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Comparative Study of Antioxidant, Antimicrobial and Inhibitory Properties of Lipoxygenase Using *Ocimum tenuiflorum* and *Ocimum sanctum* Extracts.

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ABSTRACT

Ocimum tenuiflorum and *Ocimum sanctum* were used as a medicinal plant in ancient ayurveda. The objective of this study is to investigate the phytochemical constituents, antioxidant, antimicrobial and enzyme inhibition properties to justify medicinal use of the plant. The extracts were screened for phytochemical screening. Phytochemical screening revealed the presence of carbohydrates, phenols, alkaloids, flavonoids and glycosides. Extracts were assessed for their antioxidant properties utilizing *In Vitro* antioxidant assay models, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power assay (FRAP) and total antioxidant activity for scavenging the free radicals. Minimum inhibitory concentration and minimum bactericidal concentration were performed to evaluate the antimicrobial property. The *InVitro* enzyme inhibition activity was performed for lipoxygenase. Compared to *Ocimum tenuiflorum*, which has an IC_{50} value of 259.79µg/ml, *Ocimum sanctum*'s methanolic extract showed improved antioxidant properties with an IC_{50} value of 113µg/ml. The results of antimicrobial activity revealed that the methanolic extract of *Ocimum* species had better inhibitory activity against pathogenic strains with MIC values of 600µg/ml. Lipoxygenase inhibition was maximum for methanolic extract of *Ocimum sanctum* which highlights its rolein anti-inflammatory responses.

Keywords: Antioxidant, Antimicrobial, Lipoxygenase Inhibition, *Ocimum tenuiflorum* and *Ocimum sanctum.*

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INTRODUCTION

Medicinal plants are a rich source of different types of medicines and produce various bioactive molecules. They are rich in secondary metabolites which are chemically and taxonomically extremely diverse compounds with obscure function. A large number of phytochemicals are widely used in human therapy, agriculture, veterinary and scientific research [1]. *Ocimum sanctum L.* is one such medicinal plant having numerous medicinal properties such as anti-oxidant, antimicrobial, anti-inflammatory etc. Commonly, on the basis of morphology there are different varieties of *Ocimum sp.* that have been found all over India*. Ocimum sp.,* has been used for thousands of years for its diverse healing properties [2]. It is known to possess antimicrobial activity against various bacteria. They also contain antioxidants like beta carotene that help in preventing cell damage [3]. *Ocimum sp.* also have anti-inflammatory properties by which they can block both the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism [4]. In this study, the presence of secondary metabolites, antioxidant property, antimicrobial property and enzyme inhibition activity of *Ocimum tenuiflorum* and *Ocimum sanctum* is compared and investigated.

MATERIAL AND METHODS

Extraction of the plant material

The plant materials were crushed using a blender and fine powder was collected. The fine powder was subjected to extraction using various solvents (methanol, ethanol, chloroform and aqueous). These extracts were then subjected to concentration using a water bath for 6-8 hours and the final residue was collected [5].

Phytochemical Screening

Qualitative Analysis

The residues obtained from extraction were then taken for phytochemical screening. Tests for proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids were performed as per the standard protocol [6].

Quantitative Analysis

Estimation of total phenolic contents

The amount of total phenol for aqueous, methanol, ethanol and chloroform extract were determined by the folin-ciocalteu reagent method. 2.5 ml of 10% folin- ciocalteu reagent and 2 ml of 2% Na_2CO_3 were added to 0.5 ml of plant extract. The mixture was then incubated at room temperature for 30 minutes. Gallic acid was used as standard (1mg/ml). The absorbance of the sample was measured at 765 nm. All the tests were done in triplicates and the results were determined from standard curve and were expressed as gallic acid (trihydroxy benzoic acid) equivalent (mg/g of extracted compound) [7].

Estimation of carbohydrates using anthrone method

The amount of carbohydrates was estimated using the anthrone method using glucose as standard. 5ml of anthrone reagent was added to the sample and it was incubated at 90ºC for 5 minutes. The absorbance was read at 620 nm. Standard glucose was used with the concentration of 0.2mg/ml.

Estimation of alkaloids by Harborne method

5 g of the sample was taken and 200 ml of 10% acetic acid in ethanol was added to the sample and allowed to stand for 4 hours. Then the solution was filtered and the extract was concentrated on water bath conc. ammonium hydroxide was added drop wise and the whole solution was allowed to settle and the precipitate was then washed with dilute ammonium hydroxide and filtered. The residue was dried and weighed and this was the amount of alkaloid present in the plant material [8].

Estimation of flavonoids using aluminium chloride method

Extract was separately mixed with 1.5 ml of ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 418 nm. The percentage of total flavonoids were calculated from the calibration curve of Rutin (200-1000µg) plotted by using the same procedure and total flavonoids were expressed Rutin equivalents (RE) in mg/gm.

