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Original article

IN VITRO EVALUATION OF ANTI-TRICHOMONAL POTENTIAL OF *PSIDIUM GUAJAVA* LEAF ESSENTIAL OIL AND ITS MAIN COMPONENTS AGAINST TROPHOZOITES OF *TRICHOMONAS GALLINAE*

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Summary

Akbarian, J., M. R. Youssefi & M. A. Tabari, 2024. *In vitro* evaluation of anti-trichomonal potential of *Psidium guajava* leaf essential oil and its main components against trophozoites of *Trichomonas gallinae. Bulg. J. Vet. Med.,* **27**, No 1, 68–78.

Trichomoniasis is a protozoan disease caused by *Trichomonas gallinae* that mainly affects the upper digestive and respiratory tracts of columbiforms. Metronidazole has been used for many years for the control of trichomoniasis, however, in recent years, there were several reports on resistant *T. gallinae* strains. *Psidium guajava*, a well-known medicinal plant species, possesses several biological activities including anti-protozoal effects. Considering growing interest on plant and plant-derived compounds as alternative sources of bioactive chemicals, the present study was performed to evaluate the anti-trichomonal potential of *P. guajava* leave essential oil (EO) and its main components on *T. gallinae* trophozoites. EO was extracted from plant fresh leaves by steam distillation using a Clevenger-type apparatus and gas chromatography-mass spectrometry was performed for identification of the constituents. *In vitro* susceptibility assay was done in sterile multiwell plates incubated with the trophozoites and the corresponding concentrations of tested compounds. Metronidazole was used as the standard anti-trichomonal drug. Mortality rates were evaluated by eosin staining and recorded every 12 h for 2 days. The EO composition was dominated by the β-caryophyllene (30.2%) and α-pinene (18.3%). A significant effect of concentration of the tested compound and time of exposure was noted in the toxicity of the EO and its main constituents. Based on the 12 h fifty percent lethal concentrations (LC_{50}), β-caryophyllene was the most potent anti-trichomonal agent, with LC_{50} of 0.32 µg/mL. Considering the natural source of β-caryophyllene and its reported low cytotoxicity and promising anti-trichomonal efficacy, this compound can be considered as a candidate ingredient for the development of green antitrichomonal agents.

Key words: herbal drugs, pigeon, terpenoids, trichomoniasis

INTRODUCTION

Trichomonosis, a protozoan disease, is caused by the flagellate *Trichomonas gallinae*. It mainly affects the upper digestive and respiratory tracts of columbiforms, raptors and psittaciforms. Manifestations vary from subclinical to severe infection resulting in organ necrosis, tissue invasion, and death (Youssefi *et al.,* 2017). Although this disease affects individual birds or siblings in a nest in many cases, sizable epizootics have been reported, especially among free-ranging columbiforms. *Trichomonas gallinae* is a cosmopolitan parasite and its distribution is correlated closely with that of the rock pigeon, one of its most important hosts (Hashemi *et al.*, 2021).

Psidium guajava L. (from Myrtaceae family), commonly known as guava, is found in tropical America and South East Asia. Fruits, leaves and barks of this plant species have been used for a long time in traditional medicine for different ailments. Leaf extracts have also been reported to possess biological activities, including antioxidant, anti-inflammatory, antimicrobial, and anti-plasmodial effects (Machado *et al.,* 2018). A variety of compounds, including α-pinene, β-caryophyllene, limonene, and nerolidol, are found in the essential oil (EO) of the leaves of *P. guajava* and probably these constituents are the active parts for exerting biological activities of EO. Beta-caryophyllene is a natural bicyclic sesquiterpene and a constituent of many essential oils. Studies have revealed promising antibacterial and anti-parasitic activities of this compound on some major pathogenic organisms (Soares *et al.,* 2013; Moo *et al.,* 2020). Alpha-pinene, an organic compound of the terpenoid pinene class, is found in the oils of many plant species, notably the pine tree *(Pinus* sp.), *Eucalyptus* sp., and rosemary (*Rosmarinus officinalis*). Pinenes show antibacterial, fungicidal, and insecticidal activities and exhibit some antiviral effects against infectious bronchitis virus (IBV) (Rivas da Silva *et al.,* 2012; Malekifard *et al,* 2021; Polanco-Hernández *et al.*, 2021).

