Original Article

Evaluation of Unsaponified Petroleum Ether Extract of *Lantana camara* L. leaves for Antioxidant Activity and Oxidative Stability

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ABSTRACT

Background: *Lantana camara* L. leaves have been traditionally claimed to be used in treatment of different illnesses. Leaves are rich in lower terpenes in the form of essential oil, however they also contain lipids like waxes. Both classes, lipids and terpenes are soluble in petroleum ether, but only lipids can be saponified, not terpenes. **Aim:** The present study was aimed towards preparation of unsaponified terpene-rich extract of *L. camara* L. leaves; its assessment for anti-oxidant activity and capacity to impart oxidative stability. **Methods:** In order to remove lipids and retain terpenes, in present study, petroleum ether extract of *Lantana camara* L. leaves was saponified using aqueous potassium hydroxide and unsaponified matter was standardized using β-caryophyllene as marker; screened for anti-oxidant activity, using DPPH radical scavenging model and estimated for its capacity to impart oxidative stability using Rancimat test. **Results:** Tested extract showed considerable antioxidant activity (IC_{50} value:13 μg/ml) and imparts 74.83% protection against oxidation as compared to standards used. **Conclusion:** Lipids can be removed by saponification and thereby, petroleum ether extract can be made rich in terpenes. This unsaponified petroleum ether extract of *L. camara* L. leaves shows better antioxidant results as compared to petroleum ether extract and comparable with standards used.

Key words: *Lantana camara* L., Terpenes and Tepenoids, DPPH Radical Scavenging Activity, Rancimat Test, Unsaponified petroleum ether extract.

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INTRODUCTION

Lantana camara L. (Verbenaceae) is the notorious but ornamental weed, commonly known as red sage or wild sage. It is native to tropical, subtropical and temperate regions.[1] In different parts of world, *L. camara* L. leaves are used for treatment of different diseases. In Nigeria and Senegal *L. camara* L. leaf infusions are used in treatment of cough, colds, asthma and pyrexia,[2] in West Africa the leaves are used as diaphoretic, stimulant, and treatment of jaundice and rheumatism[3] while, in Central and South America and Ghana *L. camara* L. leaves along with flowers have been used against fever, influenza and stomach-ache sores, chicken pox, measles and high blood pressure.[4] Leaves are rich in essential oil which showed antimicrobial potential,[5-6] allelopathic property,[7] anti-inflammatory activity,[8] larvicidal efficacy against Aedes larvae.[9]

L. camara L. leaves are simple, opposite, petiolate, ovate, 5 to 8 cm in length, 2 to 4 cm in width, crenate, hairy acute, surface rugosely reticulate, pubescent, petiole usually curved or bent, highly pubescent and reported to contain pentacyclic triterpenoids with carboxylic acid group; steroids, flavonoid aglycones, iridoids and some polyphenolics Table 1 however essential oil obtained from its leaves is rich in monoterpenes and sesquiterpenes hydrocarbons Table 2.

All organisms utilize oxygen and thereby generate Reactive oxygen species (ROS).[31] However, excessive ROS production leads

to oxidative stress by overcoming cellular antioxidant defenses.[32] These free radicals are responsible for causing a large number of diseases including cancer.[33] cardiovascular disease.[34] neural disorders,[35] Alzheimer's disease,[36] mild cognitive impairment,[37] Parkinson's disease,[38] alcohol induced liver disease,[39] ulcerative colitis,[40] aging[41] and atherosclerosis.[42] Antioxidants are chemical entities preventing these oxidation damage and they can be divided into two main categories: enzymatic and nonenzymatic. Enzymatic antioxidants (examples:superoxide dismutases, catalase and glutathione peroxidases.) react with reactive species and are subsequently recycled. Non-enzymatic antioxidants interact with radical species and they are consumed during the reaction. Nonenzymatic antioxidants can be divided into hydrophilic (examples: glutathione, ascorbate and uric acid) and hydrophobic (examples: α-tocopherol, carotenoids, and ubiquinol-10) antioxidants.[43]

Rao *et al*. studied *in-vitro* antioxidant potential of aqueous extract of *L. camara* L,[44] leaves while Badakhshan *et al.* evaluated antioxidant activity of methanolic extract of various parts of *L. camara* L. and found that methanolic leaf extract was more effective that other parts.[45] Few researchers have obtained essential oil from leaves; analysed it for phytochemicals present and evaluated its antioxidant activity.[46,47] The present study was aimed at phytochemical screening and evaluation of antioxidant potential of unsaponified petroleum ether (USPE) extract of *L. camara* L. leaves.

