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Phytochemical analysis, antibacterial and antioxidant activity determination of *Ocimum sanctum*

Abstract

The objective was to analyse phytochemical constituents from the leaves of *Ocimum sanctum* using suitable solvents and extraction technique and to evaluate the in-vitro antibacterial and *in-vitro* antioxidant activities of leaf extract of *Ocimum sanctum*. In present work, Soxhlet extraction and maceration extractions were applied to the fresh leaves of the *Ocimum sanctum* by using absolute ethanol. Phytochemical analysis for the important chemical constituents from ethanolic extract was carried out. Antimicrobial activity of *Ocimum sanctum* extract was carried out using Well Diffusion method by comparing the clear inhibition zone of standard antibiotic and the extracts on the Mueller Hinton agar. Antioxidant activity of *Ocimum sanctum* was carried out performing total phenolic content test and DPPH to identify the percentage of scavenging by the chemical constituents. For phytochemical analysis, only test for alkaloids, test for terpenoids and test for carbohydrates showed positive results for *Ocimum sanctum* extract. For antibacterial screening, all the concentrations of OSESE showed negative results due to low concentration of extract being used. For antioxidant analysis, total phenolic contents and DPPH radical scavenging showed antioxidant result for OSESE. It is concluded that *Ocimum sanctum* is a very essential plant medicinally. A long term research project is a must to evaluate the pharmacological uses of extracts with different solvents that can be used to isolate the pure and high yield of chemical constituents from the plants.

Keywords: soxhelt, maceration, ethanol, phytochemical, antibacterial, anti-oxidant

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Introduction

The plant's height is up to 1 m, with branches, carrying with a pungent aromatic odour smell, the branchlets and new growth pubescent with soft white hairs. The *Ocimum sanctum*'s leaves with blades elliptic to elliptic-oblong approximately 3 to 6cm long, width is1–2.5cm, cuneate to attenuate at base, obtuse to acute at apex, entire to remotely serrate at margins, pubescent on both surfaces but especially on the nerves beneath. For the flowers terminal, the slender racemes or panicles are 4 to 12cm long with the width of 1 to 1.5cm, the bracteoles are 2 to 3mm long, ovate, acuminate, ciliate; flowers in verticils, on the pedicels are 2 to 4.5mm long; at anthesis, the calyx c is 2.5mm long, in fruit up to 5mm long, glabrous within, the upper lip suborbicular, reflexed, short-apiculate, the lower lip longer than the upper lip, the teeth 4, lanceolate; corolla pale pink, pale lavender or white, to 4mm long; filaments of stamens exerted, slender, the upper pair of each with a small, bearded basal appendage. The appearance of fruit is purple-green to brown, broadly ellipsoid, approximately 0.8–1.2mm long, smooth to minutely pitted, swelling in water.[1,2] The leaves and flowers of *Ocimum sanctum* are shown in Figure 1.

Figure 1 The leaves and flowers of *Ocimum sanctum*.

Other names of *Ocimum sanctum* are Tulsi, Tulasi, Gouri, Bhuteshta, Bhutaghini, Nagamata, Surasah, Mal-Tulasi, Krsiatulasi, Indian Basil, Holy Basil, Sacred Basil, Nalla Thulasi, Raihan, Lo-Le, Basil Icum, Basilic, Basilienkraut, Selasih, Kemangi, Basilico, Meboki, Selaseh, Belanoi, Sulasi, Man Jericao, Bazilik, Albahaca, Suwenda-Tala, Maduru-Tala, Basilkort, Horopa, Manghk, Krapow, Bai Horapa, Rau Que.[3,4] It is available in India, Sri Lanka, Himalaya, Bangladesh, South West Asia, Burma, China, Thailand, Malaysia. In addition, it is also available at dry sandy areas in Hainan, Sichuan, Taiwan Cambodia, Indonesia, Laos, Myanmar, Philippines, Vietnam; Africa, South West Asia, Australia.[2] *Ocimum sanctum*, the Queen of medicinal herbs is the holiest and the most valuable of the many healing and ill-health giving herbs of the suitable way.[5,6] The blessed Basil or Tulsi is significant in the traditional Ayurvedic and Unani system.[5] In India, *Ocimum sanctum* are believed that it can be given the treatment of bronchitis, bronchial asthma, malaria, diarrhoea, dysentery, skin diseases, arthritis, painful eye diseases, increase in body temperature and also insect bite. More importantly it has anticancer, antifungal, antihypergycaemic, antibacterial, in treatment of nausea and vomiting, protection against liver and heart, analgesic, adaptogenic and diaphoretic actions. It improves the body immune system, reproductive system, CNS, CVS, gastrointestinal tract system, urinary system and also blood circulation.[4] Table 1 show the nutritional facts about *Ocimum sanctum*.

