STUDY OF EUGENIA UNIFLORA ESSENTIAL OIL FOR USE IN MEDICINAL APPLICATION

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ABSTRACT

In spite of alternative methods to decrease or to end up illnessess, which affect the human race, the multidisciplinary researches of active ingredients in plants establish an important tool in the medical chemistry. Besides, the botanical and phytochemical studies, plant researches have been developed by many research groups to elucidate Brazilian plants' main composition. However few investigations were carried out about specific active ingredients in such plants. This study was developed using a sylvestral fruit tree native of the Brazilian forest, the Eugenia uniflora L., from Mirtaceas family. The main goal was focused on the extraction methods. In this sense, Clevenger extraction was the best yield in relation to SFE and Soxhlet. The SFE method presented good yield but presented a great amount of components in the final extract. The essential oil extracted was analyzed by HRGC/FID showing a vast range of polarity and boiling point compounds. Beside it analytical solid phase extraction method was used for clean it up and obtain separated class of compound which was fractionated and studied by HRGC/FID and HRGC/MS.

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INTRODUCTION

From the historical point of view the development of chemistry took place along the study of plants, mainly in the 19th century when the first studies on plant were published on a scientific basis. The result was the isolation of some active principles of plants already known as having medicinal properties. From these studies some substances were obtained that became known as having effective active principles, such as morphine, camphor and cocaine, which are still used today in the treatment of some diseases (WHEEL-WRIGH, 1974; GOTTLIEB et al., 1996).

As a general rule, nature has produces the majority of the known organic substances. However, vegetables are the ones that have contributed most significantly to furnish useful substance to the treatment of diseases affecting man (PHILLPSON; ANDERSON, 1898). The fantastic variety and complexity of special metabolites with biosynthesis by plant could be formed and evolved as a defense mechanism of vegetables as a response to environmental conditions rich in microorganisms, insects and animals and also due to adaptative and regulatory conditions (REINBOTHE et al., 1990). In this way, plants are a vast laboratory for organic synthesis, a result of million of years of evolution and adaptation on Earth.

In the 20th century the introduction of antibiotics produced by microbial fermentation associated to the marked development of synthetic drugs by the drug industry, just after the 2nd World War, were causes for a clear decline in the use of medicinal plants and, thus, for investments in drugs with vegetal origin. In the last decades an important shift of paradigm in western societies contributed to a return of plant products as having an important role for populations in developed and in developing countries.

Detaining a very profitable market, phytodrugs turned on the interest of drug industries towards products of vegetal origin. Around 1990 it was estimated that circa 80% of the world population was seeking in plants the main source of drugs (FLEURETN; PELT, 1990). Today it is proved that a great part of the world population, mostly in developing countries, use them as drugs extracts or portions derived from plants.

From the two-hundred thousand species that may occur in Brazil, at least half of them may show some therapeutic property, but not even 1% of these have been focus of adequate studies so far (http://nutrinet.jfa.zaz.com.br/frindex.htm). Many exclusive

substances from Brazilian plants are patented by foreign enterprises or governmental organs (SATO, 2000).

Essential oils, or essences, are volatile oils of varied chemical composition originated from vegetal materials and grating them their main odors (COSTA, 1994; ROBBERS et al., 1996). Perfume, cosmetics and good aromatizing industries consume almost all the natural essences and derivates what justify the high technical level attained in this preparation and the high value that represents; paints and varnish industries consume considerable amounts of some essences. They are also used in flotation of minerals and preparation of humidifying agents; in medicine they are used in formulae of antiseptics, antispasmodics, inhalants and analgesics (COSTA, 1994; AGRONÔMICO, 1957).

Essential oils are most of the time bacteriostatic and frequently bactericides. They have a role in pollination as a protection against predators: microorganisms, parasites, insects, herbivorous. The toxic protective action on germination is enhanced by the peripheral localization of the secretory organs (COSTA, 1994; ENCYCLOPEDIA OF CHEMICAL TECHNOLOGY, 1981; BRUNETON, 1991).

