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Full Length Research Paper

Evaluation of some medicinal properties of *Ceiba aesculifolia* subsp. *parvifolia*

J. Orozco[1], M. A. Rodriguez-Monroy[2], K. E. Martínez[1], C. M. Flores[3], M. Jiménez-Estrada[4], A. Durán[1], R. Rosas-López[5], L. B. Hernández[3] and M. Canales[1]*

[1]Laboratorio de Farmacognosia, UBIPRO Facultad de Estudios Superiores-Iztacala UNAM, Tlalnepantla, Edo. Mex, Mexico.
[2]Carrera de Medicina, Facultad de Estudios Superiores-Iztacala UNAM, Tlalnepantla, Edo. Mex, Mexico.
[3]Laboratorio de Fisiologia Vegetal, UBIPRO Facultad de Estudios Superiores-Iztacala UNAM, Tlalnepantla, Edo. Mex, Mexico.
[4]Instituto de Quimica, UNAM, Circuito Exterior, Ciudad Universitaria, Coyoacan D.F., Mexico.
[5]Universidad de la Cañada, Carretera Teotitlan-San Antonio Nanahuatipan Km 1.7, Teotitlan de Flores Magon, Oaxaca, Mexico.

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The bark of *Ceiba aesculifolia* subsp. *parvifolia* is used for the treatment of diabetes, kidneys maladies, tumors, gastritis, and wounds by the people of San Rafael, Coxcatlan, Puebla. The aim of this work was to investigate antimicrobial activity, general toxicity, and antioxidant activity of the bark of this species. The methanol extract was obtained by maceration. After obtaining the methanol extract (MeOH1), methanol (MeOH2) and hexane (H) fractions were obtained. The antibacterial and antifungal activities were evaluated through the disc-diffusion method. The general toxicity test was carried out by using brine shrimp *Artemia salina.* The quenching of free radicals was evaluated by the decolouration of a methanol solution of diphenyl picryl hydrazyl (DPPH). Total phenolics were determined by Folin Ciocalteu reagent. Gram-positive and Gram-negative bacteria were sensitive to MeOH1 and MeOH2; MeOH2 had a bactericidal effect on *Staphylococcus epidermidis* (minimum inhibitory concentration (MIC) = 0.75 mg/ml) and *Vibrio cholera* Tor (MIC = 0.50 mg/ml). MeOH2 showed inhibition of DPPH radical (SC_{50} = 6.15 µg/ml), 28% of total phenolics and was toxic against *A. salina* (LC_{50} = 2.82 µg/ml). The methanol extract showed a variety of different compounds such as terpens, isoflavones and a coumarin. The antimicrobial, antioxidant, and toxicity activities and a qualitative chemical characterization of *C. aesculifolia* subsp. *parvifolia* are reported here for the first time.

Key words: *Ceiba aesculifolia*, Bombacaceae, medicinal bark, antibacterial activity, antioxidant activity.

INTRODUCTION

Plants have formed the base of traditional medicine systems that have been under existence for thousands of years, and it continues the contribution of new remedies to humanity (Gurib-Fakim, 2006). Mexico is considered one of the countries with more biota diversity in the world; it ranks in the fourth site for its diversity of fanerogam plants, with approximately 30,000 species, that represent between the 10 and 12% of the total world estimate (Alanís et al., 2004).

The Tehuacan-Cuicatlan valley has great importance for biological conservation, being one of the main reserves of biodiversity of arid and semi-arid areas in Mexico (Canales et al., 2006). San Rafael is located inside the valley of Tehuacan-Cuicatlan and the place is

*Corresponding author. E-mail: dra.margaritacanales@gmail.com. Tel: +52-5-623-11-36. Fax: +52-5-623-12-25.

subjected to regional ethnobotanical research that seeks to understand the current condition of natural resources, and to provide management tools for the use and preservation of natural resources within the boundaries of the biosphere preserve of Tehuacan-Cuicatlan. The aim of this work was to investigate the antibacterial, antifungal, antioxidant, and toxicity activities of the bark of *Ceiba aesculifolia* subsp. *parvifolia* a Bombacaceae in connection with the traditional use. This species locally known as "pochote", is used in San Rafael, Coxcatlan to treat diabetes, kidney maladies, gastritis, tumors, and wounds (Avendaño et al., 2006; Canales et al., 2005, 2006).