Table 1 Nutritional highlight of *Ocimum sanctum*

In fresh Tulsi, five leaves were tested. (2.5g)	
Calories	0.675
Protein	0.064g
Carbohydrate	0.108g
Total Fat	0.015g
Fiber	0.098g

Methods

Collection and preparation of plant materials

Green and fresh 557g of *Ocimum sanctum* was collected from the plants. The leaves were cleaned by distilled water and then leaves were separated from the branches manually. The separated leaves were weighed again and net weight was 349.64g and allowed for air drying under the room temperature to avoid destruction of active group in the leaves. The dried leaves were crushed by using hand into very small pieces.

Maceration

The 25.0g of crushed raw material was subjected to maceration with 200ml of absolute ethanol in round bottom flask and sealed with the aluminium foil and kept in the dark for seven days. The round bottom flask was shaken throughout to ensure uniform and complete extraction. The mixture was filtered by using clean Muslin cloth and the filtrate was collected in a cleaned beaker. The residue of maceration extract and filtrate of maceration were separated and being kept inside the cabinet for further screening.

Soxhlet extraction

26.0g of the crushed powder form was placed inside a thimble already fixed with the chromatographic paper. Ethanol added was 350ml for the extraction and poured into the round bottom flask of Soxhlet apparatus. The temperature was kept at 70°C and maintained throughout the process. The whole process took about 30 hours to complete till the clearance of colour extract. The residue of maceration extract and filtrate of maceration were separated and being kept inside the cabinet for further screening.

Evaporation

The evaporation was carried out from the extract (Soxhlet and Maceration) in a rotary evaporator. The temperature was set at 70°C throughout the evaporation and concentration. The extracts of Soxhlet and maceration, after evaporation, were 88ml and 25ml respectively.

Phytochemical screening[7–12]

The results of phytochemical analysis is recorded and tabulated in Table 2.[7–12]

Table 2 Results for qualitative phytochemical screening

No.	Phytochemical Tests	Ethanolic maceration		Ethanolic Soxhlet Sample
		Filtrate	Residue	
1	Alkaloids	+	+	+
2	Reducing Sugar	-	-	-
3	Saponins	-	-	-
4	Terpenoids	+	+	+
5	Antraquinones	-	-	-
6	Glycosides	-	-	-
7	Tannins	-	-	-
8	Flavonoids	-	-	-
9	Carbohydrate	+	+	+

+ presence, - absence

Methodology for determination of antibacterial activity Preparation of Luria Bertani broth media

2.0g of Luria Bertani broth was dissolved in 100ml distilled water. Then, 10ml of Luria Bertani broth was poured into each 4 universal bottles and subjected to the autoclave at high pressure saturated steam 121°C for around 1 hour in the laboratory.[13–17]

Preparation of Muller Hinton Broth

38.0g Muller Hinton Broth was dissolved into 1000ml of distilled water and poured into two 500ml of Scott bottles. After that, the two Scott bottles were taken to autoclave at high pressure saturated steam 121°C for around 1 hour in the Biotechnology laboratory. After the autoclaving have done, the sufficient sterilized quantity of Muller Hinton agar was poured into the sterilized petri plates and was allowed to solidify. Agar plates were stored in incubator at about 37°C.[13–17]

Bacteria strains cultures

Bacteria strains of *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus pyogenes* were cultured using Luria Bertani broth in 4 different universal bottles. The bacterial strains stored in the universal bottles were left shaking incubator for 24 hours at 37°C at 180rpm. The next days, the bacteria strains which has grown in Luria Bertani broth is then cultured into Muller Hinton agar.[16]

Dilution of extracts[16]

(dissolved in sterilized distilled water)

a. To prepare 1.0mg/ml of the extract, 10mg of the extract was dissolved using 10ml of sterilized distilled water.
b. To prepare 5.0mg/ml of the extract, 50mg of the extract was dissolved using 10ml of sterilized distilled water.
c. To prepare 10.0mg/ml of the extract, 100mg of the extract was dissolved using 10ml of sterilized distilled water.

