Activity of Extracts Obtained from the Flowers, Leaves and Stems of *Spilanthes oleracea* (jambú) Using High Pressure Extraction

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Abstract

Spilanthes oleracea, commonly known as jambú, is an annual flowering herb that has been identified as a natural source of alkylamides. These compounds are responsible for the typical bioactivities of jambú as an anti-inflammatory, antiseptic and anesthetic agent, being *N*-isobutyl-2E,6Z,8E-decatrienamide or spilanthol, already identified as its main bioactive compound. Based on the medicinal and industrial potential of this plant, the purpose of this work was to characterize the extracts obtained by a fractionated extraction procedure that included a supercritical fluid extraction (SFE) step, using supercritical carbon dioxide ($scCO_2$) as solvent, followed by an enhanced solvent extraction (ESE) step using pressurized CO_2 with ethanol, water and their mixtures as solvent enhancers. Continuous high pressure extractions of the flowers, leaves and stems of jambú were performed at 25 MPa and 323 K. The different extracts obtained were characterized in terms of their total spilanthol yield and phenolic composition as well as antioxidant and anti-inflammatory activities. Results showed that the SFE process was particularly selective for spilanthol which was almost completely extracted during the 1st SFE step. However the 2nd ESE step, using polar solvents, permitted to extract higher molecular weight compounds, as phenolic compounds, in higher extents, originating extracts with higher antioxidant activities. Through principal component analysis it was possible to conclude that the flower SFE extracts outstand from the others in what concerns the spilanthol yield and the antioxidant/total phenolics ratio, indirectly suggesting that spilanthol plays an important role in the antioxidant activity of the extracts. The anti-inflammatory activity of this compound was demonstrated by showing that the spilanthol richest extract was the one presenting the highest activity at the lowest concentration.

INTRODUCTION

In folk medicine, *Spilanthes oleracea* (or jambú) flowers are typically chewed to relieve toothache and affections of throat and gums, as well as to paralyze the tongue [1,2]. The plant leaves are also used in Brazilian cuisine as a spice to promote a characteristic tingling paresthesia effect in traditional regional dishes [3]. Extracts obtained from jambú flowers have demonstrated important medical applications that include activity against acute- or long-term treatment of microbial infections [4], strong diuretic activity [5], anti-nociceptive activity against inflammatory pain as well as vasorelaxant and antioxidant activities [6].

Phytochemically, it was already reported that *S. oleracea* is particularly rich in alkylamides and *N*-isobutylamides [5,7,8] being spilanthol (*N*-isobutyl-2E,6Z,8E-decatrienamide) the widest distributed alkylamide in this species. This compound is responsible for the sensorial effect that characterizes this plant with strong local anaesthetic and analgesic activities [6,9]. The topical *in vitro* and *in vivo* anti-inflammatory effects of isolated spilanthol have been recently reported, suggesting that spilanthol attenuates the induced inflammatory responses in macrophages being a potential new lead compound for non-steroidal anti-inflammatory drugs (NSAIDs) [10,11].

Supercritical fluid extraction (SFE) has been reported as an effective method for obtaining bioactive products from plant materials offering a number of well-known advantages concerning selectivity, separation, purification and contamination-free extracts [12,13]. These fluids display double advantageous properties presenting high density (offering high solvency), as well as high compressibility (offering large variability of solvency by small changes in temperature and pressure), and consequently high selectivity of separation, originating extracts with higher purities. In addition, SFE offers the possibility of extract fractionation, avoiding some post extraction purification steps, by the use of successive changes in solvent density or composition (and polarity), and which will lead to different solvent natures and capacities [14].

The objective of this work was to obtain and characterize alkylamide rich extracts from *S. oleracea* flowers, leaves and stems, using a fractionated supercritical fluid extraction methodology consisting of a 1st step using only CO_2, to remove less polar compounds from each part of the plant, followed by a 2nd step using CO_2 plus EtOH, H_2O or EtOH+H_2O mixtures as extraction solvents, for the recovery of more polar compounds. Since alkylamides are lipophilic compounds, they were expected to be preferably extracted with scCO_2 during the 1st step of the fractionated process. However, higher molecular weight alkaloids [15], together with polar fractions, are expected to be obtained from the 2nd step, originating extracts with different composition profiles which certainly affect their antioxidant and anti-inflammatory activities. Results were compared to the ones obtained by conventional extraction methods.