MATERIALS AND METHODS

Plant

The bark of *C. aesculifolia* subsp. *parvifolia* was collected in August, 2007 in San Rafael, Coxcatlan, Puebla. Voucher specimens were deposited in the herbarium, IZTA, at the Facultad de Estudios Superiores Iztacala (voucher no. RRL147).

San Rafael is a village in the municipality of Coxcatlan, located southeast of the Tehuacan-Cuicatlan valley, at coordinates 18°12' and 18°14' North and 97°07' and 97°09' West, at 957 m above sea level. The climate is dry or arid with summer rains and a mean temperature of 22°C (Fernandez, 1999).

The vegetation is a thorn scrub forest with species like *Bursera morelensis* Ramírez, *Bursera aptera* Ramírez, *Pachycereus weberi* (J. Coulter) Backeb, *Opuntia puberula* Pfeiffer, *Ceiba parvifolia* Rose, and *Acacia cochliacantha* Humb. and Bonpl. ex Willd (Rzedowski, 1978; Fernández, 1999; Casas et al., 2001).

Collections of specimens in the field were carried out with permission from the "Secretaria de Medio Ambiente y Recursos Naturales" of Mexico (SGPA/DGVS/1266).

Preparation of the extracts

The bark of *C. aesculifolia* subsp. *parvifolia* (940 g) was extracted with methanol by maceration at room temperature. After filtration, the solvent was evaporated under reduced pressure obtaining the methanol extract (MeOH1). The yield of MeOH1 extract was 84 g (8.94%). 65 g of MeOH1 extract were redissolved in methanol and afterwards hexane was added to it, in a separating funnel. After solvent-solvent extraction, the methanol fraction (MeOH2) was removed from the hexane fraction (H) phase. After solvent elimination, MeOH2 left 57.34 g (88.21%) and H 6.24 g (9.6%).

Antibacterial activity

The following strains of bacteria were used: *Vibrio cholerae* INDRE 206 (isolated from polluted water), *V. cholerae* (a clinical isolate corresponding with group 01, producing enterotoxin, serotype "Inaba", biotype "El Tor"), *V. cholera* CDC V 12, *V. cholerae* Serotype No 01 ATCC 35971, *Escherichia coli* ATCC 25922, *Enterobacter agglomerans* ATCC 27155, *Salmonella typhi* ATCC 19430, *Staphylococcus aureus* ATCC 12398, *Enterobacter aerogenes, Staphylococcus epidermidis, Bacillus subtilis* and *Sarcina lutea* (donated by the Laboratory of Microbiology of FES-Cuatitlan UNAM), *Yersinia enterocolitica* (donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala).