Well Diffusion test preparation in laminar air flow cabinet[16,17]

i. 1mg/ml of ciprofloxacin was used as a positive control and sterilized distilled water was used as a negative control.
ii. Marker pen was used to label the bottom of the prepared petri dish.
iii. 100µl of bacterial strain (*Bacillus subtilis*) was spread onto the surface of agar by using spreader.
iv. Five different spot corresponding to the label around 6 to 8mm were punched aseptically by using cork-borer.
v. Each holes was filled with ciprofloxacin, sterilized distilled water, 1mg/ml of extract, 5mg/ml of extract and 10mg/ml of extract respectively by using 100µl micropipette.
vi. The procedure was repeated for another three bacterial strains (*Pseudomonas aeruginosa, Escherichia coli and Streptococcus pyogenes*).
vii. After that, the agar plates were covered and subjected to incubate at 37°C for 24 hours.
viii. Antimicrobial activity is determined by measuring with the inhibition zone.

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Results of antibacterial activity

Result for the Well Diffusion Test. Table 3 indicates the zone of inhibition in mm. In present antibacterial study, two different extracts with three different concentrations were investigated to detect the zone of inhibition against *Bacillus subtilis, Pseudomonas aeruginosa, Streptococcus pyogenes* and *Escherichia coli*. Ciprofloxacin was used as a positive control and sterilized distilled water was used as a negative control. As a result, there is no antibacterial inhibition zone occurred in the most of the agar plates. This may be due to low concentration of the extract being used. Low concentration of the *Ocimum sanctum* was unable to produce any antibacterial effect against the bacteria strains in the agar plate. Figure 2, Figure 4, Figure 6 and Figure 8 shows the ethanolic maceration extract and Figure 3, Figure 5, Figure 7 & Figure 9 indicates the ethanolic Soxhlet extract on the agar plate. All Figures shown at lower region, from 2–9, showed that the agar plates do not have any antibacterial activity by well diffusion method. There was an absence of zone of inhibition for each of the different concentration used by the extract solution.[16,17]

Table 3 Zone of inhibition in mm

Microorganisms	Concentrated extract	1 mg/ml	5 mg/ml	10 mg/ml	Ciprofloxacin	Sterilized distilled water
Bacillus subtilis	Ethanolic Maceration	-	-	-	24	-
	Ethanolic Soxhlet	-	-	-	24	-
Pseudomonas aeruginosa	Ethanolic Maceration	-	-	-	26	-
	Ethanolic Soxhlet	-	-	-	24	-
Streptococcus pyogenes	Ethanolic Maceration	-	-	-	26	-
	Ethanolic Soxhlet	-	-	-	26	-
Escherichia coli	Ethanolic Maceration	-	-	-	24	-
	Ethanolic Soxhlet	-	-	-	24	-

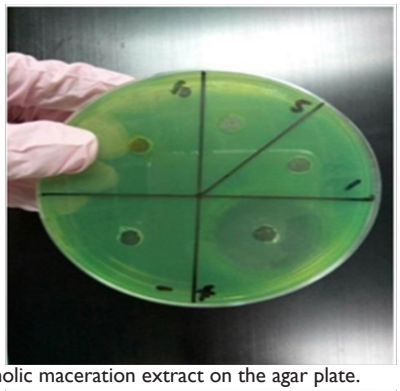

Figure 2 Ethanolic maceration extract on the agar plate.

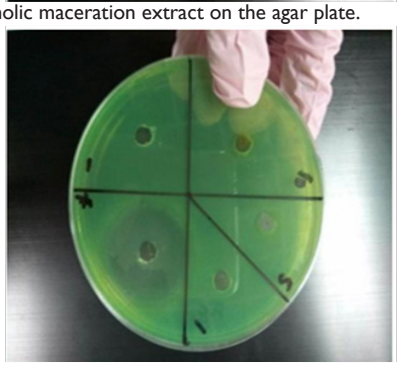

Figure 3 Ethanolic Soxhlet extract on the agar plate.

