

Full Length Research Paper

Pharmacological and toxicological evaluation of *Rhizophora mangle* L., as a potential antiulcerogenic drug: Chemical composition of active extract

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Rhizophora mangle L. is a vegetal species widely distributed in Cuba and other Caribbean countries. This species is characterized by several ethnobotanical activities as antiseptic, astringent, as well for treating skin ulcers. In the present work, we describe a pharmacological, toxicological and chemical evaluation of this plant by its use in human medicine for the treatment of gastroduodenal ulcers. The acute gastric ulcer's models were: acute gastric ulcers induced by ethanol; indomethacin; pyloric ligation; stress and immobility in cool in mice. The antisecretor effect of the extract was evaluated by pyloric ligation model. Other pharmacological tests were planned with the freeze - dried extract of *R. mangle*, as part of the evaluation on other systems to known secondary or adverse effects. These tests included the activity of the antiulcer active extract on intestinal transit, activity over arterial pressure, ileum activity and absorption of glucose in gut. The chemical profile of this extract by fatty acids was studied by Gas Chromatography/Mass Spectrometry. Some toxicological studies (genotoxicity) were carried out. The aqueous extract of *R. mangle* bark showed gastroprotective, antisecretor effects, and it induced a recovery of PGE₂ levels in doses-dependence manner comparable of knowledge antiulcerogenic medicaments. No effect was observed by arterial pressure in rats and the intestinal transit was inhibited by *R. mangle*. The intestinal motility was stimulated. Antiulcer active extract inhibit the glucose absorption in gut. This extract presented 4% of saturated and not saturated long chain's fatty acids (C10:0 at C24:0). No toxicological signs were obtained by this extract.

Key words: *Rhizophora mangle* L., antiulcer, intestinal transit, arterial pressure, ileum activity, absorption of glucose in gut, chemical composition, genotoxicology.

INTRODUCTION

Rhizophora mangle L. was widely distributed in Cuba and other Caribbean countries. This plant has several ethnobotanical uses such as antiseptic, astringent and haemostatic (Roig, 1974).

Gastric and duodenal ulcers are illnesses that affect a considerable number of people in the world and they are induced by several factors. The bacterium *Helicobacter pylori* have a high role in the etiology of this illness. Many

generic drugs are use in the treatment of gastroduodenal ulcers, but in the totally are necessary the application of combinatory therapies by the complex of this etiology.

For also, the finding of other new therapies is important, between it the traditional medicine has a high level.

By the manufacturing of phytodrugs is necessary not only probed their pharmacological effect within to study other properties of these promissory drugs in toxicological tests and to study their effect on other physiological systems, named pharmacological II.

In previous works, we report the cytoprotective effect of

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freeze - dried aqueous extract from red mangrove bark on gastric ulceration induced by ethanol - hydrochloric acid in rats (Sánchez et al., 2001). Also, we report the antiulcerogenic effect by other action's mechanism as antiselector, inhibitor of depleting of PGE2 in the gastric mucous and antioxidant (Sánchez et al., 2004; Berenguer et al., 2006).

R. mangle L. has polyphenolic structures as major components named tannins (Sánchez et al., 1998a). Tannins have many biological actions as antimicrobial, antifungal, antiviral, antioxidant, etc (Leinmüller et al., 1991).

Other preview studied performance with this extract; it had shown the presence polyphenolic structures (54.78%) and other structural components (45.22%). Polymeric tannins were the major polyphenolic component 80 and 20% were hydrolysable tannins. Epicatechin, catechin, chlorogenic acid, gallic acid and ellagic acid were monomeric structures determined in this extract. Phytosterols (0.0285%): stigmasterol, β -sitosterol and likewise campesterol were present too (Sánchez et al., 2008b). This extract present semivolatil compounds (Pino et al., 2001); fat acids and sugars (Sánchez, 1998a), for also it represent a complex mixture of secondary metabolites.

This vegetal specie with pharmacological activity as antiulcer in gastroduodenal tract was processed by analysis your possible toxic effect in acute and sub acute oral toxicology in rats. Any toxicological sign was shown in both studies (Sánchez et al., 2008a).

