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MOTHER TINCTURE OF DIFFERENT GENOTYPES OF *SCHINUS TEREBINTHIFOLIA* RADDI: NEW INSIGHTS FOR BIOTECHNOLOGICAL INNOVATION IN THE OPTIMIZATION OF EXTRACTION METHOD AND PHYTOCHEMICAL ANALYSIS

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Abbreviation List: AD1, natural certified organic adjuvant; ANVISA, Brazilian Health Regulatory Agency; EtOH, ethanol; F, female; FB, Brazilian Pharmacopeia; FHB, Brazilian Homeopathic Pharmacopeia; GMP, Good Manufacturing Practices; H, hermaphrodite; HPLC, high-performance liquid chromatography; M, male; MT, mother tincture; NP, Natural Product; PDA, Potato Dextrose Agar; PNPMF, Program of Medicinal Plants and Herbal; RENAFITO, National List of Herbal Medicines; Rf, retention factor; SD, standard deviation; SR, solid residue; ST, *Schinus terebinthifolia;* STMTLF, *S. terebinthifolia* mother tincture leaf (female plant); STMTLH, *S. terebinthifolia* mother tincture leaf (hermaphrodite plant); STMTLM, *S. terebinthifolia* mother tincture leaf (male plant); STMTFrH, *S. terebinthifolia* mother tincture fruit (hermaphrodite plant); STMTFrF1, *S. terebinthifolia* mother tincture fruit (female plant 1); STMTFrF2, *S. terebinthifolia* mother tincture fruit (female plant 2); SUS, Brazilian National Health System; SWS, stock and working solution of standards; TLC, thin layer chromatography.

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Abstract: Traditional systems of medicine have become a topic of global importance. For that matter, the species *Schinus terebinthifolia* Raddi (aroeira vermelha or Brazilian Peppertree), constitutes an important group between medicinal and aromatic species with economic value, due to its application in the food, cosmetic and pharmaceutical industry. This study reports that new insights for biotechnological innovation in the optimization of extraction method of three genotypes (male, female and hermaphrodite) of *Schinus terebinthifolia* species have profound effect on the isolation of active chemical principles and impact on quality. Physico-chemical analyzes of mother tincture from leaves and fruits presented default values for determination of dry residue (1.41% ± 0.13), alcohol content (67% ± 0.78), pH (5.6 ± 0.78) and density (0.898g.mL-1 ± 0.98) of mother tinctures. The chromatographic system - thin layer chromatography - adopted in sample screening leaves and fruits, allowed the detection of chromatographic zones of major metabolic classes for phenolic groups, flavonoid type, mainly for mother tincture's leaves. In the identification of chemical constituents of mother tincture of selected genotypes in the thin layer chromatography, was used high-performance liquid chromatography. The chromatogram for mother tincture of leaves detected metabolic groups, suggested the presence of hydrophilic compounds, as catechins and glycosylated flavonoids and for mother tincture of fruits, metabolic groups suggested the presence saponins and cardiotonic heterosides, of pyrogallic tannins (hydrolysable tannins), galotannins and tannins of ellagic acid. The best of our knowledge, this is the first report of optimization of extraction method (by extraction with "ecocertified"- AD1 adjuvant), and phytochemical analysis of different genotypes of *Schinus terebinthifolia's* mother tincture as a rich source of its components that may offer interesting potential applications in the food, cosmetic, biotechnology and/or pharmaceutical industries.

Keywords: Brazilian Peppertree.

Chromatography. Medicinal Herbs. Mother Tincture. *Schinus terebinthifolia*.

INTRODUCTION

Traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (WHO, 2011; 2014). Methods of folk healing throughout the world commonly used herbs as part of their tradition (EMA, 2011).

In Brazil, through the National Program of Medicinal Plants and Herbal - PNPMF (Brazil, 2009), which encourages research with plants native or exotic, species was included in the National List of Plants of Interest to the Brazilian National Health System (SUS-RENISUS) comprises 71 plants that could potentially derive products of interest from the most frequently used species for medicinal purposes in Brazil with potential use in primary health care (Brazil, 2010; Almeida et al., 2014) and the following year, 12 plants were selected and included in the National List of Herbal Medicines (RENAFITO), and some of them included in the "List of Herbal Medicines for Simplified Registration" (are listed in NO 5 ANVISA´s Normative Instruction (Brazil, 2009; Brazil, 2010) and under the conditions defined therein). For herbal medicines obtained from the species, *Cynara scolymus* (alcachofra), *Rhamnus purshiana* (cáscara sagrada), *Maytenus ilicifolia* (espinheira-santa) e *Mikania glomerata* (guaco), it was not necessary to validate its therapeutic indications and safety of use. The species, *Glycine max* (soja-isoflavona), *Harpagophytum procumbens* (garra-do-diabo), *Schinus terebinthifolia* (aroeira vermelha) and *Uncaria tomentosa* (unha-de-gato), were selected by safety evidence, efficiency, by searching trust database. Until the present date, were only included in the list, by ethnobotanical use and/or ethnopharmacological, the species, *Menta piperita* (hortelã), *Aloe vera* (babosa), *Salix alba* (salgueiro) e *Plantago ovata* (plantago) (Brazil, 2009; Sales, 2013).

The species *Schinus terebinthifolia* Raddi (aroeira or Brazilian Peppertree), is a small dioecious shrub-like tree native to Brazil (Figure 1), as well as presenting a wide ecological plasticity, makes up an important group among species of medicinal and aromatic plants with economic value (Silva et al., 2018; Sales et al., 2016), with potential to be used as raw materials for the food industry, have the products your primary and secondary metabolism intended for other sectors, such as the pharmaceutical and cosmetics industry.

Studies of morphological analysis of the flowers and pollination experiments of aroeira genotypes of plants hermaphrodite, female and male confirmed dioicism and obligatory xenogamy in this species. High synchrony between male and female plants occurred, an important strategy for dioecious species. The similarity between male and female flowers, added to synchrony of flowering between sexes, seems to contribute for its reproductive success, through flowers visitors' attraction to male and female plants. The occurrence of hermaphrodite individuals in populations of this species, seems to be an uncommon phenomenon, not being cited by others authors that studied the species at different places in Brazil (Cesario and Gaglianone, 2008; Ruas, 2016).