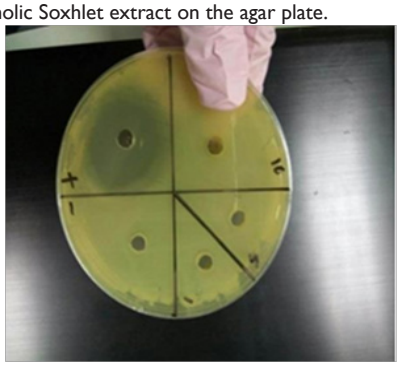

Figure 4 Ethanolic maceration on the agar plate.

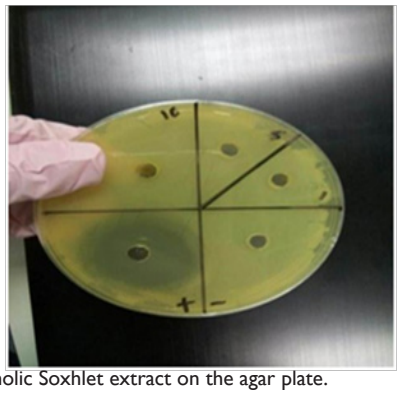

Figure 5 Ethanolic Soxhlet extract on the agar plate.

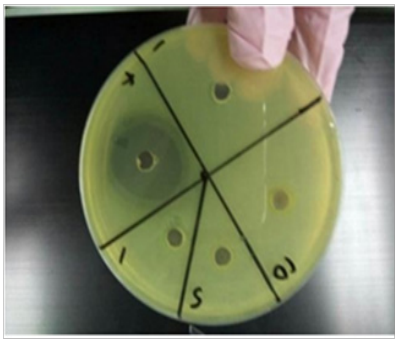

Figure 6 Ethanolic maceration extract on the agar plate.

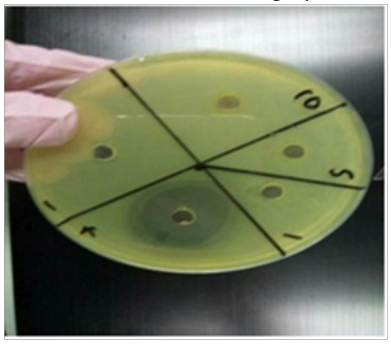

Figure 7 Ethanolic Soxhlet extract on the agar plate.

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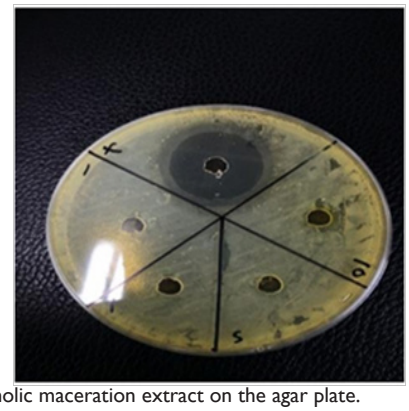


Figure 8 Ethanolic maceration extract on the agar plate.

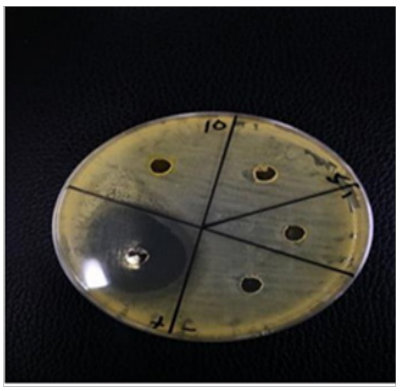


Figure 9 Ethanolic Soxhlet extract on the agar plate.

Pseudomonas aeruginosa

Figure 2 & Figure 3

Escherichia coli

Figure 4 & Figure 5

Bacillus subtilis

Figure 6 & Figure 7

Streptococcus pyogenes

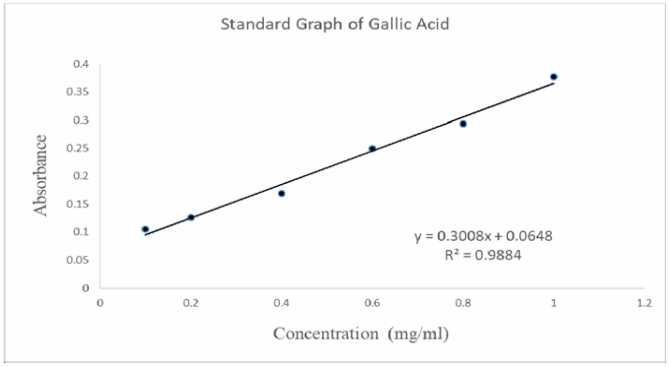
Figure 8 & Figure 9

All Figures from 2–9 showed that the agar plates do not have any antibacterial activity determination by well diffusion method. There was an absence of zone of inhibition for each of the different concentration used by the extract solution.

