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Tripanocide and antibacterial activity of *Alvaradoa subovata* Cronquist extracts

[Actividad tripanocida y antibacteriana de extractos de *Alvaradoa subovata* Cronquist]

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Abstract
We studied antioxidant, antibacterial and tripanocide activities of *Alvaradoa subovata* extracts. The ethanolic extracts showed the greatest DPPH radical scavenging capacity, especially that of bark with an IC_{50} = 4.7 ± 0.18 µg/mL. Wood dichloromethane extract displayed growth inhibition of the phytopathogenic bacteria *Xanthomona axonopodis* in the disk diffusion assay and showed a MIC value of 100 µg/ml. It also showed growth inhibition of *Trypanosoma cruzi* (IC_{50} = 0.063 ± 0.003 mg/mL). A fraction of this extract, which has emodin as the main component, showed tripanocide activity (60% of growth inhibition at 100 ug/mL). The main compounds in wood dichloromethane extract were anthraquinones, identified as chrysophanol and emodin, and coumarins, of which scopoletin was identified. These three compound s could serve as analytical markers of the extract. The results of this study show that wood extract of *A. subovata* constitute a source of bioactive compounds such as antiparasitic and pesticides agents.

Keywords: *Alvaradoa subovata*, DPPH, Picramniaceae, tripanocide, *Xanthomona*

Resumen
En el presente trabajo se estudió la actividad antioxidante, antibacteriana y tripanocida de extractos de *Alvaradoa subovata*. La mayor actividad depuradora de radicales libres se observó en el extracto etanólico de corteza (CI_{50} = 4.7 ± 0.18 µg/mL). El extracto en diclorometano de madera inhibió el crecimiento de la bacteria fitopatógena *Xanthomona axonopodis* con una CIM = 100 µg/mL. El mismo extracto mostró inhibición del crecimiento de *Trypanosoma cruzi* (CI_{50} = 0.063 ± 0.003 mg/mL). Una fracción de este extracto (100 ug/mL), cuyo componente mayoritario es emodina, inhibió en un 60% el crecimiento del parásito. Los compuestos mayoritarios detectados en el extracto de madera fueron antraquinonas, entre las cuales se identificaron emodina y crisofanol, y la cumarina escopoletina. Estos tres compuestos podrían servir como marcadores analíticos del extracto. Los resultados de este trabajo muestran que los extractos de *A. subovata* constituyen una fuente de compuestos bioactivos con potencial como antiparasitarios y plaguicidas.

Palabras Clave: *Alvaradoa subovata*, DPPH, Picramniaceae, tripanocida, *Xanthomona*

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INTRODUCTION

Alvaradoa subovata Cronquist. (Picramniaceae) is a small tree belonging to the recently established Picramniaceae family (Fernando and Quinn, 1995). This family also consists of the *Picramnia* Sw. and *Alvaradoa* Liebm. genera.

Some species of Picramniaceae family were traditionally used for the treatment of fever and malaria (Soto de Villatoro *et al.*, 1974). This parasitic disease is of low incidence in South America. However, among other problems endemic to this region is chagas disease (American trypanosomiasis) caused by the *Trypanosoma cruzi* parasite and more than 100 million people live in risk of infection (Castro *et al*., 2006).

Although the incidence of Chagas has declined over the last years, it is still a difficult disease to eradicate (Moncayo, 2003). The current therapy is based on two drugs, nifurtimox and benznidazole, that act through the generation of free radicals during their metabolism. These drugs proved to be effective in the acute phase of the illness; however their effectiveness in the chronic state is controversial. Moreover they are poorly tolerated and serious side-effects have been described in approximately 50% of treated patients (Castro *et al*., 2006). Therefore, it is still necessary to develop safer and more efficient drugs.

Anthrones and anthraquinone glycosides that act against malaria and other protozoan species, were identified on species of *Picramnia* (Solis *et al*., 1994; Hernandez-Medel & Pereda-Miranda, 2002; Camacho *et al*., 2003), thus justifying their popular use. Natural hydroxy-anthraquinones that show activity against *T. cruzi* have also been identified (Castro *et al*., 1994).

On the other hand, isolated anthraquinones from different families have shown pesticide and bactericide activities (Wei *et al.,* 2008; Godard *et al.,* 2009; Li *et al*., 2009; Yadav *et al.*, 2009; Borroto *et al*., 2010). The bacterium *Xanthomonas axonopodis* pv. citri type A (Xac) is the causal agent of citrus canker. It is a disease that weakens the plant and its fruits develop typical dark spots (cankers). This disease has a strong impact mainly on the trading of the fruit that cannot be sold in international markets and treatment is required in the packing of all fruit (Das, 2003; Gottwald *et al*., 2002). Research on the development of safer environmental methods to combat this bacterium is currently being carried out. The use of plants and their derivatives for pest control is in expansion. Most of these compounds are known to be rapidly degradable after their application, leaving minimal residues (Kambou *et al.,* 2008).