The objective of the present work was the pharmacological, toxicological and chemical evaluation to complete the study of this plant by its use in human medicine for the treating gastroduodenal ulcers. These studies including the validation of antiulcer effect of the aqueous freeze dried extract form the bark of *R. mangle* L. in mice; to complete the study of the possible adverse effects in the administration of vegetal drugs so its effects on other biological systems: arterial pressure, intestinal transit, ileum motility and the study it action on glucose absorption in gut. Other studies including genotoxicological tests and chemical composition respect to fatty acids, not described is this species at this moment.

MATERIALS AND METHODS

Preparations of aqueous extract of *R. mangle* L. bark

R. mangle L. was collected from the western zones of Cuba in 2004. The identity of the plant was authenticated by a botanist and a voucher specimen has been deposited in National Botanical Garden's Herbarium. The extract was prepared by the decoction of the bark in distillate water. The proportion of vegetal matter: water was 1:7; the decoction was made for 20 min at 90°C in lab reactor with 2 L of capacity. The plant material was separated by centrifugation and the aqueous extract was concentrated and freeze-dried to preserve it.

Pharmacological studies

Antiulcer effects in mice

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the international regulations in the working with lab animals and by our Ethical Committee.

Pyloric ligation in mice

Mice were fasted during 15 h with free access to water. The pylorus was ligated under light ether anaesthesia. After the ligation was administered the vehicle (water, 5 ml/Kg) and the aqueous extract (EA) of *R. mangle* (0.5 - 1.0 g/Kg) in the duodenal light (intraduodenal) and the abdomen were sutured. After 4 h of the administration of the test's substance, the animals were sacrificed by ethyl ether anesthesia, the stomachs were taken and the gastric contents were collected by the direct volume measure and the pH of gastric segregation. Total acidity of the collected material was determinate by NaOH 0.01 N titulation, using phenolphthalein 2% as neutralization's indicator. The number of equivalents of NaOH necessary for acid titulation was considered as indicative of total acidity (mEq [H⁺]/L/4 h).

Gastric lesion induced by ethanol

Mice with 16 fasted were treated by oral via with the vehicle (water) or with the extract of *R. mangle* (0.05 - 0.5 g/Kg). Seventy minutes after the treatments, animals received ethanol 75% / 1 ml/mice, orally). One hour after the ethanol administration the animals were sacrificed by ether anesthesia, the stomachs were removed and opened by minor curvature by the quantification of the lesions in the mucosa with optical microscope. Gastric lesions were evaluated accumulatively considerate the following aspect of the mucosa: edema presence, hyperemia, petechiae, hemorrhagic points, ulcers, performed ulcers.

Gastric lesion induced by indomethacin

Mice fasted 16 hours were treated by oral with vehicle (water, 0.1 ml/20 g) or with the extract of *R. mangle* (0.05 and 0.5 g/Kg) or positive control, ranitidine (50.0 mg/Kg). After one hour the animals received indomethacin (20 mg/Kg, orally). After 3 h of the indomethacin administration, were repeated the treatments with vehicle, extracts and ranitidine. Six hour after to indomethacin administration the animals were sacrificed with ether anesthesia, the stomachs were removed and the mucosa lesions were evaluated after was described before.

Gastric lesion induced by stress and immobility in cool (4°C)

Mice fasted by 16 hours received the vehicle (water, 0.1 ml/20 g) and the extract of *R. mangle* (0.05 - 0.5 g/Kg, orally). After 1 h the animals were immobilized and they were stored in cool (4°C) by 2 h. Animals was sacrificed by ether anesthesia and the stomachs were removed by the evaluation of the lesions of the gastric mucosa with was described before.

Activity over intestinal transit of *R. mangle*

Male NMRI mice weighing 25 - 30 g were obtained from the National Center for Lab Animals Production, CENPALAB, Cuba. Animals were fed on conventional diets and water ad libitum and

they were maintained under standard conditions of humidity, temperature and light (12 h light: 12 h dark cycle).

Thirty minutes after the different treatments administration were given orally activated charcoal (10%, 0.1 ml/10 g) in all animals. The animals were sacrificed thirty five minutes after the active carbon administration by ether anaesthesia. The stomachs were removed and small intestines were courted since cardia to ileoceal valvule. The pylorus was middle to more away place of carbon (100% total large of small intestine). The distance running by carbon was measured since the pylorus to the site more away of the small intestine with carbon content more than 1 cm continuous. If there were several sites of carbon in the area more away, we must to take the major area.