Antioxidant activity determination

Methodology for determination of antioxidant activity

Total phenolic contents analysis (TPC)[18]: For the total phenolic contents analysis, Folin-Ciocalteau (FC) assay was applied to *Ocimum sanctum* ethanolic Soxhlet extract to find out the total phenolic content of this extract. In this analysis, the standard used was Gallic acid and the measurement was done at λ max 765nm. Gallic acid standard calibration curve was generated as shown in Graph 1 and the regression value was 0.9884. From the absorbance obtained, the amount of the phenolic content measured as Gallic acid equivalent (GAE) using FC method. The total phenolic content obtained at the concentration of 1.0mg/ml was found to be the highest when compared to other lower concentration extract solution. As a result, the higher the concentration of the stock solution used for this analysis, the higher the Gallic acid equivalent (GAE).[19–25]



Graph 1 Standard graph of the Gallic acid plotted from the series of absorbance obtained from UV- Visible Spectrophotometer.

Extract preparation

i. To prepare 1mg/ml of the extract solution, 10mg of the extract was dissolved using 10ml of ethanol.
ii. To prepare 0.8mg/ml of the extract solution, 8ml of the 1mg/ml of the extract solution was dissolved using 10ml of the ethanol.
iii. To prepare 0.6mg/ml of the extract solution, 7.5ml of the 0.8mg/ml of the extract solution was dissolved using 10ml of the ethanol.
iv. To prepare 0.4mg/ml of the extract solution, 6.67ml of the 0.6mg/ml of the extract solution was dissolved using 10ml of the ethanol.
v. To prepare 0.2mg/ml of the extract solution, 5ml of the 0.4mg/ml of the extract solution was dissolved using 10ml of the ethanol.
vi. To prepare 0.1mg/ml of the extract solution, 5ml of the 0.2mg/ml of the extract solution was dissolved using 10ml of the ethanol.

Preparation of sample extract, blank and standard[16,17]

i. 0.5ml of the extract solution (1.0mg/ml) was added with the 2.5ml of the 0.75% sodium bicarbonate and 2.5ml of the 1% Folin–Ciocalteu's reagent.
ii. The sample mixtures were incubated at 45°C for 15 minutes. The UV absorbance was detected at λ max 765nm.
iii. The steps were repeated by using other concentration of extract solution (0.8mg/ml, 0.6mg/ml, 0.4mg/ml, 0.2mg/ml and 0.1mg/ml).
iv. Blank was prepared by using ethanol instead of extract solution.
v. The same steps were prepared for the Gallic acid (standard) and the calibration line was plotted.
vi. The total phenolic content was calculated in term of Gallic acid equivalent (mg of GAE/g of extrtact) by using formula of:

C= (A/B) x dilution factor

C, Total phenolic content; A, x value; x, regression line; B, concentration of extract

DPPH free radical scavenging assay[16]

i. Different concentration of extract solution (0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0mg/ml) were prepared as well as control (methanol without extract sample).
ii. Different concentration of extract solution was subjected to the uniformly mixing.
iii. 5.0ml of methanolic solution of DPPH reagent was added individually to each of the different concentration of the extract solution.
iv. The mixture samples were then subjected to vortex for few minutes.
v. The mixture samples were incubated at room temperature in the dark for around 30 minutes.
vi. The absorbance for each concentration mixture samples was measured at 517nm against a blank by using UV-Visible spectrophotometer.
vii. The percentage of DPPH scavenging activity was calculated using the following equation:

$$\%\ Scavenging\ of\ test\ sample = \frac{control\ absorbance - test\ absorbance}{control\ absorbance} \times 100$$

viii. All the procedure was repeated for BHT solution (standard) of different concentration (similar to the previous concentration).
ix. The 50% inhibitory concentration value (IC_{50}) for both extracts were calculated and it is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals.
x. The data for both extract sample and standard were obtained and tabulated.

Results of antioxidant activity

The results are shown in Table 4, Table 5, Table 6.