Although it has been reported that *Alvaradoa* species produce mainly anthraquinones and related compounds (Pearl *et al*., 1973; Soto de Villatoro *et al*., 1974; Harding *et al.*, 1999; Jacobs, 2003; Phifer *et al*., 2007), until now no studies have been undertaken on the antimicrobial or antiparasitic activities of crude extracts obtained from *A. subovata.*

Materials and methods

Plant material

Specimens from the following herbaria were examinated and used as reference materials, UNR and SI.. The fresh material was collected and checked by Dr. Ponesa and stored in the UNR herbarium. The vegetal material was collected with flowers and/or fruits to enable identification. The asterisk indicates the material used for this study.

A. subovata was collected from Argentina, Prov. of Jujuy, Dpto. Valle Grande, River Jordán, in December 1977. A voucher specimen was deposited in the herbarium of SI referring Kiesling and Ulibarry 1634; Dpto. Santa Bárbara, Abra de los Morteros, in January 1975 referring Zuloaga and Deginiani 278. *Prov. Salta*: Dpto. Orán, ruta Prov.18 a 3-4 Km del Puente Internacional Argentina-Bolivia, 22º43'S 64º43'W, 1-V-2003, referring Morrone *et al* 4536*;* Dpto. La Viña, in February1951, referring Hunziker 1226 (SI). Prov. Tucumán, Dpto. Burrucayú, in July 2007. A voucher specimen was deposited in the herbarium of UNR Gattuso M. 248*.

Preparation of extracts and partial purification

Leaf (L) (5.0 g), bark (B) (5.0 g) and wood (W) (180.0 g) of *A. subovata* were extracted three times with ethanol (EtOH) or dichloromethane (DCM) at room temperature and then concentrated under reduced pressure yielding the residues: L_{EtOH} (1.40 g), B_{EtOH} (0.53 g), W_{EtOH} (0.22 g), L_{DCM} (0.4 g), B_{DCM} (0.05 g) and W_{DCM} (0.9 g). Each of them was resuspended in the corresponding solvent or in DMSO. The DCM extracts were resuspended in EtOH to determine the antioxidant activity.

W_{DCM} extract (0.8 g) was subjected to chromatography on a Silica gel-60 column (40 cm length, 2 cm i.d.) equilibrated with hexane and eluted with a stepwise gradient of hexane and ethyl acetate. Fifty two fractions of 15 mL each were collected and analyzed using TLC aluminum sheets. On the basis of

the TLC profile similar fractions were pooled into 14 fractions: I to XIV.

Fractions I, II and IV were subjected to HPLC, under the conditions for separating anthraquinones (Ding *et al*., 2003). Briefly, 100 mg of each fraction were resuspended in 25 mL ethyl acetate. After shaking, an aliquot of 0.5 mL was taken from each one and the solvent was evaporated again. Finally, the fractions were resuspended in 0.5 mL methanol and filtered: 100 µl of each one was injected into a C18 column (Phenomenex Luna C18 column, 100 °A pore size, 5 µm particle size, 250 x 4.6 mm i.d. column). Methanol: 0.5% acetic acid (85:15) was used as the mobile phase, the flow: 0.6 ml/min and the detector (Spectra 100 UV-Vis), the chromatograms were recorded at 254 nm (Ding *et al.*, 2003). The eluted fractions were then labeled: T1 to T12 and analyzed by TLC.

DPPH radical-scavenging activity

Quantitative assay

DPPH radical-scavenging activities of the DCM and ethanol extracts were measured as previously described by Argolo *et al*., (2004).

In this assay, an aliquot (20 µL) of each extract (dissolved in ethanol, with samples ranging from 0.01 to 0.6 mg/ml) was mixed separately with ethanolic solution of DPPH (3 mM) in a final volume of 1 mL. After 20 min or the indicated time, the disappearance of DPPH radical was monitored by measuring the optical density at 515 nm. Scavenging activity was determined using the following equation: % DPPH radical-scavenging = [(control absorbance - sample absorbance)/control absorbance] × 100

The positive control was ascorbic acid, and DPPH solution without extract was used as negative control. Each determination was repeated three times.

The inhibitory concentration 50 (IC_{50}) value was obtained by extrapolation of the linear regression analysis and the concentration of the sample required to scavenge 50% DPPH radicals.

DPPH autography assay

In order to determine the number of active antioxidant compounds, a drop (5 µL) of ethanol and dichloromethane extracts (2 mg/mL) were placed individually on a TLC plate which was eluted with different mobile phases. The TLC sheet was immersed in a methanolic solution of DPPH (0.4 mM) for 10 s. Active compounds were observed as yellow spots against a purple background.

Tripanocide assay

Epimastigotes from CL Brener and Y strains were cultured in LIT medium supplemented with 10% fetal bovine serum (FBS) at 28 ± 0.5 °C in 24 wells culture flasks (Camargo, 1964). Cultures (3 - 4 × 10^6 parasites/ml) were incubated with increasing amounts of each extract in DMSO (1% final concentration). The parasites' growth was monitored by counting them in a Neubauer chamber. The percentage of inhibition was calculated as the *ratio* between parasites' growth in the presence or absence of each extract, after 72 h of culture. IC_{50} was obtained by plotting the percentage of inhibition against extract concentration. Each experiment was performed in triplicate and tripanocide drug benznidazole (BZL) was used as positive control.

