FUNCTIONAL PROPERTIES OF SUPERCRITICAL EXTRACT FROM YLANG-YLANG (*Cananga odorata*) PEEL FRUIT

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Many studies in recent years confirm that bioactive compounds have properties that are beneficial to human health, with potential physiological effects such as anticancer, vasoprotective, anti-inflammatory, and hepatoprotective, among others. On the other hand, the reported harmful effects of synthetic antioxidant compounds, that has lead the World Health Organization to imposed restrictions to their use and the population awareness of ecological issues has stimulated the research on food, drug and cosmetic industries. In this work, supercritical extracts from Ylang-Ylang (*Cananga odorata*) peel fruit were evaluated in terms of anthocyanin and total phenolic contents and antioxidant activity.

Key words: Supercritical fluid extraction, antioxidant activities, *Cananga odorata*, anthocyanins.

INTRODUCTION

Natural extracts are in increasing demand from the manufacturers of foods, cosmetics and pharmaceuticals [1]. The increasing demand for natural food antioxidants and "clean" additives has fostered worldwide research for extracting biologically active substances from a variety of vegetal raw materials [2]. Parallel, in recent years the development of new separation techniques has gained increasing importance in this industries due to the imposed of solvent-free environmental and public health regulations [2-4].

For this reason, the use of supercritical fluid extraction (SFE) appears as an advantageous technology [2,3] and has become an alternative to conventional extraction procedures, since this technique eliminates the problem of residuals solvents and allows the use of lower temperatures, which reduce the deterioration of thermally labile compounds from vegetable sources [2,3,5]. This technology exploits the high solvation power, low viscosity and high mass diffusivity of supercritical fluids [6]. SFE has been shown to be a technically viable process to obtain a series of high quality extracts [7], and more recently, it was also shown as an economically viable process [8-10].

Supercritical CO_2 (Pc = 7.28 MPa; Tc = 304.1 K) is the most frequently used solvent for SFE. It is the most appropriate for food, cosmetic and pharmaceutical industries because of its physicochemical properties (higher diffusivity, lower viscosity, and lower surface tension than conventional solvents) which facilitate mass transfer, and because its nontoxic and nonflammable character, environmental safety, huge availability, low cost at high purity, among others [2-4,7,11].

Though industrial applications have been already developed, this process is far from to be considered exhaustively studied due to the structural complexity of compounds that can be

extracted and the large variability of the raw materials to be treated [12]. Because of this, the production of antioxidant extracts from vegetal material is a research field of increasing importance [2].

Ylang-ylang (*Cananga odorata*) belongs to the Annonaceae family [13]. Of Southeast Asian origin, ylang-ylang is often found growing spontaneously in secondary forests and agro forests, where it regenerates easily [14], distributed in both tropical and subtropical regions [15]. This medium-size tree has multiple uses. The wood is often used for canoe parts, small canoes, furniture, and fuelwood. The distillate oils of the flowers are used in perfumes, shampoos, creams, and lotions but also in ice creams, candies, and baked goods flavors [14,16]. Ylang–Ylang oil is used in the food industry as a flavor ingredient. It is approved as a food additive by Food and Drug Administration (FDA) and has been determined to be Generally Recognized as Safe (GRAS) by Flavor and Extract Manufacturers Association (FEMA) [17].

In traditional medicines the decoction of bark is used in the treatment for rheumatism, ulcers and fevers and also as an anti fungal [18] and amebicidal agent [18,19] and used to treat stomach diseases and sometimes as a laxative [14]. The dried flowers are used against tinea infections [15], fever [15,20] and malaria [14,20], and the fresh flowers are used in cephalalgia, ophthalmia, gout [20], and also to treat asthma. The distillate oils of the flowers is said to have medicinal value by herbalists and aromatherapists, which claim that the oil is useful for depression, anxiety, distressed breathing, high blood pressure, as an aphrodisiac, etc [14] and to relief from frigidity, hypertension, palpitations, stress and as antiviral, antifungal and amebicidal, anti-seborrheaic, antiseptic activities [18].

Phytochemical investigations of Annonaceae revealed that the flowers, leaves, branches, barks and fruits from *C. odorata* species contain highly biologically active principles including alkaloids, terpenoids, flavonoids, acetogenins, coumarins, volatile oils, styryl lactones and other oxygen containing heterocycles [13,15,16,18,21-23] with proved functional properties as antifungal [24], antimicrobial [20], cytotoxic [15,20], minimum inhibitory concentrations (MIC) [18], antioxidant activity [1,25] that may be considered as potential lead compounds for the development of new drugs. In an investigation of potential cytotoxic constituents from the fruits of *Cananga odorata*, Hsieh et al. [15] make the general statement that ‘‘plants of the genus *Cananga* are rich in alkaloids and terpenoids.’’ The entire fruits of *Cananga odorata* gave a monoterpenic essential oil in which sabinene (34.3%), myrcene **(**24.7%), *α*-pinene (1 1.1 %) and terpinen-4-ol (8.0%) were the most abundant using hydrodistillation extraction [23].