Eugenia uniflora L., main focus of the present study, pertains to the Mirtacea family (JOLY, 1966). Pitangueira is a wild fruit tree native in the Brazilian forests, found from the border of the Guyanas down to São Paulo; in the southern states it does not survive (DICIONÁRIO DAS PLANTAS ÚTEIS DO BRASIL, 1984; BAILEY, 1966).

The leaves of the *Eugenia uniflora* are used in popular remedies as infusion against fever, rheumatism, stomach disease, diarrhea, weight loss, to treat arterial hypertension, yellow fever and gout (ADEBAJO et al, 1989; SCHMEDA-HIRSCHMANN et al, 1987; WEYERSTAHL et al, 1988; FADEY; AKPAN, 1989). Some studies proved that the odor from leaves has repellent properties (WEYERSTAHL et al, 1988). Besides that, the infusion of the dry leaves and of the green fruit is use to treat malaria and the aqueous extract of the dry leaves is used as a menstrual stimulant (SCHMEDA-HIRSCHMANN et al, 1987).

The objective of the present study is to evaluate various extraction processes for extraction of *Eugenia uniflora* essence aiming to obtain adequate samples for the analysis of the various compounds in its essential oil.

MATERIAL AND METHODS

Samples

A bunch of the plant was collected for identification of the specimen, which was identified based on the botanic archives of the UNBA herbarium at the Universidade Estadual Paulista – UNESP, Campus Bauru, SP.

After the identification it was stored in the same herbarium under the number 2148 (2).

Fresh leaves were randomly collected at the same time (7:30 am). The leaves were in an intermediate position between the apex and the vase of a tree located in an Experimental Field near the main gate of the University. They were immediately sent to the Laboratory of Chromatography and Analytic Chemistry at the same University in order to proceed to extraction with fresh and dry leaves.

Leaves were dried for seven days in a naturally aired place protected from sunlight and with controlled temperature.

A mill with steel blades spinning at high speed (3800 rpm) was used for triturating leaves. After trituration the samples were sieved (Gowmac system) with various sizes of sieves from 1 to 50 mesh and the intermediated portions were used.

Extraction

In the attempt to select the best way to extract the essential oil of *Eugenia uniflora* it was used the solid-liquid process, the Clevenger system, Soxhlet and supercritical fluid extraction (SFE).

For the solid-liquid process 20 g of grinded leaves were put into a Becker (250 mL) with 200 mL of distilled water. The mixture was left for 30 minutes under the action of a magnetic stirrer.

Then, for liquid-liquid extraction, the mixture was filtered to another Becker of same volume trough a filter paper; 80 mL of petroleum ether was added (proposed solvent). The funnel with the mixture was stirred. The operation was repeated five times aiming maximally to favor the phenomenon of partition among the two solvents of opposed polarity and then the mixture was allowed to rest for 20 minutes to attain separation between the two phases.

Then the phases were separated with the simple collection of the phase by opening the funnel register. The aqueous phase (darker and inferior) was eliminated and discharged. The organic phase, clearer, was collected in a Becker and put in the chapel under a flow of nitrogen at room temperature in order to allow volatilization of the solvent and remaining only the essential oil.

With the same finality, i.e, to extract the essential oil by the solid-liquid method as well to allow performance comparison, another procedure was done with the same solvent. In a 250 mL Becker 20 g of grinded leaves were introduced and 200 mL of distilled water was added. The mixture was put under the action of a magnetic stirrer for 20 minutes in the Becker. Only the aqueous part of the decanted mixture was put in a separation funnel. The petroleum ether (solvent) was added in two moments: initially 40 mL were added, stirring the funnel and opening the register to release gases; then, it was added more 20 mL and the same procedure was repeated. The funnel was laid to rest for 20 minutes and the method for phase separation was the same as previously mentioned. After collection the Becker was put in a cheaper under nitrogen flow for solvent evaporation, remaining the essential oil.