MATERIALS AND METHODS

Chemical

Few consumables, 1,1-diphenyl-2-picrylhydrazyl (DPPH, >95%) and Butyrate hydroxyl toluene (BHT, >99%) were purchased from Sigma-Aldrich (Bangalore, India) while, ascorbic acid (Vitamin C, >99%), potassium hydroxide (KOH) were procured from Loba Chemie Pvt. Ltd. India. Analytical grade ethanol, ethyl acetate, petroleum ether were purchased from Analab Fine Chemicals- Mumbai. Sunflower oil was bought from local market, Pune.

Plant material

L. camara L. leaves Figure 1 A & 1B were collected in September, 2016 from Medicinal Plant Garden of Padm. Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri and identified by morphology and microscopy; and standard description. Leaves were then dried under shade and pulverized in grinder.

Extraction

Dried powder of *L. camara* leaves (10 g) was extracted with Petroleum ether (30 ml) at room temperature for 6 h with frequent shaking. It was then treated with 30 ml of warm 10% aqueous KOH, shaken and polarity-based two layers were separated. Petroleum ether layer was then concentrated to dryness under reduced pressure to obtain sticky mass (0.3 g). It was then analysed phytochemically and used as unsaponified petroleum ether (USPE) extract for evaluation of anti-oxidant activity.

Phytochemical Prospection

Both petroleum ether (PE) extract and USPE extract were tested for the presence of different secondary metabolites (alkaloids, terpene, tannins, flavonoids). Test specific for each class of secondary metabolites was based on change in colour or formation of precipitate on addition of specific reagent. Further, based on colour reaction and TLC analysis, USPE extract was standardized using β-caryophyllene as marker compound by GLC.

Thin Layer Chromatographic analysis

Both PE extract and USPE extract were applied separately on a precoated silica gel 60 F_{254} TLC plate (E. Merck) of uniform thickness of 0.2 mm. Plate was developed in the mobile phase Toluene: Ethyl acetate (93: 07) in a twin trough chamber to a distance of 8 cm and then visualized by spraying anisaldehyde-sulphuric acid reagent and heating at 105°C for 5 to 10 min.

Gas Liquid Chromatographic analysis

After confirming the presence of β-Caryophyllene in USPE by TLC, USPE was analyzed as test solution for quantification of β-Caryophyllene by GLC, using β-Caryophyllene as marker. Standard solutions (0.2, 0.4, 0.6, 0.8 and 1.0 μl/ml) of β-Caryophyllene were prepared in petroleum ether. About 1 μl each of the standard and the test solutions were injected to the Agilent GC instrument with nitrogen as carrier gas at flow rate of 1 ml/min at 250 °C. It has HP-5 (5% Phenyl methylsiloxane) capillary column (30 m × 320 μm × 0.25 μm) and flame ionization detector. Content of β-Caryophyllene was calculated from the integrated areas of the peak corresponding to that of β-Caryophyllene. The estimation was carried out in triplicate.

Radical-scavenging activity-DPPH assay

The antioxidant activity of USPE extract was evaluated by monitoring their ability in quenching the stable free radical DPPH.[46] Stock ethanolic solution of DPPH (100 μM) was prepared and its 1.5 ml was added to 2.5 ml of petroleum ether solutions of different concentrations

of USPE extract (10, 20, 40 μg/ml) and incubated for 30 min in dark at room temperature. Then absorbance of samples was measured at 518 nm by spectrophotometer (Shimadzu UV-1800 UV-Vis spectrophotometer). Blank reading was previously noted and Ascorbic acid was used as standard. Percent Inhibition was calculated using a formula:

$$\% \text{ Inhibition} = 100 - [(Abs_{sample} - Abs_{blank}) \times 100 / Abs_{control}]$$

Where Abs_{sample} is the absorbance obtained in the presence of USPE extract concentrations and $Abs_{control}$ is that obtained in the absence of extracts. The concentration necessary to inhibit 50% radical formation, inhibitory capacity IC_{50} were obtained by extrapolating the graph obtained by plotting the % inhibition versus different concentrations of USPE extracts, determined in triplicate.

