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Research Article

APPLICATION OF ESSENTIAL OILS FROM TWO SPECIES OF THE RUTACEAE FAMILY AS CELLULAR OXIDATION CONTROLLER AGENT AND TRYPANOCIDAL CAPACITY

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ABSTRACT

Objective: The purpose of this study was to investigate the chemical composition of the hydro distilled essential oils (EOs) of *Citrus aurantium* and *Swinglea glutinosa.* The ability of the oils and mixtures in the control of cellular oxidation and the trypanocidal activity against *Trypanosoma cruzi* was also investigated.

Methods: The EOs were analyzed by gas chromatography-mass spectrophotometry. Radical scavenging of the EOs and mixtures was verified using 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), and antioxidant activity by the use β-carotene/linoleic acid bleaching test. Butylated hydroxytoluene (0.15 mg/ml) and ascorbic acid (0.1 mg/ml) were used as positive controls. Furthermore, protective activity against erythrocytes of degradation and trypanocidal activity against *T. cruzi* epimastigotes was evaluated.

Results: The major compounds identified in EOs of *S. glutinosa* were germacrene-D (30.8%), β-pinene (22.6%) and sabinene (11.6%), while limonene (94.4%) was the most abundant component in *C. aurantium.* In the ABTS assay, the best median inhibition concentration (IC_{50}) were found for *S. glutinosa* (86.18±4.54 µg/ml, $p<0.05$). A similar pattern to that obtained in the $ABTS^{+\bullet}$ assay was observed in the β-carotene test. *C. aurantium* EO showed the best anti-hemolytic activity (IC_{50} was 86.69±4.98 µg/ml). In the trypanocidal activity was evident that most of the treatments affect cell viability by more than 80% ($p<0.05$).

Conclusion: "Germacre-D-β-pinene" and "limonene" chemotype was found for fence lemon (nondescript by other researchers) and sour orange, respectively. Our findings demonstrate that these oils have a wide spectrum as cellular oxidation controller and a potential anti-trypanosomal activity, leading to the suggestion that may be considered as a promising future candidate in pharmacology industry.

Keywords: Essential oil composition, *Citrus aurantium*, *Swinglea glutinosa*, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), Rutaceae.

INTRODUCTION

Colombia has favorable geographical conditions for plant cultivation. An example of this is approximately 50,000 species of flora including those from horticulture. However, while the production is permanent throughout the year, it is just visible on the market during some harvest times [1]. Citrus are an important group of fruits within the market; Colombia has the 0.3% of the total world production coming mainly from the Tolima region which is actively involved within the market. Many citrus species are recognized for their medicinal, physiological and pharmacological activities including antimicrobial [2], antioxidant [3], anticancer [4], anti-inflammatory [5], and hypoglycemic [6] activities. *Citrus aurantium* and *Swinglea glutinosa*, two citrus species, have been extensively used in traditional medicine for medicinal purposes as analgesic [7], for diseases treatment such as malaria [8] and rheumatism [9], on account of the various bioactive compounds that they contain such as phenolics, flavonoids and essential oils (EOs).

Broadly, undiluted EOs are sold at a high price on the international market of aromatherapy, perfume and cosmetic industry [10]. In nature, EOs play an important role in the attraction of insects to promote the dispersion of pollens and seeds or to repel other ones [11]. In addition, EOs may also act as anti-bacterials, antivirals, antifungals, insecticides or have feeding deterrent effects against herbivores by reducing their appetite for such plants [12]. The detection of some of these biological properties needed for the survival of plants has likewise been the base for searching similar properties for the combat of several microorganisms responsible for some infectious diseases in humans and animals [13].

Furthermore, protozoal diseases, such as amebiasis, giardiasis, trichomoniasis, and trypanosomiasis, constitute a major public health problem in Latin American countries [14]. The parasitosis of major importance on the American continent is caused by the *Trypanosoma cruzi* protozoa. Chagas disease is transmitted to animals and people through insect vectors (more than 20 Leishmania species). Chagas disease also can be acquired by humans through blood transfusions and organ transfusions and organ transplantation [15], congenitally [16] and through oral contamination [17]. Acute infection can be lethal and cardiomyopathy develops in 25-30% of infected persons [18]. These potentially toxic and mutagenic effects are the main reasons why it is essential to develop alternative antiprotozoal agents with high activity, low toxicity, and high efficacy. Between 1975 and 1999 only 1% of newly approved drugs were intended for the treatment of tropical diseases. The so-called "Chagas disease," is part of a list of known tropical diseases that have been neglected. The most effective and commonly used drug in the treatment of this protozoa is metronidazole; however, this compound has unpleasant side effects such as a metallic taste, headache, dry mouth, urticaria, pruritus, and dark-colored urine [19]. That is why, the seeking of new drugs for the treatment of trypanosomiasis is necessary. Plants extracts and EOs have been used for many years in the treatment of several diseases, including parasite infections. Moreover, careful investigation of EOs from two fruit species, *C. aurantium* and *S. glutinosa,* have not been undertaken with respect to treatment of Chagas disease and have not previously been known to the scientific community in the Tolima region.

