PHARMACOLOGICAL POTENTIAL FROM *Rubus liebmanii* MICRO-PROPAGATED AND THE CALLUS BIOMASS

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Summary

Rubus liebmanii is used in Mexican traditional medicine to treat cough, nervous conditions, insomnia and dysentery. There are not biological and chemical reports from this specie; however, in other species of the genus, many compounds with important biological activity have been isolated. The ethanolic extract of aerial parts showed antiprotozoal activity against *Entamoeba histolytica, Trychomonas vaginalis* and *Giardia lambia,* with values inhibition 10, 12, and 4% at 100µg/mL, respectively. Against *G. intestinalis* presented an IC_{50} =11.75µg/mL. Low toxicological effect against Balb/c mice and Sprague-dawley rats (>5g/kg) were determined.

Key Words: *Rubus liebmanii,* antiprotozoal and toxicological effect, ethanolic extract.

Genus *Rubus* is constituted by 250 species, it belongs to the Rosaceae family, and it is distributed in different parts of the world. For Mexico, 28 species of genus have been described, and some of them are endemic (1). The *Rubus liebmanii* Focke, is known as Citun-zarza and Tsituni (purepecha language), Tunita del cerro, Zarzamora and Zarza. It is a bush 1 to 4 meters high, with short spines, 4 to 10 cm leaves, white petal flowers and blackish fruits of up to 1.5 cm diameter. It can be find in Michoacan, Jalisco, and Mexico State (2). The fruit is eaten ripe, in traditional Mexican medicine; the infusion prepared with its leaves is used to treat cough, nervous conditions, and insomnia. The infusion of new leaves is used against dysentery (1)(2)(3).

This specie has not been studied before; however, in other species of the Rubus genus terpenoides, flavonoides, galotanins, steroles, cetones, carboxylic acids, alcohols, proanticianidin, poliphenoles, antrones, and alkaloids with important biological activity have been isolated (4)(5)(6)(7)(8)(9).

Pharmaceutical studies in some species of the *Rubus* genus have allowed demonstrating that the CH_2CI_2: MeOH extract (1:1) from *R. coriifoluis* has antiprotozoal activity *in vitro* against *Entamoeba histolytica* and *Giardia lamblia* (10), *R. chamaemorus*, *R. ideaus,* and *R. umlmifolius* have antibacterial effect *in vitro* against *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Escherichia coli, Bacillus subtilis,* and *Candida albicans* (5)(7)(11)(12). *R. coreanus* has antiviral activity against Hepatitis B virus (13) and analgesic effect (8); *R. idaeus* has relaxant and antioxidant activities (14, 15). *R. chingii* has antioxidant activity (16), and *R. coriifolius* has anti-inflammatory and anti-protozoal activities *in vivo* (17, 18).

Is important to mention that gastrointestinal diseases have the second position in morbidity nationwide, and parasitic diseases, specifically intestinal amebiasis, has the fifth place, presenting more incidentally in Chiapas, State of Mexico, Jalisco, Puebla, Veracruz, and Distrito Federal. The most affected group is children between 1 and 9 years of age, its occurrence depends basically on the availability of sanitary services and hygienic habits in the population (19). For treating parasitic diseases there are several drugs as metronidazole, tinidazole, albendazole mebendazole and several antibiotics, but unfortunately they have severe secondary effects as damage to normal intestine flora, possible carcinogen effects, and they easily induce resistance (20).

Material and methods

Plant material

R. liebmanii was selected under quimiotaxononic and ethnomedical criteria. The specie is used in tradicional Mexican medicine to treat cough, pulmonia and dysentiry. Plant material was micro-propagated from axillary bud explants in the Centro de Investigación Biomédica del Sur (CIBIN), Morelos from explantes of specimen bought in the Acotzingo Greehouse, Estado de México.

Preparation of extract

Aerial parts (1500 g) were air-dried, powdered, and allowed to macerate in ethanol (3 x 12 L) at room temperature for 72 h. After filtration, the extract was concentrated under low pressure to dryness at 40º C and 250.0 g crude extract were obtained.

Microorganisms strains

The microorganisms strain used in this study were obtained from ATCC: *Staphylococcus pyogene*s (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 43071), *Salmonella thyphi* (ATCC 06539), *Candida albicans* (ATCC 10231), *Trychophyton mentagrophytes* (ATCC 28185) and *T. rubrum* (ATCC 28188).

The bacteria were maintained and tested on Trypticase Soya agar (TSA, Merck) at 37° C and the yeast and fungi were maintained on Sabourand 4% dextrose agar (SDA, Merck).