Antioxidant Activity

DPPH Radical Scavenging Activity

Different concentrations of sample (100-500µg/ml) were taken and the volume was made up to 1ml using methanol. To these aliquots 1ml of 0.1mM DPPH was added. The tubes were kept for incubation for 30 minutesin the dark. The absorbance was read at 517nm after 30 minutes. Gallic acid was used as the standard curve (1mg/ml). From the absorbance the inhibition percentage was calculated [9]. The formula used was:

$$A_0 - A_S / A_0 \times 100$$

where A_0 is control and A_S is absorbance of sample.

Reducing Power Assay

Different aliquots of sample (100-500 µg/ml) were taken in the test tube. To these tubes 1ml of phosphate buffer (pH 6.9) was added and 1ml of potassium ferricyanide was added. This was then kept for incubation at 50ºC for 20 minutes. After 20 mins, 1ml of trichloro acetic acid was added to all the tubes. To these tubes 0.5ml of distilled water and 0.5ml of ferric chloride was added. The absorbance was measured at 700nm. Gallic acid was used as the standard.

$$A_0 - A_S / A_0 \times 100$$

where A_0 is control and A_S is absorbance of sample.

Total Antioxidant Activity

Firstly, phosphomolybdenum reagent was prepared by mixing 28mM Sodium phosphate, 4mM Ammonium Phosphate and 0.6M sulphuric acid. Aliquots of sampleswere taken in the test tube. To all the tubes 1ml of the phosphomolybdenum reagent was added. The tubes were closed and it was incubated at 95ºC for 90 minutes. The absorbance was read at 695nm.

Antimicrobial Activity

Macro-dilution method

Minimum Inhibition Concentration

Primary inoculum was prepared by using Laboratory strain of *E. coli* and *Aspergillus niger* that was collected from the Department of Microbiology, The Oxford college of Science.The inoculum was cultivated using Muller Hilton broth. The culture was kept in a rotary shaker incubator. After 24 hours of incubation, the inoculum was taken and it was poured in different tubes. To these tubes, different concentrations of sample were added. These tubes were again kept in an incubator. After 24 hours of incubation the turbidity was checked for the *E. coli*strain and after 48 hours of incubation the turbidity was checked for the *Aspergillus* strain. Based on the turbidity the minimum inhibition concentration is studied [10].

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Minimum Bactericidal Concentration

The Primary inoculum is taken after 24 hours incubation and it is poured in different tubes. To these tubes various concentrations of the sample were added. Muller Hilton agar plates are prepared. The tubes are then inoculated in the plates using an inoculum loop. The plates are sealed and are kept in the incubator. After 24 hours and 48 hours of incubation, growth colonies were checked in order to identify the Minimum Bactericidal Concentration.

Extraction of Enzyme

Lipoxygenase enzyme was extracted from soybean seeds. The soybean seeds were grinded into fine powder. The powder was mixed with 3 times more volume of n-hexane. It was centrifuged at 10000 rpm for 30 minutes. The supernatant was collected and it was centrifuged again for 10000 rpm for 30 minutes. The cycle was repeated for one more time. The final supernatant was precipitated using polyethylene glycol 6000. The upper layer was collected and was stored in cold condition. This was used as the source of enzyme [11].

Inhibition of Lipoxygenase Enzyme

The Enzyme activity was performed by using linoleic acid as substrate in presence of borate buffer. The reaction mixture was kept for incubation at room temperature for 30 mins. The product (lipoxygenase hydroperoxides) is then taken in various volumes in-order to estimate its amount. The absorbance is read at 235nm using a spectrophotometer. In the reaction mixture, various concentrations of sample were added [12].

RESULT AND DISCUSSION

Phytochemical Screening

The phytochemical screening of different extracts of the two *Ocimum species* revealed the presence of phenols, carbohydrates, flavonoids, glycosides, alkaloids in all the four samples. The results are illustrated in Table 1. These are bioactive chemical constituents present in plants that have various physiological actions in the human body [13]. These phytochemicals have been well known for their medicinal and physiological activity. Tannins, Saponins and Terpenoids were present only in the methanol and ethanol extract. Proteins and Steroids showed negative results for all the four extracts [14].

Table 1: Phytochemical screening of *Ocimum sanctum* and *Ocimum tenuiflorum*

Bioactive constituents	*Ocimum tenuiflorum*				*Ocimum sanctum*			
	Methanol	Ethanol	Chloroform	Aqueous	Methanol	Ethanol	Chloroform	Aqueous
Protein	-	-	-	-	-	-	-	-
Carbohydrates	+	+	+	+	+	+	+	+
Phenol	+	+	+	+	+	+	+	+
Tannin	+	+	-	-	+	+	-	-
Flavonoid	+	+	+	+	+	+	+	+
Saponins	+	+	-	-	+	+	-	-
Glycosides	+	+	+	+	+	+	+	+
Steroids	-	-	-	-	-	-	-	-
Terpenoids	+	+	-	-	+	+	-	-
Alkaloids	+	+	+	+	+	+	+	+

\+ Indicates the presence of the compounds in the given species.
\- Indicates the absence of the compounds in the given species.