Arterial pressure

Wistar rats were use by this experiment weighing 200 - 220 g. *R. mangle* L. was administered intraarterial at doses of 25, 50 and 100 mg/Kg body weigh. The signal was registered with electronic manometer Stand MP-250 coupled to a multipurpose polygraph Nihon Kohden.

Ileum activity

Male and female wistar rats, 180 - 200 g, were used. They were fasted 24 h before the experiment beginning.

Ileum segments were collected in tiroidal solution bath at 37°C. They were washed with tiroidal solution. Atropine and acetylcholine were used as inhibitor and stimulator of the intestinal motility.

Glucose absorption in gut in presence of freeze dried extract of *R. mangle*

In the evaluation of activity of freeze dried extract of *R. mangle* on obstruction of active transport of glucose in gut were used rats Wistar, male with 200 g b.w. Animals were fasted by 36 h before the experiment. Rats were anesthetised with sodium pentobarbital 20 mg/Kg intraperitoneal then they were practiced a laparotomy media and the bile-duct were ligature in the beginning. The guts were cleaned with saline solution. It were made ligature longitudinal to gut with 4 cm of distance between each. In each segment were injected 1 ml of isotonic glucose solution 10 mM (control, group I) and in the treatment groups were injected this solution with extract of *R. mangle* in the doses 250 (group II) and 500 mg/ Kg b.w. (group III). After 30 minutes the remnant liquid were collected and were determinate glucose levels. The determinations of glucose were made with SPINREACT kit, Glucose Trinder, GOD-POD, Spain.

Chemical characterization

Fatty acids determination was made by Gas Chromatography/Mass Spectrometry. One gram of freeze- dried aqueous extract of *R. mangle* was extracted in Soxhlet equipment with 250 ml of n-hexane during 24 h. The extract obtained was evaporated to almost dry. The remnant was transfer at tube adding 3 ml of sulphuric acid in methanol at 6% (weigh/volume). It was hope during 90 minutes to 75 - 85°C, after it was cooled to atmospheric temperature. It was adding 3 ml of water at the sample and extracted with 2 ml of n-hexane (twice). Superior phase (n-hexane) was separate and it was transfer to a tube with tape proceeding to fatty acids methyl ester's analysis by GC - Mass. It was used a Gas Chromatograph coupling to Mass Spectrophotometer JEOL Model JMS -DX 300 (DEOL, Japan) with electronic impact. It was used a DB-1 column, 25 m ×

0.32 mm internal diameter × 0.25 um of picture sponsor, temperature range 80 - 280°C, with increasing of 8°C, temperature inlet 280°C, carried gas flow (helium) at 1 ml/min, mode EI and 70 eV.

The peaks identification in the chromatogram was made by comparison of retention times with patterns inject in the equipment and they mass spectrum or by these date reported in the literature (Stehagen et al., 1974).

Genotoxicological evaluation

Bone marrow micronucleus test in mice

In this experiment were used mice NMRI with 8 to 12 week of age, 23 - 25 g of body weigh (b.w.). Ten animals were used by experimental group, 5 female and 5 male. They were maintained to conventional diet and water ad libitum. They were administered by oral via 10 ml/Kg b.w. in the following doses of freeze dried aqueous extract: 500, 1000, 2000 mg/Kg b.w. in sterile water. In the positive group were used ciclofosamide, 40 mg/Kg b.w. by intraperitoneal via (nitrogen alkyl agent). The proceeding used to obtain the preparation of the bony marrow cells were the described by Schmid (1976), with this proceeding were obtain a differential tincture between polychromatic erythrocytes (PCE) and the normochromatic erythrocytes (NCE), quantifying the number of PCE porters of micronucleus (MN) which are expressing in percentage of PCE/MN respect at total of PCE observed, 1000 by animals. It was named genotoxicity index. It was accepted by micronucleus to structures round in the cytoplasm of PCE, with colour intensity similar or more debility that cellular nucleus sugaring the presence of a cellular membrane.