Assay of the antibacterial activity

Culture conditions and media

The phytopathogenic bacterial strain used was *Xanthomonas axonopodis* pv. citri type A (Xac). Cells of Xac were cultivated at 28 °C on Silva-Buddenhagen (SB) medium (5 g/L sucrose, 5 g/L yeast extract, 5 g/L casein peptone and 1 g/L glutamic acid, at pH 7), under aerobic conditions with agitation. For plate cultures the medium was supplemented with 1.5% w/v agar in the case of solid medium and 0.7% w/v for semisolid medium. The antibiotic, ampicilin, was used at a final concentration of 25 µg/mL.

Disk diffusion assay

An aliquot (50 µL) of an overnight culture was mixed with 10 mL semisolid SB-agar medium and it was poured onto a plate of SB-agar medium, both supplemented with antibiotic. Once the agar was solidified, five sterile paper discs (5 mm in diameter) were placed on the plate. Four were embedded with 7 µL W_{DCM} extract of *A. subovata*, resuspended in DCM, of different concentrations (50, 25, 10 and 5 mg/mL); and one, the negative control, placed in the centre, with the same volume of DCM. In all cases the solvent was left to evaporate before the disc was placed on the plate. The zones of growth inhibition were measured after incubating the plates in an oven at 28 ºC for 48 h.

Quantitative antibacterial activity assay by minimum inhibitory concentration (MIC)

The microplate serial dilution method was used to determine the minimum inhibitory concentration (MIC) of wood extract against Xac. Extract were dissolved in acetone (10 mg/ml) and diluted serially twofold with SB medium. 4 µL of standardized suspension of bacteria (10^9 CFU/mL) was added to each well containing different concentrations of the extract (from 12.5 to 400 µg/mL) with a total volume of 200 µL and incubated in an oven at 28 ºC for 24 h. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control acetone had no influence on the growth at the highest concentration used (2%). MIC was tested three times in duplicate.

General experimental procedures

Extracts were subjected to TLC examination on aluminum sheets pre-coated with silica gel 60 F 254 (Merck). Mobile phases were **I** ethylacetate: methanol: water (100:13.5:10), **II** ethylacetate: acetic acid: formic acid: water (100:11:11:26), **III** chloroform: acid-glacial acetic acid: methanol: water (60:32:12:8), **IV** hexane: ethylacetate: formic acid (75:25:1).

Chromatograms were first observed without chemical treatment, under UV_{254} and UV_{365} nm light, and then using the spray reagents: NP/PEG, 5% KOH, Liebermann-Burchard, anisaldehide sulphuric reagent or 3% $FeCl_3$ (Wagner and Bladt, 2001).

Detection of Tannins

An aliquot of 500 µL of B_{EtOH} was mixed with 5 µL of 5% ferric chloride solution, observing the coloration that was produced by the type of tannin present. In general, a green or brownish green color suggests catechol derivatives or non-hydrolysable tannins, and a blue color, derivatives of pyrogallol or hydrolysable tannins (Vonka, 2006).

HPLC separation was also carried out on a Waters 2690 separation module equipped with a binary pump, vacuum degasser, autosampler and a PDA detector (Waters 996). The solvent system and pump gradient program used water with 0.025% trifluoroacetic acid (solvent A) and MeCN (solvent B) at a flow rate of 0.8 ml/min. The solvent gradient was designed to decrease solvent A from 95% at 0 min to 0% at 60 min. The injection volume was 10 µL.

GC-MS analyses were carried out on an Agilent 7890A Gas Chromatograph attached to a 5975 C MSD. A DB-5 (30 m × 0.25 m mm × 0.25µm) column was used with helium (1 mL/min) as the carrier gas. Oven temperature was programmed from 60 ºC to 310 ºC at a rate of 3 ºC/min. The mass-scanning was done by electron impact (EI) at 70 eV (*m/z* range 35 - 450). The identification of compounds was based by comparison of retention indices and mass spectra with those of authentic samples, and data from the NIST GS-MS library.

Statistical analysis

The analysis was performed using the GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, **www.graphpad.com.**

When was needed, the different treatments were submitted to the ANOVA (analysis of variance) and Tukey's test for multiple comparisons.