Estimation of total phenol, carbohydrate, alkaloids and flavonoid Content

The results of total phenolic, carbohydrate, alkaloid and flavonoid contents are shown in Table 2. From the results obtained phenols, carbohydrates, alkaloids and flavonoids were higher in methanol

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extract of *Ocimum sanctum* in comparison to extracts of *Ocimum tenuiflorum*. The amount of phenol was found to be 242µg/ml for the methanolic extract of *Ocimum sanctum* which was the highest, and least value of 95µg/ml amount was observed for aqueous extract of *Ocimum sanctum*. The amount of carbohydrates, alkaloids and flavonoid were 78 µg/ml, 65%, 69µg/ml respectively for the methanolic extract of *Ocimum sanctum*. Earlier reports have confirmed the presence of alkaloids, terpenes, phenol, tenins, steroids etc in methanolic extract of *Ocimum sp.* [15],[16]. The various factors affecting the recovery of polyphenols and flavonoids include the nature of sample, solvent, time duration and temperature of extraction. Methanol has already been demonstrated to extract polar polyphenols and flavonoids more efficiently than other solvents.

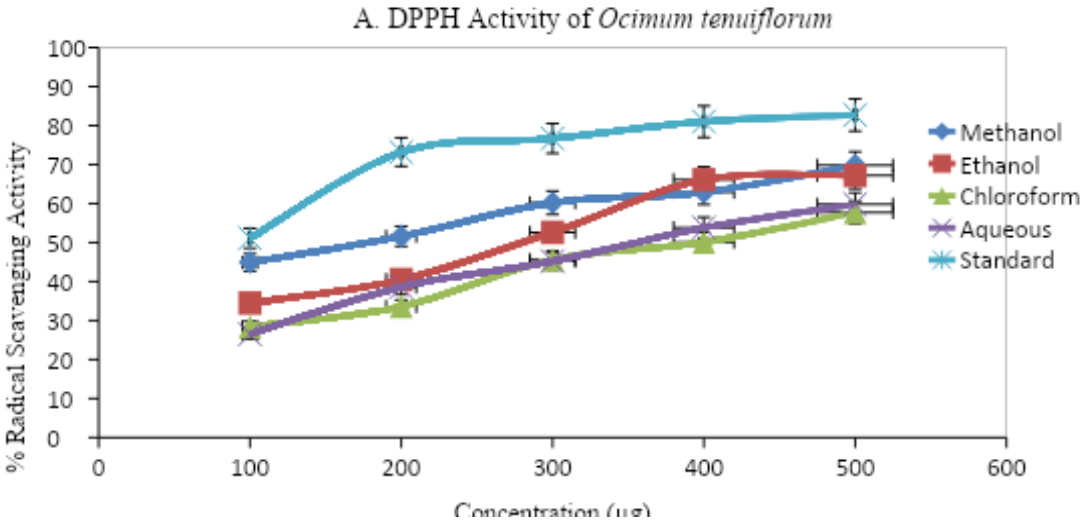
Table 2: Estimation of total Phenol, Carbohydrate, Alkaloids and Flavonoid Content of *Ocimum tenuiflorum* and *Ocimuum sanctum*

Plant samples	Solvent Extracts	Phenols (µg/ml)	Carbohydrates (µg/ml)	Alkaloids (%)	Flavonoids (µg/ml)
Ocimum tenuiflorum	Methanol	220	63	61	67
	Ethanol	215	60	60	64
	Chloroform	200	20	30	54
	Aqueous	178	30	47	62
Ocimum sanctum	Methanol	242	78	65	69
	Ethanol	225	67	62	66
	Chloroform	210	26	25	58
	Aqueous	95	37	52	59

Antioxidant Activity

DPPH Free radical Scavenging Activity for *Ocimum tenuiflorum* and *Ocimum sanctum*:

The antioxidant activity of various fractions of *Ocimum sp.* determined as DPPH free radical scavenging potential are depicted in Figure 1 and Table 3.The methanolic extract of *Ocimum sanctum* showed higher antioxidant activity with IC_{50} 113µg/ml. Antioxidant activity of medicinal plants serves as scavengers of free radicals thus playing a vital role in neutralising the free radical potentiality. The results indicate that *O. sanctum* has bioactive components that could impart hydrogen to a free radical and terminate the potential damaging effect [13]. The free radical scavenging is a major mechanism in *Ocimum sanctum* products that protect against cellular damage. The highest antioxidant activity of alcoholic extract has a linear correlation with total flavonoid and phenolic contents. The phenolic compounds, mainly rosmarinic acid, plays a major role in the antioxidant activity of *O. sanctum extract* [17]. Recently in a similar study using DPPH they have reported strong antioxidant property with IC_{50} values of 255.6ppm and 455.11ppm for *Ocimum tenuiflorum* and *Ocimum sanctum* respectively[18].