For the extractions made with the Clevenger system it was used grinded leaves, a round bottom balloon and a heating mantel for a 500 mL volume. The oil performance for extractions was calculated (quantitative analysis) relating the obtained mass with the initial mass and the time of extraction (in hours).

In all hydrodestilation with the Clevenger system the distilled water (the solvent used to generate vapor) and the vegetal material (sample) were introduced in the round bottom balloon. The proportion of plant and the volume of water used in the present study was 1:10 (m/m) being 30 g of grinded leaves to each 300 mL of distilled water.

After closure of the system it was heated to ebullition keeping the temperature of circa 98 ºC for extraction performed at 1, 2, 3 and 4 hours.

Another type of extraction, with supercritical fluid, was done with an ISCO–SFX^{TM} 220 – Supercritical Fluid Extractor and Controller SFX^{TM} 200.

It was used 2.5 g of grinded leaves for each extraction. A study of performance against extraction pressure was done under the following conditions:

- extractor fluid: CO_2
- CO_2 volume: 50 mL
- Extraction time: 10 minutes
- Restrictor: id 100 mm, od 378 mm
- collection procedure: cooled with dry ice
- studied pressures: 4000, 5000, 6000, 7000, 8000, 9000 psi.

After each extraction the obtained essential oil was collected in a previously weighed clean and dry glass flask. After collection

the CO_2 evaporation was awaited and the flask was again weighed and with the difference of the new weight in relation to the initial mass it was calculated the gravimetric performance for each extraction in a given pressure.

For solvent extraction it was used the Soxhlet system. The sample was introduced in a filter paper cartridge (10 cm^2) in which 7 g of grinded leaves were also introduced. Over the mass of leaves a signalized piece of glass wool was put (enough to fill the empty gap of the cartridge). Then the cartridge was put in the equipment to start extraction. The solvent was 92% commercial alcohol. The temperature was circa 98 ºC and the extraction time was 4 hours.

Analysis

The injected volume was 1 µL prepared as a solution 1:20, v/v with ethanol for injection. The analytical conditions were as follows: A Chromatograph HP 5890 Series II equipped with a flame ionization detector and a polyethylene-glycol chromatographic column CW-20 (Carbowax 20 M) 50 m long, inner diameter of 0.22 mm and film width of 0.35 mm.

injector temperature: 250 ºC
detector temperature: 300 ºC
initial temperature: 40 ºC
final temperature: 250 ºC
initial time: 2 minutes
final time:10 minutes
Ramp: 8 ºC/minute
Pressure at the column head: 120 kPa
Split: 1:70
Carrier gas: hidrogen 9.5, with µ=38,5 cm/s

Purification and isolation of the Active principle (Linalol)

For further use of oil in a test of Lethal Dose as well as in other biological tests the sample was purified and the active principle was isolated using the silica adsorption chromatography.

Procedure

A piece of signalized glass wool was introduced in a glass column that was packed with 10 g of silica previously activated at 140 ºC for 4 hours (cooled in a desiccator) conditioning it with 50 mL of n-hexane. After the introduction of the sample the column was eluded with

80 mL of n-hexane/ether (30:70, v/v). The fraction was collected in a round bottom balloon and it was concentrated to dry in a rotaevaporator under reduced pressure at 60 °C. The extracts were diluted in 1 mL of n-hexane and submitted to analysis by HRGC/FID.

RESULTS AND DISCUSSION

TABLE 1 shows the average gravimetric performance for the essential oil of *Eugenia uniflora* obtained through various tested extraction methods. In all instances it was observed the presence of a cerous mass in the bottom of the Becker, probably greases and hydrocarbonets of high molecular weight obtained after volatilization of the solvent. This mass was not taken into consideration for the chromatographic analysis due to the risk of contamination of the chromatographic system: mainly the column and detector.

As can be seen the greatest performance was obtained by the SFE method, almost 3.0 g and by the Clevenger system, circa 4.0 g and the small performance was that of the solid-liquid extraction, 0.015 g.