In this context, the purpose of this work was to evaluate the potentiality of supercritical extracts from Ylang-Ylang (*Cananga odorata*) peel fruit. The extracts were evaluated in terms of anthocyanin and total phenolic contents and antioxidant activity. In addition, it was evaluated the effect of pressure on the extraction yield, antioxidant activity and extract composition. To our knowledge, this is the first report of the use of supercritical fluid extraction (SFE) using Ylang-Ylang peel fruit as vegetable source of functional compounds.

MATERIALS AND METHODS

Plant Material

Ylang-Ylang fruit (*Cananga odorata*) were acquired from Agronomic Institute of Campinas (IAC) - Campinas/SP/Brazil. Immediately after acquiring, the fruit were stored in the dark in a domestic freezer (-10 ºC) (Double Action, Metalfrio, São Paulo, Brazil) until sample preparation. Before extraction, the fruit were manually peeled and Ylang-Ylang peel

fruit were dried using a forced air circulation dryer (Fanem, Guarulhos, Brazil) at 318.15 K for 24 hours. The humidity of the dried peel was determined by the AOAC method [26].

Supercritical fluid Extraction

Dried peel fruit of Ylang-Ylang were manually cut into fine particles. Approximately, five grams (12.95 ± 0.05 % of humidity) were used for the SFE. The solvent used for the extraction was carbon dioxide (CO_2) (99.5%, Gama Gases Especiais, São Paulo, Brazil). A temperature (313 K) and two pressures (15 and 30 MPa) combination was set. The CO_2 flow rate was 8.33×10^{-5} kg/s and an 5 cm^3 extraction column was used.

Extract Characterization

Antioxidant Activity (AA)

The evaluation of antioxidant activity of the extracts was based on coupled oxidation of β-carotene and linoleic acid. The technique developed by Marco [27] consisted of measuring the bleaching of β-carotene resulting from oxidation by degradation products of linoleic acid. One milligram of β-carotene (97 %, Sigma-Aldrich, St. Louis, USA) was dissolved in 10 cm^3 chloroform (99 %, Ecibra, Santo Amaro, Brazil). The absorbance was tested after adding 0.2 cm^3 of the solution to 5 cm^3 of chloroform, then reading the absorbance of this solution at 470 nm using a UV–Vis spectrophotometer (Hitachi, model U-3010, Tokyo, Japan). A reading between 0.6 and 0.9 indicated a workable concentration of β-carotene. One mililiter of β-carotene chloroform solution was added to a flask that contained 20 mg of linoleic acid (99 %, Sigma-Aldrich, St. Louis, USA) and 200 mg Tween 40 (99%, Sigma-Aldrich, St. Louis, USA). Chloroform was removed using a rotary evaporator (Laborota, model 4001, Viertrieb, Germany), with vacuum control (Heidolph Instruments Gmbh, Viertrieb, Germany) and thermostatic bath at 40 °C; then 50 cm^3 of oxygenated distilled water (oxygenation for 30 minutes) was added to the flask with vigorous agitation to form an emulsion. Five mililiter of the emulsion was added to 0.2 cm^3 of the antioxidant solution (7.5 mg of extract/1 cm^3 of ethanol) in assay tubes; (to the control solution 0.2 cm^3 of ethanol was added). A blank consisting of 20 mg linoleic acid, 200 mg Tween 40 and 50 cm^3 oxygenated distilled water was used to bring the spectrophotometer to zero. Tubes were manually shaken and absorbance measurements made at 470 nm immediately after the addition of the emulsion to the antioxidant solution. The tubes were placed in an water bath (model TE 159, Tecnal, Piracicaba, Brazil) at 50 ºC. Absorbance measurements were made at 30 minutes intervals during 3 hours. The antioxidant activities (AAs) were calculated by equation (1) [28]:

$$AA(\%) = 100X\left(1 - \frac{Abs_{extract}^{t=0} - Abs_{extract}^{t}}{Abs_{control}^{t=0} - Abs_{control}^{t}}\right) \quad (1)$$