MATERIALS AND METHODS

Materials - Jambú was acquired in Belém, Pará, Brazil. The plant flowers, leaves and stems were separated and comminuted. Particle size distribution was analyzed using a sieve series (120-18 mesh), under mechanical stirring. The humidity of each plant part was determined by the Jacobs xylol distillation method with duplicate assays.

Experimental procedure **-** High pressure extractions were performed using an apparatus previously described in the literature with some modifications as presented by Serra et al. [16]. A two-step extraction procedure was employed at 323 K and at 25 MPa, using a total solvent mass flow rate equal to 3.5×10^{-3} kg/min. The 1st step, designated by SFE(CO_2), was maintained for 180 min over dynamic conditions, in which the raw material was extracted using only scCO_2 as the extraction solvent, in order to remove the low polarity CO_2-soluble compounds. This step was followed by a 180 min dynamic 2nd extraction step, designated by ESE(EtOH), ESE(H_2O) and ESE(EtOH+H_2O) in which EtOH, H_2O or their mixture (50% v/v), respectively were added in a proportion of 70% (and 30% of CO_2) in order to enhance the extraction of more polar compounds. An adsorbent packed column was placed after the recovering flask in order to further prevent any extract losses. Ethanol-containing extracts were evaporated in the absence of light using a rotary evaporator with vacuum control. Water-containing extracts were lyophilized after being frozen with liquid nitrogen. For comparison purposes,

hydrodistillation and Soxhlet ethanolic extraction were also performed, for each part of the plant.

Extracts characterization - Gas chromatography analysis of the samples was performed on a gas chromatograph-FID equipped with a split injector and a FID operated at 523 K using a DB-1 cross-linked fused-silica capillary column according to the procedure described in detail by Dias et al. [17]. Spilanthol was isolated from an ethanolic extract obtained from comminuted jambú flowers that was purified by TLC using hexane: ethyl acetate (2:1, v/v) as the mobile phase. The total phenolic (TP) content of the extracts was determined by the Folin-Ciocalteu with some modifications [18]. The antioxidant activity was assayed based on the coupled oxidation of β-carotene/linolenic acid system according to the method optimized by Braga et al. [19] and was expressed as oxidation inhibition (OI) percentages as a function of time (1-3h). The anti-inflammatory activity of different jambú extracts was screened using an indirect *in vitro* method for the inhibition of soybean lipoxygenase, which is in many respects equivalent to the arachidonic acid cascades in animals [17,20,21]. According to the present methodology, the extracts that allow lower linoleic acid conversions are the ones that strongly inhibited the activity of the enzyme and consequently the leukotriene pathway, promoting the anti-inflammatory process. Principal Component Analysis (PCA) was used for data compression and information extraction [22]. Samples were analyzed concerning their total phenolic (TP) content, antioxidant activity after 1h of oxidative reaction (OI_{1h}), maximum anti-inflammatory activity (PI), total phenolic versus antioxidant activity ratio (OI_{1h}/TP), global yield (Y_g) and spilanthol yield (Y_s). Data were pre-treated by auto scaling and the number of principal components (defined as the number of independent variables describing the system) was chosen to be equal to 2 (PC1 and PC2), describing 68% of the variance of the experimental data. The chemometric functions included in the PLS MatLab Toolbox were used to generate the PCA model.

RESULTS

The results obtained for the global yields (on a dry basis, d.b.) showed that, for all plant parts, the yield obtained by SFE(CO_2) was higher than the one obtained by HD, being this difference particularly significant (more than 100 times) in the case of the flowers. The yields increased in the sequence flowers < stems < leaves for HD and stems < leaves < flowers for SFE(CO_2). The ratios between the yields from the 2nd and the 1st step where lower than 4 for EtOH and between 4 and 25 for H_2O, increasing in the sequence flowers << leaves ~ stems, in both cases. The solvent enhancer effect was more pronounced for H_2O (when compared to pure EtOH and despite system's heterogeneity) which may be due to the fact that aqueous extracts are more likely to contain higher molecular weight hydrophilic compounds such as polysaccharides and glycoproteins. Furthermore, results also show that the change in the solvent's mixture polarity and density induced by the use of EtOH+H_2O mixtures as solvent enhancers did not significantly improve global extraction yields, being only a slight increase observed for flowers.

The spilanthol yields in each of the different extracts, and for the different parts of the plant, are represented in Figure 1. Results show that SFE(CO_2) was the most selective extractive process for spilanthol, specially for flowers and stems for which approximately 95% of the total amount of extracted spilanthol was obtained from this 1st step. It is also clear that the extraction of spilanthol still remaining in the matrix is more efficient when using the enhancers individually (ESE(EtOH) or ESE(H_2O)) than when using their mixture. Nevertheless, the use of solvent enhancers during the 2nd

extraction step improved the TP amount extracted from each part of the plant which was ~2.5 higher when compared to the 1st SFE(CO_2) step.