The antibacterial activity was measured by disc-diffusion method (Vanden Berghe and Vlietinck, 1991). The microorganisms were grown overnight at 37°C in 10 ml of Müeller-Hinton broth (Bioxon 260-1, Estado de Mexico, Mexico). The cultures were adjusted to turbidity comparable to that of Mc Farland No. 0.5 standard with sterile saline solution. Petri dishes containing Müeller-Hinton agar (Bioxon, Edo. de Mexico, Mexico) were impregnated with these microbial suspensions. Concentrations of 200 mg/ml of each extract were prepared, discs (Whatman No. 5) of 5 mm diameter were impregnated with 10 µl of each one (final doses per disc: 2 mg of MEOH1, MEOH2 and H). Discs impregnated with 10 µl of hexane and methanol, were used as negative controls. Discs of chloramphenicol (25 µg) were used as positive controls. The plates were incubated overnight at 37°C and the diameters of any resulting inhibition zones (mm) were measured. Each experiment was repeated at least three times. The estimate of the minimal inhibitory concentration (MIC) was carried out by the broth dilution method (Vanden Berghe and Vlietinck, 1991). Dilutions of plant extracts from 2.0 to 0.125 mg/ml were used. The tubes were inoculated with microorganism suspension of 1×10^5 CFU/ml. MIC values were defined as the lowest extract concentration that prevents visible bacterial growth after 24 h of incubation at 36°C. Each experiment was repeated at least three times. The bactericidal kinetic assay was performed using the appropriate concentrations of extract (corresponding to ½ MIC, MIC and minimum bactericidal concentration (MBC)) (Lennette et al., 1987).

Antifungal activity

The fungi strains used were: *Fusarium sporotrichoides* (ATCC NRLL3299), *Fusarium moniliforme* (CDBB-H-265), *Trichophyton mentagrophytes* (CDBB-H-1112), *Aspergillus niger* (CDBB-H-179), *Rhizoctonia lilacina* (CDBB-H-306) and *Rhizoctonia solani* (donated by INIFAP, Celaya, Mexico). The assay of antifungal activity was carried out in Petri dishes (80 × 10 mm) containing Czapek Dox agar (30 ml) (Bioxon, Edo. de Mexico, Mexico). After the mycelial colony had developed, discs impregnated with 2 mg of hexane extract, were placed at a distance of 10 mm away from the Petri dish border, then were incubated at 23°C for 72 h until mycelial growth had enveloped discs containing the control and had formed crescents of inhibition around discs containing samples with antifungal activity (Ye et al., 1999). Ketoconazole (7 µg/disc) was used as a positive control.

For quantitative assays, a cultive plate of 24 wells was used. Seven dilutions of plant extract were added to Czapek Dox agar (5 ml) at 45°C, these being mixed rapidly and poured into three wells of a cultive plate. One day after, a small amount (1 × 1 mm) of mycelia, the same amount to each well, was inoculated. Dimethyl sulfoxide (DMSO) (20 µl in 5 ml of agar) was employed for a negative control. Ketoconazole was used as a positive control. After incubation at 23°C for 48 h, the area of the mycelia colony was measured, and the inhibition of fungal growth and hence the IC_{50} was determined by the following formula:

$$I\ (\%) = dc - dt/dc \times 100$$

where dc is the diameter of the colony of the control culture, dt is the diameter of the colony of the treated culture.

The IC_{50} values were calculated by rectangular hyperbola regression of plots, where the abscissa represents the concentration of tested plant extract and the ordinate represents the average percent of inhibition of fungal growth from three replicates.

General toxicity assay

The general toxicity test was done with the *in vivo* brine shrimp lethality test using brine shrimp *Artemia salina* (Leach) larvae,

according to the methodology described by McLaughlin (1991). Each plant extract was tested at 1000, 100 and 10 ppm (µg/ml) and also evaluated by triplicate. Samples were prepared by dissolving extracts in DMSO. The final DMSO concentration did not exceed 1%, which has been shown not to have any harmful effects on the larvae. As positive control, gallic acid was used (LC_{50} = 321.5 µg/ml); and as negative control, DMSO was used. Survivors were counted after 24 h. LC_{50} was determined from the 24 h counts. The general toxicity activity was considered weak when LC_{50} was between 500 and 1000 µg/ml, moderate when the LC_{50} was between 100 and 500 µg/ml, and designated as strong when the LC_{50} ranged from 0 to 100 µg/ml (Padmaja et al., 2002).