RESULTS

Antioxidant activity

Analysis of free radical scavenging capacity

Quantitative analysis

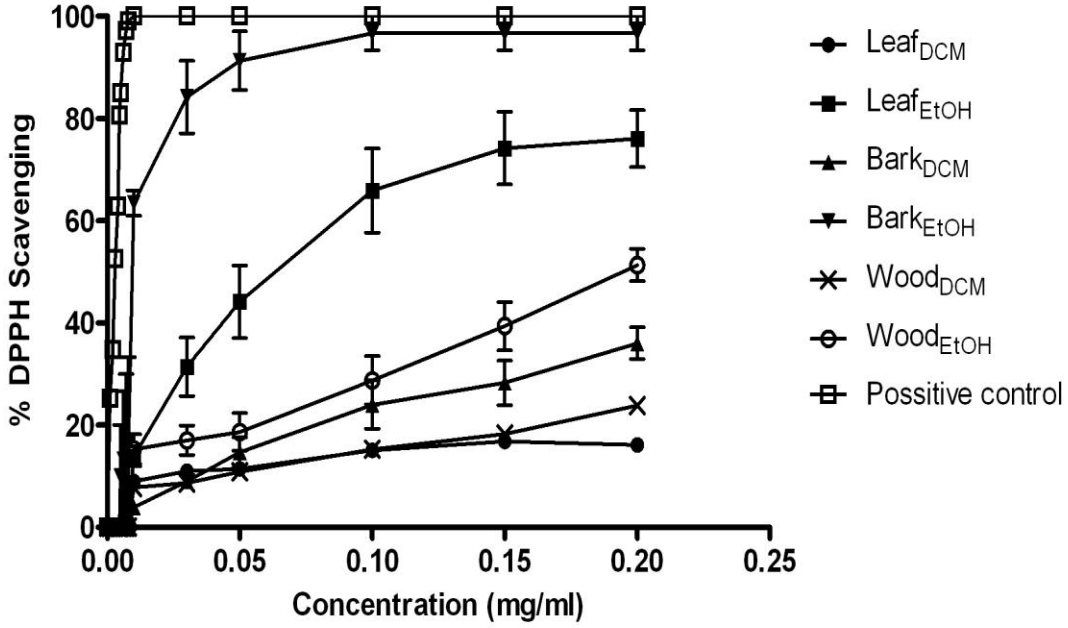
All extracts showed dose dependent scavenging activity, i.e., the higher concentration of the extract had the larger quantity of DPPH scavenged (Figure 1A). The ethanol extracts showed a greater DPPH radical scavenging capacity than the dichloromethane, which occurred in the following order:

$$B_{EtOH} >>> L_{EtOH} >> W_{EtOH} > B_{DCM} > W_{DCM} > L_{DCM}$$

for the final concentration of extract of 0.2 mg/mL. The IC_{50} value of B_{EtOH} was 4.7 ± 0.18 µg/mL similar to that of ascorbic acid (2.90 ± 0.03 µg/mL) used as positive control. The ANOVA and Tukey's test showed no significant differences for these extracts ($p > 0.05$). L_{EtOH} and W_{EtOH} showed lower IC_{50} values (62.00 ± 1.15 and 180.00 ± 2.89 µg/mL, respectively, $p < 0.001$).

In the kinetic study performed (Figure Nº 1B), it was seen that B_{EtOH} reached the maximum DPPH activity at 2 min while the rest of the extracts reach the stationary state 12 min after the start of the assay. W_{EtOH} reached that state after 20 min, although it was more active than the three DCM extracts.

A.



B.

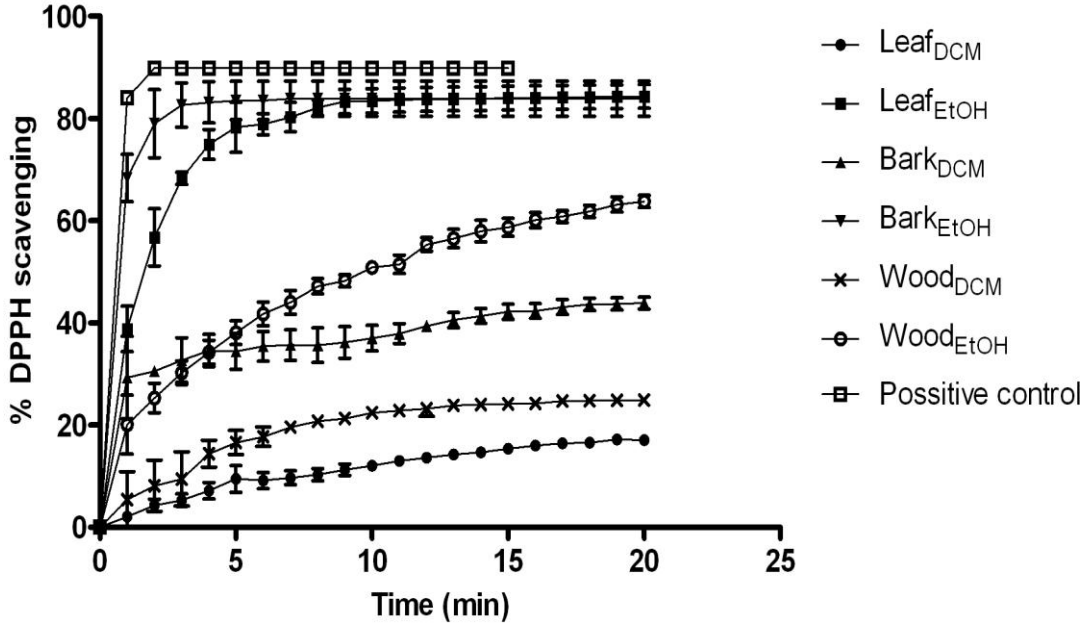


Figure Nº 1
DPPH quantitative assay determined spectrophotometrically at 515 nm, by reaction with methanolic solution of DPPH (3 mM). A: Percentage of DPPH scavenging at different extract concentration B: Percentage of DPPH scavenging at different incubation time. Extract concentration: 0.20 mg/ml, except for B_{EtOH} (0.02 mg/ml) and positive control (ascorbic acid: 0.0045 mg/ml).
The resulting values of each experiment were obtained from the average of three replicates expressed as mean and standard error.