It was evaluated the proportion PCE/NCE as estimator of toxicity on the haematopoiesis, the NCE were quantified in the cont of 250 PCE in each lamina. It was named toxicity index.

The different between negative control group (sterile water) and the treatment groups were analysed with the real cont for both index with Kolmogorov-Smirnov y Bartlett test.

The results of both index and their respective positive controls were analysed by parametric methods: Variance Analyses (ANOVA) and a lineal regression analyses.

RESULTS

Pharmacological evaluation

Antiulcer effect of R. mangle

(a) Validation of the acid gastric- antiselector activity, cytoprotection of extract of *R. mangle* in mice: The Figures 1 - 4 shows the antiulcer effect of *R. mangle*. These experiments validate the antiulcer activity in another experimental animal's models, Sánchez et al. (2001 and 2004).

The results shown that the doses testing of extract from *R. mangle* (0.1 - 1.0 g/kg, i.d.) reduce the volume of acid gastric segregation, so the total acidity was decreasing only of the high doses.

R. mangle administered orally, decrease the number of ulcers produced in the three models (ulcers induced by stress, indomethacin and ethanol), and within change significantly the lesion index of the gastric mucosa. The effect was not relation with the doses; it inferred a possible topic action of the extract. It is basic in the

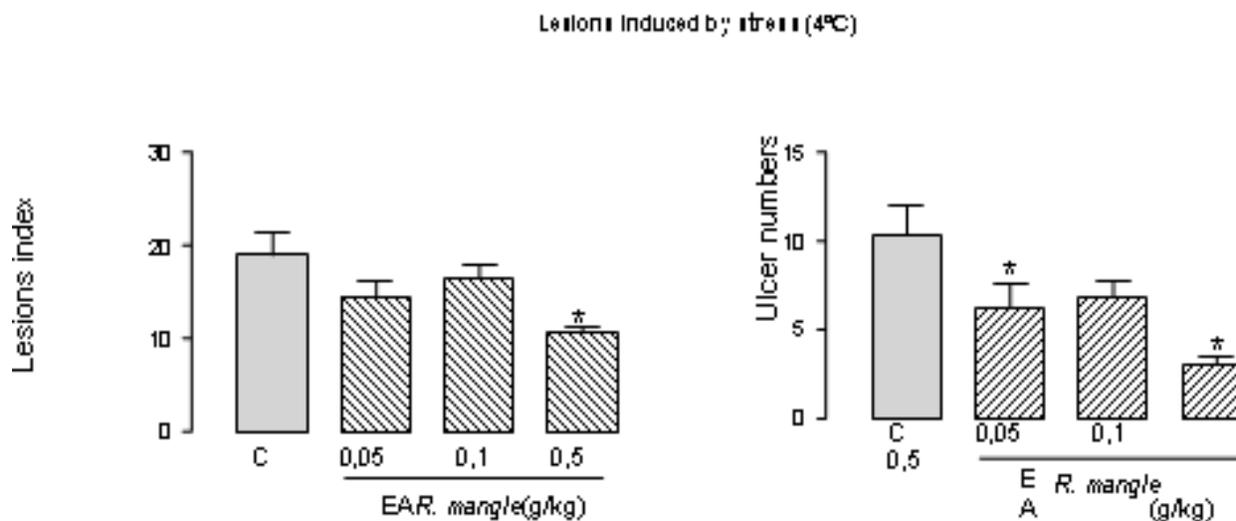


Figure 1. Effect of aqueous extract (EA) of *R. mangle* (0,1 to 1,0 g/Kg, i.d.) and ranitidine (0,05 g/Kg) administered by intraduodenal via, in the volume, pH and total acidity of acid gastric segregation in mice with pylorus ligation for 4 hour. Control group (C). Columns and vertical barras represent media \pm standard error of the media of five animals. * Different of the control (ANOVA – Dunnet, $p < 0,05$).