OSESE

Table 4

Concentration	y	x	A	B	Dilution factor	C
0.1mg/ml	0.3008 x + 0.0648	0.073mg/ml	0.073mg/ml	0.1mg/ml=0.0001g/ml	1/10000	(A/B) x dilution factor= (0.073/0.0001) x 1/10000= 0.073 mg GAE/g
	0.067=0.3008x + 0.0648					
	0.3008 x = 0.0022					
0.2mg/ml	0.3008x + 0.0648	0.084mg/ml	0.084mg/ml	0.2mg/ml=0.0002g/ml	1/5000	(A/B) x dilution factor= (0.084/0.0002) x 1/5000= 0.084 mg GAE/g
	0.090 = 0.3008x + 0.0648					
	0.3008x = 0.0252					
0.4mg/ml	0.3008x + 0.0648	0.297mg/ml	0.297mg/ml	0.4mg/ml=0.0004g/ml	1/2500	(A/B) x dilution factor= (0.297/0.0004) x 1/2500= 0.297 mg GAE/g
	0.154 = 0.3008x + 0.0648					
	0.3008x = 0.0892					
0.6mg/ml	0.3008x + 0.0648	0.513mg/ml	0.513mg/ml	0.6mg/ml=0.0006g/ml	3/5000	(A/B) x dilution factor= (0.513/0.0006) x 3/5000= 0.513 mg GAE/g
	0.219 = 0.3008x + 0.0648					
	0.3008x = 0.1542					

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Table Continued....

Concentration	y	x	A	B	Dilution factor	C
0.8mg/ml	0.3008x + 0.0648 0.269 = 0.3008x + 0.0648 0.3008x = 0.2042	0.679mg/ml	0.679mg/ml	0.8mg/ml=0.0008g/ml	1/1250	(A/B) x dilution factor= (0.679/0.0008) x 1/1250= 0.679 mg GAE/g
1.0mg/ml	0.3008x + 0.0648 0.354 = 0.3008x + 0.0648 0.3008x = 0.2892	0.961mg/ml	0.961mg/ml	1.0mg/ml=0.001g/ml	1/1250	(A/B) x dilution factor= (0.961/0.001) x 1/1000= 0.961 mg GAE/g

Table 5 UV absorbance of Gallic acid in various concentration

S. No	Concentration of Gallic acid / mg/ml	Absorbance
1	0.1	0.105
2	0.2	0.126
3	0.4	0.169
4	0.6	0.25
5	0.8	0.294
6	1	0.377

Table 6 Dilution factors for Gallic acid in total phenolic analysis

Conc. Of Sample (mg/mL)	100	10	1	0.1	0.2	0.3	0.4	0.6	0.8
Stock solution (ml)	1000	100	10	1	1	1	1	1	1
Vol. of stock solution (ml)	1	1	1	1	2	3	4	6	8
Vol. of 95% methanol (ml)	9	9	9	9	8	7	6	4	2
Total Volume (ml)	10	10	10	10	10	10	10	10	10
Dilution factor	1/10n	1/100	1/1000	1/10000	1/5000	3/10000	1/2500	3/5000	1/1250

DPPH free radical scavenging analysis[18]

Table 7, Table 8

Table 7 Absorbance value of standard (BHT) and extract of *Ocimum sanctum* at 517nm, Control (DPPH) absorbance = 0.550

Concentration (mg/ml)	UV Absorbance	
	BHT	OSESE
0.03125	0.442	0.468
0.0625	0.32	0.421
0.125	0.233	0.314
0.25	0.102	0.168
0.5	0.03	0.053
1	0.02	0.038

Table 8 Percentage scavenging of BHT and OSESE in DPPH assay

DPPH ASSAY		
Concentration (mg/ml)	%Scavenging	
	BHT	OSESE
0.03125	19.64	14.91
0.0625	41.82	23.45
0.125	57.64	42.91
0.25	81.45	69.45
0.5	94.55	90.36
1	96.36	93.09