Nitroimidazoles have been drugs of choice for the treatment of trichomoniasis for many years, however, there are several recent reports on resistant strains and failure of treatment with the recommended doses of these drugs in infected birds (Tabari *et al.,* 2021). Considering centuries of use of medicinal herbs, their robust background, and better compliance for natural compounds in the treatment of ailments, plant and plant-derived compounds can serve as an alternative source for the research and development of new anti-trichomonal agents. Bearing the above in mind, the present study was performed to evaluate the anti-trichomonal potential of a natural product *P. guajava* leaf EO and its main components, β-caryophyllene, and α-pinene on trophozoites of *T. gallinae* to find out the functional constituent of the EO and the possible underlying mechanistic interaction of the components.

MATERIALS AND METHODS

Chemicals

Beta-caryophyllene and α-pinene were purchased from Sigma (Germany). Tryptone/yeast extract/maltose (TYM) medium and foetal calf serum were also obtained from Sigma (Germany). Metrodidazole was a gift from Alborz Daru pharmaceutical company (Iran). All other chemicals were analytical grade and commercially available.

Plant material and essential oil extraction

The fresh leaves of *P. guajava* were purchased from Mirnia Nahalestan (Babol, Mazandaran, Iran). Voucher specimen was authenticated and deposited at Herbarium of Sari University of Natural Resources, Sari, Iran under No.99-2024. Essential oil was extracted from leaves by steam distillation using a Clevenger-type apparatus and yielded 0.12% (w/w). The chemical analysis of the EO was carried out on an Agilent 7890A gas chromatograph equipped with 5975C mass spectrometer. The oil sample was diluted to 1% with n-hexane, and 2 µL of the solution was injected into the GC-MS system 3 times. The carrier gas was at a flow rate of 1.0 mL/min. The injector and detector temperatures were 230 and 250 °C, respectively. The identification of the EO compounds was based on the comparison of their retention indices and mass spectra with those contained in the commercial libraries.

Parasite

By using wet swabs, samples were taken from suspicious lesions in the oropharyngeal area of 10-week-old captive pigeons reared by local pigeon breeders. Wet smears were prepared and examined under a light microscope at ×100 and ×400 magnifications to confirm the existence of *T. gallinae*. Molecular characterisation of the isolates was also done and samples were confirmed as *T. gallinae* (data in press). Parasite culture was prepared by immersing oral swabs in TYM medium supplemented with 10% foetal calf serum (Sigma, Germany) and incubated at 37 °C (Youssefi *et al.,* 2017). When the parasites showed more than 95% mobility and normal morphology, sub-cultures were done on isolates during the logarithmic phase of growth every 48 h.

In vitro assay

The method used for the *in vitro* assay was previously described (Tabari *et al.*, 2017). In brief, to examine the susceptibility of *T. gallinae* to *P. guajava* EO and its main components, β-caryophyllene and α-pinene, sterile multi-well plates were used to incubate trophozoites with the corresponding tested compounds. A volume of 100 µL of culture medium containing 1×10^4 parasites was pipetted into each well, as well as prediluted components to give final concentrations of 50, 25, 12.5, 6.25, 3.12 and 1.5 µg/mL in the final volume of 300 µL. For β-caryophyllene, and α-pinene because of showing 100% mortality at the aforementioned concentrations the diluted concentrations of 5, 2.5, 1.25, 0.625 and 0.312 µg/mL were used for *in vitro* susceptibility assays. Tween 20 (0.01% of final concentration) was used as solubilisation vehicle. Control wells received only Tween 20. Metronidazole at concentrations of 50, 25, 12.5, 6.25, 3.12 and 1.5 µg/mL was tested as the standard anti-trichomonal drug (Tabari *et al.,* 2021). For each concentration of tested compounds, assays were run in triplicate. The number of dead trophozoites in the medium were counted every 12 h (at time points of 12, 24, and 48 h) using the trypan blue exclusion assay (Tabari *et al.,* 2017).