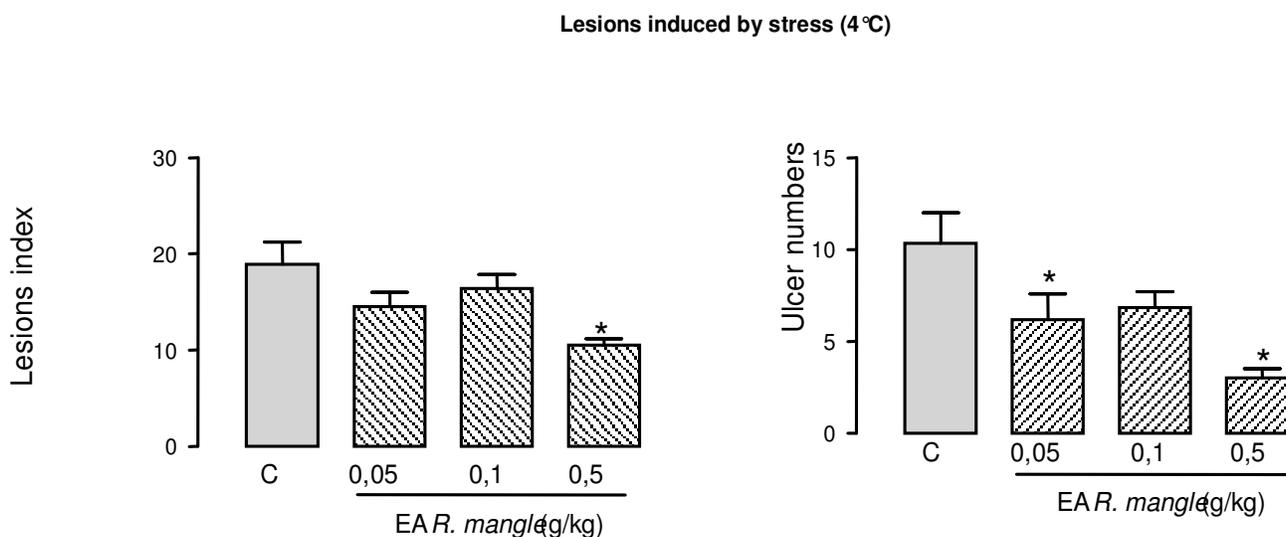


Figure 2. Effect of the oral administration of aqueous extract (EA) of *R. mangle* (0.05 and 0.5 g/kg, v.o.) and control group, C, in the lesion index and the number of ulcers induced by stress and immobility at 4°C in mice. Columns and vertical barras represent media \pm standard error of the media of twelve animals. * Different of the control (ANOVA - Dunnet, $p < 0.05$).

intensity addition of the extract in the gastric mucosa of the alls treatment animals, principally with a high dose.

Intestinal transit

Figure 5 shows the result by the freeze-dried aqueous extract of red mangrove on the intestinal transit. This extract shows inhibitory effect on the intestinal transit statically comparable with neostigmine, an inhibitory

substance. This effect explains the anti diarrheic properties described in this plant, so this property is also explained by astringent and protein precipitation capacity. The inhibitory effect found for red mangrove is superior to other plants with tannin contents, reported by Schapoval et al. (1994).