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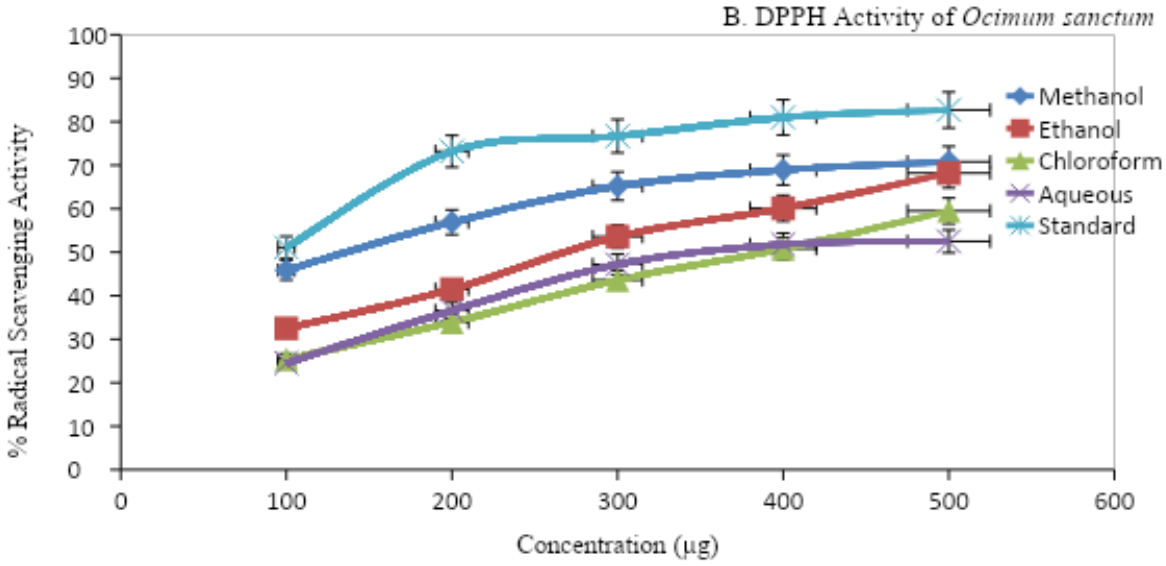

Figure 1: DPPH Activity of A: *Ocimum tenuiflorum* B: *Ocimum sanctum*

Table 3: DPPH Free radical Scavenging Activity for *Ocimum tenuiflorum* and *Ocimum sanctum*

	Percentage inhibition of *Ocimum tenuiflorum* (%)					Percentage inhibition of *Ocimum sanctum* (%)				
Concentration	Methanol	Ethanol	Chloroform	Aqueous	Standard	Methanol	Ethanol	Chloroform	Aqueous	Standard
100	44.9	34.5	28.4	26.5	51.1	45.9	32.5	25.4	24.5	51.1
200	51.6	40.5	33.6	38.6	73.2	56.9	41.5	33.9	36.6	73.2
300	60.2	52.6	45.6	45.2	76.7	65.2	53.6	43.6	47.2	76.7
400	62.9	66.1	50.1	53.8	81	68.9	60.1	50.8	51.8	81
500	69.8	67.2	57.7	59.8	82.7	70.8	68.2	59.5	52.5	82.7

Reducing Power Assay on *Ocimum tenuiflorum* and *Ocimum sanctum*

The results of FRAP assay obtained with different solvent extracts *Ocimum sp* are shown in Figure 2 and Table 4. The highest inhibition efficiency 171.03µg/ml was observed in methanolic extract of *Ocimum sanctum,* compared to other extracts. The IC_{50} was highest for the aqueous extract of *Ocimum tenuiflorum* with 459.17µg/ml, which indicates that it has the lowest inhibition efficiency. The reducing ability of *Ocimum* extracts assayed by FRAP method measured the electron donating ability of the extract. The results obtained suggest that the methanolic extract of *Ocimum sanctum* can play a protective role against oxidative damage by reactive species [19].