Likewise, the antioxidant activity of EOs is another biological property of great interest because they may preserve foods from the toxic

effects of oxidants. Moreover, EOs being also able of scavenging free radicals may play an important role in some disease prevention such as brain dysfunction, cancer, heart disease, and immune system decline. Increasing evidence has suggested that these diseases may result from cellular damage; in addition, carcinogenic, and teratogenic effects have been documented [20,21] caused by free radicals [22].

On the other hand, knowledge of the identity and relative amounts of the volatile substances released by plants is of great importance to several fields of basic and applied research in biology, chemistry and many other disciplines. Obtaining this knowledge requires overcoming many analytical challenges posed by these complex mixtures, because they normally present considerable variations in component amounts, chemical structures and functionalities.

Developing countries such as Colombia have an obligation to define and structure the strategies needed to promote and strengthen the advancement of agriculture. In such a way, the reasonable use of natural resources as well as the sustainable social growth and socially equitable development is guaranteed. Also by doing this, an added value to the natural resources is given.

The aim of the present work was to assess the ability of the EOs from *C. aurantium* and *S. glutinosa* and mixtures in the control of cellular oxidation and the trypanocidal activity against *T. cruzi* epimastigotes, a protozoan transmitter of Chagas disease, which is a systemic infection that is taking place in 21 countries, affecting about 8 million people and giving the risk to other 25 million people in Latin America of becoming infected [23]. Thereby seeking to establish alternative industrial uses for these natural products or their mixtures.

METHODS

Plant materials

The fruits of *C. aurantium*, sour orange, and *S. glutinosa*, fence lemon, were collected from different sectors of Ibague city, located in the center of the country near the central mountain range of the Colombian Andes (1,285 m.a.s.l., 27°C), including the Tolima University Campus and recreational parks. The identity of the plants was confirmed by the National Herbarium-National University of Colombia. The voucher specimens were identified as COL 576777 for *S. glutinosa* and 576778 for *C. aurantium*.

Extraction of the EOs

The fruits of *C. aurantium* and *S. glutinosa* were washed and manually cut into equal portions to remove the peels. The fruit albedo layers were peeled off carefully and discarded and were submitted to hydrodistillation (HD) using a Clevenger-type apparatus with cooling water recirculation under strict temperature control using a heating mantle and extracted with water until to ultimate exhaustion (3 hrs). The EOs were dried over anhydrous sodium sulfate and the percentage content was calculated on the basis of the dry weight of plant material. The HD of EOs was performed fivefold (n=5). The EOs were kept in a freezer until analysis (4°C).

Analysis of chemical composition

The analysis of the EO was performed on gas chromatography-mass spectrophotometry (GC–MS) on a Hewlett Packard 5890 GC with on an AT 6890 Series Plus (Agilent Technologies, Palo Alto, California, USA), using a DB-5MS (J & W Scientific, Folsom, CA, USA.) [5% - phenyl polydimethylsiloxane]), capillary column (60 m × 0.25 mm × 0.25 µm i.d., 0.5 µm film thickness); with helium as the carrier gas at a flow rate of 1 ml/min; split ratio 1: 20. The column temperature was from 70°C to 210°C at a rate of 2.5°C/min, with a final hold time of 5 min. The analysis procedure involved injecting 2 µl of EO diluted in dichloromethane (50 µl of sample:1 ml dichloromethane). Both injector and detector (flame ionization detector [FID]) temperatures were maintained at 230°C. Mass spectra were recorded in the range 50-450 amu, operating at 70 eV, and the ion source temperature was maintained at 200°C.

Identification of constituents

Identification of the components was made by comparison of their mass spectra with those stored in NIST 05 (Standard Reference Data, Gaithersburg, MD, USA) and Wiley 275 libraries (Wiley, New York, NY, USA) and literature data [24]. Relative percentages of the individual components of the EO were obtained by averaging the GC-FID peak area% reports.