Rancimat method

Ability of USPE extract to impart oxidative stability to oil was evaluated by Rancimat method[48] (EN 14112) with modifications. Samples of sunflower oil (3.5 g) with 2.5 ml of petroleum ether solutions containing different concentrations (10, 20 and 40 μg/ml)of USPE extract were heated at 110°C for 6 h (Induction period for sunflower oil). Butyrate hydroxyl toluene (BHT) was used as standard. Then, at the same time, Fourier Transformed Infra Red (FTIR) spectrums of samples were recorded.

Statistical analysis

Data of evaluation of antioxidant activity i.e. effect of different concentrations on DPPH free radicals were expressed as mean ± S.D.

RESULT

Phytochemical Prospection

Phytochemical tests indicated the presence of sterols, lipids, terpenes in PE extract while sterols and lipids were found absent in USPE extract. TLC analysis resulted in same observations where PE showed green, blue, violet, brown bands while USPE had only violet and pink bands. Based on reference finding and TLC analysis, it was confirmed that USPE contains only terpenes and β-caryophyllene was one of them.

Further, β-caryophyllene quantification by GLC generated plot Figure 2 indicated β-caryophyllene content and GLC profile of USPE. Retention time of β-caryophyllene was 16.7 min. Area of the peak corresponding to β-caryophyllene in USPE was used in calculations for determination β-caryophyllene content, which on triplicate analysis, was found to be ranging between 31.01 to 31.8%.

DPPH radical-scavenging activity

The results of DPPH radical-scavenging activity of USPE extract of *L. camara* L. leaves have been reported in Figure 3. It was found that there is increase in % inhibition on increase in concentration of both Ascorbic acid and USPE extract. Sample with higher concentration of USPE extract (40 μg/ml) had highest 93. 67%

Table 1: Chemical composition of *L. camara* leaves

Class	Phyto-constituents
Pentacyclictriterpenoids	Lantadene A,[10]Lantadene B;[10]Lantadene C,[11]Lantadene D,[12] Icterogenin,[13]Camarinic acid,[14]Camaric acid,[14]Oleanolic acid,[14]Pomolic acid,[14]Camarilic acid,[15]Camaricinic acid,[15]Lantanoic acid,[16]Lantanolic acid,[17]Lantic acid,[18,19]Lantanilic acid,[20] β, β-Dimethylacryloyl ester of lantanilic acid,[20] 3, 24- Dioxy-urs-12-en-28-oic acid[21],Lantabetulic acid,[22] lancamarolide[23]
Steroid	Lancamarone[24]
Iridoid glycosides	Theveside,[25] Camaraside,[26] Martynoside,[1] Derhamnosylverbascoside,[27] Isonuomioside A[27]
Flavonoid aglycones[28]	3-methoxy quercetin, 3, 7- dimethoxyquercetin, 3,7,4'-trimethoxyquercetin
Polyphenolics[29]	Salicylic acid, Gentisic acid, β-resorcylic acid , Coumarin, Ferulic acid, *p*-Hydroxybenzoic acid, 6-Methyl coumarin

Table 2: Chemical composition of essential oil obtained from *L. camara* leaves[30]

Class	Phyto-constituents
Monoterpenes hydrocarbons	Sabinene
	α-pinene
	γ-terpinene
Oxygenated monoterpenes	E)-citral
	(Z)-citral
	1,8 cineole
Sesquiterpenes hydrocarbons	trans-caryophyllene,
	β-caryophyllene
	bicyclogermacrene ,
	α-curcumene ,
	germacrene D

inhibition. IC_{50} value for USPE extract (13 μg/ml) was found to be comparable with that of Ascorbic acid (11 μg/ml).

Rancimat method

FTIR spectra for sunflower oil before oxidation was recorded and superimposed by those recorded for samples containing different doses of extracts and for sample with BHT after heating at 110°C for 6 h have been shown in Figure 4. In each spectra, carbonyl band was obtained at 1760 cm^{-1}. Their area under curve (AUC) was used for quantitative estimation of the oxidation level. Table 3 shows AUC of carbonyl bands of spectrum of all samples.

Induction period of sunflower oil was estimated at around 6 h. The highest oxidative stability in sunflower roil was exhibited in sample containing highest dose i.e. 40 μg/ml of USPE extract. The extent of oxidation changes monitored in all examined samples of sunflower oils containing USPE has proved that it imparts dose dependent oxidative stability.