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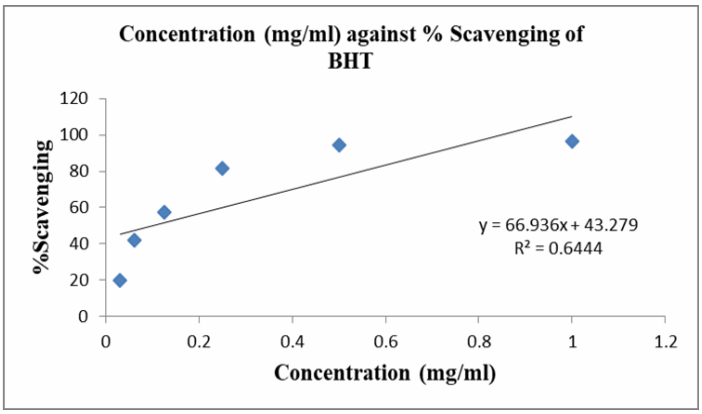
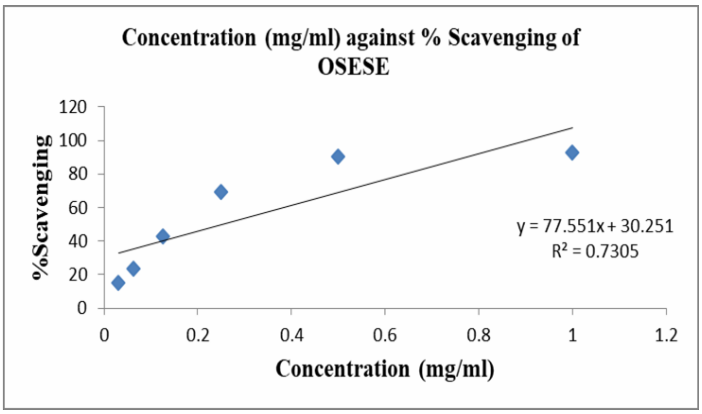
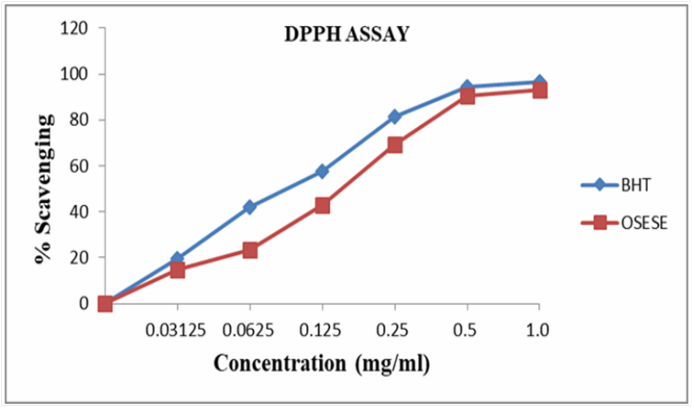


IC_{50}	0.1	0.25



Graph 2 Concentration against % scavenging of butyl hydroxyl toluene



(BHT).

Graph 3 Concentration against % scavenging of OSESE.

Graph 4 Percentage scavenging against concentration for both BHT and OSESE.

Graph 4 shows a gradually decrease in the scavenging ability of the OSESE sample to the standard BHT. OSESE shows IC_{50} of 0.25mg/ml which was comparable to BHT with IC_{50} of 0.10mg/ml. The IC_{50} of BHT was calculated by the equation which plotted from the standard graph of BHT as shown in Graph 2 and the IC_{50} of OSESE was calculated by the equation plottedfrom the graph of concentration against % scavenging of OSESE as shown in Graph 3.

For BHT:

IC_{50}	
Y = MX + C	
50 = 66.936x + 43.279	
66.936x = 50 - 43.279	
66.936x = 6.721	
x =	6.721/66.936
x =	0.1mg/ml

For OSESE:

IC_{50}	
Y = MX + C	
50 = 77.551x + 30.251	
77.551x = 50 - 30.251	
77.551x = 19.749	
x =	19.749/77.551
x =	0.25 mg/ml

In DPPH scavenging method, the result obtained for OSESE at 1.0mg/ml stock concentration was 93.09mg/ml whereas in total phenolic content analysis, the result obtained for OSESE at 1.0mg/ml stock concentration was 0.961mg GAE/g.

Conclusion

Hence, this can be proved that this extract has the antioxidant potential. The preliminary in- vitro antibacterial screening of *Ocimum sanctum* was not effectively showed any control over the growth of the test bacteria strains due to low concentration used in this study. To have more insight into the antibacterial screening, further investigation on isolates of extracts should be done perfectly. *Ocimum sanctum* has shown substantial antioxidant activity through total phenolic content analysis and DPPH radical scavenging analysis. It is concluded that there is a good antioxidant potential of *Ocimum sanctum* with ethanolic Soxhlet extraction.

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None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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