Bioactivities

Antiradical activity

The radical scavenging (antiradical activity) of the EOs and mixtures was verified using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) according to that described by Re *et al.* [25], with some modifications: 17.5 mg of ABTS were dissolved in 9.9 ml of sterile H_2O and 1 ml of aqueous potassium persulfate (34 mg, $K_2S_2O_8$). The stock solution was prepared by mixing 9.9 ml of the $ABTS^{\bullet+}$ solution (3.5 mM) and 0.1 ml of the $K_2S_2O_8$. The radical was stable in this form for more than 2 days when stored in the dark at room temperature for 24 hrs before use. Later, aliquots were diluted with propanol to adjust the absorbance to 0.7±0.05 (λ=734 nm) for spectrophotometer measurements. To evaluate EOs and mixtures antiradical capacity, concentrations from 25 to 800 µg/ml (diluted in propanol) were prepared. 40 µl from each solution were taken and diluted with 1960 µl of $ABTS^{\bullet+}$ and absorbance was measured at 734 nm. After 7 minutes in darkness, the measurement was repeated. Propanol was used as a blank (40 µl of each treatment per 1960 µl of alcohol) and butilhidroxitoluene (BHT) as standard. All determinations were performed in triplicate (n=3). The inhibition percentage was calculated according to Equation 1.

$$\%Inh=\frac{Abs_i - Abs_f}{Abs_f} \quad (1)$$

The median inhibition concentration (IC_{50}) of each sample was determined from the obtain data.

β-carotene/linoleic acid bleaching (BCLA) test

This test was performed as reported by Henao and Mejía [26], with some modifications. 10 mg of β-carotene in 10 ml of chloroform was mixed with 20 mg of linoleic acid and 200 µl of Tween 20 emulsifier mixture. After chloroform was evaporated under vacuum (40°C, 5 minutes), 50 ml of distilled water satured with oxygen were added by vigorous shaking. 2.5 ml of this mixture were transferred into deferent test tubes containing 0.1 ml of EOs and their mixtures to different concentrations (6.25-200 µg/ml). As soon as the emulsion was added to each tube, the zero time absorbance was measured (470 nm) using a spectrophotometer. The emulsion system was incubated in a water bath (50°C/2 hrs) and the oxidation of the emulsion was spectrophotometrically monitored (60 minutes). A blank devoid of β-carotene was prepared for background subtraction. A control sample was prepared by replacing the EO with water and BHT was used as a standard. The ability to control the oxidation of unsaturated fatty acyl was expressed as percentage inhibition after 60 minutes of incubation, using Equation 2.

$$AA=\frac{100\,(DR_c - DR_s)}{DR_c} \quad (2)$$

Where:

DR_c=Degradation rate of control sample: $DR_c=\frac{\ln(\frac{a}{b})}{60}$

DR_s=Degradation rate of sample: $DR_s=\frac{\ln(\frac{a}{b})}{60}$

a=Absorbance at time 0 and b=Absorbance at 60 minutes

Protective activity against erythrocytes degradation

The effect of EOs and mixtures on ferrous ion-induced hemolysis was evaluated [27]. The range of concentrations for EOs and mixtures

emulsions was from 6.12 mg/l to 100 µg/ml. 400 µl of red blood cells suspended in phosphate buffered saline (PBS) (pH 7.4), 200 µl of the EOs and mixtures emulsions, and 400 µl of $FeSO_4$ (2.5 mM) were added in a falcon tube and incubated at 37°C (30 minutes). 4 ml of PBS was added and further centrifuged (2500 rpm, 5 minutes). An aliquot of supernatant (250 µl) was added to 96-well microplates, and the absorbance was measured at 540 nm (Multiskan GO Thermo Scientific). Vitamin C was used as negative control, whereas $FeSO_4$ was used as positive control.