Table 4: Reducing Power Assay (FRAP) on *Ocimum tenuiflorum* and *Ocimum sanctum*

	Percentage inhibition of *Ocimum tenuiflorum* (%)					Percentage inhibition of *Ocimum sanctum* (%)				
Concentration	Methanol	Ethanol	Chloroform	Aqueous	Standard	Methanol	Ethanol	Chloroform	Aqueous	Standard
100	44.9	34.5	28.4	26.8	51.1	37.5	31.8	29.6	26.8	51.1
200	51.6	40.5	33.6	38.6	73.2	46.2	41.6	34.88	31	73.2
300	60.2	52.6	45.6	45.2	76.7	52.8	49	45.5	37.9	76.7
400	62.9	66.1	50.1	53.8	81	63.5	60	51.5	47.5	81
500	69.8	67.2	57.7	59.8	82.7	77.6	66.8	59.8	52.6	82.7

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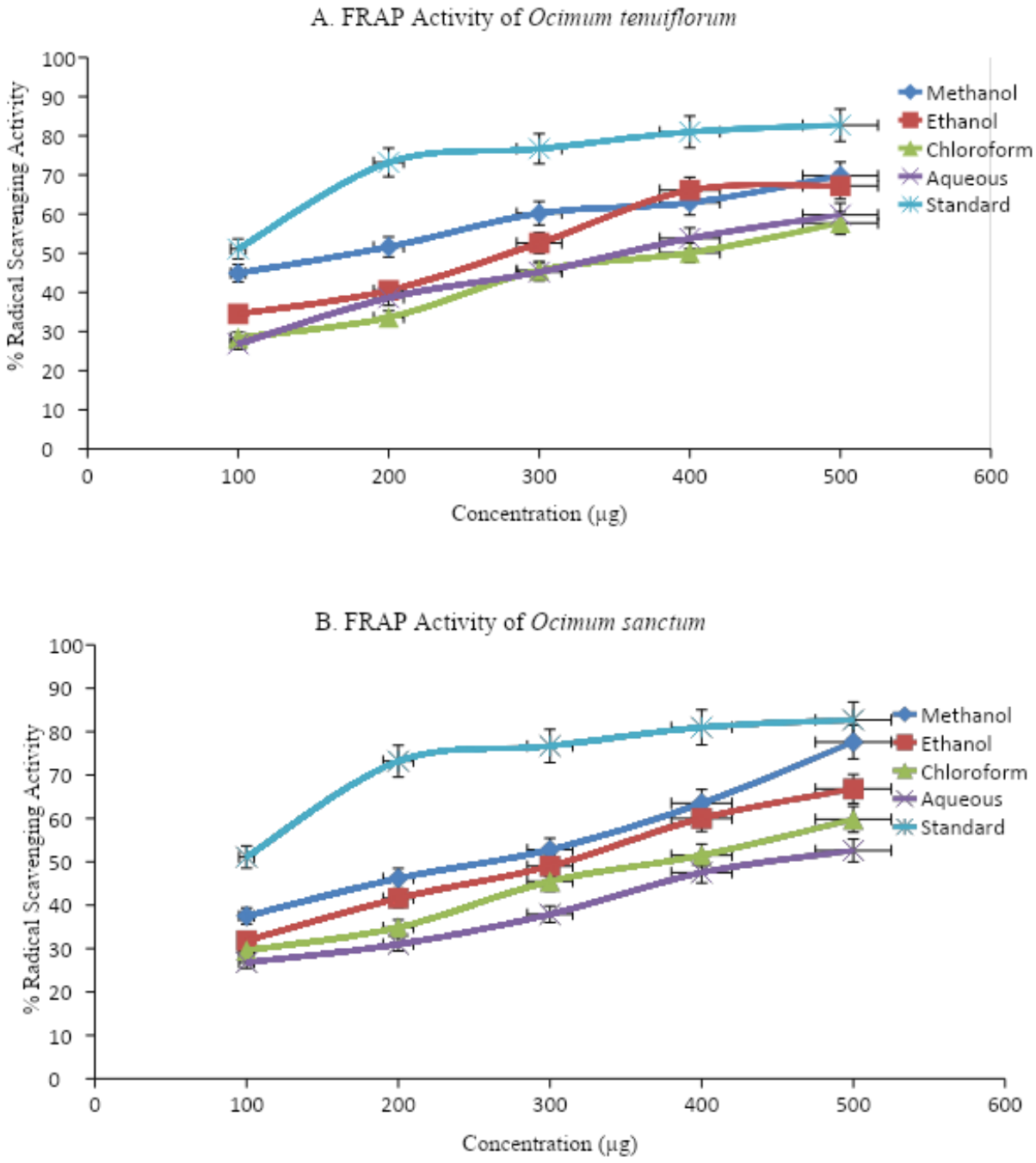

Figure 2: FRAP Activity of A: *Ocimum tenuiflorum* and B: *Ocimum sanctum*

Total Antioxidant Property of *Ocimum tenuiflorum* and *Ocimum sanctum*

Total Antioxidant concentration was 52µg/ml for methanolic extract of *Ocimum sanctum*, as compared to other extracts as shown in Table 5. Higher the concentration of phenolic and flavonoids content confirms the potent antioxidant activity, which are responsible for scavenging the free radicals [20].