Chemical studies showed that polyphenol

and flavonoid are major chemical groups of the extracts of *S. terebinthifolia* leaves and fruits (Silva et al., 2017; Silva et al., 2018; Sales, 2013; Ruas, 2016). Recent studies describe the use of various extractive forms of *S. terebinthifolia* and its pharmacological activities, the most differentiated, being the antioxidant and anti-inflammatory (Bernardes et al., 2014; Fedel-Miyasato et al., 2014; Muhs et al., 2017; Silva et al., 2017; Iwanaga et al., 2019; Maciel et al., 2019) and antimicrobian activity (Bernardes et al., 2014; Muhs et al., 2017; Silva et al., 2018). These activities are related to the presence of flavonoids (mainly in fruits, biflavonoids), tannins, triterpenic acids and, mono and sesquiterpenes in the essential oil of fruits and leaves of the species (Silva et al., 2018; Bernardes et al., 2014; Schimitberger et al., 2018; Ceruks et al., 2007; Correia et al., 2006; Oliveira, 2012; Ruas, 2016; Iwanaga et al., 2019; Maciel et al., 2019), with application in the food, cosmetic and pharmaceutical industries.

Despite the tradition of use and acceptance of medicinal plants by population, the number of herbal medicines products licensed in the country is small when compared to other nations and the licensing of medicines is regulated by the Brazilian Health Regulatory Agency (ANVISA), a federal agency responsible for heath surveillance over products and services, including processes, ingredients and technologies that pose any health risks (Carvalho et al., 2018). Brazilian sanitary legislation have been changing in recent years, bringing international harmonized concepts of quality control of herbal medicines products (Brazil, 2015), on based in WHO - Guidelines on Good Manufacturing Practices (GMP) for herbal medicines (WHO, 2007), and General Guidelines for methodologies on research and evaluation of traditional medicine, Quality control methods for medicinal plant materials, Guidelines on good agricultural and collection practices for medicinal plants, were also issued (Brazil, 2015).

The development and commercialization of medicinal plants ("bioindustries") based in the developing countries is dependent upon the availability of facilities and information concerning upstream and downstream bioprocessing, extraction, purification, and marketing of the industrial potential of medicinal plants (Singh, 2015). Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. Good practices in production and quality control, provides guidance on actions to be taken separately by production and by quality control personnel for the implementation of the general principles of quality assurance (WHO, 2007; Brazil, 2015). Studies have shown that the extraction method of medicinal plants have profound effect on the isolation of active chemical principles (Manilal and Idhayadhulla, 2014; Sales et al., 2016).

The Brazilian Herbal Formulary of the Brazilian Pharmacopeia (Brazil, 2011) defines norms for herbal drug manipulation, and its lists were established based on the plant species most frequently used in herbal medicine in diverse regions of Brazil (Almeida et al., 2014), and presents several formulations included by scientific studies and a history of use in the herbal medicine services of the country, among these formulations contained of monographs of vegetal drugs for infuses and decocts and tinctures.

The contents of the Pharmacopeias and Forms aim at guiding the medicine manufacturing and the regulation of the pharmaceutical sectors involved in the manufacturing and control of drugs, inputs and pharmaceutical specialties (FHB, 2011). The manufacturing process is one

of the key steps where quality control is required to ensure quality of medicinal products, including herbal medicines. Good manufacturing practices (GMP) is one of the most important tools for this measure (FHB, 2011; OMS, 2007; 2014; EMA, 2011). Beyond medicinal products from the herbal medicines are included the mother tincture from vegetable origin, prepared following Brazilian Pharmacopeia, by extraction method (maceration or water seepage) using ethanol in different gradations, pursuant to the drug monograph. In case there is no specification in monograph, the alcoholic content in the start-up of the extraction shall be 60% (v/v) and in the end of the extraction shall be from 55% (v/v) to 65% (v/v) (FHB, 2011; Sales et al., 2016).

The herbal material(s) or the herbal preparation(s) will be considered to be active ingredient(s) of a herbal medicine(s). However, if constituents with known therapeutic activities are known, the active ingredients should be standardized to contain a defined amount of this/these constituent(s) (WHO, 2007; 2014). Most studies are performed out with parts of medicinal plants extracted with organic solvents not suitable for human use and, in this case, the assessed form would not be the form to be applied in research with humans or animals. *S. terebinthifolia* extraction can be performed by different processes and conditions that can influence extract characteristics. Bertoldi (2006) obtained ten times greater results in the antioxidant activity of extracts from fruits of the *S. terebinthifolia*, altering the extraction solvent, from acetone to water, allowing an enrichment of the sample with constituents with higher antioxidant activity, in the case of the study, phenolic compounds. Interestingly, the use of the mother tincture, as hydroalcoholic form, becomes therapeutically very efficient and safe for use in humans.

Studies have shown that the extraction method of medicinal plants have profound effect on the isolation of active chemical principles (Manilal and Idhayadhulla 2014; Sales et. al, 2016). Quality assurance and correct identification of the starting material is an essential prerequisite to ensure reproducible quality of an herbal medicine, which contributes to its safety and efficacy (Braz et al., 2012). Pharmacotechnics has advantages in the use of extractive adjuvants, mainly on the influence of the biological activity of chemical constituents, which could be related to the increased solubility of the phenolic moieties in the lipid regions, in which protection against biological degradation or chemical oxidation is required (Gennaro, 1995).

Considering contributing to the development of an innovative technology for monitored extraction and in the identification of the chemical constituents of three genotypes (male, female and hermaphrodite) of *Schinus terebinthifolia* species, this work presents an economically viable pharmaceutical form and safe for human, animal and phytosanitary use, with the addition of eco-bioavailable and bioavailable adjuvants.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh leaves and fruits of three genotypes, male (M), female (F) and hermaphrodite (H), *Schinus terebinthifolia* species, were collected in February (leaves) and May (fruits) on 2012, in the municipality of Pedro Canário, Espírito Santo, Brazil (latitude: -18° 01' 49"; longitude: - 40° 09' 02"). All plants were georeferenced by GPS (*Global Positioning System*). The identification of botanical species was performed by Prof. Marcelo Simonelli (Instituto Federal do Espírito Santo, Vitória, Espírito Santo, Brazil) and Exsiccates are deposited in the herbarium of the Museum

of Biology Mello Leitão (currently National Institute of the Atlantic Forest), located in the municipality of Santa Teresa, Espírito Santo, Brazil (cataloged and registered under the No 41895).

SAMPLE EXTRACTION

Pretreatment of plant material: leaves and fresh fruits were previously washed with water and subsequently disinfested with sodium hypochlorite (1%) and then rinsed again with sterile distilled water. Then, the fresh vegetable drug underwent this treatment process prior to maceration including the use of a natural certified organic adjuvant (AD1) in the extraction process, aiming the formation of a stable plant biomass. AD1- is an alternative glycol, 100% natural, approved by Ecocert™ and certified by *Natural Products Association* (NPA), because, not being derived from petroleum, presents high purity.