Statistical analysis

Statistical analysis was performed by comparing the mean mortality rates in different groups by one-way analysis of variance (ANOVA) and Tukey *post hoc* test. Differences between multiple time points were analysed by Repeated measures ANOVA, Bonferroni test. The 50 and 90 percent lethal concentrations LC_{50} and LC_{90} were calculated using Probit regression analysis (SPSS software version 22

(Chicago, Illinois, USA). For all tests, values of $P<0.05$ were considered statistically significant.

RESULTS

Chemical composition of EO

The chemical constituents of *P. guajava* EO were identified by GC-MS analysis and are shown in Table 1. Thirty-one components were identified in EO, accounting for 99.5% of the total composition. The EO composition was dominated by the terpene alcohols including β-caryophyllene (30.2%) and α-pinene (18.3%) which were the most abundant components, followed by veridiflorene (11.4%) and limonene (8.6%).

Table 1. Chemical composition of the *Psidium guajava* essential oil.

No	Component[a]	Calculated RI[b]	Abundance (%) [c]
1	α-Thujone	932	0.3
2	α-Pinene	941	18.3
3	β-Pinene	970	0.4
4	Myrcene	991	0.5
5	*p*-Cymene	1019	0.3
6	Limonene	1025	8.6
7	(Z)-β-Ocimene	1037	0.5
8	γ-Terpinene	1056	0.4
9	*cis*-β-Terpineol	1130	tr
10	Terpinen-4-ol	1172	0.2
11	α-Terpineol	1185	1.3
12	Bornyl acetate	1275	0.9
13	Neryl acetate	1348	0.7
14	Geranyl acetate	1364	3.5
15	α-copaene	1388	7.1
16	β-Elemene	1389	0.4
17	α-Gurjunene	1409	0.4
18	Aromadendene	1420	1.8
19	*β*-Caryophyllene	1428	30.2
20	α-Humulene	1440	3.2
21	(*E*)-β-Farnesene	1452	0.4
22	γ-Gurjunene	1473	0.2
23	Germacrene D	1482	1.6
24	Veridiflorene	1498	11.4
25	(*E,E*)-α-Farnesene	1506	0.2
26	γ-Cadinene	1514	3.4
27	δ-Guaiol	1520	0.3
28	δ-Cadinene	1525	1.6
29	(E)- γ -Bisabolene	1539	0.8
30	β-Eudesmol	1650	0.5
31	Cadalene	1676	0.1
	Total identified (%)		99.5

[a]Compounds are listed in order of their elution from a HP-5MS column. [b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c]Relative percentage values are means of three determinations with a RSD% in all cases below 10%.

Anti-trichomonal activity

Mean mortality rates of trophozoites of *T. gallinae* after 12, 24, and 48 h of exposure to different concentrations of EO and metronidazole are demonstrated on Fig. 1. For all tested compounds, a significant effect of tested concentration ($P<0.001$), the time of exposure ($P<0.001$) and their interaction ($P=0.001$) were noted. At 50, 25, and 12.5 µg/mL guava EO resulted in higher than 90% mortality in trophozoites; no significant difference was observed in comparison of these concentrations at different time points ($P>0.05$). The same results were recorded for 50, 25, and 12.5 µg/mL of metronidazole. For EO of guava and metronidazole at the concentrations of 6.25, 3.12, 1.56, and 0.78 µg/mL, toxic effect of these concentrations increased by passing time ($P<0.05$). EO of guava showed a good anti-trichomonal activity, and at the concentration of 6.25 after 24 h killed 81% of trophozoites while the standard anti-trichomonal medication, metronidazole, killed 84% of trophozoites. At the lowest tested concentration, 0.78 µg/mL EO of guava and metronidazole resulted in 19.33 and 34.66% mortality rates, respectively.

