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Antiprotozoal activity of Mexican ethnomedicine plants against *Trichomonas tenax*, a protozoan associated with periodontal disease

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— Abstract—

Objective: To evaluate the antiprotozoal activity of extracts of *Cordia dodecandra*, *Gaultheria odorata*, *Tagetes nelsonii*, and *Talisia oliviformis*, plants used in Mexican ethnomedicine, against *Trichomonas tenax*. Materials and methods: The study plants were collected in the state of Chiapas, Mexico. Hexanolic and methanolic extracts were prepared using the sonication-assisted maceration technique. The extracts were dissolved in DMSO (0.25 % v/v finally). The trichomonodal activity of the extracts was evaluated *in vitro* at concentrations between < 500 µg/mL. Metronidazole was employed as a positive control. The percentage inhibition was estimated from cell counting with a manual hemocytometer using an untreated control culture as a reference. The assays were performed in triplicate in two independent experiments, and the IC_{50} was determined by Probit regression analysis. Results: The results were interesting for the hexane extract of *G. odorata* and *T. nelsonii* with an IC_{50} < 200 µg/mL. Conclusion: This work constitutes the first report of Mexican ethnomedicinal plants in the investigation of natural products against *T. tenax*. In addition, it enriches the ancestral knowledge of herbal resources for the treatment of infections by parasites and diseases of the oral cavity.

Keywords:

Oral health; anti-T; tenax activity; medicinal plants.

Globally, the oral health situation is alarming; diseases such as caries, gingivitis, periodontitis, and lack of teeth are considered important public and private health problems, despite being preventable. These oral pathologies are serious and debilitating, manifested in experiences of pain, inability to eat, limited communication, and low self-esteem due to the loss of function and aesthetics of the stomatognathic system, hurting the general health and quality of life of those who suffer from them[1]. As of 2021, the WHO estimated just over 3.5 billion cases of oral health conditions worldwide, making them more widespread than mental disorders, heart disease, diabetes, and cancer[2].

Among oral pathologies, periodontal disease is the second cause of oral discomfort after dental caries, with a prevalence of 19% worldwide and with a higher occurrence in the population of productive age. In Mexico, the prevalent rate is 50% of the population between 35 and 79 years of age. In 2019, this represented an economic burden between 11 and 50 US dollars per capita. Cases prevail in regions with limited access to public health services, education, social programs, and with average economic income[2,3].

Periodontal disease is a chronic inflammatory condition that is characterized by a progressive destruction of the tissues surrounding and supporting the teeth, including the gums, alveolar bone, and periodontal ligaments, leading to tooth loss if not properly treated. These include gingivitis and periodontitis. As a primary etiological factor, the formation of microbial dental plaque is reported, which, with the participation of additional factors of local, immunological, and systemic origin, causes contamination, inflammation, and destruction of the periodontium[1,4,5].

Often, the causative agent has been categorized as bacterial in origin. However, the high incidence of protozoa such as *Entamoeba gingivalis* and *Trichomonas tenax* in the conditions has led some researchers to consider them etiological agents of the disease, pointing out that the increase in the incidence of these protozoa constitutes an indicator of progression of periodontal disease[6-8].

T. tenax is a flagellated commensal protozoan that lives in the cavity of decayed teeth, between tartar and in the gingival margins of the gums of people with poor oral hygiene. It is transmitted through direct contact, saliva droplets, and contaminated fomites. When it spreads beyond the oral cavity, it can cause sinusitis, tonsillitis, and pulmonary trichomoniasis[9,10]. Its pathogenicity has been linked to the presence of tissue adhesion proteins and proteolytic activity similar to those of *T. vaginalis*[11,12].

Although Trichomonas infections are generally treated with metronidazole, reports of metallic taste, acquired resistance, and mutagenicity have been reported, ultimately exposing the need for new therapies to treat trichomoniasis[13].

