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Quality Control of *Baccharis trimera* (Less.) DC. *(Asteraceae)* Hydroalcoholic Extracts

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SUMMARY. The aerial part of *Baccharis trimera* (Less.) DC.*(Asteraceae)*, used as the raw material for the preparation of phytotherapeutic drugs, was analysed by botanical, chemical, physico-chemical and technological methods. An hydroalcoholic extract was developed and its quality was compared to an aqueous extract by physico-chemical and sensory tests. Paper, thin-layer, vacuum liquid and high performance liquid chromatography were employed in order to determine the quality of the extracts. For these purposes, eupatorin and 3-O-methyl-quercetin were used as markers. With the exception of paper chromatography, the other chromatographic techniques proved to be adequate quality control methods for evaluation of *Baccharis trimera* extracts.

RESUMEN. "Control de calidad de extractos hidroalcohólicos de *Baccharis trimera* (Less.) DC.*(Asteraceae)*". La parte aérea de *Baccharis trimera* (Less.) DC. utilizada como materia prima vegetal en la preparación de productos fitoterápicos, fue analizada por métodos botánicos, químicos, físicoquímicos y tecnológicos. Se preparó el extracto hidroalcohólico y se comparó su calidad con la de un extracto acuoso, a través de ensayos sensoriales y físicoquímicos. Para determinar la calidad de los extractos se empleó cromatografía en papel, en capa fina, líquida al vacío y líquida de alta resolución, utilizándose como sustancias marcadoras la eupatorina y la 3-O-metilquercetina. Con excepción de la cromatografía en capa fina, el resto de las técnicas cromatográficas mencionadas demostraron ser adecuadas para el control de la calidad de extractos de *Baccharis trimera.*

INTRODUCTION

Baccharis trimera (Less.) DC. *(Asteraceae)*, known as "carqueja", is used widely in Brazil and neighbouring countries for both nutritional and medicinal purposes, either as industrialised products or as popular herbal preparations. *B. trimera* shows hepatoprotective [1], antiviral [2] and anti-inflammatory activities [3]. Its effects on glycaemia and insulinaemia levels were tested by Bragança [4,5].

The presence of flavonoids has been reported by several authors [6-8]. The plants also contain *ent*-clerodan [6,7] and *neo*-clerodan [9] diterpene lactones, tetra-*nor*-diterpene lactones [8], and a volatile oil consisting of carquejol [10,11], carquejil acetate, canphene, and α- and β-pinene [12].

Although the Brazilian pharmaceutical market includes several products containing the isolated drug or its extracts [13], there are almost no published results of studies concerning production and quality control aspects and aimed at development of a phytotherapeutic product, as presently defined in Brazil [14]. The single exception is Mello [15], who has developed quality control techniques covering the entire processing cycle of hydroalcoholic extracts of the aerial parts of *B. trimera,* and has also evaluated the effect of several production factors on the characteristics of the extractive solutions.

In view of the lack of such information, the present work presents some considerations and results regarding the quality evaluation of *B. trimera,* not only as pharmaceutical raw material, but also during the technological development of its derived products.

PALABRAS CLAVE: *Asteraceae*, *Baccharis trimera*, Control de Calidad, Métodos Cromatográficos, Tecnologia.
KEYWORDS: Asteraceae, Baccharis trimera, Chromatographic methods, Quality control, Technology.

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MATERIAL AND METHODS

Plant Drug

The drug was collected in its flowering period (August) in "Sertão Santana", Rio Grande do Sul State, Southern Brazil (30° 28' 31" S and 51° 35' 25"W), and identified by Professor Dr. Lilian Auler Mentz of the Bioscience Institute of UFRGS. The voucher specimen is deposited under the number 67.228 at the ICN Herbarium in Porto Alegre. The rough-cut herb was dried in a circulating air oven at 35 °C, for nine days, and then ground in an hammer mill (Retsch, sieve nr. 1). The milled drug was then stored in the dark in paper bags at room temperature.

Chromatographic Analyses

Paper chromatography (PC) was carried out using sheets (20x20 cm) of common filter paper (138 g/m^2) in a saturated chamber with several eluent systems [15].

For thin-layer chromatography (TLC), silicagel 60 GF254 (Merck) and microcrystalline cellulose (Sigma) were used, with the aid of a Desaga apparatus to prepare 0.25 mm thick layers on glass plates (20x20 cm).

The eluent systems for the paper and thin-layer chromatographic techniques will be described when necessary. The detection reagents were: 10% (w/V) solution of ceric sulphate in sulphuric acid 2M (R1), 3% (w/V) ethanolic solution of sulphuric vanillin (R2), oxalo-boric solution (R3), and 1% (w/V) methanolic solution of ferric chloride (R4). After being sprayed with R1 and R2, the plates were heated at 110 °C for 5 to 10 min. R3 and R4 were used on cellulose plates without subsequent handling.