Fig. 2 shows toxicity of β-caryophyllene and α-pinene on trophozoites of *T. gallinae*. Main constituents of guava EO, β-caryophyllene and α-pinene displayed promising anti-trichomonal activity. At the concentration of 5 µg/mL β-caryophyllene led to 95.33% mortality after 48 h which was significantly higher relative to 84.66% mortality at the time point of 12 h ($P<0.05$). At the time points of 12, 24, and 48 h, α-pinene at the concentration of 5 µg/mL resulted in 74, 75.33, and 82% mortality rates, respectively. Beta-caryophyllene and α-pinene, at their lowest tested concentrations, led to 48 h mortality rates of 36.66 and 74.66%, respectively.

Based on the 12 h calculated LC_{50} and LC_{90} values, β-caryophyllene presented the most potent anti-trichomonal activity among all tested compounds of the present study, with LC_{50} and LC_{90} values of 0.32, and 10.31 µg/mL, respectively (Table 2). Based on the obtained LC_{50}s, α-pinene and β-caryophyllene were more potent than guava essential oil (2.70 µg/mL), however, by comparing LC_{90} values, in spite of lower value for β-caryophyllene (10.31 µg/mL) relative to guava essential oil (12.54 µg/mL), due to the overlapping confidence limits, no significant difference was noted in their anti-trichomonal efficacies. The standard anti-trichomonal drug, metronidazole led to the LC_{50} and LC_{90} values of 2.17 and 9.72 µg/mL, respectively.

DISCUSSION

A promising alternative to overcome the resistance to current parasiticides and to avoid the ecological costs of their use is searching for novel nature-derived anti-parasitic agents (Piña *et al.,* 2017). In the present study, β-caryophyllene was the major component of the EO of *P. guajava*. This finding agreed with reports showing *P. guajava* EO as an excellent source of this compound (Santos *et al.,* 1998; Arain *et al.,* 2019). However, comparison between the present study with that of Arain *et al.* (2019) showed some differences in reported minor constituents. The higher limonene and veridiflorene components of the *P. guajava* leaf EO have also been reported by Weli *et al.* (2019) from Oman. Differences in the chemical ingredients of the *P. guajava* leaf EOs are most probably due to the geographical as well as meteorological