DPPH autography assay

Qualitative analyses have shown that the ethanol extracts have greater DPPH radical scavenging capacity than the dichloromethane extracts, which is in agreement with the results of the quantitative assay (Figure Nº 2). Elution of these extracts by TLC using FM I have shown the presence of a prominent compound with antioxidant activity (R_f = 0.74) in leaf extracts and, to a lesser extent in those of wood and bark (Figure Nº 2A). It probably corresponds to anthraquinone, as it was seen as yellow band at 365nm and it was red when revealed with Bortränger's reagent.

At the same time some antioxidant components of greater polarity were not resolved in the B_{EtOH} extract (Figure Nº 2A) and so an assay was performed to determine whether tannins were present. A greenish precipitate indicated the presence of catechol derivatives or condensed tannins (non-hydrolysable).

In order to better resolve the ethanolic extracts, the FM II was tested and four compounds with antioxidant activity were observed in the $Leaf_{EtOH}$ When they were analyzed using various spray reagents these four bands could be associated with flavonoids. In Figure 2B the band with the greatest intensity (R_f = 0.37) in the L_{EtOH} revealed with DPPH coincides in the R_f and staining characteristic of the rutin marker.

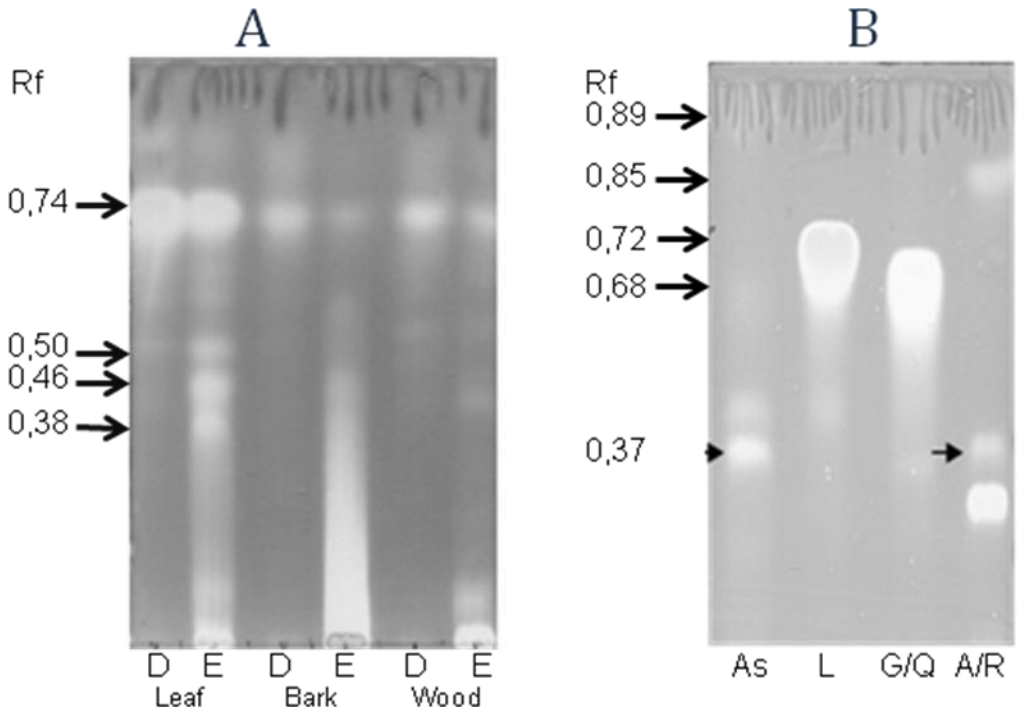


Figure Nº 2

DPPH qualitative assay using silica gel TLC plates stained with methanolic solution of DPPH (0.4mM). A: Mobile phase I ethylacetate: methanol: water (100:13.5:10). B: Mobile phase III chloroform: acid-glacial acetic acid:methanol:water (60:32:12:8)

As (H): L_{EtOH} ; L: luteolin (R_f = 0,72); G: genkwanin (R_f = 0,89); Q: quercetin (R_f = 0,68); A: apigenin (R_f = 0,85); R: rutin (R_f = 0,37).

Tripanocide activity

L_{DCM}, B_{DCM} and $W_{DCM,}$ showed antiparasitic activity (Table Nº 1). The wood extract was the most active with 80.00 ± 4.61% growth inhibition at 100 µg/mL and IC_{50} = 0.063 ± 0.003 mg/ml. The ANOVA and Tukey's test showed significant differences for the three extracts at 100µg/ml ($p < 0.05$).

Subsequently, this extract was subjected to silica gel column chromatography (see below *Fractionation of W_{DCM}*.). The antiproliferative activity of three fractions resulting from the separation and

enriched in the anthraquinones aloe-emodin and chrysophanol (F_I), chrysophanol (F_{II}), emodin (F_{IV}) and also pure emodin (Em) were analyzed. While inhibition values of F_I and F_{II} were not significant (20% and 24%, respectively) at 100ug/ml, inhibitory activity increased specially in F_{IV} (62%) and Em (60%). This could indicate that the effect observed is partially due to the presence of emodin.