The high-performance liquid chromatography (HPLC) analyses were carried out in a liquid chromatograph, model CG- 480 C, with a 20 µL loop Rheodyne injector; a CG-435 variable wavelength detector; and a CG-300 integrator-processor. A RP-18 Hibar column, 5 µm (250 x 4 mm; Merck) was used, having as the mobile phase methanol-phosphoric acid 1% (40:60), both LiChrosolv® (Merck), at a flow of 2.0 mL/min. Detection was carried out at 342 nm.

For the vacuum liquid chromatography (VLC), the apparatus shown in Fig. 1 was used. Nine grams of silicagel 60 for column chromatography (mesh 70-230, Merck) were initially dry-packed into the separation tube closed with a sintered glass (Duran 50, D2). To 1 g of the same chromatographic support, 20.0 mL of the hydroalcoholic turbo-extract were added, following evaporation in a water-bath, and then added to the upper part of the column [16,17]. Elution was performed under negative pressure (600 mm Hg) with 10 mL portions of each mobile phase, in increasing order of polarity: cyclohexane; cyclohexane-hexane (1:1); hexane; hexane–toluene (1:1); toluene–chloroform (20:1), (17:1), (14:1), (8:1), (5:1), (4:1), (3:1), (2:1); chloroform; chloroform–ethyl acetate (20:1), (17:1), (14:1), (8:1), (5:1), (4:1), (3:1), (2:1); ethyl acetate. Each fraction was collected individually.

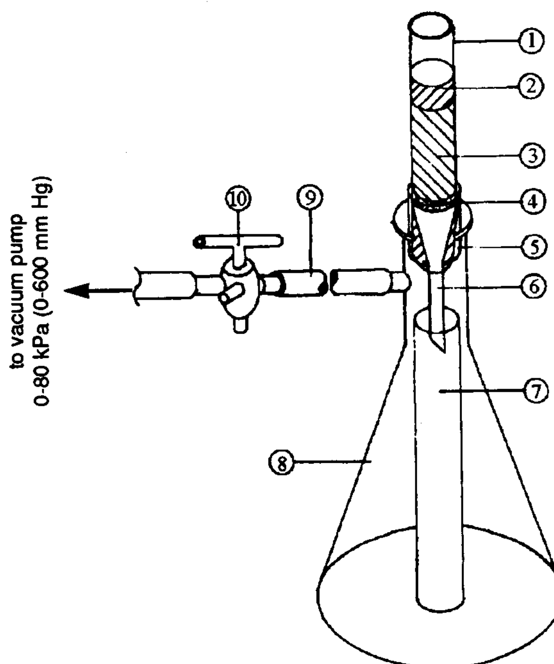

Figure 1. Laboratory VLC apparatus. **1**. Sinteres glass Büchner filter funnel. **2**. Substrate, after absorption on support. **3**. Adsorbent (silicagel 60, mesh 70-230, Merck). **4**. Fritted disk (ASTM Duran 50 D-2). **5**. Rubber gasket. **6**. Funnel. **7**. Test tube, 50 mL. **8**. Kitasato flask (1000 mL). **9**. Rubber tubing. **10**. Three-way stopcock.

Characterization of the Drug

Next, the raw drug material was evaluated according to: a) loss on drying [18,19]; b) volatile oil content [18]; c) saponin test through foam formation assay [19]; d) determination of extractives [19]; e) total flavonoids content, calculated as quercetin [20]; and f) granulometric analysis, by sieving [21].

Preparation of Extractive Solutions and their Purification

The hydroalcoholic turbo-extract was prepared in a proportion of 10% by weight of milled drug in relation to the 50% (V/V) hydroalcoholic extractive medium, in a closed container for 20 minutes, maintaining the tem-

perature lower than 40 °C (Ultraturrax T4, Janke). The extractive solution was dried under low pressure (Rotavapor, Büchi) and washed with 15 mL each of ethyl acetate and chloroform. This dried, purified residue was placed in 10.0 mL of methanol and used for chromatographic analyses. For comparative evaluation, a 10% (w/V) aqueous extract was prepared by decoction according to the Brazilian Pharmacopoeia [18], and passed through the same purification steps as the turbo-extract.

Evaluation of the Extractive Solutions

The following methods were used: a) determination of bitterness [18]; b) determination of foaming index [18]; c) pH determination [18]; d) dry residue [19]; and e) ethanol content of the turbo-extract [18].