ISSN: 0975-8585

Table 5: Total antioxidant capacity of *Ocimum tenuiflorum* and *Ocimum sanctum*

Plant Sample	Extracts	Absorbance at 695nm	Concentration [µg/ml]
Ocimum tenuiflorum	Methanol	0.20	45
	Ethanol	0.16	36
	Chloroform	0.06	13
	Aqueous	0.09	20
Ocimum sanctum	Methanol	0.23	52
	Ethanol	0.18	41
	Chloroform	0.07	16
	Aqueous	0.08	18

Anti-microbial Activity

MIC and MBC

The methanolic extract of both the species showed MIC 600µg/ml for *E. coli* and *Aspergillus niger*. The lowest inhibition was seen in the aqueous extract with 800µg/ml. However, compared with standard, the minimum inhibitory concentration was three times more efficient than the methanolic extract. The results are seen in Figure 3 and Table 6. The closer MIC result was reported for *Ocimum sanctum* [21]. The methanolic extract of both the species showed MBC 800µg/ml for *E. coli* and *Aspergillus niger*. The lowest inhibition was seen in the aqueous extract with 1000µg/ml. However, compared with standard, the minimum inhibitory concentration was more efficient than the methanolic extract. The results are seen in Figure 4 and Table 7. From the results, it suggests that the presence of alkaloids and phenolic content in the methanolic extract of *Ocimum. sanctum,* which are responsible for antimicrobial activity in the defined mode of function. The present results showed a good inhibitory effect of *Ocimum* extracts on pathogenic bacteria [22].

Table 6: Minimum Inhibition Concentration of *Ocimum tenuiflorum* and *Ocimum sanctum.*

Plant Sample	Extracts	MIC [µg/ml]
Ocimum tenuiflorum	Standard	200
	Methanol	600
	Ethanol	800
	Chloroform	800
	Aqueous	1000
Ocimum sanctum	Standard	200
	Methanol	600
	Ethanol	800
	Chloroform	800
	Aqueous	1000

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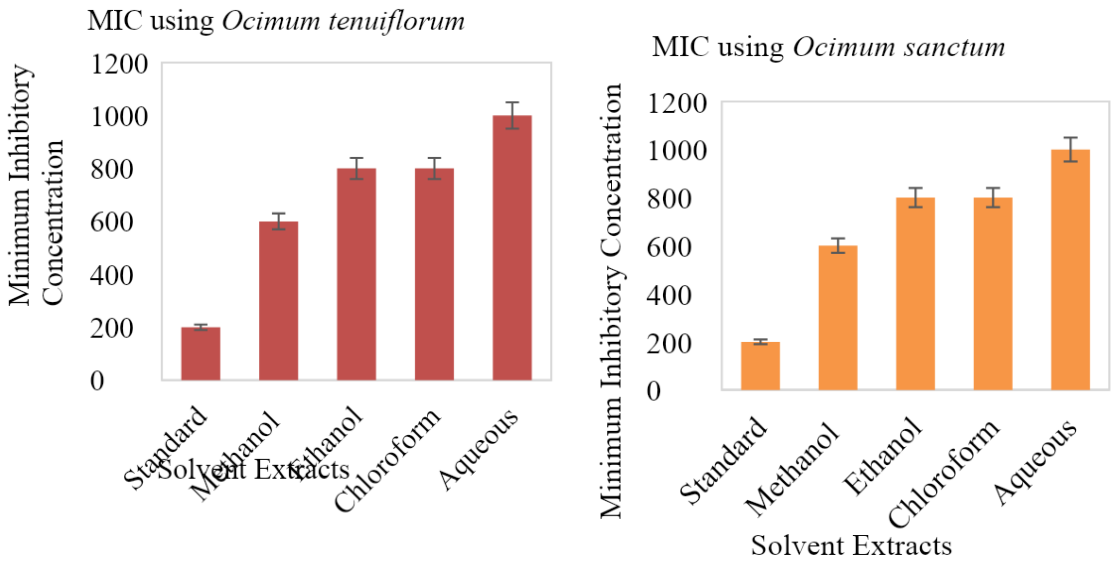

Figure 3: Minimum Inhibition Concentration of *Ocimum tenuiflorum* and *Ocimum sanctum.*

Table 7: Minimum Bactericidal Concentration of *Ocimum tenuiflorum* and *Ocimum sanctum.*

Plant Sample	Extracts	MIC [µg/ml]
Ocimum tenuiflorum	Standard	400
	Methanol	800
	Ethanol	800
	Chloroform	1000
	Aqueous	1000
Ocimum sanctum	Standard	400
	Methanol	800
	Ethanol	800
	Chloroform	1000
	Aqueous	1000

