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Contents

Phytochemical Composition and Evaluation of Antioxidant Activity in Canthium angustifolium Roxb

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ABSTRACT

Canthium angustifolium Roxb. is used for the preparation of various traditional medicines. Leaf extracts of Canthium angustifolium were taken in petroleum ether, chloroform, methanol and water and screened for phytochemicals. For the identification of different phytoconstituents, both qualitative and quantitative phytochemical analysis were done with standard procedures. Antioxidants are substances which scavenge free radicals from the body cell and prevent or reduce the damage caused by oxidation. Active principles such as Alkaloids, Glycosides, Flavonoids, Terpenoids, Phenol, Fatty acids, Quinones, Saponins, Steroids and Tannins were found as a result of qualitative analysis. Total phenols, flavonoids, alkaloids, tannins, saponins and steroids were quantitatively estimated. Antioxidant property of different extract of Canthium angustifolium was examined using DPPH and FRAP assay. From the result of the assays, it was clear that water and methanolic extracts show significant inhibitory properties and those combinations can be used as promising antioxidant drug. A higher amount of saponin, tannin and phenol is the reason behind the antioxidant activity. The study reveals the medicinal importance of Canthium angustifolium as it contains valuable phytochemicals.

Keywords : Canthium angustifolium, Antioxidant Activity, Phytochemical Screening, Qualitative Analysis, Quantitative Analysis

1. Introduction

The Rubiaceae family is composed of dicotyledonous herbs, shrubs, trees, and lianas and each genus is well distinguished by leaves simple opposite, stipules interpetiolar and gamopetalous, actinomorphic flower. It comprises of about 611 genera and 13150 species [1]. Most of the species have medicinal properties and economic importance. Therefore, many phytochemical studies have been carried out in most of the genera of the Rubiaceae family. The genus Canthium was named by Jean Basptiste Lamark in Encyclopedie Methodique [2]. Canthium was a member of the tribe Vanguerieae, a tribe that is monophyletic and easily recognized morphologically [3]. Plants have endless power to produce aromatic substances. The most common ones are phenols or their derivatives [4]. About 12,000 plant-based natural products have been isolated so far. The plant-derived secondary metabolites serve as plant defense mechanisms against microorganisms [5]. From previous studies, the plant has a great role in various common ailments. The leaf paste was applied to swelling part to treat inflammation [6]. The main aim of the present study is the phytochemical analysis of C. angustifolium because of its limitless medicinal properties.

Phytochemical analysis includes qualitative and quantitative tests of components which are higher in concentration, and also the assessment of antioxidant property. This study leads to the pharmacology of Canthium angustifolium with respect to its medicinal properties.

2. Materials and Methods

2.1. Collection of Plant Material

For this study, fresh and healthy leaves of Canthium angustifolium were collected from Kottayam District. The plant was identified by visiting the herbaria of TBGRI, KFRI and University of Calicut and authenticated by comparing it with the protologue and several local Floras.

2.2. Preparation of Plant Extract

Soxhlet extraction method is used (extract of 100 mg of powdered leaf in solvents like distilled water, methanol, chloroform and petroleum ether were taken) [7]. Polarity was thus determined based on boiling points and the ability to evaporate. Repeated the process till the solvent got colourless in the extracting chamber, finally the desired compound came out as concentrated mass which was then collected in the distillation flask.

2.3. Determination of Percent Yield

$$\% \text{ Yield} = W1/W2 \times 100 \quad (1)$$

W1 represents weight of extract residue W2 represents weight of powdered sample taken

2.4. Qualitative Determination of Phytochemicals

Several chemical tests were performed for the presence of phytochemical constituents using standard procedures. The tests for different classes of secondary metabolites are as follows:

2.4.1. Phenolic Compounds

1% of lead acetate solution was added to 2 ml of leaf extract. Presence of phenolic compound is proved as a white precipitate is formed [8].

2.4.2. Tannin

0.1% ferric chloride solution was added to 2 ml of leaf extract. Presence of tannin is proved as a brownish green precipitate is formed [9].

2.4.3. Flavonoids

2 ml of leaf extract and 2 ml of 10% Lead acetate were mixed together. Yellowish green colour is the indication of the presence of flavonoids [8].

2.4.4. Saponins

1 ml of leaf extract, 2 ml of distilled water and few drops of olive oil were taken in a test tube and shaken well. It created a foam which persisted. This indicates the presence of saponins [10].

2.4.5. Terpenoids

Mix 2 ml of leaf extract and 2ml of chloroform with 2
ml conc. sulphuric acid and allowed the mixture to evaporate. Then heat it for 2 minutes. The solution turned out grey. The result indicates the presence of terpenoids [8].

2.4.6. Alkaloids

Mix 2 ml of leaf extract with an equal amount of Wagner's reagent. A reddish-brown precipitate indicates the presence of alkaloids [8, 10].

2.4.7. Glycoside

From 5% FeCl3, one drop was taken and added to 5 ml of leaf extract, 2 ml glacial acetic acid and conc. H2SO4 was added. There appeared a brown ring. This indicates the presence of glycosides [8].

2.4.8. Quinone

Mix 2 ml of leaf extract with 3-4 drops of concentrated HCl. Presence of quinone is proved as a yellow-coloured precipitate is formed [8].

2.4.9. Fatty Acids

Mix 0.5 ml leaf extract and 5 ml ether and this mixture was allowed to evaporate on a filter paper. There was appearance of transparency on the dried filter paper and this indicates the presence of fatty acids [8,10].

2.4.10. Steroids

1 ml of plant extract was dissolved in 10 ml of chloroform and an equal volume of concentrated sulphuric acid was added to it. Two layers were formed. The top layer turned red and the bottom layer of sulphuric acid appeared yellow with green fluorescence. This result indicates the presence of steroids [11].

2.5. Quantitative Estimation of Phytoconstituents

The quantitative estimation of phytoconstituents was carried out according to the standard procedures [11,12].

2.5.1. Estimation of Total Phenol

Extracts in different solvents (in separate test tubes) made up to 3 ml by adding distilled water. To each test tubes, 0.5 ml Folin-Ciocalteau reagent and 2 ml of 20% Na2CO3 were added, which was placed in a boiling water bath (one minute). OD at 750 nm was taken, against a reagent blank, after cooling down it. The same process was repeated using gallic acid (as standard) solutions (2.5100µg/ml) [12].

2.5.2. Estimation of Tannin

Folin-Ciocalteu method is followed. 1 ml of leaf extract was made up to 7.5 ml using distilled water, and added to this 0.5 ml of Folin-Ciocalteu reagent and 1 ml sodium carbonate solution (from 35%) were added. The volume of the solution was made up to 10 ml using distilled water and the absorbance was taken at 700nm [11].

2.5.3. Estimation of Flavonoid

Aluminum chloride colorimetric assay method is applied. 0.30 ml of 5 % sodium nitrite was added to the reaction mixture (1mg of leaf extract + 4 ml of distilled water). Then added 0.3 ml of 10 % aluminum chloride after 5 minutes. 2 ml of 1M Sodium hydroxide was added to the mixture and then total volume made to 10 ml by adding distilled water. Quercetin in different concentration (20, 40, 60, 80 and 100 µg/ml) was used as standard. Using UV/Visible spectrophotometer the absorbance of solutions (both test and standard) was measured against the reagent blank at 510 nm [11].

2.5.4. Estimation of Saponins

The vanillin-sulphuric acid assay method was used to determine the total saponin content. To 1 ml leaf extract 0.25 ml of vanillin in ethanol solution (0.8% (w/v)) was added. Then 2.5 ml of Sulphuric acid

(72% (v/v)) was added. The prepared solution was placed in a shaking water bath for 15 min at 60°C. Diosgenin was used as standard. In reagent blank, extraction solvent was used instead of leaf extract. The solution was cooled down and the absorbance was measured at 544 nm [11].

2.5.5. Alkaloid Estimation

1 ml dimethyl sulphoxide (DMSO) was added to 1 ml leaf extract, to this 1ml of 2N HCl was added and filtered. 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added to a separating funnel after transferring this solution to it. This mixture was subjected to vigorous shaking with 1- 4 ml of chloroform, which was collected in a 10 ml volumetric flask, which was then diluted with chloroform. Atropine was used as standard. Then absorbance was tested against the reagent blank at 470 nm [11].

2.5.6. Estimation of Steroids

Took 1 ml of leaf extract, 5 ml of ferric chloride and 4 ml conc. sulphuric acid together in a test tube. Cholesterol in varying concentrations was used as standard. 5 ml of ferric chloride reagent and 4 ml of concentrated sulphuric acid were added to the standard. OD of the reaction mixture was measured at 540nm after incubating it for 30 minutes. To find out unknown value of steroid in the test sample, a standard graph was plotted [11].

