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Original research paper

Rapid extraction of andrographolide from *Andrographis paniculata* Nees by three phase partitioning and determination of its antioxidant activity

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ABSTRACT

Three phase partitioning (TPP) process was developed for recovery of andrographolide (AP) from the leaves of *Andrographis paniculata*. AP is a potent anti-inflammatory agent and possesses antioxidant, anticarcinogenic and antidiabetic activity. Optimization of TPP process parameters such as pH, ammonium sulfate saturation, slurry to t-butanol ratio, time and solid to solvent ratio on the extraction efficiency was performed for enhanced yield of AP. Under optimum conditions of extraction (40% w/w ammonium sulfate, 120 min time, pH 7, 1:40 solute to solvent, 1:1 slurry to t-butanol ratio, 40 °C) the maximum AP yield of 26.55 mg/g was obtained. Considering soxhlet extraction as the reference for comparison, TPP showed 62.5% yield in 120 min whereas batch extraction produced 59.89% yield in 140 min. To investigate the antioxidant potential of *A. paniculata* extract, an *in vitro* assay (2,2-diphenyl-1-picrylhydrazyl assay) of t-butanol and aqueous layer was performed. Also total phenolic content of both the layers were determined using Folin Ciocalteu reagent. Total phenolic content and antioxidant activity of t-butanol layer was higher as compared to aqueous fraction. A positive correlation of R^2=0.988 between total phenolic content and % scavenging of t-butanol layer was observed. The result showed that t-butanol extract of *A. paniculata* exhibited high antioxidant activity and has the potential to be used as a source of natural antioxidant.

1. Introduction

The need for investigation on plants of medicinal value has become interesting and relevant due to an increase in the inefficacy of many modern drugs used for the control of many infections. *A. paniculata* is one such medicinal plant belonging to the Acanthaceae family. It is popularly used as a folklore remedy for the treatment of an array of ailments such as leprosy, cancer, influenza, diabetes, high blood pressure, bronchitis, skin diseases, colic, dysentery, dyspepsia, malaria and ulcer (Chao and Lin, 2012). It is cultivated extensively in China and Thailand and grows abundantly in Asian countries like southeastern India, Sri Lanka, Pakistan and Indonesia (Datta et al., 2012). The primary medicinal component of *A. paniculata* is AP, a diterpene lactone. It is colorless and crystalline in appearance with a very bitter taste. Its leaves contain the highest content of AP, while the seeds contain the lowest (Okhuarobo et al., 2014). AP has a broad range of pharmacological effects such as anti-inflammatory, anti-viral, anti-malarial, hepatoprotective, anti-cancer, cardiovascular and immunostimulatory activity (Jarukamjorn and Nemoto, 2008; Lim et al., 2012; Bothiraja et al., 2013). Extraction of AP from *A. paniculata* has been investigated in the past focusing mainly on conventional solvent extraction. Various modern extraction techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SCFE) have also been developed recently for its efficient extraction (Rao and Rathod, 2015; Kumoro and Hasan, 2007). Sharma et al. (2013) studied the adventitious root culture of leaf explants of *A. paniculata* using different strength MS medium, supplemented by auxin and a combination of NAA+kinetin for AP production. The AP content produced was 3.5–5.5 folds higher than that of the natural plant depending on the strength of the medium (Sharma et al., 2013). They further attempted elicitor induced activation of AP biosynthesis in the cell cultures of *A. paniculata*. A particular concentration of the elicitor yielded 5.25 times more AP content after 24 h treatment (Sharma et al., 2015). Natural product extraction and purification involves a complex array of techniques. But, process development constantly demands methods that increase the purity and yield, and reduce the process time cost. Three phase partitioning (TPP) is one such recent bio