Diphenyl picryl hydrazyl (DPPH) decolouration assay

The quenching of free radicals by extracts was evaluated spectrophotometrically at 517 nm by the decolouration of a methanol solution of DPPH according to Murillo (2006). A freshly prepared DPPH solution (4 mg/100 ml methanol) was used for the assays. Samples were dissolved in methanol (1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, and 100 µg/ml) and the DPPH solution served as a control. The degree of decolouration indicates the free radical scavenging efficiency of the samples. Quercetin was used as reference free radical scavenger (SC_{50} = 4.6 µg/ml). The percentage of DPPH decolouration was calculated as follows:

$$\text{Inhibition percentage (Ip)} = [(A_B - A_A)/A_B)] \times 100$$

where A_A is the absorbance of the sample and A_B is the absorbance of the control (Yen and Duh, 1994).

The SC_{50} was calculated when allowed according to the scavenging efficiency. The SC_{50} values were calculated by rectangular hyperbola regression of plots, where the abscissa represented the concentration of tested plant extract and the ordinate the average percent of scavenging capacity from three replicates.

Determination of total phenolics

Total phenolics were determined by Folin Ciocalteu reagent (Singleton et al., 1999). A solution of methanol extract or gallic acid was mixed with distilled water, Folin Ciocalteu reagent, and aqueous Na_2CO_3 (20%). The mixtures were allowed to stand for 120 min and the total phenols were determined by colorimetry at 760 nm. The standard curve was prepared using 0.00625, 0.0125, 0.025, 0.05, 0.1, and 0.2 mg/L solutions of gallic acid in water. Total phenol values are expressed in terms of gallic acid equivalent.

Fractionation of MeOH2

The MeOH2 extract was subjected to silica gel column chromatography (silica gel mesh 70-230 Sigma 5-2509, St. Louis, MO-USA). The column was eluted with the following gradient of solvents, hexane: chloroform, chloroform: ethyl acetate, ethyl acetate: methanol; obtaining sixteen collected partitions. The purification of the active compounds was made by assay-guided isolation; the antibacterial activity of all collected partitions was measured by disc-diffusion method (Vanden Berghe and Vlietinck, 1991). The most active partitions were analyzed using high performance liquid chromatography (HPLC) on a HP Series 1100 separations module (Hewlett-Packard, Wilmington, DE, USA), equipped with an 1100 diode array detector (DAD), and operated with Instrument ChemStation A.09.03 [1417] software. Separations were carried out on a discovery C_{18} column (4.6 × 250 mm, 5 µm) with a flow rate of 1.0 ml/min. The sample volume injected was 20 µl and data were analyzed at 220 to 400 nm. The standard compounds were catechin (retention time (Rt) = 3.57 min), gallic acid (Rt = 3.03 min), catechol (Rt = 4.32 min) and quercetin (Rt = 8.44 min). The identification of the constituents was assigned on the basis of comparison of their ultraviolet (UV) spectra with those given in the literature (Rijke et al., 2006). The mobile phase for the analysis consisted of a methanol:acetonitrile:water 25:25:50 system, and was developed by isocratic way.

Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation of the three experiments were determined. The IC_{50}, LC_{50} and SC_{50} values were calculated by rectangular hyperbola model.

RESULTS

The results obtained in the evaluation of the antibacterial activity of the extract and fractions of *C. aesculifolia subsp. parvifolia* bark are shown in Table 1. The hexane fraction did not have any activity. MeOH1 and MeOH2 were active against all Gram-positive bacteria. MeOH1 was active against six Gram-negative bacteria (four strains of *V. cholerae, E. coli* and *S. typhi)*, this extract showed the lowest MIC in *S. aureus, S. epidermidis, V. cholerae* No-01, *V. cholerae* Tor and *V. cholerae* cc (MIC = 0.50 mg/ml). MeOH2 was active against five Gram-negative bacteria (four strains of *V. cholerae,* and *E. coli)*, this extract showed the lowest MIC in *S. aureus, S. epidermidis, S. lutea, V. cholerae* No-01, *V. cholerae* Tor and *V. cholerae* cc (MIC = 0.75 mg/ml).