In this regard, various natural products derived from ethnomedicinal plants have played an important role in controlling diseases caused by protozoa that affect human health[14,15]. However, the study of locally available medicinal plants to treat infections caused by *T. tenax* is limited. In relation to this, the plant species *Cordia dodecandra*, *Gaultheria odorata*, *Tagetes nelsonii*, and *Talisia oliviformis* are used by the indigenous Mexican population to treat gastrointestinal problems caused by bacteria and/or parasites[16,17], as shown in Figure 1. Previously, our working group demonstrated that organic extracts exhibit antimicrobial activity[18]. Therefore, in order to establish its integral use in therapies to maintain oral health, this work aims to determine *in vitro* the anti-*Trichomonas tenax activity* of *C. dodecandra, G. odorata, T. nelsonii*, and *T. oliviformis*.

Note. A) Cordia dodecandra (Cupapé); B) Gaultheria odorata (Arrayan); C) Tagetes nelsonii (Chilchahua), and D) Talisia oliviformis (Guaya).

Figure 1. Southeastern Mexican ethnomedicinal plants

MATERIAL AND METHODS

Chemical

Dimethyl sulfoxide (DMSO 99.5% for cell culture) and metronidazole (analytical standard grade) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The chemicals employed in the preparation of the TYI-S-33 medium (Ascorbic Acid, Ammonia Ferric Citrate, Cysteine, Sodium Chloride, Yeast Extract, and Glucose from Sigma Aldrich; Potassium Monoacid Phosphate, and Potassium Diacid Phosphate from JT Baker and Casein Peptone from DIBICO) and those employed in the extraction process (n-hexane and Methyl Alcohol from JT Baker) were of analytical grade obtained from the Fisher Scientific Chemical brand.

Obtaining plants and preparing crude extracts

The plants were collected during the winter of 2021, in Tuxtla Gutiérrez (*C. dodecandra* at 16° 43′ 46.2′′ N, 93° 06′ 0.87′′ E and *T. oliviformis* at 16° 43′ 45.7′′ N, 93° 06′ 0.70′′ E) and Zinancatán (*G. odorata* and *T. nelsonii* at 16° 45′ 34.0′′ N, 92° 43′ 18.0′′ E), Chiapas, Mexico. The specimens were identified at the species level by professional staff of the FCB-UANL herbarium, being registered as: *C. dodecandra* (025879), *T. oliviformis* (025881), *T. nelsonii* (025883), and *G. odorata* (025884). The plant material was cleaned, cut, dried at room temperature without direct lighting, and crushed, using a hand mill. Hexane and methane extracts were obtained by maceration, mixing 50g of plant material and solvent in a 1:10 ratio for 18 days at room temperature and without stirring, performing extraction processes every six days with hexane and applying sonication for 15 minutes, followed by extraction with methanol under the same conditions. On each occasion, the extracts were filtered and concentrated under reduced pressure using a Yamato Model RE2000. rotary evaporator. Finally, they were dried in an oven at 40°C until they reached a constant weight to calculate the percentage yield. The extracts obtained were stored at 4°C until they were used.

T. tenax Culture

Trophozoites of T. tenax strain ATCC-30207 were grown in TYI-S-33 medium supplemented with bovine serum to a concentration of 10% v/v. The culture was maintained under axenic conditions. For the bioassays of antiprotozoal activity, trophozoites were used in their exponential phase.

Antiprotozoal activity

A series of *in vitro* bioassays was performed to determine the antiprotozoal activity of the crude extracts [19]. Briefly, an inoculum of $1x10^5$ trophozoites of *T. tenax*/mL was incubated at 37 °C for 24 h in TYI-S-33 medium supplemented with bovine serum and in the presence of different concentrations (18.75 to 500 µg/mL) of the crude extracts and metronidazole (0.015 to 0.25 µg/mL) as a positive control. Briefly, 30 mg of each extract and 10 mg of metronidazole were first independently dissolved in 250 µL of DMSO and diluted with sterile deionized water, and inoculated into the medium to the desired concentration. The final concentration of DMSO did not exceed 0.25% v/v in the tested dilutions. Each assay had a blank (inoculated medium).

Two independent trials were performed in triplicate. In each assay, the extracts, metronidazole, DMSO (at the established concentrations), and a blank (inoculated medium) were evaluated. After incubation, a 1:10 dilution of each sample was prepared with formalin. The number of parasites was counted with a hemocytometer, and the % inhibition with respect to the blank tube was determined.