2.6. Determination of Antioxidant Activity

2.6.1. FRAP Assay Method of 20 mM FeCl3

The FRAP reagent consisting 2.5 ml of 10 mM 2,4,6tris(2pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 2.5 ml in 25 mL of 0.1 M acetate buffer pH 3.6, was prepared. This was then incubated at 37°C for 10 minutes. Adding varying concentrations of leaf extract with 2 ml of the FRAP reagent and 1 ml distilled water. A blank solution was prepared by the same method but using distilled water instead of the leaf sample. This solution was incubated for 30 minutes and absorbance was measured at 593 nm against blank [13].

2.6.2. DPPH Radical Scavenging Assay

Radical scavenging activity of the leaf extract was tested against DPPH [14]. Ascorbic acid was used as the standard (prepared in distilled water (1 mg/ ml; w/v)). 1 ml of leaf extract at various concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400,800, 1000µg/ml) was mixed with 3.9 ml, 60µM solution of DPPH in methanol. The absorbance was measured at 515 nm after incubating for 15 minutes at room temperature. Control used here consists of only DPPH solution. A blank solution used here consists of only 95% methanol.

For the calculation of radical scavenging activity, the following formula (2) was used:

$$\% \text{ inhibition} = \frac{ABS^{control} - ABS^{test} \times 100}{ABS^{control}} \qquad (2)$$

3. Results

Secondary metabolites are responsible for the therapeutic effect of plants. The present study performed on the plant Canthium angustifolium reveals the presence of bioactive compounds. The physical characteristics and percentage yield of different extract are described in table 1 and table 2 respectively. Qualitative phytochemical screening shows the presence of alkaloids and saponins in all solvents. Flavonoids, steroids, phenols and tannins were present in all solvent systems except in petroleum ether (Table 3).

3.1. The Percent Yield

Table 1. Physical characteristic of different extract of C. angustifolium

Sl. No	Extracts	Consistency	Colour
1	Petroleum ether	Powdery	Yellowish green
2	Chloroform	Sticky	Greenish black
3	Methanol	Sticky	Yellowish green
4	Water	Powdery	Brown

Table 2. Percentage yield of different extracts of C. angustifolium

Sl. No	Extracts	Weight of sample (g)	Weight of extract (g)	% Yield
1	Petroleum ether	25.033	0.322	1.29
2	Chloroform	25.033	1.264	5.05
3	Methanol	25.033	1.026	4.10
4	Water	25.033	1.367	5.46

3.2. Qualitative Analysis of Phytochemicals

Table 3. Phytochemical composition of the leaves of C. angustifolium

Sl. No.	Name of test	Extracts			
		PE	Chl	MeOH	H_2O
i	Phenols	-	++	+	+++
ii	Tannins	-	++	+	++
iii	Flavonoids	-	++	++	+++
iv	Saponins	++	+++	+++	++
v	Terpenoids	-	+++	+	-
vi	Alkaloids	++	+	+	++
vii	Glycosides	-	++	+	-
viii	Quinones	-	+++	+	-
ix	Fatty acids	-	+	+	-
x	Steroids	-	++	++	+

'+' indicates presence and '-' indicates absence, PE- Petroleum ether, Chl- Chloroform, MeOH- Methanol, H2O- Water

Table 4. Quantitative estimation of phytoconstituents of C. angustifolium

Phytochemical	Chloroform	Methanol	Water	Petroleum ether
Phenols (mg GAE/g extract)	59.08±0.44	12.09±0.37	83.56±0.25	-
Tannins (Tannic acid mg/ml)	89.16±0.30	49.22±0.29	90.86±0.30	-
Flavonoids (QE/g)	36.50±0.33	25.63±0.29	45.41±0.24	-
Saponins (µg/mg)	239.85±0.24	235.6±0.38	115.6±0.71	112.1±0.29
Alkaloids(µg/mg)	25.11±0.25	22.33±0.27	72.33±0.31	98.44±0.34
Steroids (mg/100g)	70.13±0.31	24.85±0.40	10.82±0.18	-

3.3. Quantitative Analysis of Phytoconstituents

Quantitative estimation was carried out only for selected phytochemicals according to previous literature. Thus, six of them were selected for quantitative analysis. They were phenols, tannins, flavonoids, saponins, alkaloids and steroids as this plant (Canthium angustifolium) was rich in these constituents. In Canthium angustifolium the water extract contained higher levels of total phenols, tannins, flavonoids. The highest saponin content was found in chloroform extract followed by methanol extract. The highest alkaloid content was in the petroleum ether extract, which was also followed by water extract. The highest flavonoid content was found in the water extract which was also followed by chloroform extract. From the quantification results it was found out that flavonoid, phenol and tannin contents in the order of extracts water>chloroform>methanol. Saponin content in the order chloroform>methanol> water>petroleum ether. Alkaloid content was in the order petroleum ether>water>chloroform>methanol and the steroid content was in the order chloroform>methanol>water (Table 4). 3.4. Antioxidant Analysis

3.4.1. Result of FRAP Assay

The present values occur in the range of 5.21±0.29 mg to 55.02±0.68 mg. Higher reducing power is found in higher concentration of the sample (Table 5 and 6). Water extract shows the highest reducing power out of the four.

Table 5. Absorbance of Ferrous Sulphate as standard

Sample code	Concentration (mg/ml)	OD at 593nm
FeSO4 (Standard 3.6µM/ ml)	10	0.231
	20	0.410
	40	0.822
	60	1.364
	80	1.831
	100	2.090

Table 6. Reducing power of different extracts of Canthium angustifolium in different concentration (FRAP assay)

Sl. No.	Sample code	Concentration (mg/ml)	OD at 593nm	Mm Fe2+/ (mg)
1	Petroleum ether	20	0.116	5.21±0.29
		40	0.175	7.93±0.33
		60	0.195	8.85±0.38
		80	0.212	9.63±0.58
		100	0.217	9.86±0.08
2	Chloroform	20	0.148	6.68±0.24
		40	0.185	8.39±0.21
		60	0.205	9.31±0.37
		80	0.289	13.18±0.41
		100	0.327	14.93±0.73
3	Methanol	20	0.244	11.11±0.39
		40	0.307	14.01±0.54
		60	0.504	23.09±0.65
		80	0.637	29.22±0.61
		100	0.756	34.70±0.56
4	Water	20	0.473	21.66±0.61
		40	0.634	29.08±0.09
		60	0.879	40.37±0.71
		80	1.011	46.45±0.46
		100	1.197	55.02±0.68

3.4.2. Result of DPPH Assay

The leaf extract exhibited a dose dependent inhibition of DPPH activity. The absorbance of the sample was lower at higher concentration. The radical scavenging activity was higher (higher % of inhibition) in higher concentration (Tables 7 and 8).

Table 7. Absorbance of different extract of Canthium angustifolium at 515 nm by UV-Visible Spectrophotometer (DPPH assay)

	OD at 515 nm				
Conc. (µg/ml)	Ascorbic acid	PE	Chl	MeOH	H_2O
Control	1.28	0.8716	0.8716	0.8703	0.8703
1.56	1.20	0.8625	0.8518	0.8548	0.8854
3.12	1.19	0.7812	0.8174	0.8444	0.8033
6.25	1.12	0.7532	0.7937	0.8214	0.7574
12.5	0.96	0.7233	0.7510	0.7867	0.7625
25	0.65	0.7032	0.7074	0.7020	0.7041
50	0.09	0.6945	0.6866	0.6494	0.6647
100	0.09	0.6607	0.6713	0.5763	0.5136
200	0.08	0.6564	0.6552	0.4686	0.3569
400	0.08	0.6213	0.6243	0.3026	0.3025
800	0.09	0.5845	0.5904	0.2706	0.2706
1000	0.09	0.5506	0.5533	0.1544	0.1906

Table 8. Percentage inhibition of different extracts of Canthium angustifolium with ascorbic acid as standard

	Percentage inhibition				
Conc. (µg/ml)	Ascorbic acid	PE	Chl	MeOH	H_2O
1.56	6.0	1.05	2.28	1.78	1.74
3.12	6.8	10.38	6.21	2.98	7.70
6.25	12.2	13.58	8.94	5.61	12.97
12.5	25.0	17.01	13.83	9.60	12.38
25	48.7	19.32	18.84	19.34	19.09
50	93.0	20.32	21.22	25.38	23.63
100	93.2	24.20	22.98	33.78	40.99
200	93.5	24.69	24.83	46.16	58.99
400	93.2	28.72	28.37	65.23	65.25
800	93.2	32.94	32.27	68.91	68.91
1000	93.2	36.82	36.52	82.26	78.10

The methanolic extract inhibited DPPH with a half maximal inhibitory concentration (IC 50) value of 253.61 µg/ml and which is 155.10 µg/ml in water extract.