DISCUSSION

There are various phytoconstituents of different classes present in leaves of *L. camara* L. Out of these, oils, fats, sterols, terpenes are soluble in non-polar solvent like petroleum ether. Accordingly, petroleum ether extract showed presence of sterols, lipids and terpenes. In the present study, saponification of petroleum ether extract was carried out. This lead to saponification of oils and fats which were solubilized in petroleum ether extract to their corresponding Potassium salts. At the same time, saponification had no effect on other petroleum ether soluble compounds like terpenes. Ultimately, USPE extract showed presence of only terpenes. This is again justified by β-caryophyllene content of USPE extract (31.01 to 31.8%.). Different researchers have reported different β-caryophyllene content in of essential oil isolated from *L. camara* L. leaves. Sonibare *et al.* found that its content as 8.9%[5]; Zoubiri *et al.* found it is 35.70%;[49] Alitonou *et al.* estimated it as18.5%;[50] while Randrianalijaona *et al.* found it is 30.85%.[51] However, Unnithan *et al.*[52] found β-caryophyllene content of petroleum ether extract of *L. camara* L. leaves to be 0.06%. These findings indicates that saponification of petroleum ether extract of *L. camara* L. leaves removed fats and oils contents of leaves and retained terpenes in unsaponified matter.

USPE extract of *L. camara* L. leaves showed DPPH radial scavenging activity comparable to standard Ascorbic acid. DPPH exist as free radical stabilized by delo-

Figure 1(A): *Lantana camara* L. a) Habitat.

Figure 1(B): *Lantana camara* L. b) Leaves.

Table 3: Area under curve (AUC) of Carbonyl band

Sample	AUC of carbonyl band at 1760 cm^{-1}	% Protection
BHT	298	84.66
USPE (40 μg/ml)	489	74.83
USPE (20 μg/ml)	634	67.37
USPE (10 μg/ml)	1052	45.86
Without USPE	1943	0.00

calization of unpaired electron over the whole DPPH radical. This imparts violet colour, in ethanol which has absorbance maximum at 518 nm. As it receives hydrogen atom from anti-oxidant compound, it gets reduced to colourless DPPH hence absorbance of DPPH–antioxidant solution decreases.[53] IC_{50} values of both Ascorbic acid and USPE extract indicates that nearly same quantity of both Ascorbic acid and USPE extract is sufficient to make 50% of DPPH free radicals to colorless DPPH.

Results of Rancimat tests were also found to be dose-dependent in imparting oxidative stability to sunflower oil. In sample containing no USPE, oxidative stability was imparted by naturally occurring range of antioxidants such as tocopherols, phenolics and sterols.[54] In Rancimat test, it was important that antioxidant to be tested or added should be soluble in sunflower oil. Hence, Ascorbic acid, a standard used in evaluation of DPPH radical scavenging activity was replaced by BHT as it is soluble in sunflower oil. Area under curve (AUC)