Figures 1 and 2 show the effect of the MeOH2 in the survival curve on *S. epidermidis* and *V. cholerae* Tor. MIC (0.75 mg/ml) had bacteriostatic effect and MBC (1.00 mg/ml) had a lethal effect on the bacterial population of *S. epidermidis* at 12 h; the same is shown in *V. cholerae* Tor (MIC = 0.50 mg/ml and MBC = 0.75 mg/ml) at 7 h. MeOH1, MeOH2 and H did not show any antifungal activity.

MeOH2 was toxic against *A. salina* (LC_{50} = 2.82 µg/ml). MeOH2 presents a potent antioxidant activity, efficiently scavenging the DPPH free radical with a SC_{50} value of 6.15 µg/ml. MeOH2 showed 28% of the total phenolics as gallic acid equivalents.

For MeOH2, sixteen fractions were obtained from silica gel column chromatography; only fractions 4 to 8 had antibacterial activity against *V. cholerae* Tor, fraction 6 presents the higher halo (11 mm). The effect of fraction 6 on the survival curve of *S. epidermidis* was bacteriostatic (MIC = 0.75 mg/ml), the same was observed on *V. cholerae* No-01 (cell mediated immune (CMI) = 0.50 mg/ml). Fraction 6 presented an antioxidant activity of 3.57 µg/ml and showed 50% of the total phenols as gallic acid equivalents. According to the HPLC analysis, fractions 5, 6, and 7 show a variety of different compounds like terpens (UV λ_{max} = 236 nm), isoflavones

Table 1. Antimicrobial activities of *C. aesculifolia* subsp. *parvifolia*.

Bacteria	Positive control Chloramphenicol	MeOH1 (mm)	CMI (mg/ml)	MeOH2 (mm)	CMI (mg/ml)
Sa	10.00 ± 1.00	9.00 ± 1.00	0.50	10.00 ± 0.00	0.75
Se	6.66 ± 1.15	8.70 ± 1.15	0.50	9.00 ± 0.00	0.75
Sl	32.00 ± 0.50	16.70 ± 1.5	0.75	16.30 ± 0.60	0.75
Bs	29.33 ± 2.62	8.00 ± 1.00	1.00	8.30 ± 0.60	1.00
Vch Indre	8.33 ± 0.58	14.00 ± 0.00	1.00	6.00 ± 0.00	1.00
Vch No-01	7.33 ± 0.58	7.70 ± 0.60	0.50	6.00 ± 0.00	0.75
Vch Tor	7.33 ± 0.58	7.70 ± 1.15	0.50	6.00 ± 0.00	0.75
Vch cc	27.67 ± 0.47	8.70 ± 1.15	0.50	6.00 ± 0.00	0.75
Ec	21.67 ± 1.70	7.30 ± 0.60	1.00	6.00 ± 0.00	1.5
St	28.00 ± 1.63	7.60 ± 1.15	1.00	Na	Na

Sa, *Staphylococcus aureus*; Se, *Staphylococcus epidermidis*; Bs, *Bacillus subtilis*; Sl, *Sarcina lutea*; Vch No-01, *Vibrio cholerae* No-01; Vch cc, *Vibrio cholera* (clinical isolate); Vch Indre, *Vibrio cholera* (isolated from water); Vch Tor, *Vibrio cholera* CDC V12; Ec, *Escherichia coli*; St, *Salmonella typhi*. Na: no activity.