Arterial pressure and ileum activity

The freeze-dried aqueous extract of *R. mangle* did not

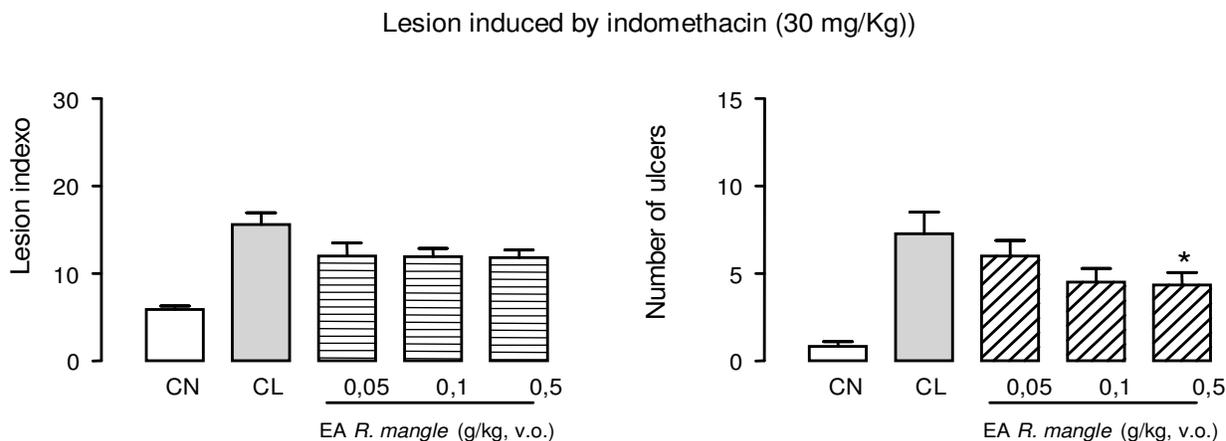


Figure 3. Effects of the oral administration of aqueous extract of *R. mangle* (0.05 and 0.5 g/kg, v.o.) in the lesion index and in the ulcers number induced by indomethacin (30 mg/kg, s.c.) in mice. CN, represent a negative control within ulcer and CL represent a control with lesion. Columns and vertical barras represent media \pm standard error of the media of twenty four animals. * Different of the control (ANOVA - Dunnet, $p < 0.05$).

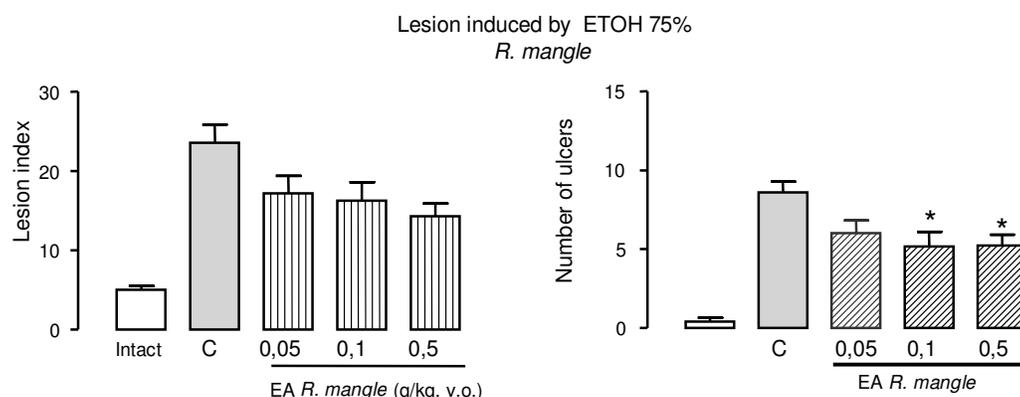


Figure 4. Effects of the oral administration of aqueous extract (EA) of *R. mangle* (0.05 and 0.5 g/kg, v.o.) in the lesion index of the ulcers number induced by ethanol 75% in mice. Control group with lesion, C. Columns and vertical barras represent media \pm standard error of the media of eighteen animals. *Different of the control (ANOVA - Dunnet, $p < 0.05$).

affect the arterial pression by intraperitoneal administration, only a little increase was shown in high doses. In this case is oportune to mention that the administration was intraperitoneal, for also in the oral administration not effect on arterial pressure would be present in therapeutically doses. The intestinal motility was stimulated, where would be wait decrease the astrigent effect of the extract in oral administration in the treatment of gastroduodenal ulcers.

Glucose absorption in the gut in presence of freeze dried extract of *R. mangle*

In the Table 1 shown the results of the effect of *R. mangle* on glucose absorption in gut. In this case we

could appreciate the inhibition of glucose absorption in the presence of *R. mangle*. The major inhibition of this absorption had been shown in the minor doses 250 mg/Kg b.w. In this aspect is important to relieve the contribution of glucose free by extract, because this extract has a quantity of carbohydrate between as glucose is identified in previous chemical characterization.

Chemical characterization

R. mangle was a high composition of fatty acids in freeze dried aqueous extract. They were long chain fatty acids that including it so C 10:0 to C 24:0 with a high percentage of unsaturated. Mass spectrum of methyl derivate of fatty