Statistical analysis

The concentration that inhibits trophozoite growth by 50% (CI_{50}) was determined by Probit linear regression, as seen in Figure 2. In addition, the data were subjected to a bifactorial analysis of variance, and the Tukey test was applied to determine the statistical difference between treatments, using SPSS Statistics Version 18.0 (IBM SPSS Statistics for Windows, Armonk, NY).

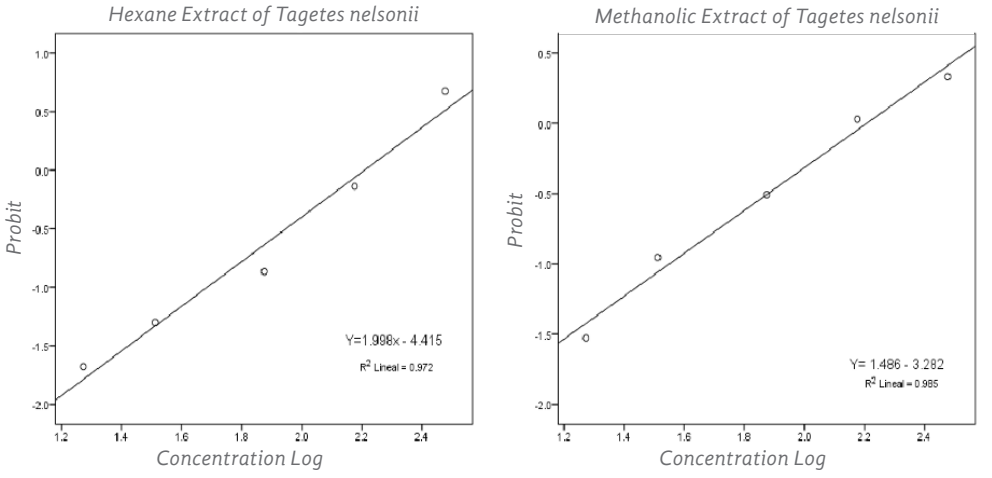

Figure 2. Probit graphs of the anti-protozoan activity of Tagetes nelsonii extracts versus T. tenax

RESULTS

A total of eight crude extracts, four methanolic, four hexanic, and metronidazole, were evaluated against *T. tenax*. The extraction yield and *in vitro* antiprotozoal activity (CI_{50}) of the extracts obtained from the four medicinal plants evaluated are shown in Table 1. The results show a higher extraction yield when using methanol as solvent, with yields between 5,0% for *C. dodecandra* and 14% for *G. odorata*. Statistical analysis revealed significant effects of both plant type ($F= 151.54$, $p < 0.05$), extract type ($F= 61.08$, $p < 0.05$), and their interaction ($F= 27.57$, $p < 0.05$) on antiprotozoal activity. Multiple comparisons using Tukey's test showed a significant difference ($p < 0.05$) between the plants tested. In particular, *T. nelsonii* exhibited the highest anti-*T. tenax* activity was significantly higher than the rest of the species, while *T. oliviformis* showed the lowest activity. The results suggest that both the plant species and the type of extract have a significant influence on antiprotozoal activity, and that there is a specific interaction between these two factors.

Table 1
Antiprotozoal Activity of selected medicinal plants on T. tenax

Plant Name (Family)	Part of the Plant Used*	Antiprotozoal Activity (CI50= µg/mL)			
		%**	Hexane	%	Methanolic
Cordia dodecandra (Boraginaceae)	C y T	0.4	315.83± 16.55^{c}	5.0	323.42 ± 22.11^{c}
Gaultheria odorata (Ericaceae)	H	1.6	185.00 ± 19.57^{b}	14.0	243.44 ± 19.54^{b}
Tagetes nelsonii (Asteraceae)	C y T	0.7	162.90 ± 5.26^{a}	6.9	161.70 ± 13.87^{a}
Talisia oliviformis (Sapindaceae)	H	2.4	278.46 ± 20.75^{d}	8.0	449.71 ± 23.64^{d}
Metronidazol					0.013 ± 0.003

*C= bark, H= leaves, T= stem; **dry matter yield %; Results: Mean + DS, means followed by different letters are statistically different, p<0.05.