Determination of solid residue (S.R.) of fresh vegetable: fresh leaves (10g) and fruits (10g) were fractionated in reduced fragments and placed in an oven at 100 ° C - 105 ° C until constant weight, as described in the Brazilian Homeopathic Pharmacopeia (FHB) (2011).

Determination of extractive liquid content (EtOH): the extraction content was determined by determining the solid residue (FHB, 2011): a) EtOH 90% (p/p) for S.R. ≤ 29% (for plants with high water content); b) EtOH 80% (p/p) for S.R.: 30% - 39% (plants with medium water content); c) EtOH 70% (p/p) for S.R. ≥ 40% (plants with low water content). The EtOH volume was equivalent to the final volume of mother dye to be obtained subtracted from the volume of water contained in the fresh vegetable.

MOTHER TINCTURE'S (MT) EXTRACTION

Mother tincture samples (MT) of the species *S. terebinthifolia* were prepared from the plant material stabilized by the pre-treatment process, according to standardized techniques in official compendia (Brazilian Homeopathic Pharmacopeia, 2011; British Pharmacopeia, 2009), modified by the authors.

STOCK AND WORKING SOLUTION OF STANDARDS (SWS)

An aliquot of 10mL, each MT, was taken to the Bain-marie (95° a 105°C), for evaporation of the EtOH, for 40 to 60 min. After evaporation of the solvent, the residue was reconstituted in 2.0 mL of EtOH (SWS) and applied in chromatographic plates with the aid of micropipettes or capillaries, in which they were dispensed 5μL and multiples of this volume. Each SWS was packed in amber glass of 5.0mL capacity, properly identified and kept out of light and heat. The samples were identified by the origin of the plant tissue and its genotypes: leaves of the hermaphrodite plant (STMTLH), female (STMTLF), and male (STMTLM) and of fruits, hermaphrodite plants (STMTFrH) and two samples of the female plant (STMTFrF1 e STMTFrF2).

PHYSICAL AND PHYSICAL-CHEMICAL DETERMINATIONS OF MT

Physico-chemical analyzes of MT from leaves (STMTLH, STMTLF e STMTLM) and fruits (STMTFrH, STMTFrF1 e STMTFrF2) of *S. terebinthifolia*, were performed to determine color and appearance, dry residue, ethanolic title of MT, pH and density as stated in the Brazilian Pharmacopeia (FB) (2010), FHB (2011) and Bacchi (1996). All experiments were performed with three replicates at the appropriate temperature (20 °C ± 2 °C).

DETERMINATION OF THE CHROMATOGRAPHIC PROFILE

THIN LAYER CHROMATOGRAPHY (TLC)

The experiment was performed in the thin layer chromatography system (TLC), using silica gel aluminum plates GF254 (Merck®) and glass plates, DC- Fertiglatten® ADAMANT for TLC (Marcherey Nagel®), according to methods described in literature (FB, 2010; Wargner and Bladt, 1996; Braz et al., 2012; Cardoso, 2009) and adapted by the author, for reconstituted samples of SWS, from MT de *Schinus terebinthifolia*. All the chromatograms were developed in an exhaust chamber. The mobile phases employed in this study were selected or developed according to the SWS (Table 1). The plate was developed at a distance of 80 mm, at room temperature in an exhaust hood. After the development of the chromatography and the evaporation of the solvents, the spots were visualized sequentially with visible light and in a darkroom (Clinlab®), to UV of 254 and 365 nm, and then sprayed with specific chromogenic reagents according to the chemical groups analyzed. The final position of each spot was designated by Retention factor (Rf) (Rf = distance from the origin/ distance traveled by the solvent from the origin).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) APPPARATUS AND CHROMATOGRAPHIC CONDITIONS

In the identification of chemical constituents of mother tinctures (MT) of the leaf and fruit of selected genotypes in the TLC (STMTLH e STMTFrH), of the species *S. terebinthifolia* it was used high-performance liquid chromatography (HPLC), described in relevant literature (Bacchi, 1996; Cimpan; Gocan, 2003; Renger, 2000), with adaptations for the use of stock and working solution of standards (SWS). The reverse phase chromatography was performed in HPLC/ Shimadzu Proeminence LC20 AT. The column used was the C18 (Phenomenex: Phenosphere (Next 5µ) with reverse phase silica (250 mm x 4,6 mm) and the elution solvents were: a) water: acetic acid (90: 10) and b) acetonitrile (Cimpan; Gocan, 2003). The samples, eluted at EtOH (PA), were injected and subjected to a gradient 0 - 100% of water/acetic acid: acetonitrile (0- 10min.: 20%B, 10- 30min.: 30%B, 30- 50min.: 100%B), at a flow rate of 0,6mL/min. for 50 min. of racing, monitored by the detector (SPD 20A) spectrophotometric (UV/Vis) with reading at two wavelengths, 220nm e 350nm, simultaneously.

RESULTS AND DISCUSSION

PRETREATMENT OF PLANT MATERIAL

The strategic treatment procedure for the fresh vegetable drug of *S. terebinthifolia*, together with the use of the adjuvant, in the process of botanical extraction, efficiently maintained the stability of the plant drug, as it allowed the maintenance of the physical-chemical vegetal integrity necessary for the accomplishment of the maceration technique by exhaustion. The use of fresh plant material was indispensable for the detection of specific components, since its use has the advantage of avoiding the presence of substances originating from the metabolism of plant failure. Besides that, the addition of bioavailability adjuvants as AD1, promoted a synergism with the phytoactive compounds present in *S. terebinthifolia* by improving the process of extracting the plant drug and supporting action of physicochemical stability of the chemical constituents of medicinal plants.

OBTAINING MOTHER TINCTURE (MT) AND STOCK AND WORKING SOLUTION OF STANDARDS (SWS)

The values obtained and presented (Table 2) established the pharmacopeial determinations contained in the raw material extraction standards and technical processes for obtaining vegetable dyes, specialy for mother tinctures (Bacchi, 1996; Farias, 2010; FB, 2010; FHB, 2011; HPSUS, 2000). The preparation of the SWS, from each MT, was performed according to proposed methodology and represented an important strategy in the preservation of the sample and in the identification of chemical clusters in the TLC.