Anthocyanin Content

The pH differential method described by Giusti and Wrolstad [29], which relies on the structural transformation of the anthocyanin chromophore as a function of pH, was used to identify the presence and determined the anthocyanin content. A UV–Vis spectrophotometer (Hitachi, model U-3010, Tokyo, Japan) was used for spectral measurements at maximum absorbance wavelength and 700 nm, using distilled water as a blank. For this purpose, 20 mg of extract was dissolved in 10 cm^3 of distilled water. Two dilutions of the sample were prepared: one with hydrochloric acid/potassium chloride buffer pH = 1.0 and the other with sodium acetate/acetic acid buffer pH = 4.5. The pH values of the buffers were measured using

a pH-meter (Digimed, model DM-22, São Paulo, Brazil) calibrated with buffers at pH 4.01 and 6.86 and they were adjusted with HCl (99.5 % Ecibra, Santo Amaro, Brazil). Aliquots of extract were brought to pH 1.0 and 4.5; 15 min later, the absorbance scan were then made from 800 to 190 nm in order to identify the presence and determined the anthocyanin content.

Total Phenolic Content

Total phenolic content was estimated using the Folin-Ciocalteau method for total phenolics, based on a colorimetric oxidation/reduction reaction of phenols [30]. Briefly, 1 cm^3 of Folin and Ciocalteu's phenol reagent was added to 1 cm^3 of sample (0.75 mg of extract/1 cm^3 of a solution containing 10% v/v of ethanol and 90% v/v of distilled water) in assay tubes. After 3 min, 1 cm^3 of saturated sodium carbonate solution (50 % w/w) was added to the mixture and the volume adjusted to 10 cm^3 with distilled water. The reaction was kept in the dark for 90 min at room temperature, after which the absorbance was read at 725 nm with a UV–Vis Spectrophotometer Hitachi, model U-3010 (Tokyo, Japan). For control sample, 1 cm^3 of the solution containing ethanol and distilled water was taken. The results were calculated on the basis of the calibration curve of gallic acid (GA) and expressed as milligrams of gallic acid equivalents (GAEs)/g of extract.

Thin-Layer Chromatography (TLC)

The supercritical extracts were fractionated by thin-layer chromatography (TLC). The TLC was performed using silica plates (20 x 20 cm, 1-mm height, Merck, Darmstadt, Germany) and three different sprays to identify the composition of the extracts. The mobile phase used was composed by hexane 70% (96%, P.A., Merck, Darmstadt, Germany) and ethyl acetate 30% (99.5%, P.A., Merck, Darmstadt, Germany). To observe the compounds of the volatile oil on the visible and ultraviolet (365 nm) light spray of anisaldehyde solution was used. To observe compounds as flavonoids on the ultraviolet light (365 nm) the spray solution of 2-aminoethyl diphenylborinate was 1% (NP) (Sigma, lot 123k2512, U.S.A.) in methanol. Finally, to observe alkaloids on ultraviolet (254 nm) light no treatment was done and to observe alkaloids on visible light the spray solution of Dragendorff was used [31].

Statistical Analysis

For establishing the statistical significant differences or similarities between the values of total phenolic content, the Tukey's test was used. A confidence coefficient of 95 % was used for the comparison of all the mean's pairs.

RESULTS

The effect of pressure on the extraction yield, antioxidant activity, and extract composition was evaluated. Two pressures (15 and 30 MPa) were studied.

It is well known that at a constant temperature an increase of pressure (increasing the carbon dioxide density) cause, in general, an increase in the extraction yield. Nonetheless, this phenomenon was not observed in this work (Table 1). At 313 K an increase in pressure caused a decrease in the extraction yield. This behavior has been observed in other works being this phenomenon associated with the retro-degradation behavior of compounds in pressurized solvents [9].

The knowledge of the influence of pressure and temperature conditions on the extraction yield is important when an economic evaluation of the industrial process is desired.

But the choice of the best condition obeys mainly to other considerations, like biological activities of the products to be obtained.

In table 1 are shown the values of the antioxidant activity in percentage of the extracts obtained at 15 MPa/313 K and 30 MPa/313 K. In this figure can be seen that the lower pressure resulted in an extract with higher antioxidant activity. The pure standards' antioxidant activity presented higher values than the reported for the extracts, as expected. These values were, approximately: 96.89 % for the BHT (a synthetic antioxidant) and 74.45 % for the Quercetin. Comparing the results of the extracts with the standards it is verified the potential of the supercritical extract obtained, mainly, at 15 MPa as a source of natural antioxidants.

The study of the composition of vegetable extracts has demonstrated that usually different extraction conditions introduce different combinations of bioactive compounds in the extract, resulting in different functional properties.