4. Discussion

Alkaloids, Glycosides, Flavonoids, Terpenoids, Phenol, Fatty acids, Quinones, Saponins, Steroids and Tannins were present as a result of phytochemical analysis of the leaf extract of Canthium angustifolium. Phytochemical analysis of Canthium parviflorum in various extracts shows the presence of alkaloids, flavonoids, steroids, tannin, saponins [15]. Alkaloids in plants regulate plant growth and give protection from predators [16]. Therapeutically, alkaloids were important for their anesthetic, cardioprotective and anti-inflammatory activities [17]. Glycosides were important in the treatment of congestive heart failure [18]. Flavonoids are potential active principles, which is anticancerous, antioxidant, anti-inflammatory and antiviral in property. They also have neuroprotective and cardioprotective effects [19]. Terpenoids were reported as good antiulcer and antioxidant compound. Tannin acts as a diuretic agent [20]. Phenolic compounds are chemo preventive [21]. According to modern clinical studies, steroids are anti-inflammatory and analgesic agents. Saponins have various medicinal applications like antiinflammatory, antimicrobial, antioxidant, antidiabetic etc. as well as emulsifiers or sweeteners in food and cosmetic industries [22,23,24,25]. According to another study, they noticed that saponins also have strong antioxidant activity which depends on their free radical scavenging abilities [26,27]. According to a study [28], saponins have strong antioxidant activity in vitro than in vivo condition. This result also supports the present study. From the quantitative analysis, it was observed that many of the phytochemicals were abundant in methanolic and aqueous extracts. As successive serial extraction was done, the phytochemicals were dissolved maximum in polar solvents such as chloroform and methanol than non polar solvents. These results were in accordance with the study conducted by Sasikala [29] that phenols and flavonoids are higher in methanolic extract than others. In most of the studies ethanolic extract was more preferable than other solvents. But unlike the studies conducted so far, phytoconstituents in the present study were highly concentrated in methanol and chloroform extracts. As a result, it is clear that methanolic and chloroform extracts are also a good option economically (instead of ethanolic extract). The value of percentage yield of Canthium angustifolium was higher than that in Tabernaemontana divaricata [30].

The antioxidant activity of the leaf extract of Canthium coromandelicum is reported as it contains flavonoids and tannins [31]. According to a study, only the water soluble phenolics was important as antioxidant compound [32]. Both the methanolic and water extract show higher free radical scavenging activity. The study conducted by Amalraj reported that methanolic and aqueous extracts showed significant free radical scavenging abilities [33] and it also supports the present study.

5. Conclusions

This study provides useful knowledge about the antioxidant properties and responsible active principle in Canthium angustifolium. The qualitative analysis was very useful to find out the presence of various chemical compounds which leads to their quantitative estimation. The result of the present work may vivify the standardization process of phytochemicals from the medicinally important plant, Canthium angustifolium. The abundance of saponins, tannins and phenols may be the reason for the antioxidant activity. The plant is a promising source of potential antioxidants. As saponins are present in a higher amount, this study also leads to the provision for the isolation of the specific compound responsible for the antioxidant activity.

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Impact of Chronic Paraquat Toxicity on Lipid Peroxidation Activity of Indian Major Carp Cirrhinus Mrigala Ham.

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ABSTRACT

Paraquat Dichloride, a broad-spectrum herbicide, used for the killing of aquatic weeds in aquaculture ponds and reservoirs was tested on extensively cultured Indian Major Carp (IMC) Cirrhinus mrigala Ham.. For this purpose, fingerlings of this economically important fish were exposed to the sub-lethal concentrations (LC1/20th and LC1/10th) of paraquat continuously for 30 days by using the static renewal bioassay method. Post Exposure, the lipid peroxidation activity in 4 vital organs of the fish viz. gill, muscle, liver, and brain were estimated by E. D. Wills's method to rectify paraquat's toxicity. Annotated findings specified, a highly significant ($p<0.001$) chronological increase in lipid peroxidation activity (LPO) of gill, muscle, and liver tissues respectively in the LC1/10th concentration group, while a moderately significant ($p<0.01$) increase in LPO activity of brain tissue in the same group. While the gill and the brain tissue of the LC1/20th group showed moderately significant ($p<0.01$) and significant ($p<0.05$) increases in the LPO activity respectively. All changes showed their dependency on the time and concentration factor of the toxicant. Concluding the study, it was stated that, chronic paraquat sub-lethal exposure significantly increased the LPO activity in the vital tissues of Cirrhinus mrigala Ham. thus supporting its highly toxic nature and an immediate need to restrict its use as much as possible.

Keywords Paraquat Dichloride, Chronic Toxicity, Cirrhinus Mrigala Ham., Lipid Peroxidation Activity

1. Introduction

Paraquat Dichloride (C12H14Cl2N2) 75-305-73-0 (CAS) is a quaternary nitrogen bipyridyl weedicide produced commonly in the form of brown color concentrated liquid consisting of 10-30% strong dichloride salt of it, sold under the brand name Gramoxone [1] by agrochemical company 'SYNGANTA'. In humans, paraquat ingestion causes hazardous effects in different organ systems. Such paraquat toxicity has no firm existing antidote therapy [2] [4]. The direct spray of paraquat on matured food crops to desiccate them for better marketability reasons is the current most alarming issue that has the highest chances of residual paraquat in the daily diet of humans and animals causing large-scale epidemic issues [5] [6]. Such extensive application of paraquat across various platforms of terrestrial and aquatic ecosystems has led to its far-reaching residues in soil and water that conclusively enter into the food chains [7]. Paraquat enters into aquatic ecosystems through surface run-offs, leaching, atmospheric deposition, drifting, etc., and accumulates into various organisms that reside in waters profoundly the fishes [8]. Numerous water bodies viz. Bois d'Orange River, Choc River, Cul-de-Sac River, Roseau Dam, Roseau River, Choc River, Cul-de-Sac River, Soufriere River, Soufriere Dam, Cannelles River, have been found to contain paraquat [9]. The overall health and equilibrium of aquatic creatures may be negatively impacted by the direct application of paraquat to weeds in aquatic

ecosystems [10]-[13] thus leading to severe acute as well as chronic toxicity in aquatic organisms. Fish's digestive system, skin, and gills may absorb paraquat from the residual surface waters [11] hence the study of its effects on fish is an important marker for the assessment of its eco-toxicity.

Pesticide poisoning has a key molecular mechanism called Lipid Peroxidation Activity (LPO) [14]. It is a detrimental attack of reactive oxygen species (ROS) that damages tissues and organs by oxidative stress [15]-[17].

Biota exposed to ambient pollutants may experience significant ROS activity, which may prevent the protective antioxidant system from eliminating them, resulting in oxidative stress and damage [18]-[21]. LPO is crucial for aquatic species since they have a much higher concentration of polyunsaturated fatty acids (PUFA) than terrestrial animals [22]. This study documented the changes in LPO activity as a consequence of malondialdehyde (MDA), a byproduct of PUFA peroxidation that occurs inside cells. The present study was intended to investigate such toxic effects of paraquat dichloride on the lipid peroxidation activity in the fingerlings of commercially important Indian Major Carp Cirrhinus mrigala Ham., as grassroots biomarkers of pesticide nuisance to the health of aquatic animals useful in the assessment of environmental risks.

2. Materials and Methods

2.1. Procurement and Rearing of Experimental Animal

The Government Fish Seed Production Center, Dhom (Wai), Satara District, Maharashtra State, India, supplied Cirrhinus mrigala Ham. fingerlings (mean weight- 3.18±0.21 gm. and mean length- 4.72±0.44 cm) for this study in large plastic bags sustained by oxygen. Fish were sanitized in a lab setting by being dipped for two minutes in a 0.1% KmNO4 solution. After that, they were placed in well-aerated glass aquariums with regular dechlorinated faucet water, where fish underwent 15 days of acclimatization to room temperature. Standard procedures as illustrated in APHA [23] were used in the experiment to determine the physicochemical parameters of the water. Obtained values were as follows: Temperature 26.2 ºC, pH ranges 7.1-7.6, Dissolved oxygen (DO) content 5.61-6.13 mg/L, liberal CO2 14.27 ± 0.47 mg/L, hardness 119.38 ± 3.72 mg/L, phosphate content 0.5 ± 0.03 mg/L, nitrates content 1.11 ± 0.26 mg/L. During acclimatization and experimental procedures, 2 percent fish food (Taiyo Discovery) of the fishes' average body weight was fed to them every day. Natural photoperiod was maintained. During the acclimatization, the aquarium water was changed every 24 hours to discard food remnants and fecal matter that can cause unnecessary stress in the enclosed water system. The water quality parameters were checked weekly to ensure normal conditions. Removal of any dead fish was done immediately to avoid possible water quality deterioration. After 15 days of acclimatization, the fish to be used for the experiment were screened critically for any indication of physical damage, disease, stress, and mortality.

Any suspected fishes were discarded immediately and only the healthy fishes were selected for the study. Before initiation of experimental protocols, the fish were acclimatized to well-aerated 22-liter capacity plastic containers for 7 days, during which they were to be exposed to the toxicant. 24 hours before the test, feeding was discontinued to reduce the effect of vomiting and excess animal excreta due to the toxicant, but later, after 24 hours of exposure, the feeding was restored at 2% of their body weight.