Antimicrobial assay

The inoculum for each microorganism was prepared from broth cultures containing 10^7 colony forming units (CFU)/mL, equivalent to 0.5 McFarland standards for the bacteria and 10^6 CFU/mL for yeast. The extract for testing was dissolved en DMSO (Merck) and added to melted agar culture medium in Petri dishes (100 x 15 mm) at the following final concentrations: 1, 2, 4, and 8 mg/mL. The antimicobacterial assay was carried out on Mueller-Hinton agar (MHA, Sigma), *S. pyogenes* sheep blood (5%) was added to Mueller-Hinton medium, and for the yeast Sabouraud 4% dextrose agar was used. The diluted inoculum was added to a Steer replicater, calibrated to deliver 10^7 or 10^6 CFU and then they were incubated in Petri dishes for 24 hr at 37° C. Gentamicin (for bacterial) and nystatin (for yeast) (5-20 µg/mL) were used as reference standards. The assays were performed by duplicate and repeated twice, the results were expressed as the lowest concentration of plant extract that produced a complete suppression of colony growth, the minimal inhibitory concentration (MIC) (21).

Antiprotozoal assay

The microorganisms used for this test were trophozoites of *Entameba histolytica* (HM1:IMSS), *Giardia lamblia* IMSS0989 and *Trichomona vaginali*s, using the method previously described by Said-Fernández and Meckes (22)(23). *E. histolytica* was grown axenically at 37º C in PEHPS medium supplemented with 10% heat inactivated bovine serum, *G. lamblia* and *T. vaginali*s were grown in TYI-S-33 modified medium and subcultured twice a week. Cell viability was established as previously reported (24). Trophozoites, in the log phase of growth ($6X10^3$ and $5X10^4$ cell for *E. histolytica* and *G. lamblia*, respectively), were distributed into tubes containing 1 mL of the media and increasing concentrations of the crude extracts dissolved in DMSO. Controls with only DMSO or medium were included in each assay. After an incubation period of 48 h at 37º C, the trophozoites were washed and 50 µL inoculum of each tube were subcultured for another 48 h in medium without the plant extract. Afterwards, parasites were detached from the tubes by immersion in an iced-water bath for 10 min and samples were counted in a haemocytometer. On the other hand, the tubes were centrifuged and the pellets were incubated at 37º C for 45 min in saline phosphate buffer containing 0.075% tetrazolium salt (MTT) and 250 mg phenazine methasulphate (PMS). Tubes were centrifuged for 5 min at 1200 rpm and supernatants were discarded. The pellets were resuspended in 0.5 mL of 0.04 M HCL in isopropanol to extract and dissolve the dye (formazan) from inside the cells. After 5 min, the tubes were vigorously mixed, centrifuged and the absorbance of the supernatant at 579 nm was determined in a spectrophotometer. Experiments were

performed by triplicate and metronidazol and emetine were included as a positive control.

Acute toxicity *in vivo*:

The acute toxicity was determined in male and female Balb/c (± 28 gr) mice and Sprague Dawley rats (± 230 g) following the methodology previously described by Lorke (25). The study with animals was performed according the guidelines of the local Ethics Committee for Experimentation in Animals in Mexico, maintained in standard environmental conditions, and were allowed to have free access to food and clean water, at 12 h light/dark photoperiods. The animals were randomly divided into five groups of three animals per sex. Group 1 was the control vehicle (Tween 20: H_2O), groups 2-5 were orally treated with the extract at 1000, 1600, 2900, and 5000 mg/Kg. The extracts were solubilized in tween 20:H_2O (3:7) and were administered intragastrically in a volume not higher than 10mL/kg of weight. The extracts were administered at single doses.

The general behaviour of mice and rats was observed after the administration at the first, second, fourth and sixth hour and once, daily, for 14 days. The animals were further observed for up to 14 days following treatment for any signs of toxicity and death. After, the animals were sacrificed and the internal organs (lung, kidney, heart, spleen and liver) were extracted and the gross pathological observations were performed. The DL_{50} value was determined according to the method of probit (26).

Results and discussion

The search for new antimicrobial and antiprotozoal agents is necessary and urgent due to the resistance that microorganisms have developed to common actual drugs. The increase in cases is due to socioeconomic problems and to the limited access to governmental health institutions. On the other hand, it is important to point out that medicinal plants are constantly used to treat different parasitic and infectious diseases. The WHO reports that approximately 80% of the population in developing countries including Mexico uses this resource to resolve their health problems. It is important to mention that medicinal plants constitute an important source of pharmacologically active compounds, for example arteether (derivative of artemisinene), galantamine, nitisinone, and triotropium, which were recently approved by the FDA (27).