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separation process which is inexpensive and scalable. It has been previously used for the extraction and purification of a number of biomolecules (Choonia and Lele, 2013; Sagu et al., 2015). TPP was used to recover alkaline proteases from farmed giant catfish viscera (Ketnawa et al., 2013). Vidhate and Singhal (2013) reported the optimization of three phase partitioning for efficient extraction of kokum kernel fat. Also, TPP was used as an efficient bioseparation technique, to purify nattokinase from fermentation broth of Bacillus natto NRRL-3666 (Garg and Thorat, 2014). TPP was majorly used for the isolation and purification of proteins, enzymes and lipids. More recently it was used to separate many natural compounds from plant sources. It can be used to recover biomolecules directly from large volumes of crude suspensions (Rachana and Lyju Jose, 2014). TPP comprises of salt addition (ammonium sulfate) to the crude extract followed by the addition of t-butanol. The advantage of using ammonium sulfate is that it is highly water soluble and relatively inexpensive. Tertiary butanol is indefinitely miscible with water but on salt addition at sufficient concentration, the solution separates into a lower aqueous phase and an upper t-butanol phase (Avhad et al., 2014). Tertiary butanol is the preferred solvent as it is less flammable in comparison to hexane, acetone, methanol and ethanol which are commonly used in conventional solvent extraction. Also it works as a kosmotrope and a crowding agent at room temperature, whereas solvents like ethanol exhibit this property at near or below zero temperatures. In TPP, polar molecules get concentrated in the lower aqueous phase whereas lipids, enzymes and pigments are accumulated in upper t-butanol phase. Separation of any compound by TPP is governed by an association of a number of factors which include salting out, isotonic precipitation, cosolvent precipitation, osmolytic and kosmotropic effect, protein hydration shifts and electrostatic forces (Dennison and Lovrien, 1997). The current paper exemplifies the use of three phase partitioning (TPP) for the recovery of AP from *A. paniculata*. Furthermore, various parameters that affect the partitioning efficiency of AP by TPP such as ammonium sulfate concentration, pH, temperature, soaking time, solute to solvent ratio were optimized. Nowadays, there have been attempts to replace commercial antioxidants with natural ingredients due to the possible ill effects of synthetic antioxidants like butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), which are now being discreetly used in foods as they are suspected to be carcinogenic. Currently, the use of plants and herbs with antioxidant activity is gaining importance (Andallu et al., 2014). The extract of *A. paniculata* is reported to show antioxidant activity. *A. paniculata* is a potent scavenger of a variety of reactive oxygen species including superoxide anion, hydroxyl radical, singlet oxygen, peroxynitrite and nitric oxide (Niranjan et al., 2010). So to investigate the antioxidant potential of *A. paniculata*, an in vitro test called DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay of the t-butanol as well as the aqueous layer was performed. DPPH is a stable free radical because of its free electron delocalization over the whole molecule. The delocalization results in a deep violet color with λ_{max} around 520 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable non-radical form of DPPH is obtained, with a change of violet color to pale yellow (Pyrzynska and Pekala, 2013). Also, the total phenolic content of both the layers was determined using Folin Ciocalteu reagent. This kind of study on the extraction of AP from *A. paniculata* using TPP and a further inspection of the antioxidant activity of its extract has not been studied and reported in the past to the best of our knowledge and information.

2. Materials and methods

2.1. Materials

A. paniculata leaves powder was purchased from a local store in Mumbai. The average powder size of the leaves was 0.6–1.1 mm. The powder was sun dried and its moisture content was determined. The moisture content was found to be approximately 7%. The standard for AP (Aldrich 365645-100 mg, 98% HPLC grade) was procured from Sigma-Aldrich. Mixture of acetonitrile (HPLC grade) and acidified water was used as the mobile phase for HPLC. Acetonitrile, ethanol, gallic acid and DPPH were purchased from High media whereas; tertiary butanol (AR grade) and ammonium sulfate were procured from S. D. Fine Chemicals Limited, Mumbai. Distilled water used as one of the mobile phases, was obtained from Millipore Milli Q 50 HPLC grade.

2.2. Analytical method

AP content was analyzed from its extract using HPLC (Agilent 1260 infinity high performance auto sampler) equipped with XBD-C18 reversed-phase column. Acetonitrile and acidified water in the ratio 60:40 v/v was used as the mobile phase for HPLC. Isocratic elution was performed for the analysis where the flow rate was set at 1.0 mL/min throughout the run. 5 μL of sample was injected into the HPLC system. The peak for AP was identified at 223 nm with a retention time of 7.6 min. Fig. 1(a) and (b) shows the HPLC chromatograms of standard andrographolide and t-butanol extract of *A. paniculata* respectively.

2.3. Statistical design

All experiments were performed in triplicate to check the reproducibility and their average values have been reported. For each experiment the data has been expressed as mean value ± standard deviation. Statistical analysis was done using one-way ANOVA to study the statistical significance of the process parameters and *p*-values were obtained. The values were considered statistically significant if the *p*-values were less than 0.05.

3. Experimental work

3.1. Soxhlet extraction

Around 2 g of leaves powder was fed into the soxhlet apparatus with 130 mL aqueous ethanol as the solvent. Soxhlet extraction was carried out for 240 min. The thimble was placed in an extraction chamber, which was attached to a container containing aqueous ethanol and a condenser. As the solvent was heated, it evaporated and moved up into the condenser where it converted into a liquid that trickled into the extraction chamber containing the sample. As the solvent moved up it extracted the desired components from the solid. This cycle was continued for the desired time period. The liquid obtained after condensation was collected and analyzed for AP content by HPLC after every 30 min to ensure that complete extraction was achieved.

3.2. Batch extraction

A glass reactor of 100 mL capacity having a diameter of 4.5 cm and height 10 cm along with six bladed (pitched blade) glass turbine impeller was used for agitation. *A. paniculata* powder (1.2 g) was taken in a glass reactor and the required amount of aqueous ethanol (60 mL) was added to it. The mixture was agitated for 140 min. Other experimental conditions used were – 1:50