From the leaves of the species *S. terebinthifolia*, collected in February 2012, (total weight plant material: 930g), were obtained 960 mL mother tincture (MT) from 300g of the hermaphrodite plant (STMTLH) (with concentration of 32 $mg.mL^{-1}$), 1160 mL de MT from 350g of the female plant (STMTLF) (with concentration of 29 $mg.mL^{-1}$) e 728 mL de MT from de 280g of the male plant (STMTLM) (with concentration of 26 $mg.\ mL^{-1}$). Of the fruits, collected in May 2012, (total weight plant material: 270g), were obtained 441 mL mother tincture (MT) from 90g of the hermaphrodite plant (STMTFrH) (with concentration de 49 $mg.mL^{-1}$), 336 mL of MT from 80g of the female plant 1 (STMTFrF1) (with concentration de 42 $mg.mL^{-1}$) e 470 mL of MT from 100g of the female plant 2 (STMTFrF2) (with concentration de 47$mg.\ mL^{-1}$). One part (100 mL), of each prepared tincture, was used to determine the solid residue (S.R.) and the rest was reserved for use in bioassays and preparation of the test solution of dye mother (SWS), used in the art of TLC. The averages for solid waste (S.R.) and are within the limits allowed by legislation (Bacchi, 1996; Prista and Alves, 1990), which made it possible to determine the content EtOH (%) of each MT sample analyzed.

There was a difference in the results presented in the determination of extractive liquid content (EtOH) for each genotype of the species, determined by the part of the plant utilized in the preparation of MT. The leaves of the hermaphrodite and female plants, which have medium and high water content, respectively, demanded an alcoholic content for the liquid extracting (EtOH), between 80 and 90%. This value, bigger than EtOH (70%) used in the extraction of the fruits, aware of, has a low water content. The use of methanol in the phase of extraction of vegetal drugs, although it is considered the best solvent for phenolic compounds (Simões et al., 2010) and for medium and low polarity metabolites (Gennaro, 1995), would be an indication, mainly for the extraction and identification of the *S. terebinthifolia*. However, it was not used because of its toxicity (Lieberman; Lachman; Schwaztz, 1990; Prista and Alves, 2003). Furthermore, if used, it should be completely removed after extraction to be used as a MT vehicle, which would make the whole process difficult or impossible (Degáspari, 2004; Farias, 2010).

The authors of this work emphasize the importance of carrying out the analyzes with the extrinsic forms that will be applied in human health, as animal and vegetable. This characteristic highlights the importance of mother tincture with extractive pharmacopeia with a potential content in bioactive constituents, even if in its diluted form (10% p/V) according to standardized methods in traditional compendia.

PHYSICAL-CHEMICAL ANALYZES OF MT

The results of the analysis of the genotype *S. terebinthifolia* (Table 3), presented mean values and calculation of standard deviation for determination of dry residue (1.41% ±

0.13), alcohol content (67% ± 0.78), pH (5.6 ± 0.78) and density (0.898g.mL-1 ± 0.98) of mother tinctures' (MT), within the technical specifications proposed by the appropriate literature, when available (Brazil, 2004; FB, 2010; Gennaro, 1995; HPSUS, 2002).

According to procedures of analysis and interpretation of results of analytical methodology (Brazil, 2010; Lieberman; Lachman; Schwaztz, 1990; Prista and Alves, 1990), provided that in the description of a plant drug the percentage of the loss by desiccation is not indicated, a maximum of five percent is tolerated (Brazil, 2010; Gennaro, 1995; Prista and Alves, 1990). The determination of the dry residue is a fundamental and preliminary parameter when it is aimed to achieve the efficacy of a herbal formulation (Sharapin et al., 2000; Cardoso, 2009), as this test involves the quantification of the substances extracted from the plant by eliminating the solvent extractor, thus, this percentage is indicative of the concentration of the tincture (Brazil, 2010; Prista and Alves, 1990).

The mean values of pH and density showed no significant difference between samples and the standard allowed by the legislation. The pH value of the distilled water was also verified (5.32 ± 0.03), due to the fact that it is used in the procedure to obtain the extractive solution. The value presented in the method (5.6 ± 0.78), within the limits allowed (Brazil, 2004; FB, 2010; Prista and Alves, 1990), was compatible with the water's, not having acidity levels. In aqueous solutions, the acids are partially dissociated into ions, and this acidity can be measured by the concentration of the hydrogens in the solution (Costa, 2001). The presence of water beyond the permitted limit, leads to errors in the determination of extractor liquid content and allows the occurrence of enzymatic reactions, as hydrolysis, which promote changes and / or degradation of its active constituents, consequently leading to deterioration of the plant drug (Sales, 2013).

The average of the results of the analyzes developed with MT, presented the mean value of density of 0.898g.mL-1 ± 0.98 which remained within the recommended limit for tinctures, 0.87 e 0.98 g.mL-1 (Brazil, 2010; Prista and Alves, 1990). It is worth noticing that such testing is a requirement established by ANVISA (RDC nº 14/2010) (Brazil, 2010) and the results presented for the samples, from MT of the *S. terebinthifolia* analyzed signaled for a standardization in the process of extraction of the species, according to the evaluation of physicochemical characteristics of technologically processed products.

CHROMATOGRAPHIC PROFILE FOR *S. TEREBINTHIFOLIA*

THIN LAYER CHROMATOGRAPHY (TLC)

The chromatographic system adopted in SWS sample screening leaves (STMTLH; STMTLF; STMTLM) and fruits (STMTFrH; STMTFrF1; STMTFrF2), allowed the detection of chromatographic zones of major metabolic classes for phenolic groups mainly for MT' leaves (Table 4), selected through reports of the presence of active chemical groups, typical of phenolic compounds, characterized by colorings ranging from orange-green to yellow (Nunes et al., 2009; Wagner and Bladt, 1996), showing predominance in relation to the presence of saponins and terpenes, characterized by the coloration orange-brownish to purplish, respectively. The presence of gallic acid and catechin was detected, which, presented significant similarity to spots detected by Braz et al. (2012) using the standard of gallic acid (Rf= 0.20) and catechins (Rf= 0.81) in the identification of metabolites present in the extract of the shell of the *S. terebinthifolia*. Studies show active chemical constituents such as ethyl gallate, methyl gallate, myristylrine,

quercitrin, myricetin (Ceruks et al., 2007; Ruas, 2016) and the identification of phenolic compounds as, caffeic acid, coumaric acid, ellagic acid, gallic acid and catechin (El-Massry et al., 2009; Ruas, 2016).