2.2. Exposure to Paraquat

The herbicide paraquat dichloride commercially sold under the brand name Gromoxone (24%w/w) by Syngenta was used as a toxicant in the present study. Before the experiment, a study was conducted to determine the mortality rate of fishes exposed to various concentrations of paraquat by the static renewal bioassay method. The data so obtained was processed by Finney's Probit analysis to obtain the LC_{50} (105

ppm) value for the toxicant used. Both the sub-lethal concentrations viz. LC1/20th and LC1/10th concentrations used in this study were derived from this LC50 value. For the current experiment, three clear, openmouthed, cylindrical plastic jars with a volume of 22 liters were arranged side by side in a row. To sustain the desired level of dissolved oxygen present in the water, each jar received continuous, appropriate aeration. All containers were filled with 20 liters of clean dechlorinated tap water and 10 fish were released in each of them. The fish in the first jar functioned as a control group because they weren't subjected to any toxicant. The fingerlings in the second and the third jars were subjected to paraquat doses of LC1/20th (pre-calculated: 5.25 ppm) and LC1/10th (pre-calculated: 10.5 ppm) for 30 days each (chronic toxicity). At every 24 hours, the water medium and the toxicant in all the containers were replaced with fresh water and toxicant, to maintain the optimum concentrations throughout the experiment. Any dead fish if observed were removed immediately from the container and buried underground in follow land away from domestic areas. Live juvenile fingerlings from all three jars were euthanized after 30 days of exposure, and the LPO activity in their gills, livers, muscles, and brains was examined in each organ separately.

The remains of the euthanized fish too were buried underground in same area where dead fish were buried.

2.3. Analysis of the Lipid Peroxidation Activity (LPO)

In the current investigation, the changed levels of lipid peroxidation in all 4 tissues were estimated using E. D. Wills's protocol [24]. The reaction mixture necessary to homogenize the tissues was presumably prepared fresh. To make the reaction mixture, 1 ml of Phosphate Buffer Saline (PBS, pH 7.4), 1 ml of 1 mM FeCl3, 0.01 ml of Chlorotetracycline, and 1 ml of 75 mM Ascorbic Acid (AA) were blended together. Following that, 10 ml of this reaction mixture was used to homogenize 100 mg of fresh tissue. This mix was used as a stock. Following that, 1 ml of this stock solution was divided into three test tubes (triplicates), and 1 ml of distilled water, 1 ml of 20% TCA, and 2 ml of 0.67% thiobarbituric acid (TBA) were all added to each of these triplicates. In an additional test tube, a blank was simultaneously made by mixing 2 ml of distilled water with 1 ml of 20% TCA and 2 ml of 0.67% TBA. Following that, for 15 minutes, all test tubes were submerged in a bath of boiling water. The test tubes were cooled for 15 minutes and centrifuged at 1000 g for 10 minutes. Then, using a spectrophotometer, the absorbance readings of the supernatants so acquired were measured against the blank at 532 λmax. Using 1.56 x 105 M-1 cm-1 as its molar extension coefficient, the lipid peroxidation levels were estimated as nano-moles (nM) of MDA generated per milligram (mg) of tissue (nmol MDA/mg tissue). The following formula was used to determine the level of MDA.

$$\text{MDA / mg tissue} = \frac{\text{O.D. of the sample}}{(0.156)\ (1)}$$

Where,

0.156 = 1 mM Malondialdehyde solution's absorbance at 532 nm in a cell that is 1 cm thick. 1 = Amount of tissue taken in mg, present in 1 ml of a sample.

The final data from all the groups was expressed in Arithmetic Mean (AM) ± Standard Deviation (SD) format. Utilizing the unequal variance (2-sample) (heteroscedastic) approach of "student's T-Test" with "two-tailed distribution", the significance level was determined. If $p < 0.05$ it means it showed a significant change. If $p < 0.01$ it showed a moderately significant change and, if $p<0.001$ it showed highly significant change.

3. Results

The readings representing the effects of paraquat poisoning on the lipid peroxidation activity of Cirrhinus mrigala Ham. gills, brain, muscle tissue, and liver in the control group, LC1/20th concentration set, and LC1/10th concentration group after chronic exposure (30 days) are represented in Table 1. In the control group fish Cirrhinus mrigala Ham., the LPO activity was found to follow the Brain > Liver > Muscle > Gills sequence. The lipid peroxidation activity in gill tissue showed a moderately significant ($p<0.01$) increase in the LC1/20th group (t.stat = -7.9009) while it showed a highly significant ($p<0.001$) increase in the LC1/10th group (t.stat = -17.901).

The lipid peroxidation activity in muscle tissue showed a highly significant ($p<0.001$) increase in the LC1/10th group (t.stat = -16.1532). The lipid peroxidation activity in liver tissue showed a significant ($p<0.05$) increase in the LC1/20th group (t.stat = -3.6360) while in the LC1/10th group (t.stat = -13.026) it showed a highly significant ($p<0.001$) increase.

The lipid peroxidation activity in brain tissue showed a significant ($p<0.05$) increase in the LC1/20th group (t.stat = -3.5901) while it showed a moderately significant ($p<0.01$) increase in the LC1/10th group (t.stat = -4.884). The postexperimental lipid peroxidation activity in the four tested tissues was in the order Liver > Brain> Gill > Muscle in the LC1/20th group while in the LC1/10th group it was in the order, Brain > Liver > Gill > Muscle.

Table 1. Effect of Paraquat Dichloride on the Lipid Peroxidation activity in different tissues of the fish Cirrhinus mrigala Ham. after chronic exposure

Groups	Lipid peroxidation activity (nM of MDA/mg wet wt. of tissue)			
	Gill	Muscle	Liver	Brain
Control Group	1.76±0.14	1.92±0.14	4.98±0.12	5.12±0.33
$LC_{1/20th}$	3.15±0.26 **	3.03±0.72	6.01±0.47 *	5.9±0.16 *
$LC_{1/10th}$	4.84±0.26 ***	4.6±0.24 ***	6.8±0.2 ***	7.43±0.74 **

(Values in table no 1 are expressed as Arithmetic Mean of (n= 6); ±SD), *= $p<0.05$ (significant), **= $p<0.01$ (moderately significant), ***= $p<0.001$ (highly significant)

The results of the current study illustrate the significance levels of MDA activity that are directly proportional to LPO activity in vital tissues of fish after exposure to paraquat. LC1/10th group show highly significant MDA activity in comparison to control group while LC1/20th group shows a just significant impact on MDA activity as compared to control. Thus the higher concentration (LC1/10th) of paraquat tends to make a highly significant impact on LPO activity in vital tissues of exposed fishes as compared to the lower concentration (LC1/20th) of paraquat.

4. Discussion

Lipid peroxidation results from oxidative injury driven by ROS action that disrupts cell anatomy and physiology [25] [26]. Fish are useful markers of pollutants that allow for early detection of aquatic

issues related to environmental health [27] [28]. Internal cellular constituents of fish are harmed when toxicants promote the aberrant creation of ROS and it surpasses the intrinsic defense system of the fish. The term "oxidative stress" refers to this phenomenon [29]. Pesticide impacts causing oxidative stress had been widely investigated as a potential mechanism illustrating their toxicity and degradability, in a variety of tissues [30]. Malondialdehyde (MDA) is a byproduct of LPO. MDA synthesis is a crucial sign of induced oxidative stress driven by free radicals that harm biological membrane constituents [31]. The findings of the current study reveal noticeably higher rates of MDA activity in all four tissues, i.e. the gill, muscle, liver, and brain subjected to Paraquat at both LC1/20th and LC1/10th concentrations. These elevated levels of MDA are a sign of the peroxidation of lipids set on by Paraquat poisoning in key fish tissues. The harmful effects of Paraquat may have resulted in the oxidation and redox-cycling of cellular NADPH that potentially releases a large amount of ROS, which ultimately led to oxidative stress and damaged essential tissues via lipid peroxidation. The generated malondialdehyde also reacts actively with other biomolecules like proteins, changing their structures, properties and functions [32]. Lipid peroxidation reduces the nutritional value of edible fish meat, creating health issues as well as the monetary loss for the stakeholders [33]. Persch et al. [34] showed similar results, that Rhamdia quelen fingerlings exposed to multiple paddy herbicides used in integrated rice-fish farming demonstrate a comparable rise in lipid peroxidation rate in their gill, liver, kidney, and muscle tissues. Similarly, [35] studied cadmium induced physiological alterations in Nile tilapia and reported increased lipid peroxidation activity with elevated levels of toxicant as compared to control group.

5. Conclusions

The present study's findings suggest that paraquat poisoning can have a significant detrimental effect on non targeted creatures like fish because it elevates the levels of lipid peroxidation activity in their vital organs. Increased LPO activity has the potential to impair fish's ability to operate optimally and maintain its internal homeostasis, which might result in fish death, growth retardation, and poorer-quality fish meat, which might harm the stakeholders' nutritional needs as well as their health and exacerbate aquatic pollution. LPO activity can also function as a reliable biomarker of how anthropogenic stresses affect unintended organisms.