DISCUSSION

This study demonstrated the antiprotozoal activity of extracts from selected plants used in Mexican ethnomedicine against *T. tenax*. It is important to mention that research into medicinal plants for treating *T. tenax* infections is almost nonexistent. However, anti-*Trichomonas* activity of *Origanum mejorana* has been reported on *Pentatrichomonas hominis*, a human commensal protozoan causing intestinal *trichomoniasis*[20], as well as *Carica papaya*, *Cocus nucifera*, and *Persea americana* against *Trichomonas vaginalis*, a causative agent of human vaginal trichomoniasis[21,22].

Regarding *T. tenax*, anti-*Trichomonas* activity of *Punica granatum* ethanolic extract has been reported with an effective concentration of 12.5 µg/mL[23]. Likewise, the antiperiodontitis activity of *Punica granatum* has been confirmed [24]. The *in vivo* study of *Eugenia cariophylla* infusion on *T. tenax* has been reported to be effective at a concentration of 300 mg/mL[25].

On the other hand, the anti-gingivitis activity of *Aloe vera*, *Camellia sinensis*, *Punica granatum*, and *Salvadora persica* incorporated into dentifrices has been reported with a notable reduction in periodontal disease[26].

The above points out the relevance of the results obtained in this research, since the four plants evaluated showed anti-*T.tenax* effect, being interesting the activity shown by *T. nelsonii* and *G. odorata*, whose biological property could be extended towards the systematic search for trichomonicidal metabolites through a biodirected fractionation that increases antiprotozoal activity and its subsequent use in the development of dentifrices, oral antiseptics, and mouthwashes for oral health care.

In relation to this, the potential of the *Tagetes* plant is highlighted. Research indicates that this genus harbors species that exhibit insecticidal, antimicrobial, acaricidal, antifungal, and anthelmintic activity, attributed to the presence of terpenes such as: allylenasol, β-caryophyllene, *trans*-anethole, and tagetone[27-29]. In addition, it has been determined in *T. nelsonii* essential oil to *cis*-tagetenone, β-tagetone, and dihydrotagetone as major compounds and, to a lesser extent, the occurrence of α-terpineol, *trans*-β-ocimene, limonene, α-pinene, myrcene, mesitylene, α-terpinene, eucalyptol, linalool, and β-copaene[30]. Eugenol, carvacrol, menthol, and thymol have been found in the essential oils and organic extracts of *Tagetes*; their antiprotozoal activity could be related to changes in the permeability of the cell membrane [31]. On the other hand, the presence of gallic acid, quinic acid, syringic acid, ellagic acid, quercetin, kaempferol, patuletin, isorhamnetin, axillarin, and their glycoside derivatives has been documented in methanolic extracts of *T. erecta* compounds with potent chelating activity of Fe^{+2} ions, an essential cofactor of Fe-S proteins, which participate in the energy metabolism of trichomonads.[32, 33] Sesquiterpenes, sterols, triterpenes, and tetraterpenes

have been isolated from the leaves of members of the Asteraceae family and have been reported to prevent the growth progression of *Leishmania amazonensis*, *Plasmodium falciparum*, and *Trypanosoma brucei* possibly by alteration of mitochondrial activity[34], perhaps a similar effect occurs in the hydrogenosomes of *T. tenax* of finding this type of metabolites in the hexane extract.

Regarding *C. dodecandra*, the occurrence of phenylpropanoids such as rosmarinic acid, syringin, and salvianolic acid B; flavonoids derived from quercetin and alkaloids such as allantoin in extracts[35] has been evidenced. It is possible that anti-*T. tenax* activity exhibited by *T. nelsonii* and *C. dodecandra* extracts in this work is due to a particular metabolite or the synergistic activity thereof. Regarding *G. odorata* and *T. oliviformis*, the chemical composition of their extracts is still unknown.

CONCLUSION

Considering the multi-microbial origin and health and economic impact of periodontal diseases on human health, new strategies and adjuvants are needed to maintain oral health. In this regard, this study adds to the scientific knowledge of natural products with anti-*T. tenax* activity, showing *T. nelsonii* to have the best trichomonacidal effect, and as a candidate for the search for possible compounds with antiprotozoal activity. In addition, the results obtained enrich the ancestral knowledge of herbal resources in Mexican ethnomedicine for the treatment of parasitic infections and diseases of the oral cavity.

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