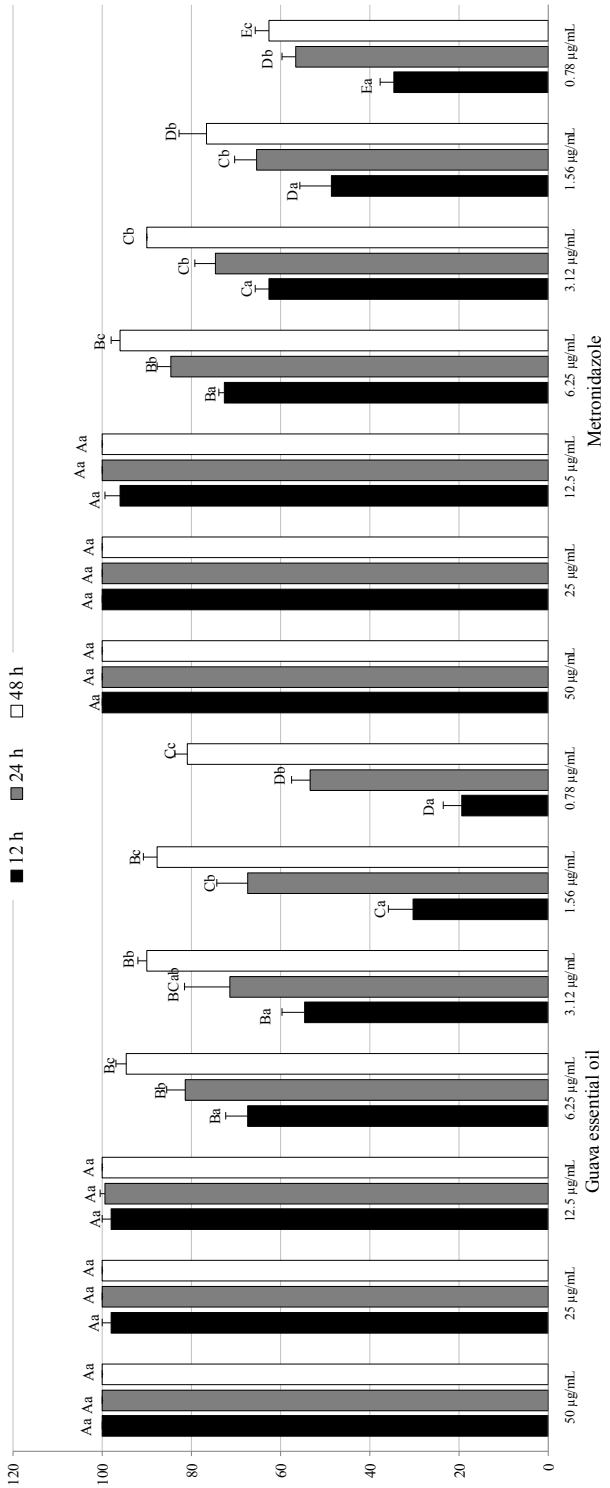

Fig. 1. Mortality (%) of trophozoites over different times of exposure to essential oil of guava in comparison to metronidazole. Data are shown as mean ± standard error. Columns marked with different lowercase letters are significantly different between times of exposure (Repeated measures ANOVA, Bonferroni test, $P<0.05$). Columns marked with different uppercase letters at each time of exposure (12, 24, and 48 hour) are significantly different between concentrations (ANOVA, Tukey's HSD test, $P<0.05$).