The protective activity of the volatile components against erythrocytes hemolysis was calculated using Equation 3.

$$\% \text{ Inhibition} = 100 \times (1-OD_{sample})/OD_{control} \quad (3)$$

In vitro test for trypanocidal activity

The trypanocidal activity against *T. cruzi* epimastigotes was determined using the methodology proposed by Araya *et al*. [28], with some modifications. After culturing, the *T. cruzi* epimastigotes in liver infusion tryptose (LIT) medium and counting the parasites in a Neubauer chamber (2×10^6 parasites/ml), 50 µl of each parasitic solution were put in 96-well microplates. Samples (EOs and mixtures) were dissolved in LIT medium, and 50 µl of the solution (6.12-100 µg/ml) was incubated (24°C, 28 hrs). Then, 10 µl of thiazolyl blue tetrazolium bromide (MTT) (5 µg/ml) was added, and the samples were incubated again (4 hrs, 37°C). After the incubation process, 100 µl of 10% sodium dodecyl sulfate (SDS) was added to extract the formazan from the parasitic cells. Immediately, the samples were incubated for 18 hrs at 26°C and then the absorbance was measured (570 nm) in a microplate reader (Multiskan GO Thermo Scientific). The IC_{50} was calculated by analysis from the log sigmoid curve of the dose versus percentage viability. The viability was calculated using Equation 4.

$$\% \text{ Viability} = \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (4)$$

Pharmacological interactions

To determine the type of drug interactions between the two oils from two species, extracts mixtures were prepared in different proportions: 1C:1S, 3S:1C, 1S:3C, where: C=*C. aurantium* and S=*S. glutinosa.* The type of drug interaction was determined by the values of combination index (CI), estimated through the free Software CompuSyn [29].

Chemical reagents

All tests were conducted using high purity chemicals of analytical grade. Specialized reagents such as ABTS, β-carotene, MTT, SDS and linoleic acid were supplied by SIGMA USA.

Statistical analysis

The experiments were done in triplicate. The results are given as mean±standard deviation. Analysis of variance and Pearson correlation were carried out with the Statgraphic program to the comparison of more than two means and between tests. A difference was considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Physical characteristics and efficiency

EOs, from the two species showed similar physical typologies to those shown by citrus products such as pleasant odour and pale (*C. aurantium*) or yellow (*S. glutinosa*) color. The EOs extraction efficiency was between 0.2% and 0.3% v/w, in relation to the dry weight of the plant material, being lower than that found in other plants (lower than 1%).

The physical properties of EOs are related to the diversity of its constituents; those features may become a fast instrument for the characterization and evaluation of the degree of purity or origin of a product, considering it as a fingerprint [30]. These parameters, among others, need to be determined by the pharmacopoeias to check the EOs purity.

Most of the diverse number of species from the citrus genus produced and stored their EOs in schizolysigenous glands located in the outside of the fruit mesocarp, a particular location that allows an easy EOs retrieval and in some way justifies the use of the applied method (expression) to extract the EOs from the citrus with efficiencies between 5% and 10% but also with the risk of removing the non-volatile compounds.

It is known that the majority of heat-sensitive compounds are the most bioactive and contribute to the product aroma but also they may be the most affected by the use of elevated temperatures. For that reason, HD was used as the extraction method of the volatiles from the two species; the risk of losing these valuable chemical constituents can be corrected using chilled water.

There is no doubt that the chemical constituents of the EOs are the ones that determine their solubility in some organic solvents and/or insolubility in water. However, both oils unlike the vast majority were not soluble in ethanol at any of the evaluated ratios, they were only soluble in propanol due to the low presence of oxygenated compounds in these EOs. Although the obtained efficiency values can be considered low (0.2-0.3% v/w), they are consistent with those reported in the literature for other citrus species [31].

EO composition (Table 1)

Although both volatile oils consisted of monoterpenes and sesquiterpenes compounds, differences were found in their quantitative composition. The EO obtained from *C. aurantium* showed a characteristic chemical profile comprised almost entirely of monoterpenes (98.7%), while the oil from *S. glutinosa* contained both sesquiterpenes (50.4%) and monoterpenes (47.1%) in almost equal proportions. The low content of aromatic compounds and high terpenes accumulation is dependent on the balance between photosynthesis during the day and the use of the resulting products at night [32]. This fact may be a consequence of the high brightness and low nutrients available at the collection zone (Ibagué urban area) that can influence the activation of the plant enzymatic mechanism increasing the biosynthesis of compounds derived from mevalonate [33]. A subsequent demise of those bioproducts (probably as a defence mechanism) may induce the activation of the shikimate pathway as a collaborative process, but in low proportion.

The different compositions obtained from the quantitative analyses of the EOs from *C. aurantium* and *S. glutinosa* are presented in Table 1. About 10 main components of *C. aurantium* EO represent about 99.95%, while those from EO of *S. glutinosa* were 24, although there was marked difference in the chemical composition of their EOs: Limonene is the most abundant terpenic compound found in the EO of *C. aurantium* (94.4%). Oils obtained from distinct citrus fruits have in common a high amount of terpenes, which are volatile low molecular weight compounds. Given that, limonene can be considered as a primary solvent for scented compounds present in essential *C. aurantium* oil. Some isoprenoids such as d-limonene and others such as farnesol, and geranial have been evaluated on their chemo-protective activity [34].