Enzyme Inhibition

The results of lipoxygenase inhibition by *Ocimum* extracts are shown in figure 5 and Table 8. *Ocimum sanctum* produced highest $IC_{50}$of 286.34µg/ml for methanolic extract in the enzyme inhibition activity. Previous study on Lamiaceae family demonstrated IC_{50} values ranging from 15 to 30µg/ml (leaf extract) for the inhibition of Lipoxygenase [23]. Lipoxygenases are enzymes that catalyse the oxidation reaction in polyunsaturated fatty acids to produce hydroperoxides [24]. Overproduction of Lipoxygenase enzymes lead to the production of leukotrienes that stimulate inflammatory reactions in the human body and are also associated with various disease development including cancer, neurodegenerative, cardiovascular diseases etc [23]. From the present study, lipoxygenase inhibition was maximum for methanolic extract of *Ocimum sanctum.* Further the isolation and identification of these potent compounds can pave the way for the development of anti-lipoxygenase inhibitorsfor suppressing the inflammatory reactions in various disease conditions.

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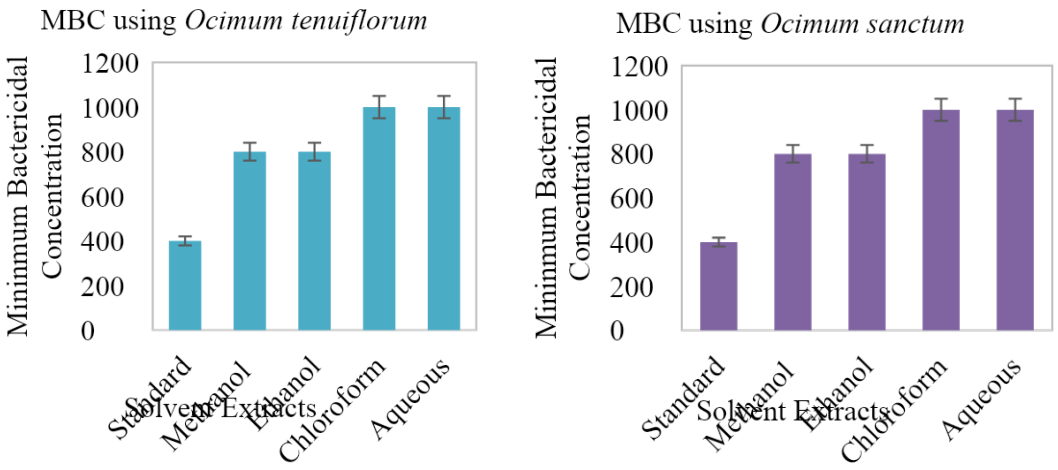
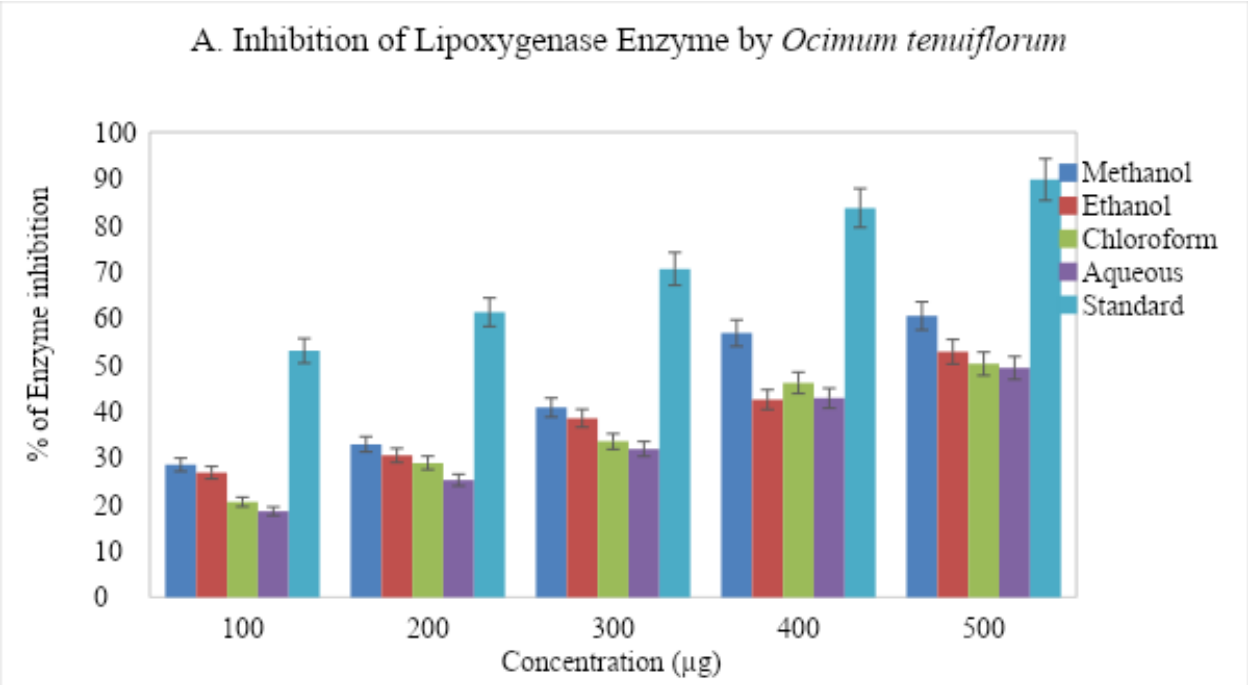