Our interest for search of new pharmacologically active compounds in the medicinal flora in Mexico is to contribute with the exploration of the pharmacologic potential of this natural resource.

Rubus genus is importance for its medicinal properties attributed to some species of Mexican traditional medicine and of other parts of the world (4)(6)(8). Also, up to date, the anti-protozoal *in vitro* and *in vivo*, antimicrobial, anti-inflammatory, and antioxidant effects of some species as *R. coriifolius, R. chamaemorus, R. idaeus*, and *R,*

ulmifolius have been described. However, the *R. liebmanii* has not been researched chemically or biologically. Among the secondary metabolites with antiprotozoal activity are (+)-catechin, (-)-epicatechin, ellagic acid, gallic acid and β-sitosterol (10); the antimicrobial and antifungal effects are due to the presence of ellagic acid, gallic acid, ferulic acid, tiliroside, and other flavonoids (active against *S. aureus, S. epidermidis, M. luteus, B. subtilis, B. cereus, P. aeruginosa, E. coli, S. cerevisiae, C. albicans, A. niger*) (5)(7). The anti-inflammatory and analgesic effects are due to poliphenoles, niga-ichigoside, and 23-hydroxytormentic acid (8)(17). The antioxidant activity *in situ, in vivo,* and *in vitro* is due to anthocyanins (as particularly cyanidin and pelargonidin derivatives), ellagitannins and proanthocyanidins (15)(28)(29). Other biological activities described by Rubus genus are lipolisis, inhibit the hyaluronidase activity and angiogenesis (30(31)(32). *Rubus chingii* has relaxing activity in the guinea pig ileum, hepatoprotector activity, and HVB antiviral activity (13)(14)(16).
The EtOH extract from the aerial parts of *R. liebmani*i inhibited moderately the growth of *S. aureus* (CMI=1 mg/mL), *T. mentagrophytes* and *T, rubrum* (CMI=2 mg/mL) and was inactive against *S. pyogenes, E. faecalis, E. coli, P. mirabilis* and *S. typhi* (CMI=8 mg/mL), and *C. albicans* (Table 1). Previously search described that *R. chamaemorus, R. ideaus, and R. umlmifolius* have antibacterial effects *in vitro* against Gram+ and Gram- strain and yeast and this effect is due to poliphenoles, antocianin and ellagic acid (5)(7)(11)(12).

Additionally, the extract showed moderate antiprotozoal activity against *E. histolytica, T. vaginalis* and *G. lambia,* with values inhibition 10, 12, and 4% at 100µg/mL, respectively. Against *G. intestinalis* presented an IC_{50} =11.75µg/mL. In literature, has been reported that CH_2CI_2:MeOH extract from the aerial parts of *R coriifolius* showed important antiprotozoal activity against *E .histolytica* and *G. lambia* and the bioguided fractionation from active extract allowed to obtain the responsible compounds of the anti-protozoal effects, being (-)-epicatechin, (+)-catechin, and ellagic acid the responsible of the biological effect (10).

The DL_{50} from EtOH extract from *R. liebmanii* was >5 g/kg, for both species of rodents, and no physical alterations in the liver, kidney, lung, nor weight variation were observed. Until now, the toxicity (DL_{50}) for any of the species of the Rubus genus has not been described. Nowadays, the EtOH extract from *R. liebamanni* has been fractionated by bioguided assay and in some primary fractions, the presence of poliphenoles and terpenoids have been detected, now in the process of separation and evaluation.

The results obtained up to date allow conclude that the *R. liebmanii* species contains secondary metabolites active against bacteria, fungi, and parasites, which require being isolated and identified.

Table1. Antimicrobial activity of ethanolic extract from *R. liebmanii.*

Species	ATCC	MIC (mg/mL)
Staphylococcus aureus	29213	1
Enterococcus faecalis	29212	8
Escherichia coli	25922	8
Proteus mirabilis	43071	8
Salmonella typhi	06539	8
Candida albicans	10231	> 8
Trychophyton rubrum	28185	2
T. mentagrophytes	28188	2

Conclusions

The ethanolic extract of micropropagated material of *R. liebmanii* showed antiprotozoarial activity and low toxicological effect. The extract can be a source of active compounds or carry out like material of reference for development of possible pharmaceutical preparations.

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