Table Nº 1
***In vitro* tripanocide activity of *A. subovata* extracts**

A. subovata Dichloromethane extract	Concentration of the extracts			
	100 μg/mL	50 μg/mL	25 μg/mL	12.5 μg/mL
Bark	25.00 ± 1.73	0	0	0
Wood	80.00 ± 4.61	41.00 ± 2.96	17.00 ± 1.00	10.00 ± 1.15
Leaf	60.00 ± 2.88	35.00 ± 2.33	0	0

***In vitro* tripanocide activity of *A. subovata* extracts. Results are expressed as percentage of parasites' growth inhibition (mean and standard error).**

Antibacterial activity

W_{DCM} showed an inhibitory capacity of Xac in the disk diffusion assay. The inhibition zone diameters were 11.00, 8.5, 8 and 7.5 mm for 350, 175, 70 and 35 μg of the extract. Streptomycin sulfate (50μg/ml) as positive control showed 18 mm of inhibition zone diameter. Around all disks a second inhibitory zone, of bigger diameter, was noticed and characterized by grooves or ramifications (Figure Nº 3), which could be related to some bacterial movement of unknown cause.

Besides, remarkable differences were not detected in the diameters of the halos despite the difference in extract concentrations, probably because there is a diffusion limitation of the extract. On account of this, minimal inhibitory concentration assay was evaluated in which this effect could be reduced. Test was performed in triplicate and the mean MIC value for the extract was 100 μg/mL.

Fractionation of W_{DCM}

In order to characterize W_{DCM} which was the most active in antiparasitic and antibacterial assays, it was submitted to HPLC/DAD. The presence of chlorogenic and 3,4-dihydroxybenzoic acids was detected. The UV spectrum compared with UV library spectra also indicated the presence of aloe-emodin, emodin and chrysophanol chromophores. Analysis by GC-MS confirmed the presence of emodin and chrysophanol. In addition scopoletin was also detected by this method.

W_{DCM} was subjected to silica gel column chromatography and 52 fractions of 15 mL each were collected. On the basis of the TLC profile similar fractions were pooled into 14 fractions: I to XIV. The tripanocide activity of the first fractions obtained (I, II and IV) were carried out and the results were shown in antiparasitic activity. In adition, these fractions showed characteristic bands of anthraquinones when were analyzed by TLC and so they were subjected to HPLC in order to isolate and identify the main compounds. Twelve subfractions were obtained: T1 to T12 which were further analyzed by TLC. The fractions T1 and T7 showed a band with an R_f, staining characteristic and elution time in HPLC (16 min) (Figure Nº 4), which coincided with the anthraquinone chrysophanol. In fraction T2, a band corresponding to a compound that has not yet been identified was eluted in HPLC at 22 min. On account of the staining characteristics it was probably an anthraquinone. T3 elution time in HPLC was found to be identical to aloe-emodin. A band was also seen in T11, with an R_f and elution time (12.5 min) that matches those of emodin.