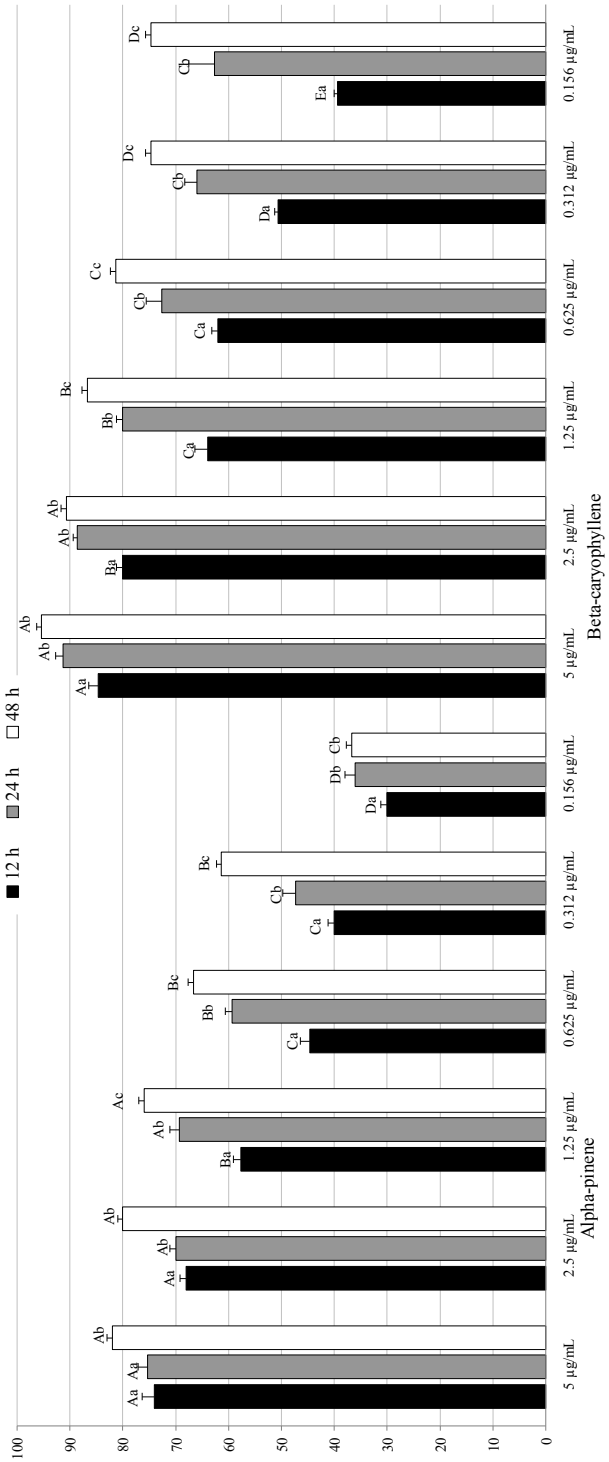

Fig. 2. Mortality (%) of trophozoites over different times of exposure to main constituents of essential oil of guava, alpha-pinene and beta-caryophyllene. Data are shown as mean ± standard error. Columns marked with different lowercase letters are significantly different between times of exposure (Repeated measures ANOVA, Bonferroni test, $P<0.05$). Columns marked with different uppercase letters at each time of exposure (12, 24, and 48 hour) are significantly different between concentrations (ANOVA, Tukey's HSD test, $P<0.05$).

Table 2. Probit analysis of lethal concentrations 50 and 90 (LC_{50} and LC_{90}) for the essential oil of guava, its two main constituents, alpha-pinene and beta-caryophyllene, and the standard antitrichomonal chemotherapeutic, metronidazole for their activity against trophozoites of *Trichomonas gallinae*

Components	Concentration (µg/mL)	12 h mortality (%) ±SE	LC_{50} (µg/mL) (LCL–UCL)	LC_{90} (µg/mL) (LCL–UCL)	χ^2 (df)[b]
Alpha-pinene	0.156	30.00±2.00	0.73	30.50	0.64
	0.312	40.00±2.00	(0.52–0.99)	(13.94–51.81)	(4)
	0.625	44.66±3.05			n.s.
	1.25	57.66±2.51			
	2.50	68.00±2.00			
	5.00	74.00±4.00			
Beta-caryophyllene	0.156	39.33±1.15	0.32	10.31	1.88
	0.312	50.66±1.15	(0.20–0.44)	(5.76–16.22)	(4)
	0.625	62.00±2.00			n.s.
	1.25	64.00±4.00			
	2.50	80.00±2.00			
	5.00	84.66±3.05			
Guava essential oil	0.78	19.33±4.16	2.70	12.54	7.58
	1.56	30.33±5.50	(2.32–3.11)	(10.24–16.10)	(5)
	3.12	54.66±5.03			n.s.
	6.25	67.33±5.03			
	12.5	92.66±4.61			
	25.0	98.00±2.00			
	50.0	100.00±0.00			
Metronidazole	0.78	34.66±3.05	2.17	9.72	3.86
	1.56	48.66±7.02	(1.30–2.90)	(8.42–11.65)	(5)
	3.12	62.66±7.02			n.s.
	6.25	72.66±1.15			
	12.5	96.00±3.46			
	25.0	100.00±0.00			
	50.0	100.00±0.00			

LCL: lower confidence limit; UCL: upper confidence limit; χ2: Chi2; df: degree of freedom; n.s. not significant.

differences of the regions of collected plant material. It has been well documented that sunlight, temperature, relative humidity and altitude in different locations are the key factors that could change physiology and EO production in plants (Khalid *et al.,* 2020).