Table 4 shows a tenuous violet color D2 (Rf ~0.50), was not conclusive for the presence of metabolites, however the result presented by the chromatographic zone in D3, possibly "saponin zones" (sapogenins), which have "yellow-brown" (Rfs that varies from 0.20 – 0.75), according to the core aglycone, such as steroidal saponins and triterpene saponins (Wagner and Bladt, 1996). Triterpenes are easily found in nature, however, when using the usual chromatographic techniques it is rarely possible to isolate these pure triterpenes, being these, therefore, obtained almost always in mixtures of difficult resolution. Results with significant evidence for the detection of phenolic compounds, flavonoid type in the samples of SWS (STMTLH, STMTLF and STMTLM), from *S. terebinthifolia leaves*, showed clear chromatographic areas with five Rfs of interest. For the spot G1 (Rf ~0.12), no references were found in the literature consulted. In G2 (Rf ~0.47), a blue fluorescent stain was detected, attributed to the possible presence of benzoic acid derivatives, in particular, chlorogenic acid (Rf= 0.50), identified in the literature consulted (Wagner and Bladt, 1996; Nunes et al., 2009; Degáspari, 2004).

The most common flavonoid heterosides are 3-rutinoside quercetin (rutin) and 7-glycoside luteolin. The rutina (3- rutinoside quercetin) belongs to flavonols class (R= OH) and theirs *O*- heterosides, which are part of a large group of flavonoids, together with the flavones (R= H). The genins most commonly associated with sugars are apigenin and luteolin in flavones and quercetin, kaferol and myricetin in flavonols. Studies support the presence of phenolic structures, in isolated compounds of the species *S. terebinthifolia* Raddi, such as flavone apigenin, ellagic acid, flavanone naringin, ethyl gallate, methyl gallate, myristylrine, myricetin, quercitrine, rutin, isoquercitrin and phenolic lipids (Ceruks et al., 2007; Degáspari; Wasczynsky and Prado, 2005; Queires et al., 2006; Ruas, 2016). A recent study, (Silva et al., 2017) obtained from leaves (*S. terebinthifolia*) seven previously known compounds, including one steroid, sitosterol-3-*O*-ß-glucopyranoside; two gallic acid derivatives, 1,2,3,4,6-penta-O-galloyl-*O*-ß -glucopyranoside and methyl gallate; and the four following flavonoids: robustaflavone, quercetin, quercetrin and luteolin.

HPLC AND IDENTIFICATION OF CHEMICAL CONSTITUENTS IN MT DE *S. TEREBINTHIFOLIA*

The accomplishment of the HPLC in the experiment allowed the separation by partition of the chemical constituents present in the standard MTs leaves (STMTLH) and fruits (STMTFrH) of the hermaphrodite plant of the species *S. terebinthifolia*, the results presented by these dyes in the physico-chemical analyzes and the chromatographic profile by TLC, mainly for the phenolic group of flavonoids. Based on the retention time interval, peak significance (by the concentration of the chemical compound in the sample), the chromatogram (Figure 2A) for mother tincture of leaves (STMTLH) detected metabolic groups identified here as L1 to L7 with retention time interval (t), of 4.8 to 21.5 min. generally suggestive of flavonoid type phenolic compounds, considering the hydrophilic polarity of the STMTLH analyzed.

Interval measurements without retention time (t) among 4.8 e 9.0 min., and in the chromatogram of the sample, leaf mother tincture (STMTLH) of the hermaphrodite plant (Figure 2A), for the peaks presented

by L1 (t~5.0), L2 (t~6.8) e L3 (t~8.85), suggested the presence of hydrophilic compounds, as catechins, for an initial racing system, of 80% acid water and 20% of acetonitrile. These results were supported by the chromatographic profile by TLC for dyeing leaves and fruits (STMTL e STMTFr), with the detection of a chromatographic zone suggestive of the presence of catechins (Rf~0.81). Sample L4 showed retention time ranging from 10.8-11.2 min., suggestive of the presence of glycosylated flavonoids (such as hesperidin, naringin and rutin). This was evidenced by qualitative HPLC/UV, of the dye of *Calendula officinalis* L., using the standard rutin, detected a retention time for this flavonol class, of t= 11.33 (Nunes et al., 2009). However, the results and the comparative profile with other studies, still does not allow us to associate with a specific chemical constituent. The chromatogram developed for the mother tincture of fruits of the hermaphrodite plant (STMTFrH), detected metabolic groups, identified here as Fr1 to Fr5, with retention time (t) ranging from 2.8 to 30.5 min., generally suggestive of phenolic compounds (Figure 2B).

The retention time range for the 50 min. run was greater than that presented in the leaf chromatogram, even because after t= 30 min. at 50 min., the mobile phase is only solvent acetonitrile, providing a way of apolar and the elution of certain non-polar molecules. The samples, Fr3 e Fr4, exhibited retention time varying in a range of 27.5-28.1 min. and 29.4-29.6 min., respectively (Figure 2B). Considering the physical-chemical properties presented, for the mobile phase and the stationary phase, with an apolarity characteristic and the retention times presented, we suggest that these metabolic groups belong to the class of flavones (possibly genins such as luteolin and apigenin). Similar studies (Degáspari et al., 2004; 2005), demonstrated that the sample obtained from the alcoholic extract of fruits of *S. terebenthifolia*, determined by HPLC analysis, the presence of flavone apigenin (four peaks) at retention times of 25 to 27 minutes, which justifies its yellowish coloration. The four apigenin peaks are at different retention times due to the fact that they are bound to different sugars or in different positions. It also showed a peak of ellagic acid at the retention time of 14 min. Retention time measurements (t) over 27 min. in the fruit chromatogram (Figure 2B), for the peaks presented by Fr3 (t ~ 27.8), Fr4 (t ~ 29.5) and Fr5 (t ~ 30.3), could suggest the presence of saponins and cardiotonic heterosides (characterized by the presence of steroids and triterpenoids); presence of pyrogallic tannins (hydrolysable tannins), galotannins (union between gallic acid units via meta-depsidic bonds) and tannins of ellagic acid.

Considering the presented results, physico-chemical factors could have influenced the chromatographic separation of the majority constituents of *S. terebinthifolia* by HPLC, which depend on the chemical nature of the separate metabolic groups, such as phenolic groups of the species *S. terebinthifolia*. Variations in the physicochemical characteristics of the plant material occur mainly due to the polarity of the plant extract, composition and flow rate of the chosen mobile phase, composition and surface area of the stationary phase (Bacchi, 1996; Farias, 2010; Renger, 2000). Regarding the physicochemical factors for flavonoids, an important observation regarding solubility is observed, which may have influenced greatly the results of HPLC chromatogram. While heterosides are generally water soluble and dilute alcohols, but insoluble in the usual organic solvents, the aglycones are usually soluble in apolar organic solvents (Zuanazzi and Montanha, 2007; Lieberman; Lachman; Schwaztz, 1990). Flavones and flavonols are poorly soluble in water, while dihydroflavonols

are more soluble (3-hydroxyflavans (catechins) and 3,4-flavonoids). Hydrolysis, alkaline and acidic, facilitate the identification of flavonic nuclei (Costa, 2001; Zuanazzi and Montanha, 2007).