TABLE 1 – Average gravimetric performance of essential oil of *Eugenia uniflora* obtained through all extraction methods.

Extraction method	average performance (%)
Solid-liquid	0.015
Sohxlet	0.140
Clevenger	0.416
SFE	2.950

The extraction made by the Clevenger system offer, in addition, different result in what regards the time of extraction (TABLE 2). As can be seen, time is an important variable in the process to obtain oil. Indeed, it was observed an increase of 350% in the performance of oil gain after 5 hours of extraction if compared to the 1 hour period.

TABLE 2 – Extraction time and essential oil performance obtained through the Clevenger system for fresh leaves.

Time (h)	Oil performance (%)
1	0.168
2	0.328
3	0.433
4	0.556
5	0.600

Aiming to attain the best condition for extraction, that is, the best oil performance, it was studied the granulation of the ground leaves. TABLE 3 shows the result obtained for the Clevenger extraction system using varied granulations (5, 15 and 30 mesh). In the present study it was observed that influence of granulation was greater in periods of 1 to 3 hours with a performance difference of up to 45%. However, after this period, that is, 4 and 5 hours of extraction, smaller variations were observed in what concerns the oil extraction (5 to 10%). In all experiments it was verified an increase in the performance of oil extraction what can be related to the increase of the contact surface and, therefore, more compounds and a greater amount of oil will be extracted (FIGURE 1).

TABLE 3 – Time of extraction x oil extraction performance by the Clevenger system for different particles.

Time (h)	performance %		
	5 mesh	15 mesh	30 mesh
1	0.080	0.098	0.102
2	0.200	0.225	0.289
3	0.322	0.336	0.394
4	0.498	0.512	0.527
5	0.522	0.558	0.569

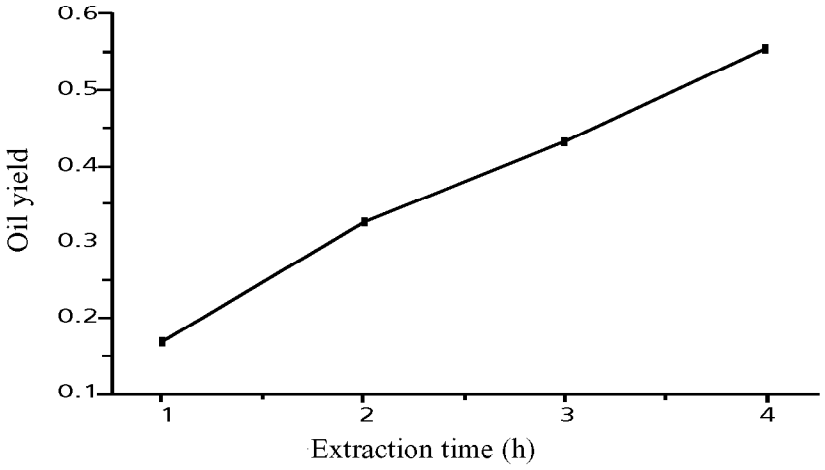

FIGURE 1 – Performance of the essential oil of *Eugenia uniflora* obtained by the Clevenger system for dry leaves.

To the extraction under the SFE system the results are presented in TABLE 4 and FIGURE 2. The latter shows graphically the oil

performance through SFE in different extraction experiments. The fluid pressure is the main parameter to influence the SFE process (CHARPENTIER; SEVEENANTS, 1988). In the present study, when the pressure increased from 4000 to 8000 psi oil performance increased in circa 300%. This can be associated to an increase in density that increases the solvatation power of the extraction fluid leading to a greater oil extraction performance. However, greater densities, or grater pressures (9000 psi) the diffusion coefficient decreases provoking a diminution in the extraction performance due to the kinetics of the extraction process, as can be seen in TABLE 4 and FIGURE 2.