Germacrene-D (30.8%) and β-pinene (22.6%), were the most abundant sesquiterpene and bicyclic monoterpene compounds found in *S. glutinosa* EO. Other important compounds were: Sabinene (11.6%), γ-elemene (5.5%), trans-β-caryophyllene (5.2%), α-pinene (5.1%) and limonene (3.9%).

Germacrene-D has been found not only in angiosperms and gymnosperms but also in bryophites, yet despite its wide distribution, its biological function in plants is still not well understood. It has been proposed that germacrene-D plays a role as a precursor of various sesquiterpenes such as cadinenes and selinenes [28]. It has also been suggested that germacrene-D, by itself may have deterrent effects against herbivores and it has been reported to have insecticide activity against mosquitoes, as well as repellent activity against aphids [32]. β-pinene is important flavor and fragrance constituents of many EOs [35,36].

Chemotype of EOs

According to Grayer's chemotype classification system [37], of the major constituents (more than 20% content), the chemotypes for these two fruit species utilized in this investigation were stablished (Table 1). *C. aurantium* exhibited the "limonene" chemotype and *S. glutinosa* presented two major components: Germacrene-D (30.8%) and β-pinene (22.6%); moreover, a not negligible sabinene content (11.6%) suggests as another main component. Therefore, this variety is characterized as germacrene-D-β-pinene chemotype and "germacrene-D-β-pinene-sabinene" subtype.

Several studies on the chemical composition of the EOs extracted from *C. aurantium* L. seem to be in agreement with our findings, indicating that the main components of peel oil are monoterpene hydrocarbons such as limonene and myrcene, linalyl acetate, and α-terpineol predominate in the leaf oil (petitgrain) [38]. Nevertheless, the chemotype of the EO extracted from *S. glutinosa*, was different to that found in other studies, indicating that the main component of peel oil is sesquiterpene hydrocarbons like carvacrol [39], β-pinene [40], β-cubebeno and β-pineno [41].

A chemotype is a chemically distinct entity with differences in the composition of the secondary metabolites. Besides plant genetics, the good climate and soil conditions in Ibagué-Tolima-Colombia may contribute to the appearance of those chemotypes. Some minor variations (environmental, geographical, genetic, etc.) can have little or no effect at a morphological level but originate major changes on the chemical phenotype. Quantitative differences may arise due to different genotype, the pedoclimatic conditions in the growing areas, etc.

A constituent-based approach can provide a simple guide in predicting the bioactivity of an EO, but the actual activity cannot be known until a sample is tested and even then the results can be surprising. Nevertheless, given the complexity of the EOs chemical composition everything suggests that their mode of action is multifaceted, and it is very likely that each of their constituents has its own mechanism of action. Therefore, it is must be accepted that the action of these complex mixtures can generally explained in terms of its main components or may sometimes, be produced via a molecular interaction between multiple compounds that could be of both lipophilic and hydrophilic character.

Antioxidant activity (Table 2)

The antioxidant activity of the peel oils extracted by hydrodistillation from fully developed and ripe fruits of sour orange, *C. aurantium* and fence lemon, *S. glutinosa*, collected from Ibagué-Province of Tolima was determined by three different test systems, namely: The scavenging ABTS radical (antiradical) activity, BCLA test and antihemolytic activity (protective activity against erythrocytes degradation). These results are presented are presented in Table 2.

In the ABTS assay, the best IC_{50} were found for the BHT (2.48±0.24 µg/ml) and *S. glutinosa* oil (86.18±4.54 µg/ml). This activity was followed by the 3S:1C mixture (IC_{50}=117.43±3.89 µg/ml), 1S:1C mixture (IC_{50}=183.20±7.45 µg/ml), 1S:3C mixture (IC_{50}=372.39±12.4 µg/ml) and *C. aurantium* (IC_{50}=1311.21±6.41 µg/ml) EO. Although the EO from fence lemon (*S. glutinosa*) exhibited the highest antiradical activity, it was observed that there was no significant difference between it and its mixture, for example (3S:1C; p≥0.05). The volatile extracts of the sour orange (*C. aurantium*) are just opposite; its activity decreases considerably compared to other treatments. Therefore in the mixture in which the orange oil is at high prevalence (1S:3C), the activity tends to decrease.

