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# Three phase partitioning coupled with ultrasound for the extraction of ursolic acid and oleanolic acid from *Ocimum sanctum*

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ABSTRACT

In this study, three phase partitioning (TPP) is coupled with ultrasound for the extraction of ursolic acid (UA) and oleanolic acid (OA) from *Ocimum sanctum* leaves and process has been optimized to obtain maximum recovery. TPP is a relatively novel bioseparation technique used for the extraction, concentration and purification of enzymes and natural products. The technique of TPP was explored for the extraction of ursolic acid (UA) and oleanolic acid (OA) from *O. sanctum* leaves. The influence of various process parameters (pH, ammonium sulfate saturation, crude extract to t-butanol ratio, time and feed loading) on the extraction efficiency was investigated to get highest yield. The optimized conditions were found to be as follows: time – 120 min, pH – 7, ammonium sulfate saturation – 50% w/v, crude extract to t-butanol ratio – 1:1 and feed loading – 7.5% w/v. The highest yield obtained for UA and OA was 79.48% and 80.67% respectively under optimized conditions of TPP. Compared with TPP higher yield (83.36% and 85.58%) was obtained by ultrasound assisted TPP (UATPP) at 40 kHz and 180 W power and the time required was only 14 min as compared to 120 min of TPP. The extraction yield obtained was also compared with conventional solvent extraction and TPP-ultrasound was found to be an attractive technique for the extraction of UA and OA from *O. sanctum* leaves.



*Keywords:* Three-phase partitioning; Ursolic acid; Oleanolic acid; *Ocimum sanctum*; Ultrasound; Extraction

## 1. Introduction

In recent years, extraction and purification of bioactive compounds from natural sources received great attention as phytochemicals which are used in diverse sectors such as foods, perfumery, cosmetics, paints, pharmaceuticals. Hence, isolation of natural products and identification of new natural sources of bioactive compounds have gained scientific and industrial importance. Extraction and purification of these phytochemicals are the basic steps to fulfill these increasing demands. Holy basil (*Ocimum sanctum* Linn.) or "Tulsi" possesses valuable and wide spectrum of medicinal uses viz. anti-carcinogenic, anthelmintic, antirheumatic, antibacterial, antidepressant, antiepileptic, hepatoprotective, radioprotective (Dharmani et al., 2004; Pemminati et al., 2007). Ursolic acid (UA) and oleanolic acid (OA) are important pentacyclic triterpene phytochemicals found in *O. sanctum* with significant biological potential. These acids are based on the structure of isoprene and contain 30 carbon atoms. These triterpenoid show diverse pharmacological activities such as anti-inflammatory (Safayhi and Sailer, 1997), antiulcer (Nishino et al., 1988), hepatoprotective (Udayama et al., 1998), antitumor (Prakash and Gupta, 2000), hypoglycemic (Ortiz-Andrade et al., 2007), and antihyperlipidemic (Suanarunsawat et al., 2009). UA, OA and their derivatives have antiviral potential and claim to inhibit the growth of several viruses including HIV (Kashiwada et al., 2000).

Applications of various conventional techniques such as soxhlet, precipitation, column chromatography (Silva et al., 2008) and novel technique like counter current

*Abbreviations:* UA, ursolic acid; OA, oleanolic acid; TPP, three-phase partitioning; UATPP, ultrasound assisted three phase partitioning.

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chromatography for the extraction and purification of UA and OA from *O. sanctum* leaves have been already reported in the literature (Frighetto et al., 2008). UA has also been isolated from several other sources such as apple peels and hawthorn fruit (Yamaguchi et al., 2008; Cui et al., 2006; Xie et al., 2001). Hua-Bin Li et al. (Xia et al., 2012, 2011) has been reported ultrasound assisted and microwave assisted extraction of UA and OA from *Ligustrum lucidum*. A kinetic study of extraction of UA and OA from the *O. sanctum* leaves powder has been reported earlier (Vetal et al., 2012).

In the present work, the novel extraction process i.e. three-phase partitioning (TPP) has been used for UA and OA recovery. TPP is a simple and fairly recent biosepartion technique which involves the addition of a salt (ammonium sulfate) to the aqueous suspension of the feed or crude extract followed by the addition of t-butanol. Tertiary butanol is completely miscible with water but on the addition of ammonium sulphate at sufficient concentration, the solution separates into a lower aqueous phase and an upper t-butanol phase. This is the basis of TPP which is responsible for isolation proteins. Though exact reason is not clear, TPP employs collective operation of principles involved in numerous techniques for protein precipitation. It is assumed that the separation of compound happened possibly due to combination of salting out, isotonic precipitation, cosolvent precipitation, osmolytic and kosmotropic effect. In TPP generally lipids, enzyme and pigments are accumulated in upper t-butanol phase while polar molecules get concentrated in the lower aqueous phase. TPP is a simple, inexpensive, scalable technique which can be directly used with crude suspensions. It is simple and rapid process which is carried out at room temperature. t-butanol is less flammable as compared to hexane, acetone, methanol and ethanol which are generally used in conventional solvent extraction (Harde and Singhal, 2012).