Despite the optimum quantitative performance of SFE for brut oil, the qualitative aspect was not convenient in what regards the analites present in the composition. The diversity of the compounds extracted were probably due to the force of penetration of the CO2 and the used conditions, which showed to be poorly selective to the proposed objective, besides compromising and making more difficult the chromatographic analysis.

TABLE 4 – Performance of the essential oil obtained by the SFE system.

Pression (PSI)	performance (%)
4000	0.832
5000	0.500
6000	1.044
7000	1.548
8000	2.512
9000	1.268

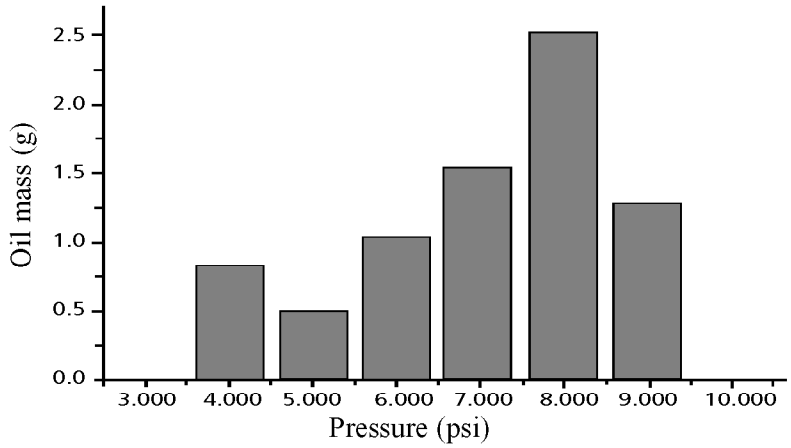

FIGURE 2 – Performance of the essential oil of *Eugenia uniflora* obtained by SFE.

To the chromatographic analyzes it was used the oil extracted by the Clevenger system, FIGURE 3 shows the chromatography for the brut essential oil and many chromatographic peaks can be observed. This result reveals a great amount of compounds with different polarities and intermediate volatility since the main mechanism of separation of the chromatographic column is by dielectric constant. Another possibility is to infer the presence of an ample band of points of ebullition based in the temperature program of the chromatograph oven.

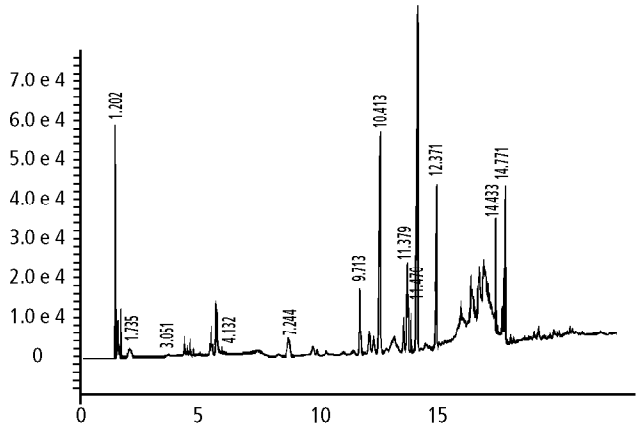

FIGURE 3 – Chromatogram for brut essential oil obtained by the Clevenger system and analyzed by HRGC/FID.

The results obtained through the fractioning of the essential oil of *Eugenia uniflora* are presented in FIGURES 4 and 5. The results show that the high polarity of silica used in opposition to the polarity of the petroleum ether (which is a mixture of hydrocarbonets) allowed the retention of polar compounds and the elution of the linalol in the fraction of 2 to 5. On should note that the fraction 1 and from 6 to 11 did not elude compounds that could be detected to this level of attenuation of the equipments.