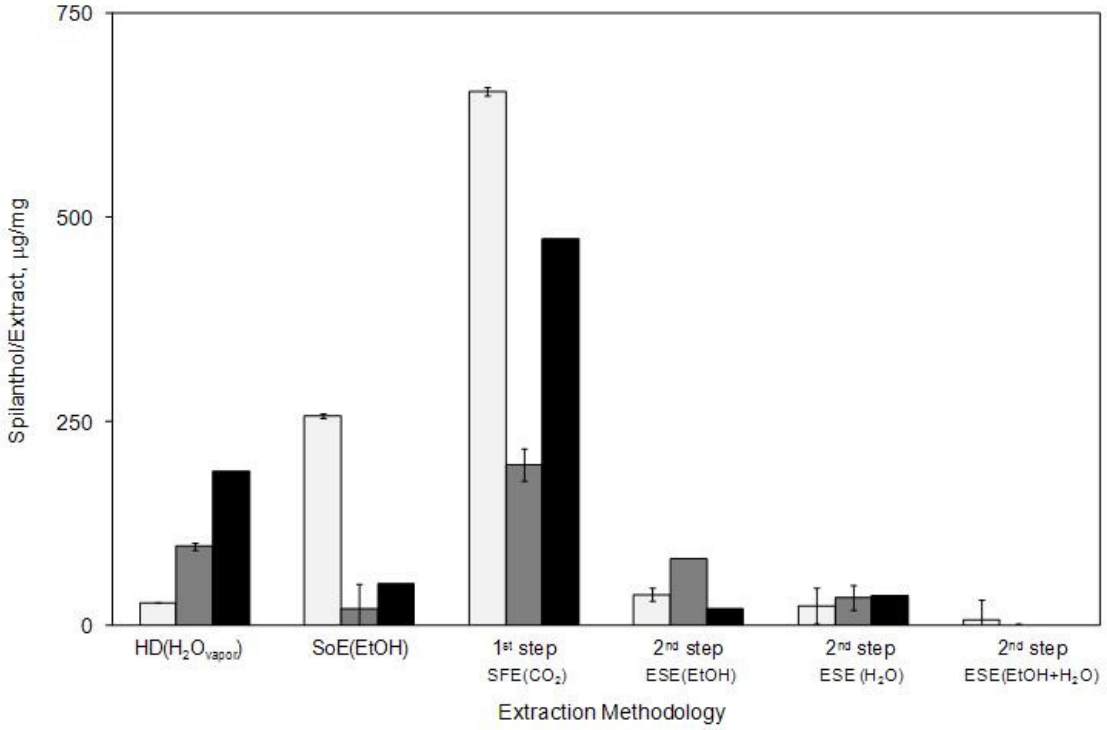

Figure 1. Yield of spilanthol obtained from flowers (■), leaves (■) and stems (■) by using different extraction methodologies.

According to the biplot analysis shown in Figure 2 it is possible to distinguish to main groups, one including mostly extracts obtained from HD and SFE(CO_2) (Group I) and the other including extracts with higher TP content and higher antioxidant activity, corresponding to the extracts obtained from SoE(EtOH) and 2nd step ESE (Group II). Extracts from the 2nd group correspond to the ones presenting the highest global yields, antioxidant activities and TP amounts. Clear exceptions were found for HD extracts from leaves and SFE(CO_2) and SoE extracts from flowers. The former may be justified by the fact that polyphenols in leaves are chemically different from those in flowers and stems being probably more soluble and easier to extract by HD. Moreover, chlorophyll based molecules present in the leaves may be contributing to the higher TP quantification.

The highest OI_{1h}/TP ratios were observed for SFE(CO_2) extracts from flowers and stems and ESE(EtOH+H_2O) extracts from leaves. As the SFE extracts presented low TP amounts, the high OI_{1h}/TP values observed for SFE(CO_2) for flowers and stems most probably indicate that the high amount of alkylamides present in these extracts, mostly spilanthol, is responsible for this enhanced antioxidant activity. The extracts that presented the highest lipoxygenase inhibitions (corresponding to highest anti-inflammatory activities), at the lowest concentrations, are the ones obtained from SFE(CO_2) from flowers (~ 50% inhibition), followed by SFE(CO_2), ESE(EtOH) and SoE(EtOH) from stems. In the 1st case, alkylamide based compounds seem to be here again the main responsible agents for the inhibitory capacity of these extracts, as SFE(CO_2) from flowers were the extracts presenting higher spilanthol yield (and in agreement with the PCA analysis where the anti-inflammatory activities and the spilanthol yields appear to be related as both variables are located in the same quadrant in Figure 2). The significant activities observed for some of the extracts obtained from

stems is most probably due to a synergetic effect achieved by a suitable composition ratio between phenolic and alkylamide compounds with important anti-inflammatory activities.