Although the Ylang-Ylang peel fruit present a strong green-black coloration, anthocyanins, one class of phenolic compounds, were not detected in these extracts (Table 1) On the other hand, the extracts presented other phenolic compounds. The correlation between total phenolic content and antioxidant activity was explored. An apparently correlation was found between them. This possible correlation could indicate that higher values of antioxidant activities are related to higher yields of phenolic compounds.

Dragović-uzelac et al. [32] observed a direct correlation between phenolic content and antioxidant capacity of several fruit extracts. Two different methods were used for measuring the antioxidant capacity, ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay] and ORAC (oxygen radical absorbance capacity), obtaining also a significant correlation between them.

Table 1: Effect of pressure (15 and 30 MPa) on the extraction yield, antioxidant activity and extract composition.

Extraction Condition	Extraction Yield (%) (dry weight)	Antioxidant Activity (%) after 3 hours	Anthocyanin Content	Total Phenolic Content (mg GAEs/g of extract
15 MPa/313 K	3.04	53.67	Not detected	63.44
30 MPa/313 K	2.16	29.26	Not detected	61.25

When comparing the average values of the total phenolic content of the extracts obtained at the different pressures, the Tukey's test found that the difference between them is significant, demonstrating that pressure affected the recovery of phenolic compounds. In contrast, this difference is not as large as the difference between the antioxidant activities of the same extracts. Thus, this fact indicates a possible presence of other compounds with antioxidant properties.

Correlations between the compound content in the material submitted to tests and the antioxidant activity are not easy to explain [33]. According to Skerget et al. [34], synergism may occur when different antioxidant compounds are present.

Therefore, in order to verify the presence of other compounds in the supercritical extracts they were fractionated by thin-layer chromatography (TLC).