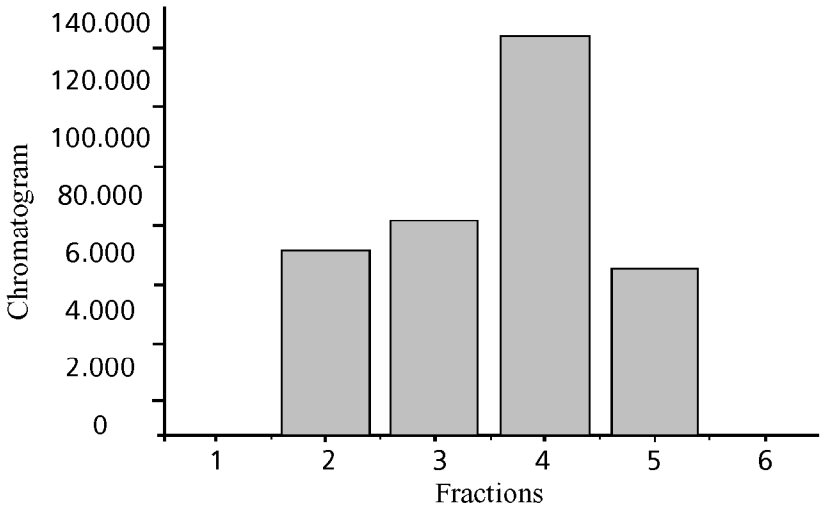

FIGURE 4 – In silica column.

The chromatogram in FIGURE 5 shows the sample of the fraction 4 as an example to illustrate the effect of the purification process, which shows a high efficiency and capability for concentration of the targeted compound (majoritarian peak).

It is intended to better study the extraction and purification process first and then quantitatively study the target compound with the help of primary standards.

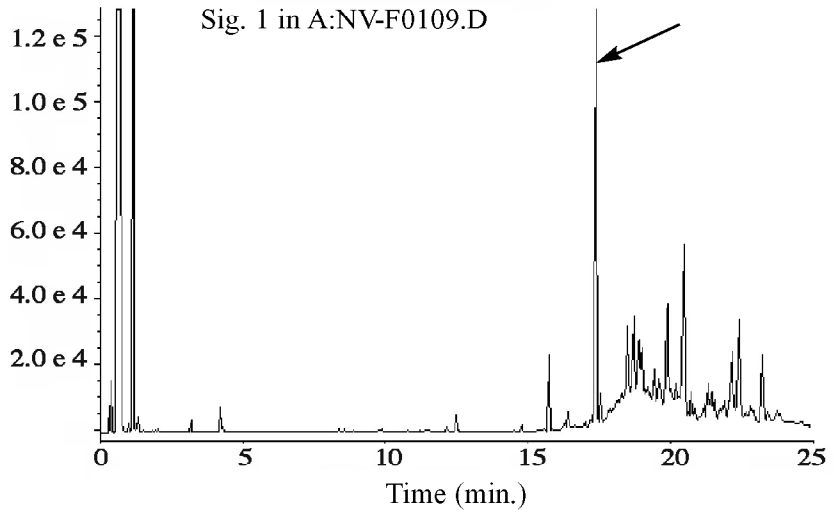

FIGURE 5 – Chromatogram of the fractioning of the oil extracted from *Eugenia uniflora* in LM column – 100 (10m x 0.18 x 0.18). Tinj: 250 ºC; Tdet: 300 ºC; column 60 ºC - 8 ºC (5 min.).

CONCLUSION

The present study showed various methods for extraction of the essential oil of *Eugenia uniflora*. The method showing better selectivity and effectiveness was the extraction by the Clevenger system. More contemporary methods also studied and, in particular, the extraction with Supercritical Fluid (SFE) is an excellent alternative mainly due to the performance and by the extraction speed. Furthermore, it is a method that spares solvents. However, it showed low selectivity being necessary an optimization to fulfill the study objectives. The purification of the oil using silica was of notable importance in the phase of isolation of the active principle (linalol) and/or in cleaning to avoid the toxic effect of co-extratives.

In further phases the use of adjustments of the conditions by multivariated biochemicmetric methods with computerized artificial intelligence may assist in to obtain better condition of extraction and purification of the studied oil to be submitted later to *in vitro* and *in vivo* biological tests.

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