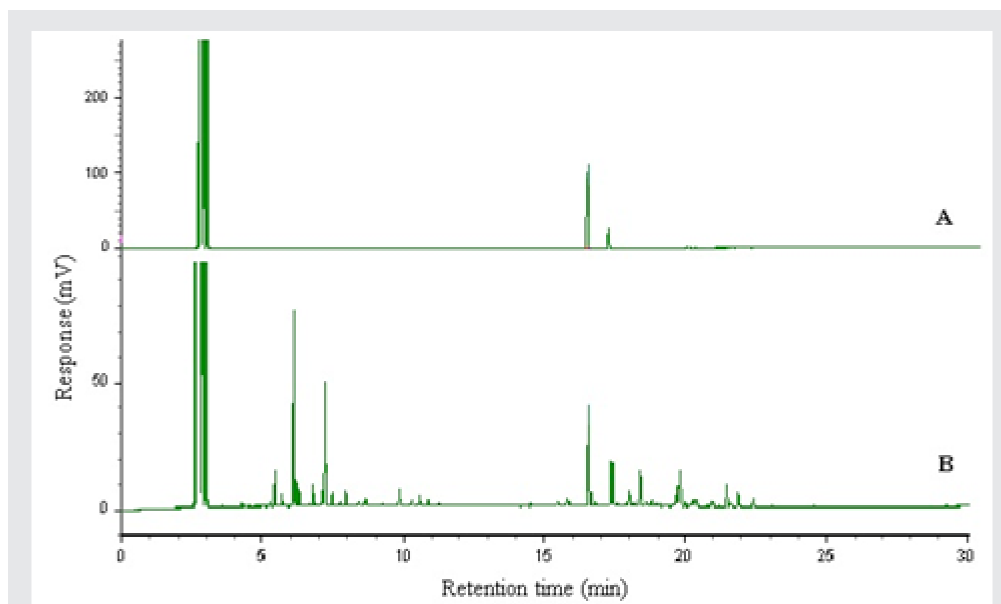

Figure 2: GLC fingerprint profile A. β-Caryophyllene standard; B. Test solution.

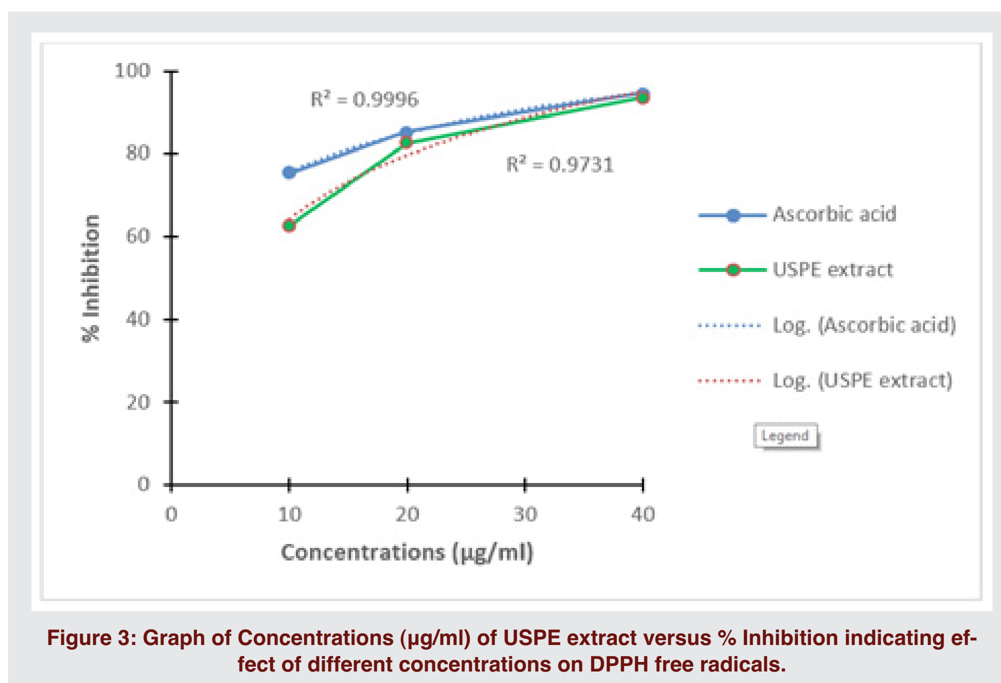

Figure 3: Graph of Concentrations (µg/ml) of USPE extract versus % Inhibition indicating effect of different concentrations on DPPH free radicals.