RESULTS AND DISCUSSION

The acceptance of a plant-derived drug as pharmaceutical raw material presupposes the existence of well-established quality specifications. These have never been established for *Baccharis trimera*. Therefore, standard pharmacognostical and pharmacopoeial control techniques were used in order to develop such specifications, in a attempt to ensure a product with constant quality. Loss on drying, indicative of water content, for the fresh drug was of 33.24 + 0.97%, while for the dried was 5.75 ± 0.06%. Storage of the dried drug at room temperature resulted in increased loss on drying until it reached equilibrium, after 9 months, at 10.44 ± 0.12% water. This technique measures not only the loss of residual water from the drug, but also the loss of volatile substances. For this reason the analysis of volatile substances must be taken into account. From the technological point of view, loss on drying may indicate the efficiency of the drying operation, in this case about 80%. The maintenance of the water content during storage indicates that *B. trimera* is a stable drug under ambient with high relative humidity.

The presence of volatile oils can be considered not only as an indication of the drug's quality, but also of its stability during storage. The volatile oil content was 3.13% in the fresh drug, and the drying process did not significantly alter this value. Nevertheless, storage under ambient conditions caused a significant decrease in the quantity of volatile oils, reaching stability after the fifth month of storage, at a concentration of about 0.68%.

The saponin test resulted in formation of a 1.2 cm high foam layer, which persisted for 10 min. and did not disappear after addition of 2M HC1, in agreement with previous observations [22].

In quality control of raw plant material, it is important to determine the extractive content. This can be considered as a characteristic of the drug quality, either as raw material or during its processing cycle. The quantity of water-extractable substances for the plant drug was 26.05% (w/w).

Although flavonoid is not the only class of substances that might be responsible for the activity of *B. trimera,* its broad pharmacological effects are well known. Therefore it can be considered as an adequate quantitative marker for the drug. The raw material used in this study had a total flavonoid content of 0.71 ± 0.021% (w/w), calculated as quercetin. The flavonoid content should be measured at the time of extraction, in order to obtain a final extract with the desired concentration.

The extraction yield is greatly influenced by granulometry of the raw plant material. To characterise the milled drug, the retention and passage curves were determined. The mean diameter (d_{50}) could then be calculated, and the granulometric homogeneity inferred from the slope of the curve. The milled *B. trimera* used in this study had a d_{50} of 0.640 mm and a slope of 36º52', suggesting that the granulometric classes were distributed homogeneously. The presence of particles over d_{50} influences negatively the flavonoid yield, as shown by Petrovick and Mello [23] for *B. trimera.*

For evaluation of the quality of extracts obtained by turbo-extraction and by decoction, a bitterness test was performed *(B. trimera* is known to be very bitter). The calculated index, using brucine as the reference substance, was 43.0 for the turbo-extract and 31.3 for the decocte. The higher value for the turbo-extract might also have been caused by the presence of ethanol, which is known to injure the mucous cells, sensitising the lingual papillae and consequently reducing the threshold for bitterness. However, this sensory test, even allowing errors from the individual variability of the people tested, resulted in a high coefficient of variability, and did not produce statistically reliable information.

The foam test, used to characterise the raw plant material, was also used to check for possible differences between the two extraction procedures. For the decocte it was 141 ± 70.44, and for the turbo-extract it was lower than 100. The

difference between the two extracts is probably resulted from the presence of ethanol, known for its anti-foaming property [24], in the turbo-extract. The high variability of the results did not permit any conclusion regarding the influence of the technological parameter studied.

The pH values of the turbo-extract and decocte were respectively 6.02 ± 0.12 and 5.12 ± 0.006. Considering that the water initially had a pH of 6.07 and the hydroethanolic mixture 6.73, in both cases the extracted constituent stimulated a slight acidification of the corresponding extracts.

One method of measuring extraction efficiency is to determine the dry residue. Comparison between the values obtained for the turbo-extract (29.95 ± 0.37%; w/w) and for the decocte (14.05 ± 0.16%; w/w) showed that the turbo-extraction in hydroalcoholic medium gave a superior yield. Another advantage of this method is the absence of heat, which protects the constituents of the extract from possible thermal degradation.

The final ethanol content of the turbo-extract was about 10% of the initial solvent mixture value (40%). This could be explained by evaporation of ethanol during the extraction, in spite of all the care taken during the procedure. Transfer of the residual moisture (10.14%) in the drug to the extraction medium could also explain this result.

The evaluation of the quality of the extractive solutions was complemented by additional tests, analysing qualitatively and quantitatively the original constituents of the plant raw material.

For the volatile oils, a clear separation was obtained using TLC on silicagel 60 GF254, with benzene(methanol (15:1) as the mobile phase and R2 as the detection reagent.