Thus, the fact that the EOs of our study do not show significant antiradical activity can be explained, since both oils are composed almost entirely of monoterpene and sesquiterpene hydrocarbons; the cited factors can be considered as the main limitation of this assay for measuring the antioxidant activity of lipophilic samples, like many EOs.

Based on these results, isobolograms were constructed to determine the type of pharmacological interaction among the components from the evaluated mixtures. The CI (CI>1.10), indicated for all cases the existence of a strong antagonism between the mixtures prepared (1C:1S, 3S:1C, 1S:3C). Data are not shown.

On the other hand, the main advantage of the $ABTS^{*+}$ test is that it can be used either in liposoluble and water-soluble samples. Nevertheless, it is not found in living organisms resulting in a disadvantage when using it as an indicator owing to its low mimic ability *in vivo* situations. The radical can be thermodynamically reduced by compounds that have a lower redox potential (0.68 V), and the end of the reaction is governed by the type of antioxidant substance used; setting short or very high times that may interfere with the final results [42].

According to the results obtained, it can be said that the EOs showed better values for the antioxidant activity in the β-carotene/linoleic acid system oxidation assay. This method can be especially useful for investigations of lipophilic antioxidants and is appropriate for the investigation of antioxidant activity of EOs. A similar pattern to that obtained in the $ABTS^{+\bullet}$ test was observed in the following order: BHT>S>3S:1C>1S:1C>C>1S:3C, which implies that the joint action of germacrene-D (sesquiterpene), β-pinene and sabinene (monoterpenes), main components of the fence lemon (*S. glutinosa)* EOS, is more powerful than the electron transfer process between β-carotene and a peroxyl radical. Terpenoid effect is reflected by time reduction for carotenoid bleaching; the power (rate) and capacity (chemical ability) of the terpene trilogy are affected when limonene (94% of the sour orange EOs) was added.

The BCLA test was selected because the samples were emulsions, a very common condition in foods and in living organisms, which allows greater contact between the β-carotene/linoleic acid system components and the apolar compounds EOs. This test evaluates the ability of a compound to slow down oxidation and measures the ability of the sample compounds to inhibit lipid peroxidation. In general, both free radical scavenging and inhibition of linoleic acid oxidation are desired in the food, pharmaceutical and cosmetic industries.

In antihemolytic model, it was found that *C. aurantium* EO showed the best antihemolytic activity than other treatments (IC_{50} was 86.69±4.98 µg/ml), which was not comparable ascorbic acid (1.28±0.32 µg/ml). The mixture 1S:3C showed greater inhibitory capacity than the other two mixtures (1S:1C or 3S:1C).

The data given in Table 2 confirmed that terpenes contained in *S. glutinosa* EO are more active in stabilizing free radicals such as peroxyl ($ROO^{\bullet}$), that is formed when the linoleic acid is degraded; conversely, the limonene from the orange EO was the one that controlled in the best way the hemolysis of human erythrocytes. It was observed that blending these two EOs is not very favorable. The mixture 1S:1C showed the best correlation between the antiradical test against $ABTS^{+\bullet}$ and decolourization of the β-carotene/linoleic acid (r=0.975), and an antagonistic correlation between the anti-hemolytic activity and the other two activities mentioned before.

Erythrocytes are vulnerable to oxidative stress due to its high content of polyunsaturated lipids and transition metals (particularly iron) that act as a catalyst of free radicals generation via the Fenton reaction [27]. Iron is associated with the oxidative degradation of membrane lipids and in the subsequent hemoglobin modification, causing hemolysis and increasing the severity of some organic disorders such as chronic kidney disease and sickle cell anemia [43]. The anti-hemolytic activity test may be then taken as a tool to validate the interaction between the constituents EOs and the biological entities at cellular level. The major constituents of the EOs from fence lemon and sour orange are terpenes (mainly unsaturated terpenes hydrocarbons), metabolites that "sacrificed" their stability and get oxidized in order to give electrons

Table 1: EOs composition of *S. glutinosa* and *C. aurantium* peels obtained within hydrodistillation process