TPP has been used for the extraction and purification of various biomolecules and enzymes such as invertase from baker's yeast and tomato (Akardere et al., 2010; Ozer et al., 2010), α-galactosidase from pepino (*Solanum muricatum*) (Sen et al., 2011), α-amylase (Wanga et al., 2011), protease (Chaiwuta et al., 2010). Saxena et al. (2007) reported 20.1 and 16-fold purification with 39.5% and 32% yield of amylase inhibitor and trypsin inhibitor, respectively. TPP has also been reported for soybean oil extraction (Sharma et al., 2002), Gaur et al. (2007) reported TPP for the extraction of edible oil from mango kernel, soybean and rice bran. Apart from enzymes and oil, TPP has also been used for the extraction of some natural products. Kurmudle et al. (2011) reported TPP as an extraction method for the oleoresin extraction from turmeric. The crude extract obtained by TPP would be subjected to further purification of targeted compounds. Although TPP is an efficient technique for the extraction of enzymes and natural products, it requires more time due to mass transfer limitations inefficient mixing. Ultrasound has been found to be useful in increasing the yield and mass transfer in many solid–liquid extraction processes. Ultrasound assisted extraction technique has several advantages and has been employed for the extraction of different phytochemicals like cinchonine, rauwlfine, digitalin, atropine, berberine (Vinatoru, 2001).

As no work is reported in literature on the extraction of UA and OA from *O. sanctum* using TPP, TPP has been used for the extraction of UA and OA from *O. sanctum*. Further, we tried to explore the application of ultrasound to enhance the mass-transfer in TPP process. TPP of UA and OA from *O. sanctum* leaves was analyzed on HPLC using photodiode array detector. The different experimental parameters, such as time, pH, ammonium sulphate loading, ratio of slurry to t-butanol, feed loading have been optimized to obtain maximum yield.

## 2. Materials and methods

### 2.1. Materials

*O. sanctum* leaves powder was purchased from Total Herbal Solutions Pvt. Ltd., Mumbai. The *O. sanctum* powder was greenish brown in color with characteristic odor, moisture content NMT 5%, bulk density-0.30 g/cc and particle size 0.50–1.00 mm. Methanol, t-butanol (AR grade) and ammonium sulphate were procured from S. D. Fine Chemicals Limited, Mumbai, India. Methanol of HPLC grade was purchased from Hi Media Ltd., Mumbai, India. Standard UA and OA acid were purchased from Sigma Aldrich Chemical Company, USA. The stock solutions of OA and UA (10 mg/mL and 5 mg/mL) were prepared in methanol, and stored at 4 °C. The calibration standards were prepared from the stock solution by the serial dilution of methanol.

### 2.2. Apparatus

Ultrasound assisted extraction has been carried out in a dual frequency ultrasound cleaning bath (Model 6.5l200 H, Dakshin, India) of internal dimensions 230 mm × 150 mm × 150 mm and tank capacity 6.5 L approx, with an ultrasonic power of 200 W and frequencies of 25 kHz and 40 kHz, equipped with heater and digital temperature controller/indicator. Temperature control was achieved by water cooling recirculation system attached to the ultrasonic bath. A selector switch is provided on the panel to select one operating frequency at a time. Power variation is possible by varying input AC voltage through auto-transformer. Based on earlier mapping study, the position of vessel in bath and the shape of the vessel have been fixed and used for all the experiments (Vetal et al., 2013).

### 2.3. Three phase partitioning (TPP)

TPP was optimized by varying the ammonium sulphate loading and ratio of slurry to t-butanol. Slurry was prepared by dispersing 0.75 g (5% of aqueous phase) of *O. sanctum* leaves powder in 15 mL of distilled water by gentle stirring using a magnetic stirrer. 4.5 g of ammonium sulphate (30% of aqueous phase) was added to the prepared slurry and vortexed gently, followed by addition of 15 mL of t-butanol. The extraction was carried out for 1 h using magnetic stirrer and 3 cm magnetic bar at 500 rpm. The mixture was allowed to stand for 1 h for the formation of three phases. Afterwards, the mixture was centrifuged at 5000 × *g* for 20 min to facilitate the separation of phases. The upper organic layer was collected and the solvent (t-butanol) was evaporated on a rotary vacuum evaporator (Equitron Roteva, India) under reduced pressure at 50 °C for 2 min. The extract so obtained was quantified for UA and OA contents using HPLC. Ammonium sulphate loading was varied from 20% to 60% w/v of slurry while ratio of slurry to t-butanol was varied from 1:0.5 to 1:2.5 by keeping all the other extraction conditions constant. Further evaluation of extraction time from 30 to 300 min was carried out for maximum yield of UA and OA. To check the effect of pH on extraction of UA and OA, pH of the system was varied from 4 to 9 by the addition of 1 N HCl and 1 N NaOH after addition of ammonium sulphate to the slurry. All experiments have been performed