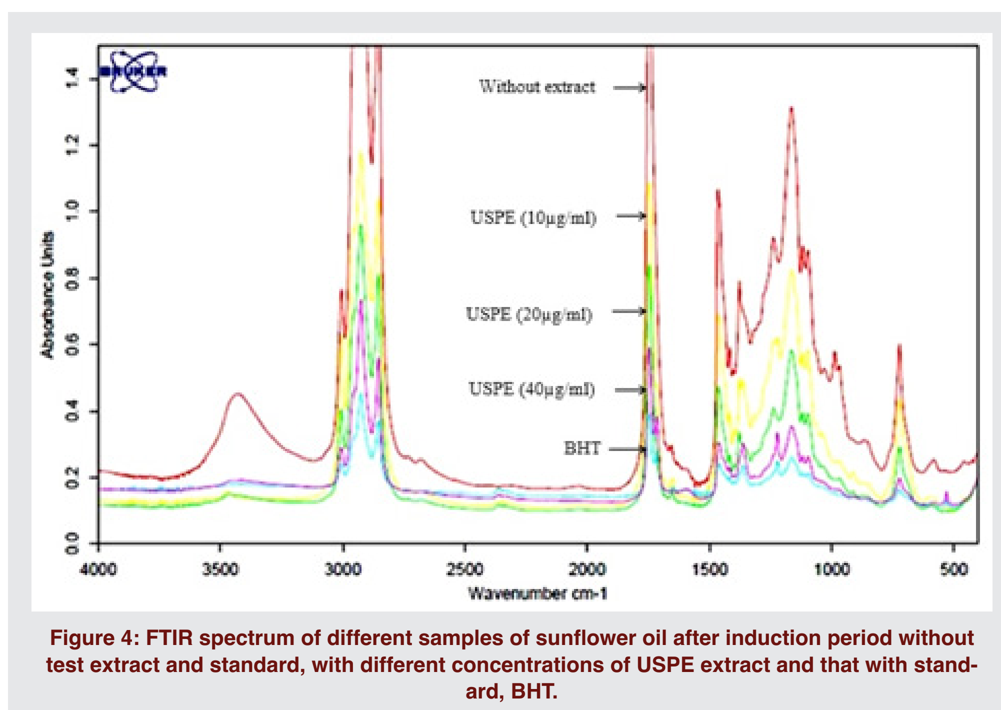

Figure 4: FTIR spectrum of different samples of sunflower oil after induction period without test extract and standard, with different concentrations of USPE extract and that with standard, BHT.

values indicate the extent of oxidation by formation of carbonyl bonds. Hence lesser the AUC value, lesser the extent of oxidation and higher the oxidative stability. Terpernes present in USPE extract may impart oxidative stability to sunflower oil.

CONCLUSION

In the present study, unsaponified petroleum ether extract (USPE) of *L. camara* L. leaves was tested for antioxidant activity and oxidative stability. Extraction was performed by simple cold maceration and saponifiable matter was separated. Unsaponified extract was then analyzed phytochemically and standardized using β-caryophyllene. Its DPPH radical scavenging activity was determined and oxidative stability was evaluated by modified Rancimat test. It was found that USPE extract has anti-oxidant potential and imparts oxidative stability to sunflower oil comparable to standards used i.e. Ascorbic acid and BHT. Based on these results, it could be noted that there is future scope for evaluation of USPE extract for *in-vivo* pharmacological activities exhibited by terpenes for research in phyto- pharmacology.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATION

DPPH: 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl); **FTIR:** Fourier Transformed Infra Red spectroscopy; **AUC:** Area Under Curve; **USPE:** Unsaponified petroleum ether; **BHT:** Butyrate hydroxyl toluene; **GLC:** Gas Liquid Chromatography; **TLC:** Thin Layer Chromatography.

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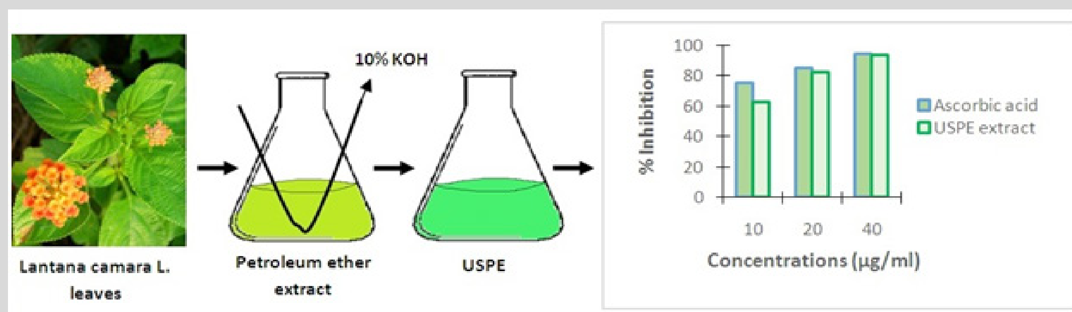
PICTORIAL ABSTRACT



SUMMARY

- Unsaponified petroleum ether (USPE) extract is prepared from petroleum ether (PE) extract of *Lantana camara* L. leaves by treatment with 10 % aqueous KOH.
- Saponification retained terpenes and removed fats and oils from petroleum ether extract.
- USPE extract has shown to possess antioxidant activity and imparts oxidative stability higher than that of PE extract.

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