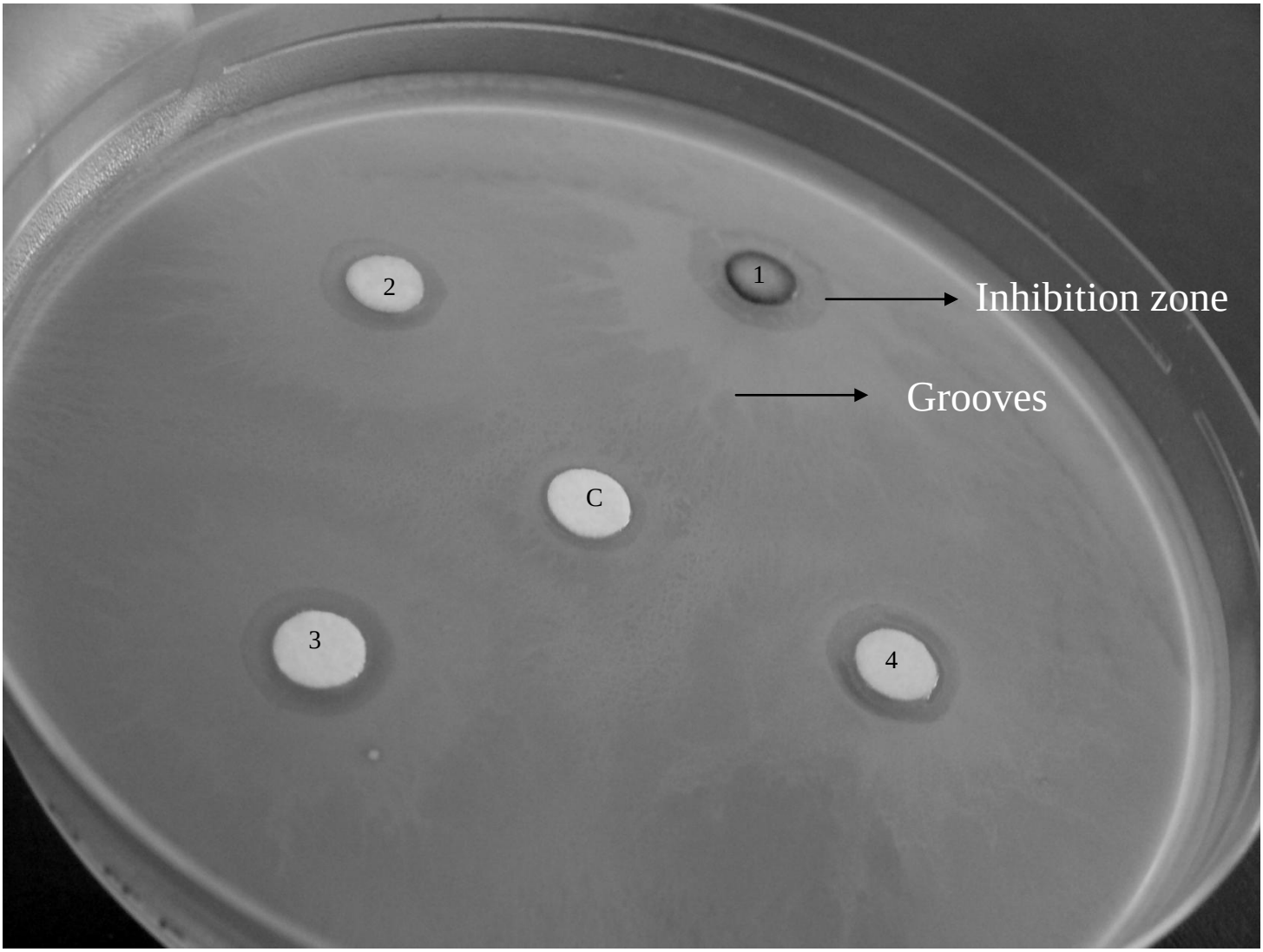


Figure Nº 3
Disc diffusion assay. Arrows are indicating the halos observed. W_{DCM} quantities: 1) 350 µg 2) 175 µg 3) 70 µg 4) 35 µg C) 0 µg (control with solvent DCM).

DISCUSSION

Various tests were performed on the biological and phytochemical activities of *A. subovata* in order to look at its potential as a phytotherapeutic drug or as a biopesticide and to identify chemical constituents that would serve as analytical and chemotaxonomic markers of the extract of interest, as there have been no previous reports on these issues.

All evaluated extracts showed DPPH radical scavenging activity. However, the ethanol extracts showed the greatest capacity, especially that of bark with an IC_{50} = 4.70 ± 0.18 µg/mL. Low magnitudes of IC_{50} indicate a greater scavening capacity. When the extracts were analyzed by autography assay, at least four flavonoids with DPPH radical scavenging capacity were observed in the L_{EtOH} and one of them was identified as rutin. This was not very striking since a large number of flavonoids are usually found in the photosynthetically active tissues. However, what is interesting is that the B_{EtOH} shows the greatest antioxidant activity, surely due to tannins (Amarowicz *et al.,* 2009; Hai-Feng *et al.,* 2008) which presence was confirmed in this extract.

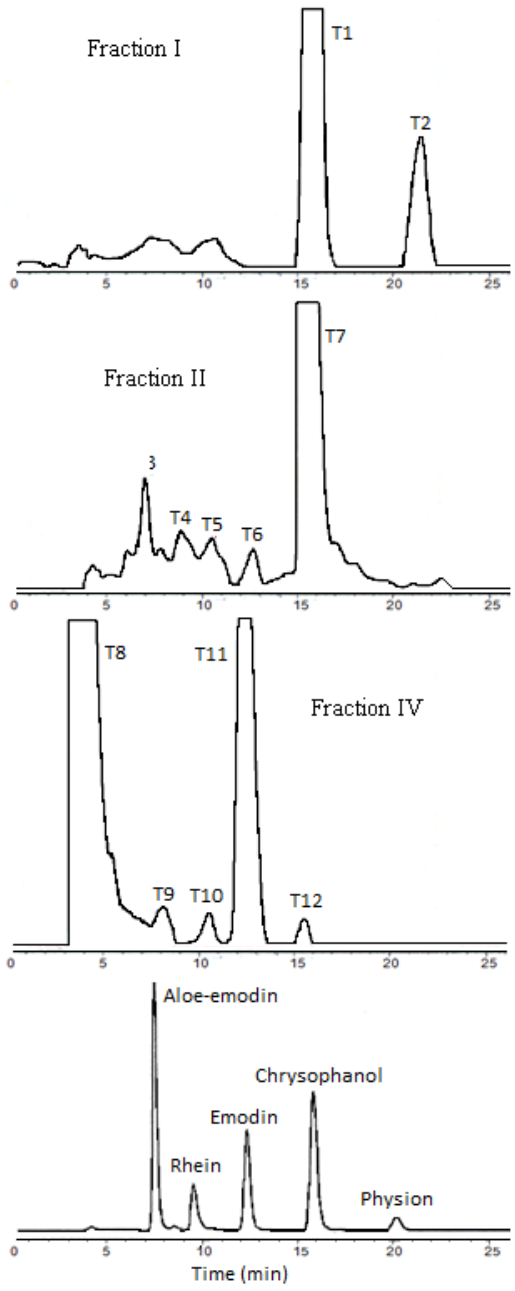


Figure Nº 4
HPLC chromatograms with the identification of the principal anthraquinone molecules. Fractions I, II and IV, obtained after silica gel chromatography of W_{DCM}, were subjected to HPLC. Twelve subfractions were obtained: T1 to T12. Peak T1 and T7 show an elution time (16 min) which coincided with the anthraquinone chrysophanol. T11elution time (12.5 min) matches those of emodin.