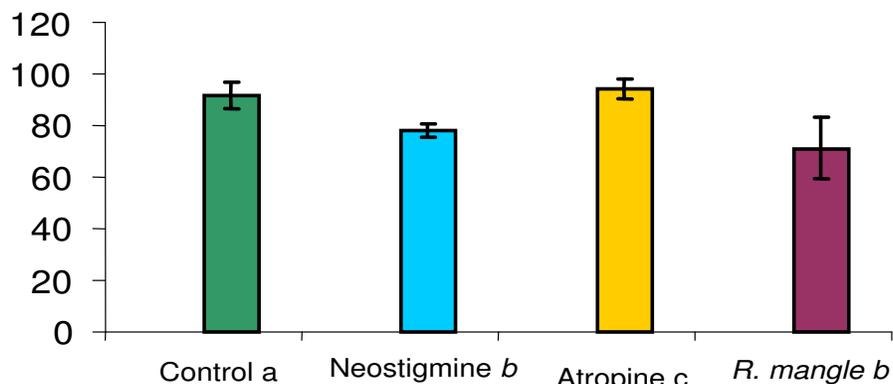


Figure 5. Effects on intestinal transit of freeze-dried aqueous extract of *Rhizophora mangle* L. (Difference letters represent statistical differences, $p < 0.05$).

Table 1. Glucose absorption in gut in presence of extract from *R. mangle*.

Treatment	Glucose level (mmol/L)
Glucose 10 mM	8.56
Glucose 10 mM + 250 mg/kg m.c. extract from <i>R. mangle</i>	1.77
Glucose 10 mM + 500 mg/kg m.c. extract from <i>R. mangle</i>	4.44

Table 2. Bone marrow micronucleus test in mice of extract from *R. mangle*.

Doses	Sex ^b	IT (X ±DE) ^c	IG (X ±DE) ^d
		PCE/NCE	MN-PCE
Sterile water ^a	M	1.13 ± 0.37	0.14 ± 0.06
	F	1.44 ± 0.51	0.08 ± 0.05
500 mg/kg b.w..	M	1.53 ± 0.22	0.10 ± 0.03
	F	1.53 ± 0.4	0.09 ± 0.1
1000 mg/kg b.w..	M	2.77 ± 1.25	0.08 ± 0.03
	F	1.33 ± 0.14	0.09 ± 0.06
2000 mg/kg b.w.	M	1.88 ± 0.47	0.07 ± 0.06
	F	2.30 ± 0.52	0.10 ± 0.06
Ciclofosfamide	M	0.40 ± 0.03**	1.84 ± 0.99**
	F	0.36 ± 0.03**	0.86 ± 0.14**

Negative control; M: male, F: female; IT: Index of toxicity; IG: Index of genotoxicity; ** Significant difference respect to negative control ($p < 0.01$).

acids was characterized by equal relation of fragment ions typical in these types of compounds, m/z 43, 66, 87, 129, 143 with different intensity.

The major proportion of these fatty acids was represented by palmitic acid (C 16:0) following by cis and trans isomers of oleic acid. In minor proportion were fatty acids with C22:? (No determined the unsaturation), linolenic

acid (C18:2) and miristic acid (C 14:0).

Genotoxicological evaluation

In Table 2 had been shown the results of bone marrow micronucleus test in mice. Our results showed not toxicity

from these aqueous extract of *R. mangle* because there are not cytotoxicity affectation of bony marrow of mice in both sex treatment. In this test a decreasing of PCE/NCE proportion is an indicator of marrow toxicity.

Though some compounds are absorbed, the plasmatic concentration had not pharmacological relevance. Statistical analysis of percentage of young micronucleus erythrocytes (MN/PCE) shown that were not found statistical difference between sex, employed doses nor a doses/ answer for also this indicate that this product is not genotoxicity. This negative answer have been relationship with tannins presence in the extract which modulate the repair of DNA in *E. coli* and chlorogenic, gallic and ellagic acids are reports a high antimutagenic, anticancer capacity Duke (2005).

DISCUSSION

The major active principles of the red mangrove are polyphenols, represented in their majority by polymeric tannins (80%) and hydrolysable tannins (20%) and special emphasis has been given to the presence of epicatechin, catechin, chlorogenic, gallic and ellagic acids, as well as galotannins, elagitannins and condensed tannins Sánchez et al (1998a) and Sánchez et al. (2008b). These substances characterized by their polyphenolic nature, have shown cytoprotective properties (González et al., 2000) and have been associated to antiulcerogenic activity in other plants König et al. (1994), Tebid et al. (1996) and Ramírez et al. (2003). Tannins or polyphenols have a number of physical and chemical properties in common, which underlie their physiological and pharmacological actions: their antioxidant and radical scavenging activities and their ability to complex with other molecules such as proteins and polysaccharides (Haslam, 1996). Vegetable polyphenols are known to inhibit lipid peroxidation *in vitro* and there is evidence about their ability to scavenge radicals such as hydroxyl, superoxide, and peroxy, which are important in cellular prooxidant states. Incidentally, it has been shown that chlorogenic acid, has and antioxidant effect as high as DL-tocopherol (Fernandez et al., 2002).