The best of our knowledge, this is the first report of optimization of extraction method and phytochemical analysis of different genotypes of *Schinus terebinthifolia* mother tincture of plants hermaphrodite, female and male, as a rich source of its components that may offer interesting potential applications in the food, cosmetic, biotechnology and/or pharmaceutical industries. The rescue of traditional knowledge combined with technological innovation in the extraction technique of this relevant medicinal plant with phytotherapeutic potential evidenced by the previous treatment of the vegetal material with the proper use of suitable adjuvant for extraction, presented peculiar and relevant aspects, evidenced by the results in the physical-chemical analyzes, besides the development of new methodologies for stabilization of the final product. The results presented in the physical-chemical analyzes indicated quality in the process of extraction of the vegetal material, probably guaranteed by the accomplishment of the procedures executed before the maceration, by the previous treatment and addition of a new adjuvant, in the vegetal material, which gave promising results in the determination of the chromatographic profile of the analyzed samples.

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AUTHOR CONTRIBUTIONS

F.G.R.: Participation at field's collect and selection of vegetable raw materials; M.D.S., and J.A.V.: Generated the experimental study protocol, coordinated the laboratory analyses, collected data and participated in data management of the laboratorial trial and participated in data analysis and interpretation; F.G.R., M.C.S., M.C.C. and D.D.M.: assistance with structuring the review, writing, and literature review; D.D.M.: performed the statistical analyses. D.D.M., M.D.S., M.C.C., F.G.R., and J.A.V. wrote the manuscript and did the literature search; D.D.M. submitted the manuscript to the International Journal of Biological and Natural Sciences/Editora Atena; all authors critically reviewed and approved the final version.

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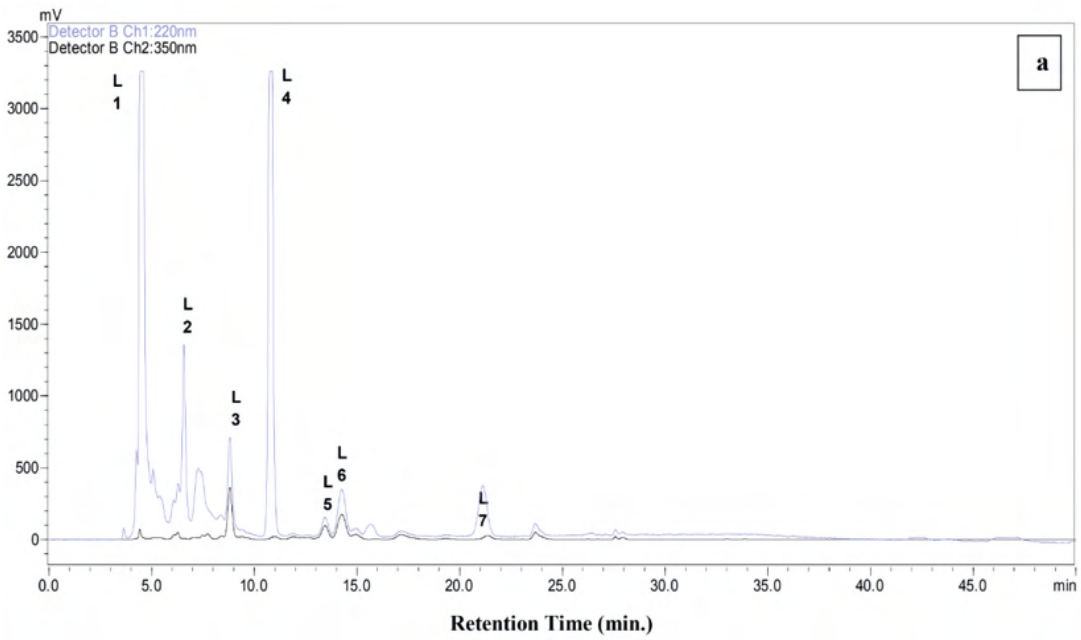
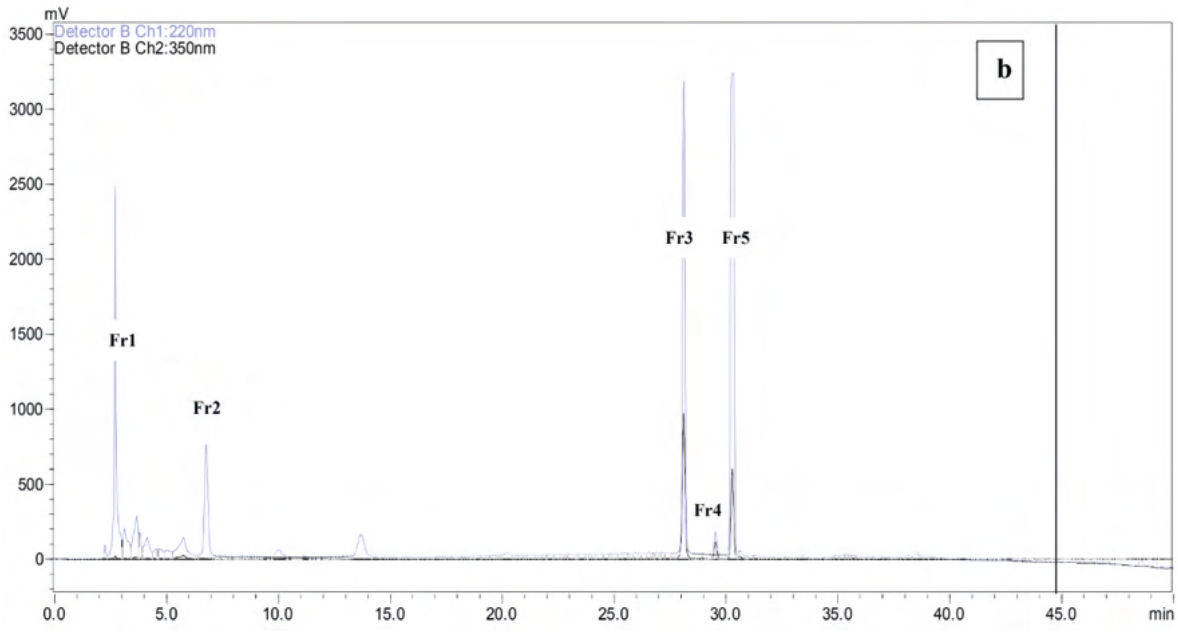
FIGURES WITH LEGENDS

Figure 1. *Schinus terebinthifolia* from Pedro Canário (Espírito Santo), Brazil: a) *S. terebinthifolia's* tree and b) *S. terebinthifolia's* fruits, flowers and leaves (in detail).