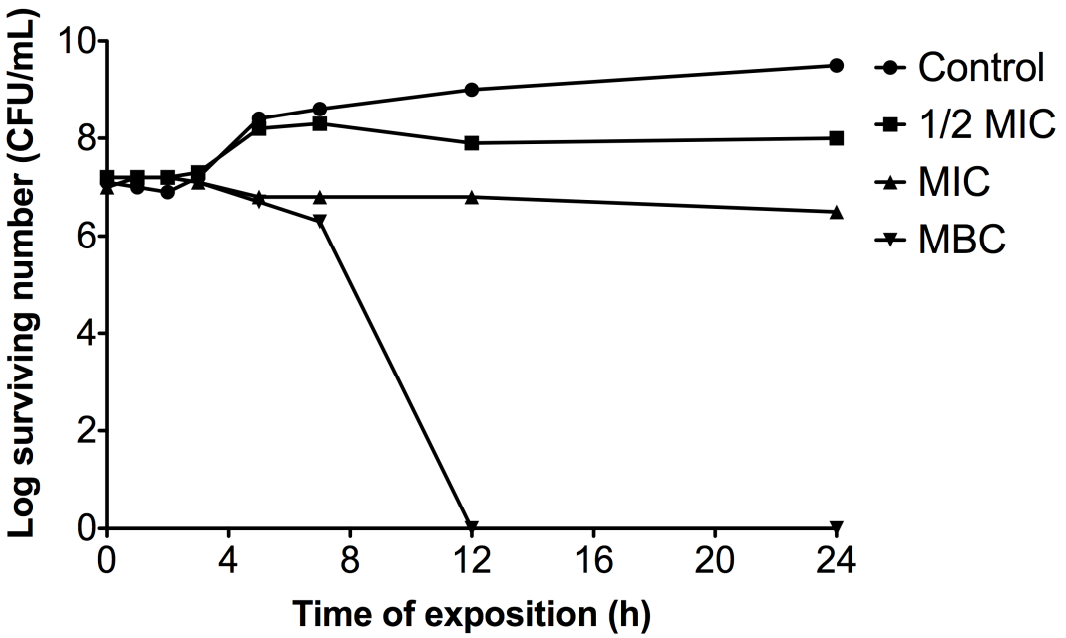

Figure 1. Survival curve of *S. epidermidis* exposed to MeOH2 of C. aesculifolia subsp. parvifolia. MeOH2 was added to each experimental culture in zero time. The concentrations used were: 0.375 mg/ml (½ MIC), 0.75 mg/ml (MIC), 1.00 mg/ml (CBM), the control tube did not contain MeOH2.

(UV λ_{max} = 236, 262, and 282 nm) and phenylpropanoids (UV λ_{max} = 278 to 280 nm) (Table 2).

DISCUSSION

The bark of *C. aesculifolia* subsp. *parvifolia* is used in traditional medicine in San Rafael, Coxcatlán, Puebla for the treatment of diabetes, kidney maladies, tumors, gastritis, and wounds (Canales et al., 2005, 2006). It is important to note that the antibacterial, toxicity, and antioxidant activities are reported here for the first time.

MeOH1 and MeOH2 showed antibacterial activity. The effect of MeOH2 extract on the bacterial population of *S. epidermidis* and *V. cholerae* Tor was bactericidal (Figures 1 and 2). The wounds can infect with Gram-positive and negative bacteria and the principal causes of the kidneys maladies are renal calculi, cancer, kidney failure and Gram-negative bacterial infection (Madigan et al., 2009), this was the reason why the extracts were evaluated in