Figure 4: Minimum Bactericidal Concentration of *Ocimum tenuiflorum* and *Ocimum sanctum.*

Table 8: Inhibition of Lipoxygenase Enzyme by *Ocimum tenuiflorum* and *Ocimum sanctum*.

	Ocimum tenuiflorum					*Ocimum sanctum*				
Concentration	Methanol	Ethanol	Chloroform	Aqueous	Standard	Methanol	Ethanol	Chloroform	Aqueous	Standard
100	28.5	26.8	20.5	18.5	53	32.3	28.1	20	19.5	53
200	32.9	30.5	28.9	25.2	61.3	41.7	31.4	28.5	26.8	61.3
300	40.8	38.5	33.5	31.9	70.6	50.5	37.8	36.9	32.5	70.6
400	56.8	42.5	46.1	42.8	83.7	62.5	40.9	45.7	42.5	83.7
500	60.5	52.8	50.2	49.3	89.8	69.5	51.6	53.2	50.1	89.8



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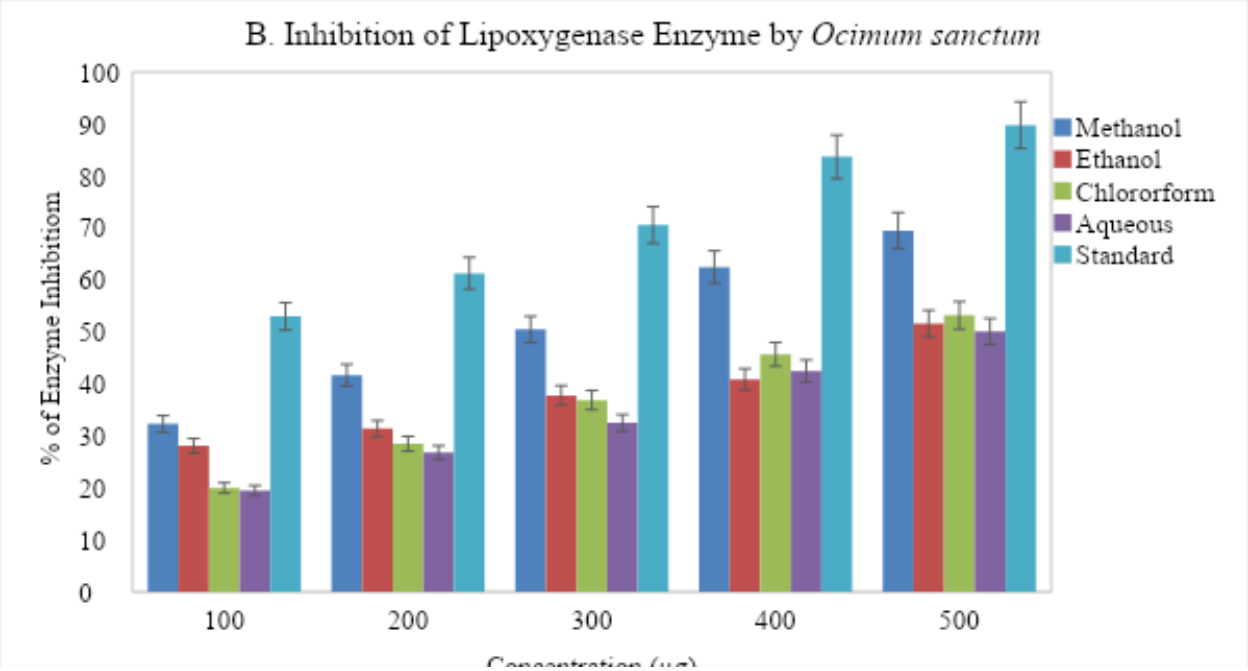

Figure 5: Inhibition of Lipoxygenase Enzyme by A. *Ocimumtenuiflorum*and B. *Ocimum sanctum*

CONCLUSION

The present study demonstrates efficient extraction of potent phytochemical compounds from methanolic extract of *Ocimum. sanctum.* The data obtained reveals the synergistic activity of phytochemical compounds by scavenging the free radicals, inhibiting the growth of microorganisms and suppressing the enzymes responsible for inflammation. The results obtained from the present study clearly indicates that *Ocimum. sanctum* is a potential source of antioxidants and lipoxygenase inhibitors which could have protective effects for lipoxygenase mediated diseases.

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