Clasification	tr	Compound	Conentration (%)	
			***S. glutinosa* (24)**	***C. aurantium* (10)**
Monoterpenes	18.64	α-pinene	5.1	0.4
	20.39	Sabinene	11.6*	0.2
	20.68	β-pinene	22.6*	1.9+
	20.98	β-mircene	0.4	1.1+
	22.85	Limonene	3.9	94.4+
	24.04	γ-terpinene	0.6	-
	22.30	α-terpinene	0.3	-
	22.95	β-felandrene	0.6	0.4
	22.64	o-cimene	0.2	-
	23.43	trans-β-ocimene	-	▪0.3
Sesquiterpenes	38.28	trans-β-cariofilene	5.2	-
	38.70	Sesquisabinene	▪0.3	-
	39.47	α-humulene	▪0.7	-
	40.10	α-muurolene	▪0.5	-
	40.27	Germacrene-D	▪30.8*	-
	40.70	Biciclogermacrene	▪0.6	-
	42.67	Germacrene B	2.4	-
	37.02	β-elemene	0.8	-
	38.39	γ-elemene	5.5	-
	34.99	δ-elemene	3.4	-
	38.58	β-gurjunene	0.2	-
Alcohols	29.57	α-terpineol	0.6	0.1
	29.06	Terpinen-4-ol	1.4	-
	25.63	Linalool	0.3	▪0.8
	23.02	1,8-cineol	1.8	-
Other	31.52	Oxigenated compound (M 136)	-	0.4

*Major components of the essential oil of *S. glutinosa* (>10%), +Major components of the EO of *C. aurantium* (>10%), ▪Components not previously reported.
EOs: Essential oils, *C. aurantium: Citrus aurantium, S. glutinosa: Swinglea glutinosa*

Table 2: IC_{50} (µg/ml) of EOs in antioxidant tests

Treatment	ABTS	BCLA	Antihemolytic activity
S. glutinosa	86.18±1.68	1.21±0.59	105.44±1.06
C. aurantium	1311.21±1.80	358.72±1.23	86.69±0.86
3S: 1C	117.43±1.50	15.88±0.97	115.26±1.03
1S: 3C	372.39±1.32	808.82±1.12	92.42±0.42
1S: 1C	183.20±1.01	26.06±0.43	91.08±0.63

EOs: Essential oils, *C. aurantium: Citrus aurantium, S. glutinosa: Swinglea glutinosa*, BCLA: β-carotene/linoleic acid bleaching, IC_{50}: Median inhibition concentration, ABTS: 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)

to the ABTS*+ radical, protecting and retarding substrate oxidation (linoleic acid) or inhibiting metal species such as iron (chelating activity) that induce ROSS.

In general, EOs from many plants have recently gained tremendous popularity and scientific interest owing to their large number of biological activities. Products obtained from citrus species (2100 BC) have been recognized as "safe" because of their antimicrobial, antioxidant, anti-inflammatory, and anxiolytic ability; they also can be used in aromatherapy and food industry [26].

Particularly the term antioxidant is commonly applied to those inhibitory substances that can retard the oxidation and so the deterioration of many organic molecules including food [44]. In some cases, the antioxidant activity has been associated with phytophenols that have the greater capacity to stabilize free radicals [45]. Nevertheless, there are some monoterpenes with antioxidant activity; for instance, limonene and pinene inhibit the production of cholesterol and protect the activity of certain enzymes [46]. In the autoxidation mechanism, an antioxidant function is to deflect the free radical preventing chain processes. Other potential antioxidants react with the hydroperoxides (autocatalytic chain initiators) and block the initiation of more autoxidation chains [47].

Trypanocidal activity (Fig. 1)

The assessment of the trypanocidal activity of the volatiles and their mixtures is illustrated in Fig. 1. It is evident that most of the treatments affect cell viability by more than 80%, nevertheless the orange EO at 6.25 µg/ml decreases its activity over 70%. This trend suggests that very low concentrations of these two EOs and mixtures (at any proportions) may control the population of *T. cruzi* epimastigotes in more than 50%.

The anti *T. cruzi* activity has been tested within a relatively high number of EOs from the same number of plant species belonging to different families [23,48]; nevertheless, the EO extracted from species of the Rutaceae family has received little attention in this respect.

One of the possibilities for action is the generation of irreversible damage to the membrane of bacterial cells, that induce material losses (cytoplasmic), leakage of ions, loss of energy substrate (glucose, ATP), leading directly to the lysis of bacteria (cytolysis) and therefore to its death. Another possibility of action is inhibition of production of amylase and protease which stop the toxin production, electron flow and result in coagulation of the cell content [11,49].