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Pongamia pinnata Seed Oil Efficacy as an Antifeedant against the Larvae of Papilio demoleus L. (Lepidoptera: Papilionidae)

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ABSTRACT

The efficacy of Pongamia pinnata seed oil as an antifeedant against the late instars of the Lemon butterfly, Papilio demoleus L., was evaluated in the present study conducted at the Department of Zoology of Osmania University, Hyderabad, Telangana, India. The study period was from June 2022 to November 2022. In the non-choice method used for the antifeedant bioassays, citrus leaf discs measuring 30 sq. cm were dipped for 1 minute in different concentrations of P. pinnata seed oil emulsions, including 0.125%, 0.25%, 0.5%, 1%, and 2%. Distilled water and an emulsifier were used as the control solution. The dipped leaf discs were dried at room temperature. A single pre-starved larva was placed on each leaf disc to feed on it. After 24 and 48 hours of introducing the larvae on leaf discs, the leaf area consumed by the larvae was measured using the graph sheet method. The antifeedant activity of the test concentrations exhibited dose-dependent results, with the 2% oil emulsion demonstrating the highest antifeedant activity of 84.65% and 70.30% after 24 and 48 hours of treatment, respectively. P. pinnata seed oil was found to possess the potential to be utilized as an antifeedant against P. demoleus larvae.

Keywords ***Citrus Butterfly, Swallowtail Butterfly, Antifeedant Activity, Feeding Inhibition, Feeding Deterrents, Biopesticides***

1. Introduction

Citrus species are believed to have originated in Southeast Asia and India, with evidence of cultivation in India dating back over 4,000 years [1]. As of 2015, Southeast Asian countries accounted for 38% of global citrus fruit production, with China being the top producer at 36.6 million tons. Citrus fruit production in India is estimated to be 7 million tonnes per year. The commercially grown citrus species in India include orange, sweet orange, lemon, and lime/acid lime, with the regions of Maharashtra, Andhra Pradesh, Telangana, Punjab, Karnataka, Uttaranchal, Bihar, Orissa, Assam, and Gujarat being the major producers [2, 3].

Citrus plants in China are affected by 800 species of pests, while in India, 250 species of pests infest citrus, with most of them attacking young trees and affecting the growth and productivity of the plants. The most damaging pests include the Citrus butterfly, leaf miners, blackflies, whiteflies, psylla, and scales. The Citrus butterfly or swallowtail butterfly (Papilio demoleus) larvae, which are leaf-feeding pests on citrus plants, are commonly called orange dogs as they release orange-colored osmeterial secretion when disturbed [4].

P. demoleus is a holometabolous insect with 4 stages - egg, larvae, pupa, and imago in its life cycle. The larval stage has 5 instars. In the study made by [5], the mean period for egg incubation was 2.99 ± 0.13 days, for the 1st instar 4.00 ± 0.08 days, for the 2nd instar 4.20±0.06 days, for the 3rd instar 4.98 ± 0.10 days, for the 4th instar 3.98 ± 0.08 days and for the 5th instar, 4.99 ± 0.09 days were observed. The pre-

pupal stage lasted for 1.84 ± 0.04 days. While the pupal stage lasted for 4.04 ± 0.09 days, the post-pupal stage lasted for 1.53 ± 0.10 days. The male adult had a mean longevity of 36.18 ± 0.39 days, while the female adult had a mean longevity of 39.11 ± 0.37 days.

The larvae of P. demoleus, especially the older instars are considered the most destructive pests in Citrus nurseries as they prefer young flush and in heavy infestations, plants can be completely defoliated [2, 3]. The purpose of using pesticides is to protect crops from pests and thus increase agricultural productivity and the quality of agriproducts [6]. The history of pesticides dates back to 4500 years ago when the Sumerians used elemental sulfur dustings [7]. In the US, $40 billion per year is spent on synthetic pesticides [8]. European countries have a share of 45%, the USA 25%, and India 4% in this pesticide market [9]. Formulations of natural origin that have pesticidal action are known as biopesticides [10]. All types of living organisms or their products may be called biopesticides if they are used to suppress pest populations [11]. Beneficial endophytes were added to the list of organisms that are considered biopesticides [12]. Many plant products, such as Neem, have been used to protect plants from pests for 4000 years [7]. Since the discovery of DDT in the 1950s, the usage of botanicals in crop protection has decreased as synthetic pesticides are cheap and effective against insect pests. Due to recent research that brought the detrimental effects of synthetic pesticides into the limelight, using biopesticides has once again started gaining popularity [10]. Advantages of biopesticides are biodegradability, eco-friendliness, the presence of multiple active ingredients resulting in synergistic effects, and a wide range of effects on the pest, as in the case of neem tree extracts - they exert antifeedant, repellent, growth retardant, and anti-oviposition effects [7]. Additionally, applying synthetic pesticides demands special safety equipment and procedures that are not required for applying biopesticides [13, 14]. Several efforts were made by researchers to evaluate the antifeedant effects of various plant extracts against P. demoleus. Antifeedant efficacy of several plant essential oils was tested against 5-day and 10-day-old larvae by [15]. Methanol extracts of Azadirachta indica and Mentha piperita were tested by [16] against the 3rd instar larvae. Neem fruit extract was tested by [17] against the 2nd instar larvae. Feeding deterrence of Betulinic acid, Andrographolide, and Azadirachtin was tested by [18, 19, 20] against the 4th instar larvae. The antifeedant efficacy of Pongamia pinnata seed oil was also reported against Plutella xylostella [21], Western flower thrips Frankliniella occidentalis [22], Colorado Potato Beetle [23], Spodoptera litura [24], and Epilachna dodecastigma beetle [25]. P. pinnata seed oil was found to exert a repellent effect on the oviposition of the common greenhouse. However, no work has been done yet on the antifeedant efficacy of P. pinnata seed oil against P. demoleus larvae. The 4th instar larvae were used in the present antifeedant bioassays as they cause much more damage to citrus orchards than the early instars.

2. Materials & Methods

2.1 .Rearing P. demoleus Larvae

Eggs and early instars of P. demoleus were collected from sweet orange (Citrus sinensis) plantations in PA Pally village, Nalgonda district, Telangana State, India (16.706923, 79.075680). 0.02% sodium hypochlorite was used to disinfect the collected eggs before they were allowed to hatch. Along with the newly hatched larvae, the larvae collected from citrus plantations were reared on C. sinensis leaves in the laboratory. As the older instars are more damaging than the younger instars, fourth instar larvae were used for the antifeedant bioassays.

2.2. P. pinnata Seed Oil Extraction

Healthy seeds of Pongamia (Millettia) pinnata were collected from the botanical garden located in Tarnaka, Hyderabad, Telangana State, India. The collected seeds were cleaned thoroughly and shade-

for two weeks. Virgin oil was collected from the seeds using the cold-pressing method, and the collected oil was filled in an airtight bottle and stored in the refrigerator until usage.

2.3. Preparation of Test Solutions

Using Tween 80 and distilled water, five different concentrations (0.125, 0.25, 0.5, 1.0, and 2.0%) of oil emulsions were prepared. Tween 80 and distilled water were used to prepare the control emulsion excluding the P. pinnata extracts.

2.4. Antifeedant Bioassay

To assess the antifeedant efficacy of the test emulsions, leaf discs, and no-choice methods were used. Fresh C. sinensis leaf discs of 30.00 sq. cm diameter were punched and separately dipped in 0.125, 0.25, 0.5, 1.0, and 2.0% test emulsions, or in the control emulsion. After drying, the leaf discs were placed on wet tissue papers in separate Petri dishes. A single-fourth instar larva of P. demoleus was placed on leaf discs of each Petri dish. Leaf areas consumed by the larvae were measured by the graph sheet method after 24 and 48 hours of treatment. For each treatment, five separate plates were maintained in a single trial. A total of 150 larvae were used in the experiment that was repeated five times. Mean values were used to calculate the percentage of antifeedant activity at the test concentrations. The following formula was used to calculate antifeedant activity index.

$$AFI = \frac{C-T}{C+T} \times 100$$

Where: AFA = Antifeedant Activity,
C = Mean Leaf area not eaten in controlled disc.
T = Mean Leaf area not eaten in the treated disc.

2.5. Statistical Analysis

The obtained results underwent a one-way analysis of variance (ANOVA) using Microsoft Excel software. The level of significance was set at $p<0.05$, and a Tukey's-HSD post-hoc test was performed.