The analysis by PC and TLC on cellulose did not show resolution for the separation of the marker substances, either for the turbo-extract or for its purified residue. TLC on silicagel 60 GF254 using toluene-ethyl acetate-methanol (75:25:5) as the mobile phase, showed the best resolution among the tested systems tested. Eupatorin showed a Rf = 0.60 and 3-0-methyl-quercetin 0.34. The corresponding spots in the hydroalcoholic showed Rf values of 0.57 and 0.35, respectively.

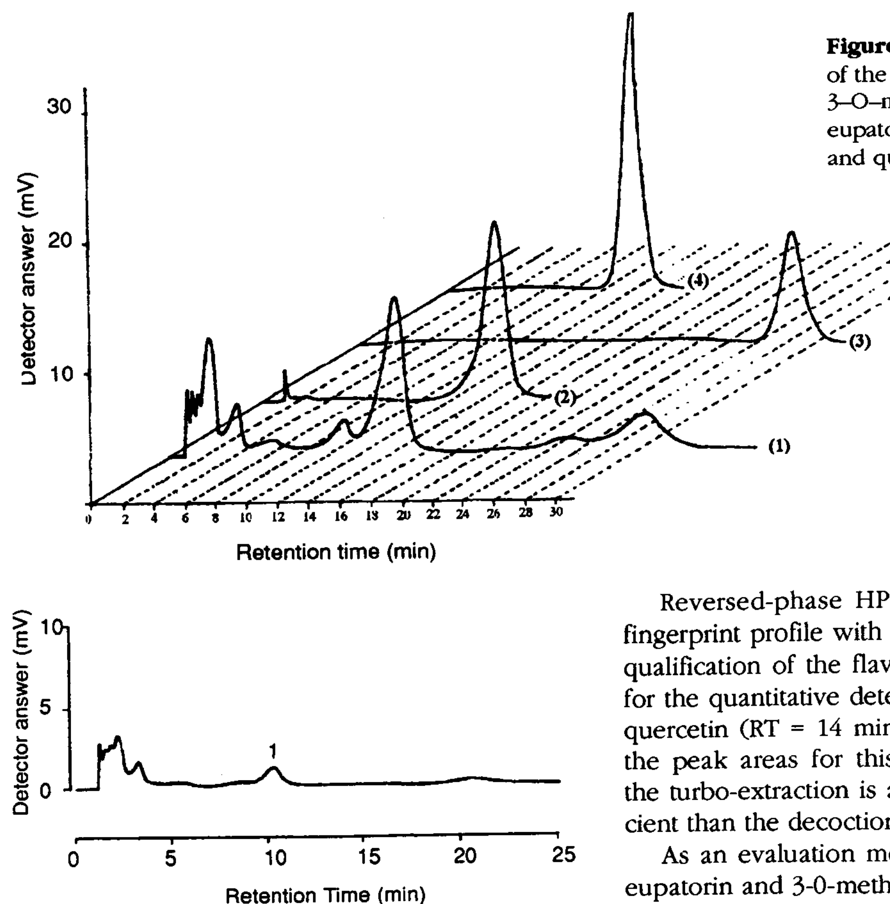

Figure 2. Reversed phase HPLC of the turbo extract (**1**), 3-O-methyl-quercetin (**2**), eupatorin (**3**), and quercetin (**4**).

Figure 3. Reversed phase HPLC of the decocte [3-O-methyl-quercetin (1)].

Reversed-phase HPLC allowed to obtain a fingerprint profile with acceptable resolution for qualification of the flavonoid markers, and also for the quantitative determination of 3-0-methyl-quercetin (RT = 14 min; Fig. 2). Comparison of the peak areas for this substance showed that the turbo-extraction is about 19 times more efficient than the decoction (Fig. 3).

As an evaluation method of the presence of eupatorin and 3-0-methyl-quercetin in the turbo-extract, vacuum liquid chromatography monitored by TLC was introduced. The first eluted

substances were detected when using chloroform-ethyl acetate (17:1), corresponding to fraction 16. Eupatorin was eluted separately in fractions 19 and 20, and mixed with 3-0-methylquercetin in the fraction 21. 3-O-methylquercetin was found as an isolated substance in fractions of greater polarity (fractions 22 and 23).

CONCLUSIONS

Although there are no studies establishing limits of acceptance or quality criteria for *Baccharis trimera,* the practicality of evaluating its quality by physical and chemical methods was demonstrated. The techniques employed also proved suitable for control of technological processing of extracts and final products, which allows to suggest their applicability as part of total quality control of phytotherapeutic products.

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