Some authors have found that the EOs action against parasites may be through interaction within their cell membrane [50]. At least, part of this activity is due to the hydrophobic nature of the cyclic hydrocarbons, which allows them to interact with the cell membrane and accumulate in the lipid bilayer of the parasitic structural unit, occupying some space among the fatty acid chains [51]. That interaction leads to conformational changes in the structure of the membrane, resulting in its thinning and expansion causing loss of stability, which in turn leads to the leakage of ions and ultimately in a decrease of the ion gradient [52]. Then, it is likely that their anti *T. cruzi* activity is not due to a single mechanism, but to several sites of action at the cellular level.

As mentioned before, the two EOs under study are comprised of aliphatic terpenes, from which more than 90% are cyclic in nature. The high hydrophobicity of these plant products and their low solubility in

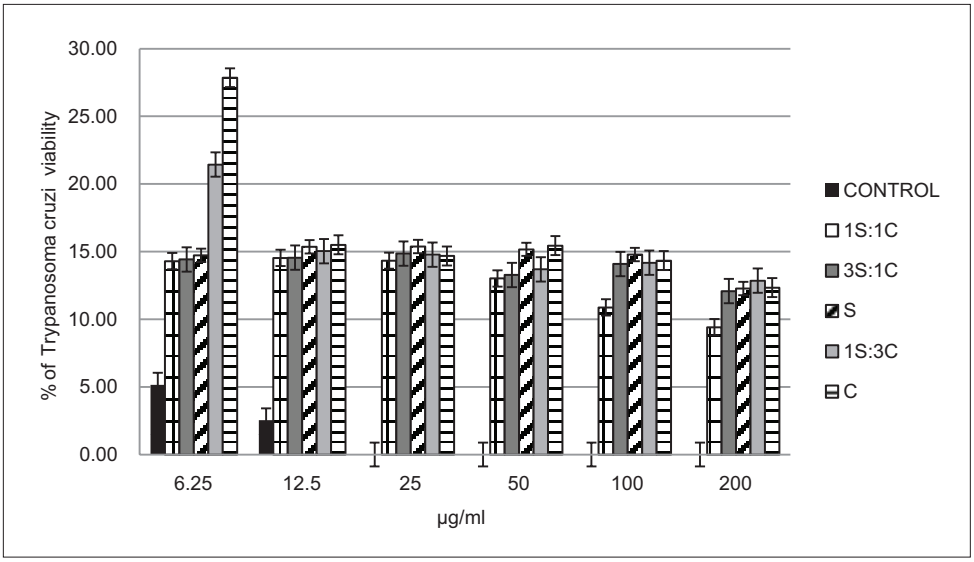

Fig. 1: Percentage of *Trypanosoma cruzi* viability

organic solvents such as ethanol, hampering the choice of a suitable solvent for the biotests; fact that can explain why the trypanocidal activity (inhibition of parasite viability) of oils and mixtures was more than 80% [53]. Various biological properties are credited for sesquiterpenoids and their derivatives, including antimicrobial, antioxidant, antiparasitic, and insecticidal activities, as well as cytotoxicity against tumor cell lines [54-56].

Monoterpenes, sesquiterpenes and diterpenes have shown antioxidant activity. Grassmann *et al*. [57], state that one mode of action of monoterpenes is to strengthen other antioxidants and meanwhile Milde *et al*. [58], argues that the cooperation of fat-soluble antioxidants can be accomplished by synergistic action. This explains the antioxidant functionality displayed by the components of the EOs in the tests applied in this work.

CONCLUSIONS

Concerning the chemical composition of *S. glutinosa* and *C. aurantium* growing in Ibagué-Tolima-Colombia, it could be stated that the "germacre-D-β-pinene" and "limonene" chemotype was found for fence lemon and sour orange, respectively. Under experimental conditions, the EOs from *S. glutinosa* and *C. aurantium* showed ability to control cell oxidation and also *in vitro* effect against *T. cruzi*, capacities that could be directly associated to the chemotype of the EOs, one of which (*S. glutinosa*) appears not to be found in other research. The significant of the EOs of two species of the Rutaceae family confirm that are natural source of biologically active compounds. Our work suggests that EOs from spices can be considered as a promising future candidate in pharmacology industry. These properties are also very much needed by the cosmeceutic industry in order to find possible alternatives to synthetic antioxidant preservatives. This study updates the data in the literature on the EOs of sour orange and fence lemon, and provides information on the composition of the oils for a further evaluation of this product and may be of interest from a functional point of view and for the valorization of *C. aurantium* L. and *S. glutinosa* in Tolima region and the wider Colombia.

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