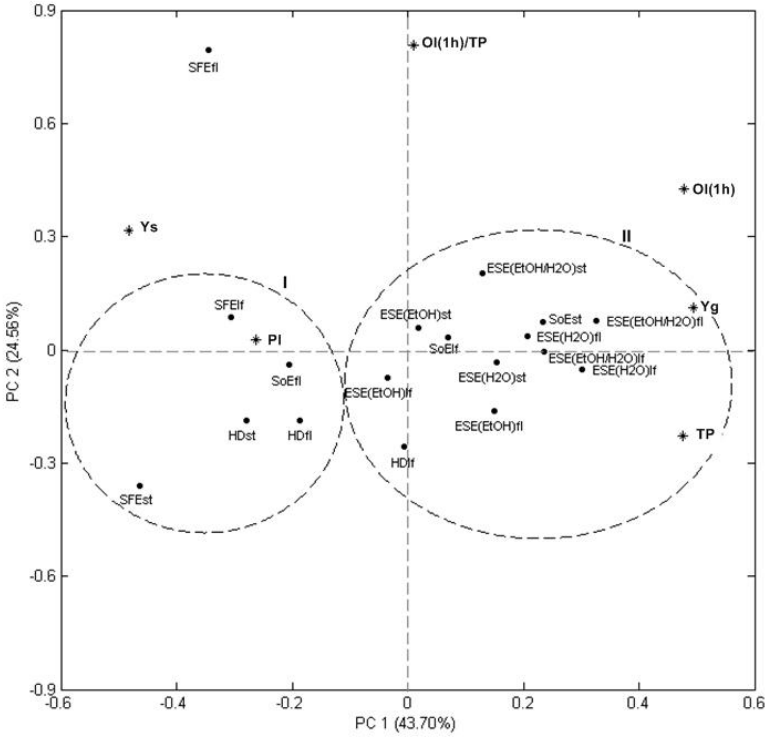

Figure 2. Biplot from Principal Components Analysis (PCA) of data obtained for the different extracts of jambú studied represented in terms of two principal components describing 68% of the experimental data. The analyzed variables are total phenolic (TP) content, antioxidant activity after 1h of oxidative reaction (OI_{1h}), maximum anti-inflammatory activity (PI), antioxidant activity *versus* total phenolic ratio (OI_{1h}/TP), global yield (Y_g) and spilanthol yield (Y_s).

CONCLUSIONS

This work studied the influence of different extraction methods and solvents, as well as different parts of jambú (flowers, leaves and stems) on the composition, and consequently on the bioactivity of the extracts. It was observed that all factors affected the chemical composition of the extracts, both quantitatively and qualitatively, concerning their total phenolic and spilanthol contents. When comparing the results obtained from the different parts of jambú, it was clear that the flowers are richer in spilanthol, which justifies the highest OI_{1h}/TP ratio as well as the highest anti-inflammatory activity using the lower amount of extract, validating the use of flowers for pharmaceutical/medical purposes. SFE(CO_2) proved to be the most effective extractive process for spilanthol, from all plant parts, particularly selective for this bioactive compound mainly in the case of the flowers, originating solvent free extracts with a yellow colour, adequate to be used without further time consuming and solvent dependent purification processes. Higher extraction yields and higher total phenolic amounts were obtained when using organic/polar solvents as enhancers which also improved the extraction of compounds with higher antioxidant activities, as was the case of ESE(H_2O) and ESE(EtOH+H_2O) from flowers, ESE(H_2O) from leaves and SoE(EtOH) from stems. Together with the flowers, leaves were an important source of phenols. As leaves presented relatively low or even pro-inflammatory responses, the valorisation of this part of the plant may focus the food industry. Finally, the stems of

jambú proved to contain significant amounts of spilanthol (with values between those of flowers and leaves) and other alkylamides that were easily and selectively extracted from SFE(CO_2), presenting anti-inflammatory activities in some cases similar to the ones observed for the flowers. Therefore, jambú stems may be considered as a valuable plant residue.

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