3. Results

Table 1 and Figure 1 present the results of the current investigation. The mean leaf areas not eaten by the test insects in the control batch were 13.28 ± 0.40 sq. cm and 8.21 ± 0.28 sq. cm after 24 and 48 hours, respectively. With a 0.125% concentration, mean leaf areas of 18.69 ± 0.45 sq. cm and 13.07 ± 0.24 sq. cm were not eaten after 24 and 48 hours, respectively. With 0.25% and 0.5% concentrations, the mean leaf areas not eaten by the test insects after 24 hours were 20.98 ± 0.32 sq. cm and 22.81 ± 0.30 sq. cm, and after 48 hours, they were 15.91 ± 0.23 sq. cm and 18.61 ± 0.42 sq. cm, respectively. Mean values of the leaf areas not eaten by the test insect were 26.30 ± 0.41 sq. cm and 21.98 ± 0.57 sq. cm with 1% test emulsion. With 2% emulsion, 28.61 ± 0.24 sq. cm and 26.20 ± 0.26 sq. cm leaf areas were not eaten, respectively, for the same periods.

Table 1. Mean and SD of Protected leaf area (sq. cm) and antifeedant activity with different concentration treatments of Pongamia pinnata seed oil against the 4th instar larvae of Papilio demoleus

Conc. in %	No. of insects	Mean ± SD After 24 hrs	Mean ± SD After 48 hrs	AFA % after 24hrs	AFA % after 48 hrs
2	25	28.61 ± 0.24*	26.20 ± 0.26*	84.65	70.30
1.0	25	26.30 ± 0.41*	21.98 ± 0.57*	63.76	46.19
0.5	25	22.81 ± 0.30*	18.61 ± 0.42*	39.86	31.34
0.25	25	20.98 ± 0.32*	15.91 ± 0.23*	29.91	21.46
0.125	25	18.69 ± 0.45*	13.07 ± 0.24*	17.54	12.55
Control	25	13.28 ± 0.40	8.21 ± 0.28	-	-

All the values are significant at P<0.05.
*All the results were significantly different from each other as per Tukey's -HSD post-hoc test.

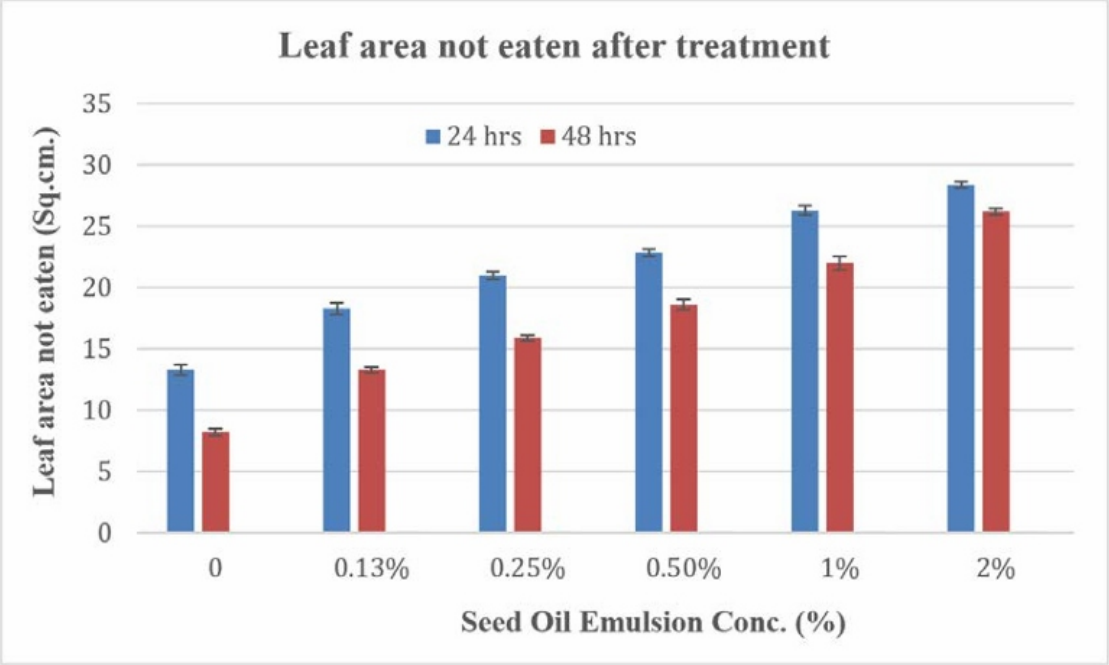

Figure 1. P. pinnata seed oil emulsion treated leaf areas not eaten by the 4th instar larvae of P. demoleus. For each treatment and check time, the mean and standard errors are reported

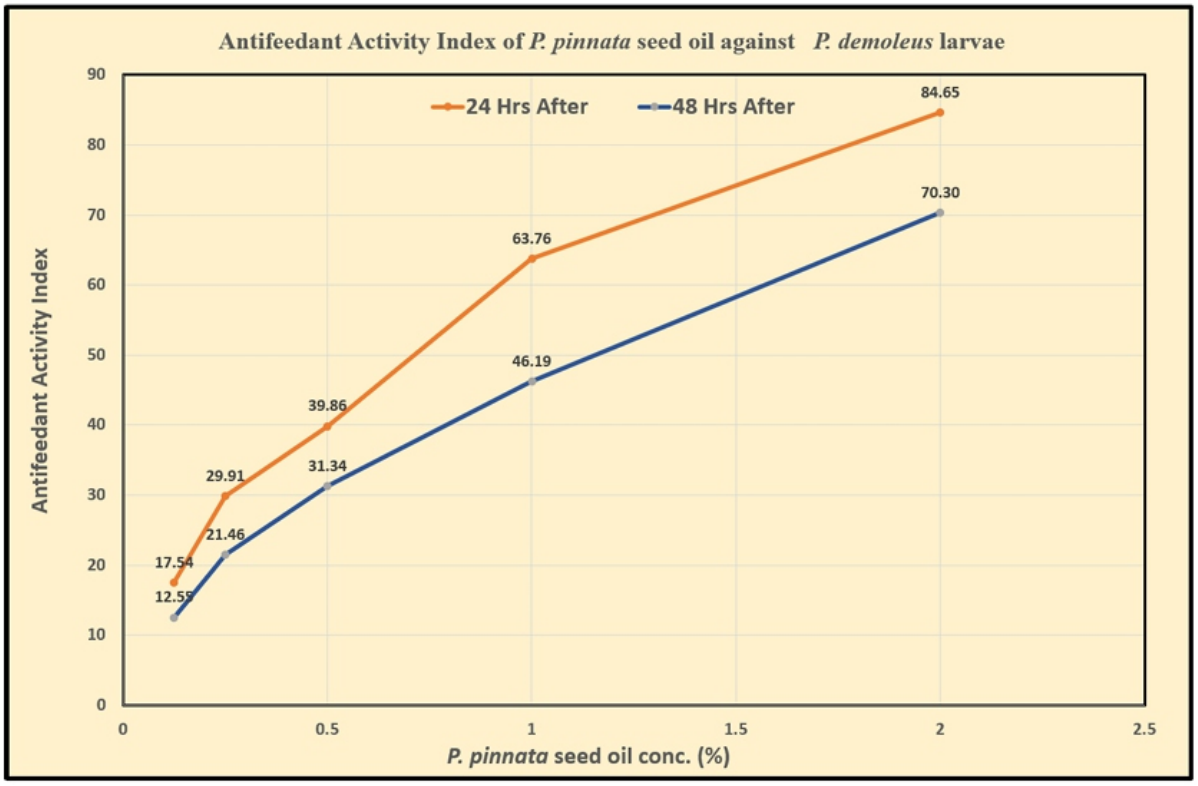

Figure 2. Antifeedant Activity Index of P. pinnata seed oil emulsion against the 4th instar larvae of P. demoleus. Y-axis values represent the percentage of feeding inhibition, X-axis values represent the percentage concentrations of test solutions

Figure 2 shows the Antifeedant Activity Index (AFI) values of different concentrations of P. pinnata seed oil emulsions. After 24 hrs. of treatment, 84.65% and 63.76% of AFI were exhibited by 1% and 2% test emulsions respectively. For the same period, 0.5%, 0.25%, and 0.125% test emulsions showed 39.86%, 29.91%, and 17.54% AFI respectively. After 48 hrs. of treatment, 2% emulsion showed 70.30% AFI. With 1%, 0.5%, 0.25%, and 0.125% emulsions, 46.19%, 31.34%, 21.46%, and 12.55% AFIs were obtained respectively.

The obtained results from the ANOVA conducted on SPSS software version 29.0 were found to be significant at $P<0.05$. Table 2 presents the ANOVA results of the 24 Hrs. antifeedant bioassay, while table 3 presents the ANOVA results of the 48 hrs. antifeedant bioassay. Tukey's-HSD post-hoc test was performed to determine the significant differences among the different test groups. The results of all test groups were found to be significantly different from each other, according to the Tukey's-HSD test.