Retention Time (min.)

Figure 2. Chromatogram obtained in HPLC from the mother tincture of the hermaphrodite plant: **a)** Leaf samples (L1 to L7); **b)** fruit samples (Fr1 to Fr5), *S. terebinthifolia* specie, using column C18 and gradient system (0-100% of water: acetonitrile), with flow velocity 0.6 ml/min., during 50 min.

TABLES

Classes of Metabolites (µL)	Aliquot	MT Test Solution[1] (STMTLH, STMTLM, STMTLF, STMTFrH, STMTFrF1, STMTFrF2) Chromatographyc System (PDA) [2]	
		Eluent System (v/v)	Chromogenic Agent
Gallic Acid	10/ 25 Plate 1[(6)]	1. Toluene: Ethyl Acetate: Methanol: Formic Ácid (75:25:10:6)	A. Ferric Chloride 1% in methanol[3]
Saponins	10/ 25 Plate 1[(6)]	2. Chloroform: Acetic Acid: Methanol: Water (60:32:12:8)	B. Sulfuric Anisaldehyde + 105°C/5 min.
Terpenes	10/ 25 Plate 1[(6)]	3. Ethyl Acetate: Toluene (7:93)	C. Sulfuric Vanillin + 105°C/5min.
Flavonoids	5 Plate 2[(7)]	4. Ethyl Acetate: Formic Acid: Acetic Ácid: Water (100:11:11:26)	D. NP/PEG 4000[4] + UV 365nm[5]

Table 1. Phytochemical screening of the main classes of secondary metabolites, through a chromatography system for MT of leaves and fruits of *S. terebinthifolia* (aroeira).

[1]MT (mother tincture) Test Solution: STMTLH: *S. terebinthifolia* MT leaf (hermaphrodite plant); STMTLM: *S. terebinthifolia* MT leaf (male plant); STMTLF: *S. terebinthifolia* MT leaf (female plant); STMTFrH: *S. terebinthifolia* MT fruit (hermaphrodite plant); STMTFrF1: *S. terebinthifolia* MT fruit (female plant 1); STMTFrF2: *S. terebinthifolia* MT fruit (female plant 2).

[2]Source: PDA (Potato Dextrose Agar): Wagner and Bladt (1996).

[3]Reagent: Fe_2Cl_3 1% in methanol (Braz et al., 2011) and in water solution (Wagner; Bladt, 1996).

[4]NP: Natural Product: -2- aminoetil- difenil- borinato/ PEG: polyethylene glycol 4000.

[5]365nm: UV light 365nm (darkroom).

[6]Plate 1: Glass plates, DC- Fertiglatten® ADAMANT for CCD (Marcherey Nagel®).

[7]Plate 2: Silica gel aluminum plates GF_{254} (Merck®).

Plant material PM (g)	Sample[1] (g)	S.R. (%) ± SD[2]	Total S.R.[3] (g)	EtOH content[4] (%)	MT vol. (mL)
Leaves PM: 930g	STLH 300	32 ± 1.05	96	80	960
	STLF 350	29 ± 0.58	101.5	90	1160
	STLM 280	26 ± 2.52	72.8	90	728
Fruits PM: 270g	STFrH 90	49 ± 0.52	44.1	70	441
	$STFrF_1$ 80	42 ± 1.15	33.6	70	336
	$STFrF_2$ 100	47 ± 1.08	47	70	470

Table 2. Determination of solid residue (S. R.) (%), EtOH content (%) and MT volume (mL) from samples of plant material (PM) of *S. terebinthifolia.*

[1]STLH: *S. terebinthifolia* leaf (hermaphrodite plant); STLF: *S. terebinthifolia* leaf (female plant); STLM: *S. terebinthifolia* leaf (male plant); STFrH: *S. terebinthifolia* fruit (hermaphrodite plant); STFrF1: *S. terebinthifolia* fruit (female plant 1); STFrF2: *S. terebinthifolia* fruit (female plant 2).

[2]Solid residue (S.R.) (%) ± standard deviation (SD).

[3]Total solid residue (S.R.): total solid residue of plant material (g).

[4]EtOH content (%): alcohol content: determined by solid residue content.

[5]MT vol. (mL): final volume of mother tincture (10x residue).

Extractive Form[1]	Color/ Aspect	S.R. (%) ± SD[2]	EtOH content ± SD[3]	pH ± SD[4]	Dens. ± SD[5] (g/mL)
STMTLH	Green/Characteristic	0.82 ± 0.06	68 ± 0.50	5.01 ± 0.21	0.895 ± 0.58
STMTLF	Green/Characteristic	1.14 ± 0.21	69 ± 0.35	5.38 ± 0.32	0.889 ± 0.54
STMTLM	Green/Characteristic	0.89 ± 0.20	70 ± 0.62	5.10 ± 0.51	0.894 ± 0.98
STMTFrH	Reddish-Brown/ Characteristic	1.57 ± 0.08	65 ± 0.87	5.88 ± 0.81	0.902 ± 1.18
STMTFrF1	Reddish-Brown/ Characteristic	2.04 ± 0.16	65 ± 1.12	5.89 ± 0.88	0.902 ± 1.25
STMTFrF2	Reddish-Brown/ Characteristic	1.99 ± 0.09	65 ± 1.14	6.01 ± 1.92	0.901 ± 1.32
Mean±SD[6]	-	1.41 ± 0.13	67 ± 0.78	5.6 ± 0.78	0.898 ± 0.98

Table 3. Results of physico-chemical analyzes of mother tinctures (MT) prepared from leaves or fruits of species of *S. terebinthifolia*.

[1]Extractive Form: STMTLH: *S. terebinthifolia* MT leaf (hermaphrodite plant); STMTLF: *S. terebinthifolia* MT leaf (female plant); STMTLM: *S. terebinthifolia* MT leaf (male plant); STMTFrH: *S. terebinthifolia* MT fruit (hermaphrodite plant); STMTFrF1: *S. terebinthifolia* MT fruit (female plant 1); STMTFrF2: *S. terebinthifolia* MT fruit (female plant 2).

[2]S.R. (%) ± SD: solid residue (S.R.) (%) ± standard deviation (SD).

[3]EtOH content ± SD: mean of determination of final alcohol content of mother tincture ± standard deviation (SD).

[4]pH ± SD: mean of determination of hydrogen potential (pH) of mother tincture ± standard deviation (SD).

[5]Dens. ± SD: mean of determination of density of mother tincture ± standard deviation (SD).