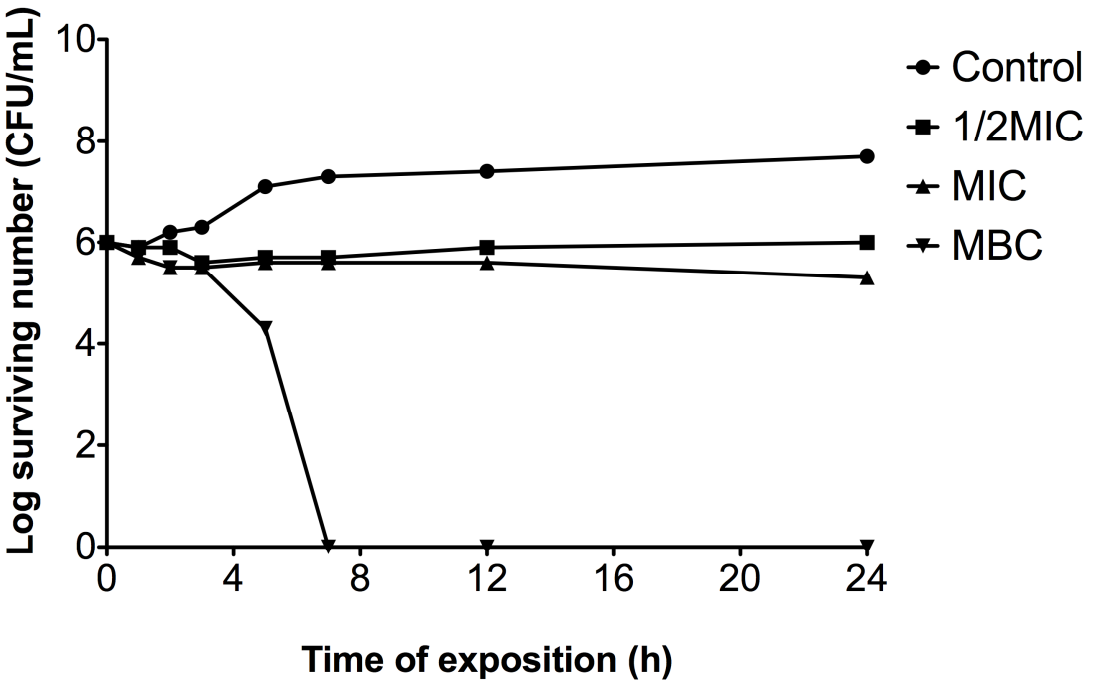

Figure 2. Survival curve of *V. cholerae* Tor exposed to **MeOH2** of *C. aesculifolia* subsp. *parvifolia*. The MeOH2 was added to each experimental culture in zero time. The concentrations used were: 0.25 mg/ml (½ MIC), 0.50 mg/ml (MIC), 0.75 mg/ml (MBC), the control tube did not contain MeOH2.

Table 2. HPLC of the fractions of the MeOH2.

Fraction	Rt (min)	UV λ_{max} (nm)
5	2.676	236
	3.822	236
	5.003	278
	5.503	280
	6.309	282
	7.609	232,282,310
6	6.379	284
	7.732	236,262,282
	10.659	258
7	3.849	264,280
	4.615	236,280
	5.282	236,276
	6.075	236,262,282

two types of bacteria.

In the antioxidant activity, MeOH2 of *C. aesculifolia* subsp. *parvifolia* showed a strong antioxidant activity (SC_{50} = 6.15 µg/ml) and it has a good content of the total phenolics (28% as gallic acid equivalents). The antioxidant activity is important, because it is a control of the reactive oxygen species that are implicated in many diseases and most probably plays a part in the wound healing properties (Kumary and Kakkar, 2008). With respect to the toxicity, MeOH2 was strong over *A. salina* (CL_{50} = 2.82 µg/ml) (Padmaja et al., 2002). This assay is a preliminary study to evaluate toxicity and in this way validate the use against tumors (McLaughlin et al., 1993).

The HPLC chromatogram obtained of 220 at 400 nm

for methanol extract showed a variety of different phenolic compounds such as terpens, phenylpropanoids, isoflavones, and a coumarin; there is only one study of the genus *Ceiba*, and Nogounou et al. (2000) isolated some isoflavones from the stem and bark of *Ceiba pentandra.*

In conclusion, our results suggest that *C. aesculifolia* subps. *parvifolia* has different compounds with anti-bacterial, antifungal and antioxidant activities that probably are responsible for this species bark medicinal properties against kidney maladies and tumors as well as wound healing capacity.

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