Table 2. ANOVA results of Antifeedant Bioassay after 24 Hours

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1695.69	4	423.92	736.69	<.001
Within Groups	69.05	120	0.575		
Total	1764.74	124			

Table 3. ANOVA results of Antifeedant Bioassay after 48 Hours

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2635.37	4	658.84	1020.88	<.001
Within Groups	77.44	120	0.645		
Total	2712.81	124			

4. Discussion

In the current study, P. pinnata seed oil emulsion demonstrated a concentration-dependent antifeedant effect. At a concentration of 2%, it was found to be an effective antifeedant with more than 50% AFI after 24 and 48 hours (84.65% and 70.30%, respectively). However, at a concentration of 1%, it exhibited more than 50% AFI only after 24 hours (63.76%) and failed to maintain this level of AFI after 48 hours (46.19%). At a concentration of 0.5%, it produced moderate results with AFI values of 39.86% and 31.34% after 24 and 48 hours, respectively. On the other hand, at concentrations of 0.25% and 0.125%, it showed poor AFI values.

These results are consistent with those obtained in a previous study [24], where 2% P. pinnata oil was found to exhibit the highest AFI (44.86%) against the larvae of S. litura (Fab.). Several oil emulsions were found to exhibit antifeedant activity against P. demoleus larvae. A 1% emulsion of lemon grass-Chirharit oil was reported to demonstrate 94.34% antifeedant activity, followed by 78.11% for lemon grass-Krishna oil emulsion [15]. Similarly, a 1.5% emulsion of neem oil was found to be effective in inhibiting feeding by 3rd, 4th, and 5th instar larvae [26]. In comparison to a synthetic insecticide, Voliam flexi 300SC, which resulted in 6% leaf consumption by the 4th instar larvae, a 1% emulsion of neem oil-based Bioneem Plus, at a rate of 1.0 ml/l of water, led to 8% leaf consumption [27]. Various insect pests were found to be susceptible to the antifeedant properties of P. pinnata oil emulsions. In particular, P. pinnata oil demonstrated superior antifeedancy compared to neem and rohituka oils against the larvae of the Epilachna beetle (E. dodecastigma) [25].

Furthermore, repellent and toxic effects were observed with 0.75% P. pinnata oil against both the western flower thrips F. occidentalis [22].

During the Mid-Ordovician period (450 million years ago), vascular plants emerged from their aquatic ancestors [28]. Concurrently, a significant arthropod speciation event also occurred [29]. To defend against herbivorous arthropod pests, plants developed protective mechanisms, including the production

and storage of secondary metabolites [30]. Insect pests locate their host plants through olfactory signals and physical characteristics [31].

Contact chemoreception of plant allelochemicals plays a critical role in pest food selection [32, 33]. Through their dietary experiences, pests develop the ability to differentiate between suitable and unsuitable food [34]. Antifeedants act on sensory cells, known as antifeedant receptors, to prevent feeding by insect pests at low concentrations. They can also block the function of feeding-stimulant receptors [35].

In the current study, a 2% seed oil emulsion of P. pinnata proved to be an effective antifeedant against the 4th instar larvae of P. demoleus. Additionally, P. pinnata seed oil was found to be a good antifeedant against many other pests. The presence of secondary metabolites in the seed oil may have blocked the feeding stimulants of P. demoleus larvae, resulting in feeding inhibition. Further research is needed to identify which secondary metabolite is responsible for this antifeedant property and to elucidate the mechanism of action.

5. Conclusions

The present study is the first effort to study the antifeedant activity of the seed oil of P. pinnata against the larvae of P. demoleus. The results revealed that P. pinnata seed oil has the potency of antifeedant activity against the 4th instar larvae of Papilio demoleus. Hence it may be used to control the larvae of Papilio demoleus in citrus orchards in place of synthetic pesticides which cause many ill effects.

Declarations

Conflict of Interest

We declare that the authors have no conflict of interest that might be perceived to influence the results and/or discussion reported in this paper.

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Avifaunal Diversity of Bhopar Gaon Wetlands, Thane, Maharashtra, India

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ABSTRACT

The study area of Bhopar Gaon Wetlands is situated in Kalyan Tehsil of Thane district in Maharashtra. The avifaunal diversity of the area was investigated using the point count method from the first week of February to the last week of April. In total, 56 species from 25 families were spotted during the study, including resident birds, migrant birds as well as birds belonging to vulnerable and near threatened categories of the IUCN Red list. A checklist of observations has been compiled. Construction activities and deforestation pose a threat to the bird diversity. There is increasing awareness about bird diversity and its significance nowadays. A checklist of the said area does not exist and will be very helpful in getting a complete picture of the biodiversity status of the state. Ornithological data is also a useful indicator of effects of environmental changes on an area, and the impact on bird migration, nesting and breeding can all be assessed with this data. Avifaunal cataloging plays a significant role in understanding the complex ecological and evolutionary patterns of the biodiversity of an area. The present study represents a modest step in that direction.

Keywords ***Avifauna, Biodiversity, Birds, Wetlands***

1. Introduction

Birds are rightly defined as 'Feathered bipeds' by Dr Salim Ali [1]. They serve a variety of functions for the ecosystem, including those of scavengers, pollinators, insect pest predators, and bioindicators of various types of environments, including urban and industrial belts, human disturbance, and illumination [2]. Birds are not only sensitive to their environment, but conspicuous and therefore relatively easy and inexpensive to monitor as indicators of immediate ecosystem health [3]. Since the census techniques are well developed and affordable and there are plenty of experienced volunteers eager to count birds, they are generally simple to detect and identify [4]. At present, there are more than 10,000 species of birds that exist all over the world [5]. There were 1364 species in India as of 2021 [6] which is over 13% of the bird species worldwide [7]. Regrettably, after Brazil and Indonesia, India ranks third among the nations with the highest number of threatened and rare species [8]. Due to its extreme diversity, India offers a wide range of habitats for birds, including forests, grasslands, mountains, shorelines, and wetlands [9].

Wetlands in India cover an area of 58.2 million hectares [10] and are known to support over 310 avian species [11]. Wetlands are defined as "areas of marsh, fen, bog, or water, whether natural or manmade, permanent or temporary, with water that is static or flowing, fresh, brackish, or saline, including areas of sea water depth of which at low tides does not exceed six metres". While some wetlands contain water that is always visible above ground, others are seasonal, with water that comes and goes. Wetlands may have water levels that fluctuate from several metres deep to a few centimetres below ground, but as long as they feature wetland plants, at least occasionally throughout the year's wet and dry seasons, they are still considered wetlands. [12]. They are lands transitional between terrestrial and aquatic eco-systems

where the water table is usually at or near the surface or the land is covered by shallow water [13]. Wetlands are among the most productive ecosystems on earth and are essential for controlling erosion, flooding, recharging aquifers, and absorbing nutrients. In addition, wetlands provide home for a huge diversity of wildlife such as birds, mammals, fish, frogs, insects and plants [14]. Wetlands are crucial bird habitats because they provide them with resources. Birds utilize them for breeding, nesting, and rearing young, as a source of drinking water and for feeding, resting, shelter, and social interactions [15]. Wetlands are one of the most crucial habitats, yet they are also among the most threatened ecosystems in the world. They are prime targets for human activities like drainage, reclamation, pollution, and biological alteration through biotic and abiotic introductions, which can lead to their exploitation, modification, and destruction [12]. Therefore, it is urgent to create conservation action plans and policies to safeguard wetlands and their rich variety of birds. Very little is known about the ecology of wetland-related birds, their diversity, and their relationship to the trophic state of wetlands in India, despite a few studies being conducted by a number of scientists [16]. The proposed study aims to document the bird diversity at Bhopar village, Dombivli situated in Thane district of Maharashtra, India. The population of Dombivli is increasing rapidly and there is a consequent demand for land. Majority of the previously existing grasslands and wetlands have been replaced by buildings either already developed or under development and farms [17]. Around 30% of land is partially reclaimed for large town planning projects, industrial waste and solid waste dumping, cattle grazing, farming, fishing and poaching [17]. The goal of this study is to gather data on the diversity of various bird species in Bhopar wetlands.

2. Materials and Methods

2.1. Study Area

The village of Bhopar is situated in Kalyan Tehsil of Thane district in Maharashtra, India (figure 2). The total area of the village is 447.30 hectares. The wetland of Bhopar village (figure 1) is located at coordinates 73°04'21.4"E, 19°11'31.7"N and it resembles tropical climatic conditions. The mean annual temperature of the study site is 24.3oC (min) to 32.9oC (max) with the hottest and driest part of the year is April-May when temperature rises to 38oC for a few days. The humidity typically ranges from 58 to 84%. The average annual rainfall in the region ranges from 1286 to 1233 mm.