On the other hand, tannins may prevent ulcer development due to their protein precipitating and vasoconstriction effect Aguwa and Nwako (1988). Their astringent action can help precipitating microproteins on the ulcer site, thereby forming an impervious layer over the lining that hinders gut secretions and protects the underlying mucosa from toxins and other irritants (Nwafor et al., 1996, 2000; Al-Rehaily et al., 2002). This propensity to bind to proteins also explains the fact that polyphenols inhibit enzymes tested *in vitro*.

The topical action of the aqueous extract of *R. mangle* in accelerating wound healing has been explained by several mechanisms, such as coating the wound, forming complexes with proteins of microorganism cell wall,

chelating free radicals and reactive oxygen species, stimulating the contraction of the wound and increasing the formation of new capillaries and fibroblasts Fernandez et al. (2002). In our study, a thick coating of *R. mangle* extract was found macroscopically adherent to the gastric mucosa, which suggests that in addition to antioxidant mechanisms, we could be inferred that the formation of a physical barrier with similar properties as observed in topical wounds may contribute to the gastroprotective action of the drug.

The presence of fatty acids in *Rhizophora* genera was not reported before. For also, it was the first reported. This finding completes the chemical characterization of compounds present in the aqueous extract. This finding was taxonomical important for this specie. Other plants have similar fatty acids partner, for example *Triticum aestivum* L. present a distribution of fatty acids so C 12:0 (lauric acid) to C 18:0 (stearic acid) determined by HPLC (Tsuyama et al., 1992).

Essential oils, phytosterols and glycosides of *Marslemia condurango* are effectiveness in ulcer gastric carcinoma and bleeding. Phytosterols have antiulcer effect, with protective action against *Helicobacter pylori*. In recent study phytosterols esters of an herb of *Dolichus* genera was protective in a model of pyloric ligation ulcer (Jayaraj et al., 2003).

Fatty acids have protective effect against peptic ulceration in several experimental models Hobsley et al. (2001). Fatty acids have antimicrobial activity. For example the presence of capric, lauric, miristic, palmitic, behemic acids in the flowers of *Moltikia caerulea* have a significant antimicrobial activity at a concentration of 100 µg (Meshkatal and Parekh, 1990).

For also, we could be inferred that the presence of fatty acids and other functional chemical groups present in this aqueous extract as semivolatile compounds, phytosterols, carbohydrate joint at the major functional group, tannins Sánchez et al. (1998a) are the responsible of pharmacological activities of this plant as combinatory complex chemistry in the pharmacological finding.

The aqueous extract of *R. mangle* not has toxic effect in acute and sub acute toxicity study (Sánchez et al., 2008). In this case was shown not genotoxicity effect in bony marrow micronucleus in mice.

In the last decade the antimutagenic study had been associated to modulation of enzymes from xenobiotic's metabolism. In this case the plants had shown antimutagenic capacity by its heterogeneous compounds. Between them have a high potential antimutagenic the polyphenols of green tea. Epigallocatechin 3-gallate is an inhibitor of some enzymes of fase I as the citocrom P450, CYP1A, 2B1 and 2E1 and the ellagic acid as inductor of detoxification enzyme (fase II) as the GSTS and NAD (P)H quinine reductase. These polyphenol decrease significantly the incidence of carcinoma produced by the exposition at aromatic polycyclic hydrocarbons because they reduce the activity of benzo (a) piren hydroxylase (Cancino et al., 2001).

Conclusion

The freeze-dried aqueous extract of *R. mangle* is promissory by the preparation of phytodrugs by the treatment of gastroduodenal ulcers. This plant showed pharmacological activity and not toxic effect was present.

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