[6]Mean ± SD: mean ± standard deviation (SD).

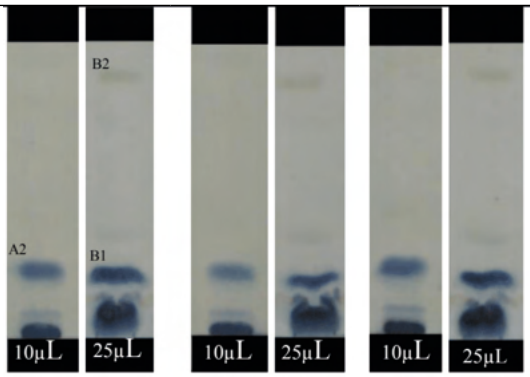
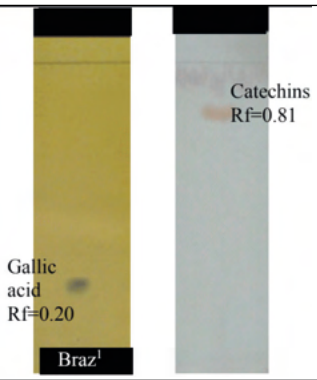
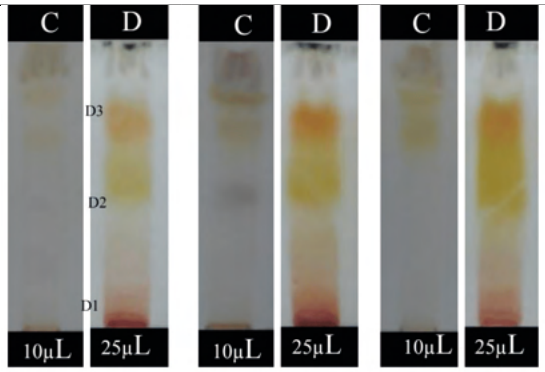
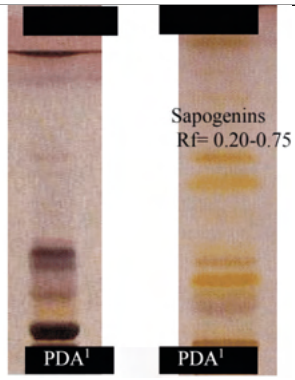
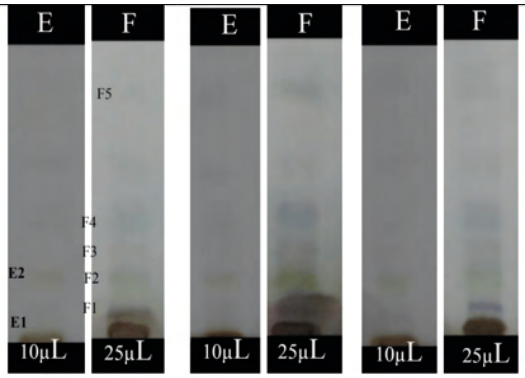
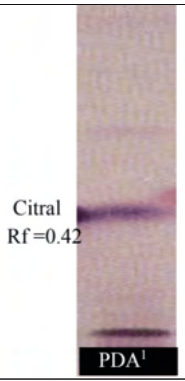
Chromatographic Systems	*S. terebinthifolia*			Literature (standard plate)[1]
	STMTLF[2]	STMTLH[3]	STMTLM[4]	
Gallic acid ES[9]: (1) CA[10]: (A) Rf[8]: A1 – 0.22 A2 – 0.38 B1 – 0.38 B2 – 0.87 Literature[1]: aroeira. Braz et al. (2011).				
Saponins ES[9]: (2) CA[10]: (B) Rf[8]: C – There was no significant run. D1 – 0.12 D2 – 0.50 D3 – 0.82 Literature[1]: (PDA) Wagner and Bladt (1996).				
Terpenes ES[9]: (3) CA[10]: (C) Rf[8]: E1 – 0.16 E2 – 0.22 F1 – 0.16 F2 – 0.22 F3 – 0.32 F4 – 0.41 F5 – 0.84 Literature[1]: (PDA) Wagner and Bladt (1996)				

Table 4. Determination of the chromatographic profile by TLC, for gallic acid/catechins, saponins, terpenes, flavonoids, of mother tinctures (MT) prepared from leaves or fruits of *S. terebinthifolia*.

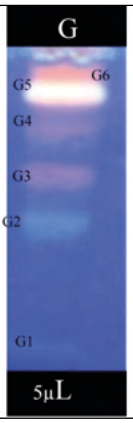
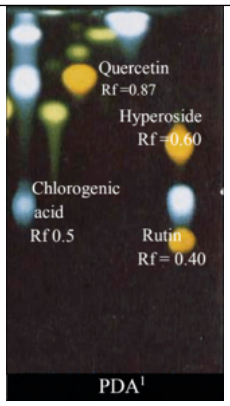
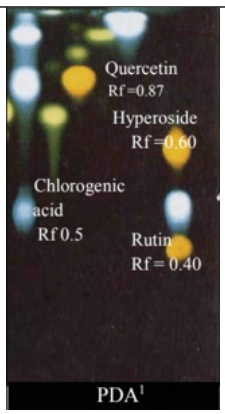
	STMTLF[2]	STMTLH[3]	STMTLM[4]	
Flavonoids ES[9]: (4) CA[10]: (D) Rf[8]: G1 – 0.12 G2 – 0.47 G3 – 0.62 G4 – 0.75 G5 – 0.87 G6 – 0.94 Literature[1]: (PDA) Wagner and Bladt (1996).				

Table 4. (continuation)

	STMTFrH[5]	STMTFrF1[6]	STMTFrF2[7]	
Flavonoids ES[9]: (5) CA[10]: (D) Rf[8]: H1 – 0.87 Quercetin Literature[1]: (PDA) Wagner and Bladt (1996).				

[1]Literature: PDA (Potato Dextrose Agar): Wagner and Bladt (1996) and Braz et al. (2011).

[2]STMTLF: *S. terebinthifolia* mother tincture leaf (female plant).

[3]STMTLH: *S. terebinthifolia* mother tincture leaf (hermaphrodite plant).

[4]STMTLM: *S. terebinthifolia* mother tincture leaf (male plant).

[5]STMTFrH: *S. terebinthifolia* mother tincture fruit (hermaphrodite plant).

[6]STMTFrF1: *S. terebinthifolia* mother tincture fruit (female plant 1).

[7]STMTFrF2: *S. terebinthifolia* mother tincture fruit (female plant 2).

[8]Rf: retention factor.

[9]ES: eluent system (v/v).

[10]CA: chromogenic agent.