Considering that certain species of the Picramniaceae, to which *A. subovata* belongs, are traditionally used as antiparasitic; in the present study an evaluation was made of the activity of *A. subovata* against the *Trypanosoma cruzi* parasite, the causal agent of Chagas disease that is endemic to South America. Moreover, the geographic distribution of this plant species is near to the areas with the greatest occurrence of cases of Chagas, which makes it very significant as the local population would have access to this plant material. The wood extract was the most active, reaching 80.00 ± 4.61% growth inhibition at 100 μg/mL and even presenting a certain percentage of activity at the lower concentration on trial (17.00 ± 1.00% inhibition at 25 μg/mL). A fraction of this extract, enriched in the anthraquinone emodine, showed 60% of inhibition of the parasite growth. Anthraquinones isolated or identified on *Picramnia antidesma* showed activity against malaria and other protozoan species (Solis *et al*., 1994; Hernandez-Medel and Pereda-Miranda, 2002; Camacho *et al*., 2003), In our work, as in the *Picramnia* specie studied, there is probably a correlation between the tripanocide activity and the anthraquinone content, which, until the present time, are the main metabolites found in the *Alvaradoa* species (among others Jacobs, 2003).

Anthraquinones isolated from different species, showed bactericide and pesticide activities (Chukwujekwu *et al.*, 2006; Wei *et al.*, 2008; Kambou *et al*., 2008; Godard *et al.*, 2009; Li *et al*., 2009; Borroto *et al*., 2010). However, the antimicrobial activity of *A. subovata* has not yet been studied. In our work the activity against the phytopathogenic bacteria *Xanthomonas axonopodis* pv. citri type A (Xac), causal agent of citrus canker was analyzed.

Plant sanitation from Xac was achieved so far with copper compounds, chlorine derivatives or broad spectrum bactericides (Sarah *et al.*, 2012). Even though these methods are effective, they cannot be used in plants for human consumption. Hence alternatives from biological sources will be highly useful in the management of these pathogens in an ecofriendly way both in field and in the storage (Sarah *et al.*, 2012). The present investigation clearly demonstrates the significant antibacterial activity of W_{DCM} of *A. subovata* against Xac *in vitro*. The extract exhibited an inhibition zone diameter ranging from 7.00 mm to 11.00 mm and a MIC value of 100 μg/ml. Although streptomycin sulfate showed higher inhibition (with 18 mm of inhibition zone diameter and MIC value of 30 μg/ml), this chemical method is

expensive and can affect the beneficial microbial population present in the ecosystem. In addition, to our knowledge only a few studies have been done on plant pathogens using plant extracts. Antibacterial activity of *Psidium guajava* (Sarah *et al.*, 2012), *Vitex negundo* and *Allium sativum* (Jeyaseelan *et al.*, 2011) against *Xac* were found. The MIC values of *P. guajava*, *V. negundo* and *Allium sativum* were 1.56 mg/ml, 2.5 mg/ml and 40 mg/ml, respectively. All these values were higher than the value obtained in our work with *A. subovata* wood extract (0.1 mg/ml). These results suggest the possibility of this plant in the management of plant pathogenic bacteria in an ecofriendly way, since compounds of biological origin are known to posses' minimal residual effect.

A phytochemical study was carried out on the W_{DCM} of *A. subovata* on account of its interesting biological activities, as there are no previous reports on this issue. Anthraquinones and coumarins were determined among the main compounds coinciding with that reported for *Alvaradoa* genus and the *Picramniaceae* family (Jacobs, 2003). In our work chrysophanol and emodin were identified among the first and scopoletin and umbelliferone among the second by TLC, HPLC and GC-MS. Those components could be used as analytical markers of that extract.

The identification of emodin is of special interest as emodin in particular, or extracts rich in this compound, were shown to be candidates for the development of biological pest control agents as they show the most pesticide activity (bactericide, insecticide and fungicide) among the different anthraquinones and in combination (emodin with other anthraquinones) induce defense reactions in the infected plants (Chukwujekwu *et al*., 2006; Kambou *et al*., 2008; Godard *et al*., 2009). It is interesting that the W_{DCM} of *A. subovata* has this important combination which is very promising for further, more specific, studies on this issue.

CONCLUSION

The results of this study show that the extracts of *A. subovata* constitute a potentially rich source of bioactive compounds such as antioxidants, especially in the ethanol bark extract, and antiparasitic agents and pesticides, especially in the dichloromethane extract of wood.

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