The toxicity of guava on several flagellated protozoa including: *Giardia*, *Trichomonas*, *Leishmania*, and *Trypanosoma* sp. has been previously reported (Muelas-Serrano *et al*., 2000; de Souza *et al*., 2017; Machado *et al*., 2018). It has been shown that the viability of *G. lamblia* trophozoites was affected by guava leaf decoction at 5 and 10% dilutions, and resulted in the reduction of viability to 40% in the treated trophozoites. On the other hand, metronidazole at 10 µg/mL showed higher activity and led to about 20% viability in trophozoites (Birdi *et al*., 2011; Morais-Braga *et al*., 2016).

The aqueous and dichloromethane:methanol extracts of leaves of guava have been reported to be active against protozoa *Trichomonas vaginalis* at the concentrations of 4 and 1 mg/mL, respectively (Van Vuuren & Naidoo, 2010). In line with these findings, the present study demonstrated *in vitro* toxicity of guava on trophozoites of *T. gallinae*. Among the studies on toxicity of natural plant-derived preperations on *T. galline*, Youssefi *et al.* (2017) have reported toxic effects of *Artemisia sieberi* on *T. gallinae* with minimum inhibitory concentration (MIC) of 10 µg/mL. Four days of treatment with *A. sieberi* EO at dose of 50 mg/kg in pigeons experimentally infected with trichomoniasis resulted in the total eradication of the infection and full recovery of birds (Youssefi *et al.,* 2017). Anti-trichomonal effects of *Lavandula angustifolia* and *Zingiber officinale* extracts were also measured on *T. gallinae* and compared with metronidazole. The 24-h MIC values of 25 µg/mL, and 50 µg/mL were reported for *Z. officinale* and *L. angustifolia,* respectively. The MIC value obtained for metronidazole in 24-h was 50 µg/mL (Malekifard *et al.,* 2021). In the present study, guava EO at a concentration of 12.5 µg/mL caused more than 90% mortality in *T. gallinae* after 12 h of exposure, and resulted in LC_{50} value of 2.7 µg/mL.

It should be noted that in spite of several studies on plant derived extracts on *T. gallinae*, no market penetration for these preparations has been reported; probably, due to the inconsistency in the chemical compositions of taxonomically same species growing in different parts of the world. One way to overcome this problem is isolation of the active ingredients and introducing them as compounds for further research and development of active pharmaceuticals. Based on this approach, in the present study main chemical components of *P. guajava* EO were tested for their anti-trichomonal efficacy. Among tested compounds, β-caryophyllene was the most potent anti-trichomonal agent with 12-h LC_{50} values of 0.32 µg/mL. In line with this finding, a strong leishmanicidal activity of β-caryophyllene against *L. amazonensis* with 12-h IC_{50} value of 5 µg/mL was reported (Soares *et al*., 2013). It has also been demonstrated that mixture of 4:1 of β-caryophyllene and lupenone were active against promastigotes of *L. amazonensis, L. braziliensis, L. mexicana, L. tropica*, and *L. aethiopica*, with inhibitory concentrations ranging from 14 to 39.3 µg/mL. The mixture of terpenoid compounds was more potent than the β-caryophyllene *per se*. The mixture led to increased production of NO, H_2O_2, and cytokines; also caused an immunomodulatory effect, without cytotoxicity (Polanco-Hernández *et al*., 2021). On the other hand, in the present study, mixture of β-caryophyllene in the whole *P. guajava* EO did not cause higher anti-trichomonal activity of the whole EO relative to the main ingredients, β-caryophyllene and α-pinene. Synergistic or additive effects between the ingredients of *P. guajava* EO probably does not exist and some antagonistic interactions are responsible for the lower anti-trichomonal activity of the whole EO. The same antagonistic interactions of the chemical ingredients have been previously reported for some other EOs. Considering the natural source of β-caryophyllene, its reported low cytotoxicity and promising anti-trichomonal efficacy, after further toxicological studies this compound can be considered as a candidate ingredient for development of novel anti-trichomonal agent.

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