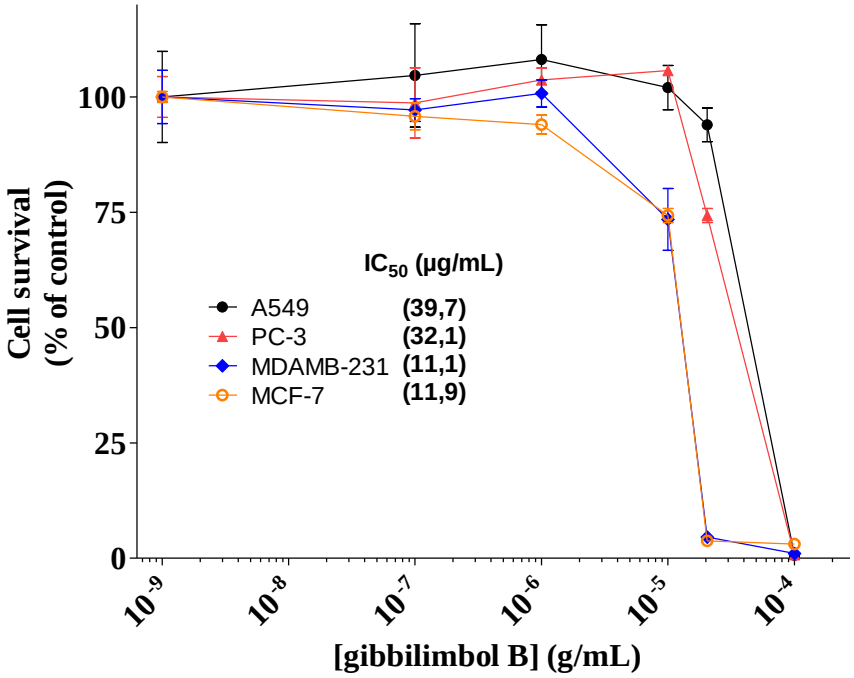

Figure 3. Inhibition of cell growth by gibbilimbol B and IC_{50} values for lung (A549), prostate (PC-3) and breast (MDAMB-231 and MCF7) cancer cells. The data is expressed as mean ± SEM (n = 4).

native plant from Papua New Guinea used in traditional medicine to treat fever, abscesses, ulceration of the skin and the juice from the bark is taken by patients with suspected cancer (Worth and Kerenga, 1987). Recently, this alkenylphenol was isolated too in small quantities from *Piper malacophyllum* and showed promising antitrypanosomal activity against *Trypanosoma cruzi*, with an EC_{50} of 17.49 µg/mL (de Oliveira et al., 2012; Varela et al., 2016). However, the limited amount of gibbilimbol B in nature restrict the possibility to perform new biological studies, prompted in scientists the interest to search a different synthetic strategies to obtain more quantities of this compound (Abe et al., 2001; Vyvyan et al., 2002; Zhou et al., 2004; Wang et al., 2009; Varela et al., 2016). The present results clearly show that gibbilimbol B is the majority component of the leaves from *P. eriopodon*, corresponding to 8% of the total ethanolic extract, indicating that *P. eriopodon* can be considered for further investigation as a natural source to obtain easily and in large amounts this bioactive alkenylphenol.

Some alkenylphenols reported as promising anticancer agents are 4-nerolidylcatechol and climacostol. 4-Nerolidylcatechol was isolated from *Piper umbellata* and induces apoptosis in SK-Mel-28 (melanoma) and in multidrug-resistant human chronic myeloid leukemia K562 cells with an IC_{50} of 24.5 µM (Cortez et al., 2015; Benfica et al., 2017). Climacostol is another potent cytotoxic alkenylphenol produced by the ciliated protozoan *Climacostomum virens* that inhibits selectively

the growth of tumor cells and induces apoptosis in cancer cells *in vitro* and *in vivo* assays (Buonanno et al., 2008; Perrotta et al., 2016).

Regarding to the IC_{50} values in cell lines tested, it was found that gibbilimbol B have partial selectivity for breast cancer cells MDAMB-231 and MCF7. The genetic differences between those cells lines include that MCF7 is estrogen and progesterone receptor positive (ER+ and PR+), HER2 negative (HER2-) and P53 wild-type, while MDAMB-231 cell line is triple-negative breast cancer (ER-/PR-/HER2-) and P53 mutant, an aggressive form of breast cancer with limited treatment options (Hahm and Singh, 2013; Neve et al., 2006). Because differences in IC_{50} values are not significant, the present study results suggest that the molecular basis implicated in the cell death are hormone independent and may be more associated with the inhibition of common pathways related to promote the oncogenic activities like cell cycle progression, senescence and metastasis in both cell lines as the PI3K/AKT pathway (Lin et al., 2009; Gao et al., 2009) or even the inhibition of the high expression levels of XIAP reported for both cell lines (Hahm and Singh, 2013; Nikolovska-Coleska et al., 2004; Obexer and Ausserlechner, 2014).

In conclusion, the findings of current study showed that *P. eriopodon* extracts present the highest cytotoxic activity among the others *Piper* selected Colombian plants. The alkenylphenol gibbilimbol B is the majority component in *P. eriopodon* and the results of the present study showed that gibbilimbol B present highly cytotoxic activity in human cancer cells, partially selective for breast cancer cells MCF7 and MDAMB-231. Though molecular basis involve in the triple-negative breast cancer is unclear, the findings of this study show that gibbilimbol B is a promising cytotoxic compound and is crucial in the future to perform molecular analysis to understand the mechanism involve in the induced cell death mediated by this alkenylphenol.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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