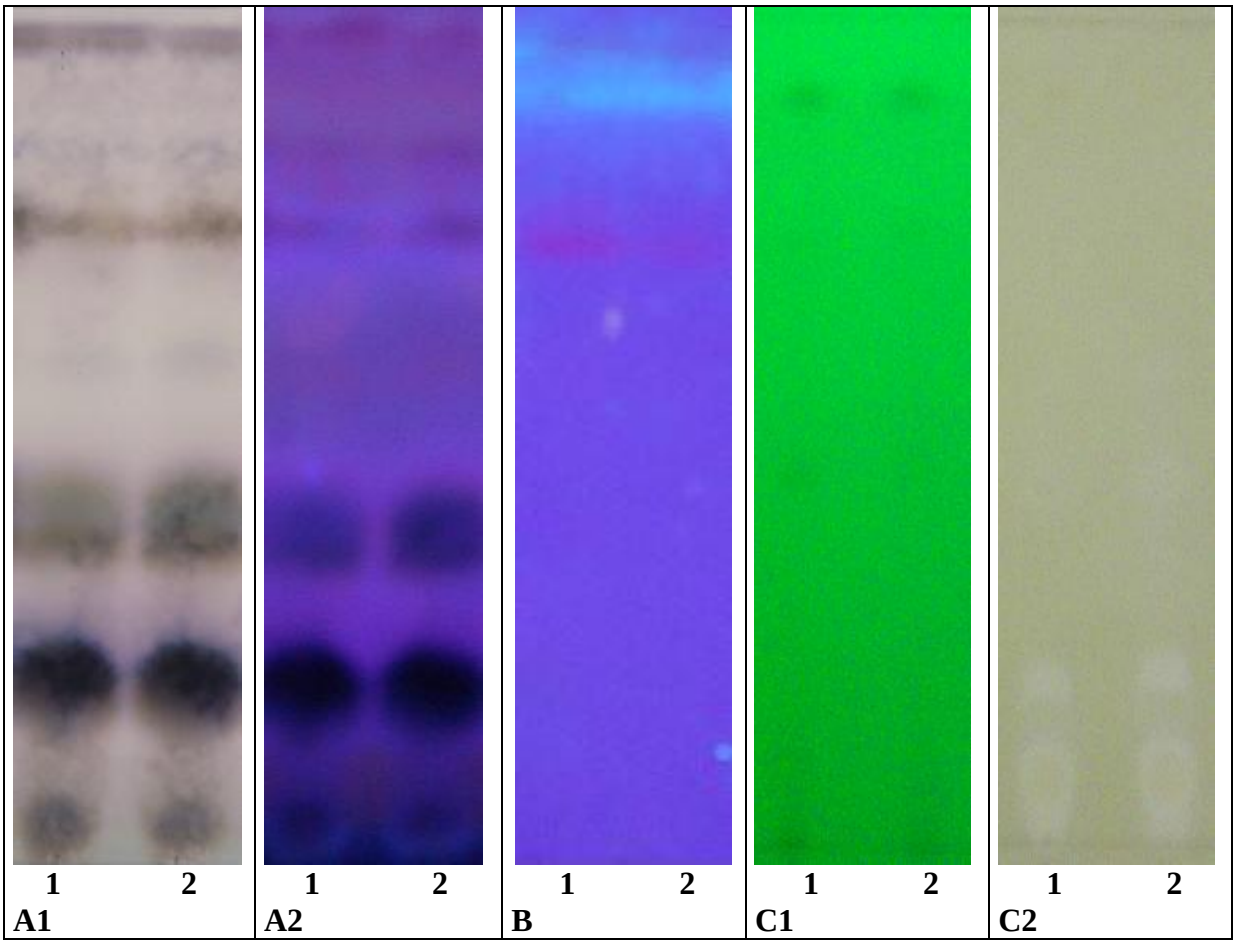

Figure 1: Thin-layer chromatography (TLC) plates (1 – Extract obtained at 15 MPa/313 K; 2 Extract obtained at 30 MPa/313 K; A1 - Revealed using anisaldehide-sulphuric acid reagent on visible light; A2 - Revealed using anisaldehide-sulphuric acid reagent on ultraviolet light (365 nm); B - Revealed using Natural products (NP) reagent on light (365 nm); C1 - Without treatment on ultraviolet light (254 nm); C2 - Revealed using Dragendorff reagent on visible light.

Figure 1 shows that both the extracts present similar phytochemical profile in the TLC plate indicating that pressure did not affect the extract composition qualitatively.

Essential oils are volatile, odorous principles consisting of terpenes alcohols, aldehydes, ketones and esters (> 90%) and/or phenylpropanoid derivatives [31]. The anisaldehyde-sulphuric acid reagent revealed (Figure 1-A1) four to five brown, green, grey or violet zones that can be terpenoids, phenylpropanoids, pungent and bitter principles, saponins due their coloration characteristics on visible light [31]. According to Wagner and Bladt [31], most of these compounds develop fluorescence under UV-365, which was observed for these extracts (Figure 1-A2). Boyom et al [23] observed that the hydrodistillation extract from Ylang-Ylang fruits gave a monoterpenic essential oil, therefore probably the compounds identified by TLC could be terpenoids.

Flavonoid extract often contain phenol carboxylic acids and coumarins that has characteristics to form blue fluorescent zones in UV-365nm [31]. Natural products (NP) reagent revealed blue fluorescence zones in Ylang-Ylang peel fruits extract near the solvent front, which can be seen in figure 1-B. According, Wagner and Bladt [33] depending on the structural type, flavonoids show dark yellow, green or blue fluorescence. Probably, among the

phenolic compounds present in the extracts the flavonoid class is the major one contributing to the antioxidant activity of them.

Most plant alkaloids are derivatives of tertiary amines, while others contain primary, secondary, or quaternary nitrogen [33]. In figure 1-C1, weak quenching zones were detected in UV-254nm near the solvent front without chemical treatment, and in figure 1-C2 minor yellow-orange zones were detected on visible light near the solvent start after treatment with Dragendorff reagent, indicating the presence of alkaloids in the two extracts according Wagner and Bladt [33]. Alkaloids also were found by Hsieh et al. [15] in the fresh fruit extracts from Ylang-Ylang.

Thus, the extract characterization revealed the presence of functional compounds such as terpenoids, flavonoids, and alkaloids. A synergistic effect between the phenolic compounds and other antioxidant compounds such as terpenoids and alkaloids could increase the antioxidant activity.

Moreover, a difference in concentration of terpenoids and alkaloids in the extracts can affect significantly the antioxidant capacity. Further experiments could measure if supercritical CO_2 at low pressures can extract higher concentration of these compounds and/or some types of these compounds with more antioxidant properties.

CONCLUSION

It was verified the potential of the supercritical extract obtained, mainly, at 15 MPa as a source of natural antioxidants.

At 313 K an increase in pressure caused a decrease in the extraction yield being this behavior associated with the retro-degradation behavior of compounds in pressurized solvents.

It was identified in the extracts terpenoids, flavonoids, and alkaloids and anthocyanins were not detected.

The correlation between total phenolic content and antioxidant activity was explored indicating that a synergistic effect between the phenolic compounds and other antioxidant compounds such as terpenoids and alkaloids could have increased the antioxidant activity.

ACKNOWLEDGEMENTS

The authors are grateful to CNPq for the doctorate fellowships (562766/2008-1, 580401/2008-1) and for the financial support.

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