Figure 1. Urban Landscape [view of selected wetland]

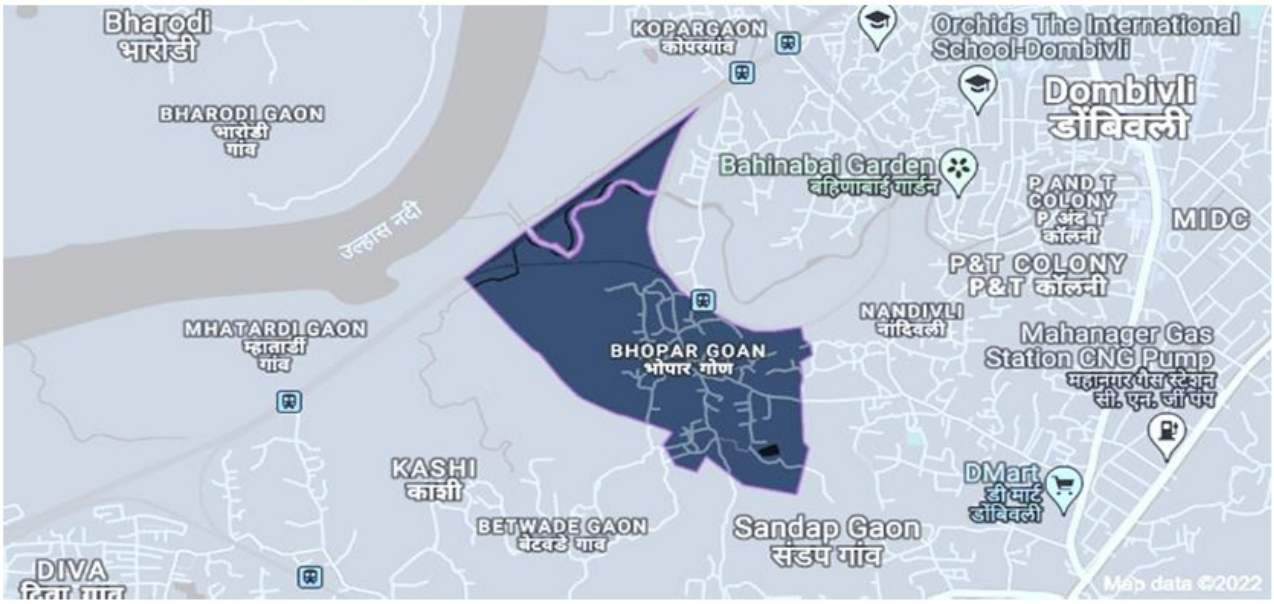

Figure 2. Map of Bhopar Gaon, Thane, Maharashtra, India [Image courtesy: Google Maps]

Figure 3. Locations of Stations [Image courtesy: Google Earth]

Beginning in the southeast near the Kalyan-Dombivli railway station, the estuary portion of the Ulhas River meanders for about 40 kilometres. The wetland of Bhopar is formed because of the backwaters of Betawade Khadi which emerges from the Ulhas River. The surrounding area is a mix of different habitats which includes marshland, grasslands, forest patches and water bodies.

The entire area of Bhopar wetland was divided into 4 stations as shown in figure 3. These stations were chosen to cover maximum area and variety of habitats

Station 1

Station 1 is Ganesh Ghat where immersion of Ganesh Idols is done. The place is also a cremation ground. This station has the highest level of human disturbance. Small scale fishing is also carried out at this point. Though the station is rarely crowded, people and two-wheeler vehicles are often present on this station. This station has wetland habitat on one side and grassland habitat on the other. When disturbance is at a minimum during the day, cormorants and painted storks can be seen foraging freely. Pond herons can be seen foraging and roosting in large numbers.

Station 2

This station has some ponds, marshland and a grassland in its vicinity. The lowest level of human disturbance can be seen on this station. Hence, flocks are usually seen roosting and foraging here.

Station 3

Wetland is present on one side and grassland is present on the other. This station is close to a forest patch and an ongoing construction activity. Railway track is close to this station thus suffers from noise pollution.

Station 4

Station 4 is a transitional habitat between grassland and the wetland. A forest patch is also present on this station. This station is closest to an ongoing construction activity. Thus, it deals with noise pollution and continuous human disturbance throughout the day. Yet, during the times of the day when disturbance is less, waders as well as grassland and forest birds could be seen.

2.2. Methodology

The study area was surveyed from the first week of February through the last week of April 2022, thrice every week. The method used in this study was point count method wherein bird count is done from a fixed place for a set amount of time [18]. The bird species observed were noted with point count method since the study area is uneven [19]. Observations were made using binoculars (Solognac 8x25/ Nikon 8x42). Digital point and shoot cameras (Nikon P950) and DSLR Camera were used for photo documentation. The birds were identified by their visual cues. The Field Guide of Indian birds by Salim Ali [20] and Birds of the Indian Subcontinent: India, Pakistan, Sri Lanka, Nepal, Bhutan, Bangladesh and the Maldives by Grimmet, Inskipp and Inskipp [21] were used as a reference to identify bird species. Species are categorized according to the latest IUCN (International Union for Conservation of Nature) Red List status, migratory and resident status, waders and other birds and their population trend. Checklists were made on the field by using eBird applications on smartphones as well as field data sheets were used to note observations and remarks.

3. Results and Discussions

A total of 56 species (Table 1) belonging to 25 families were observed during the study. Dominant family is Ardeidae with 8 species; followed by families Anatidae, Accipitridae, Muscicapidae, Scolopacidae with 4 species each; followed by families Ciconiidae, Sturnidae, Threskiornithidae, Motacillidae with 3 species each; followed by families Hirundinidae, Alcedinidae, Rallidae, Phalacrocorcidae, Phalacrocorcidae with 2 species and rest 10 families with single species. The vegetation at Bhopar supports the population of waders, swimming birds, divers, aerial foragers and terrestrial birds. As shown in figure 4, the highest total number of all species 52 was seen in March. In February 49 species were seen. Whereas, the lowest total number 40 was seen in April. During all the months of study species

from the family Ardeidae, Rallidae and Phalacrocorcidae were found to be present.
Total number of migratory species seen were highest in the month of February, followed by March, then followed by April. This is because migrant species return to their homeland in March-April. Northern Shoveler and Northern Pintail were the species observed until early- March. Common Sandpiper and Marsh Sandpiper were the migratory species observed during all the months of the study period Total number of resident species was highest in March, followed by February, then followed by April. This may be because after the departure of migrants, the residents returned to the wetland. Highest average number of birds was found in the month of February, which was followed by March and then April.
Diversity in February could be attributed to the presence of winter migrants residing in the study area as well as suitable temperature for resident birds.
As shown in figure 6, the average population of waders was highest in February, which was followed by March and then April. Following the trend of monthly total count, the number of waders decreased from the month of February to April. Painted Storks, Grey Heron, Purple Heron and Egrets were consistently present during the study period.

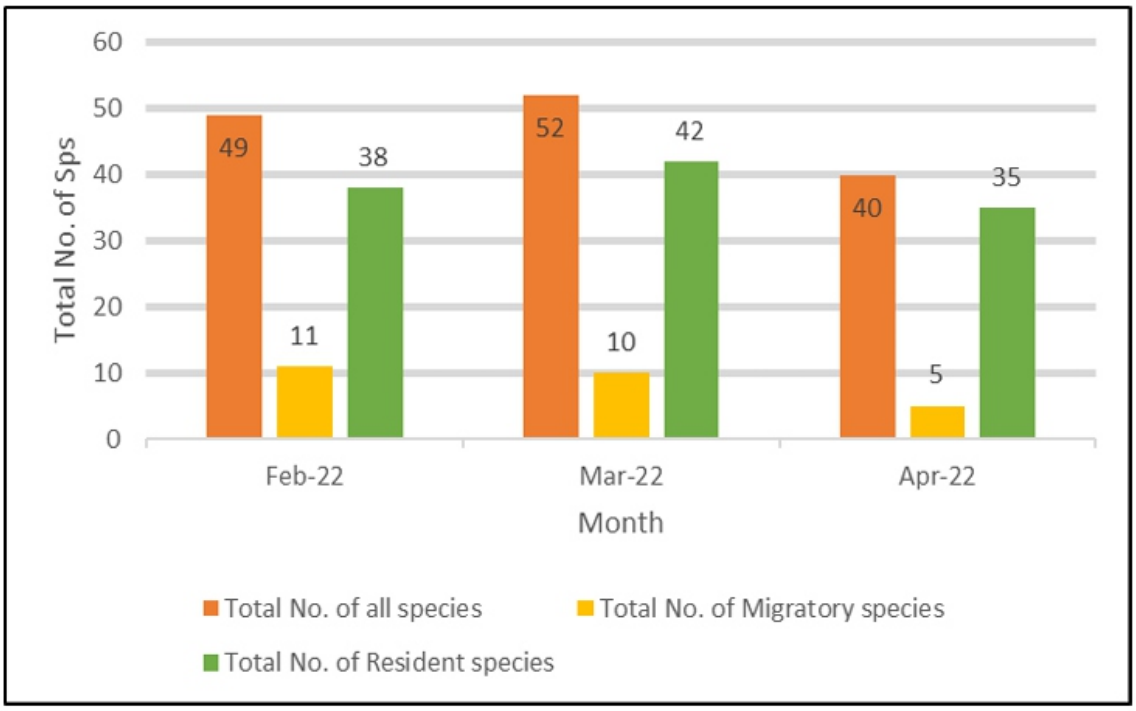

Figure 4. Month Wise Variation in no. of bird species

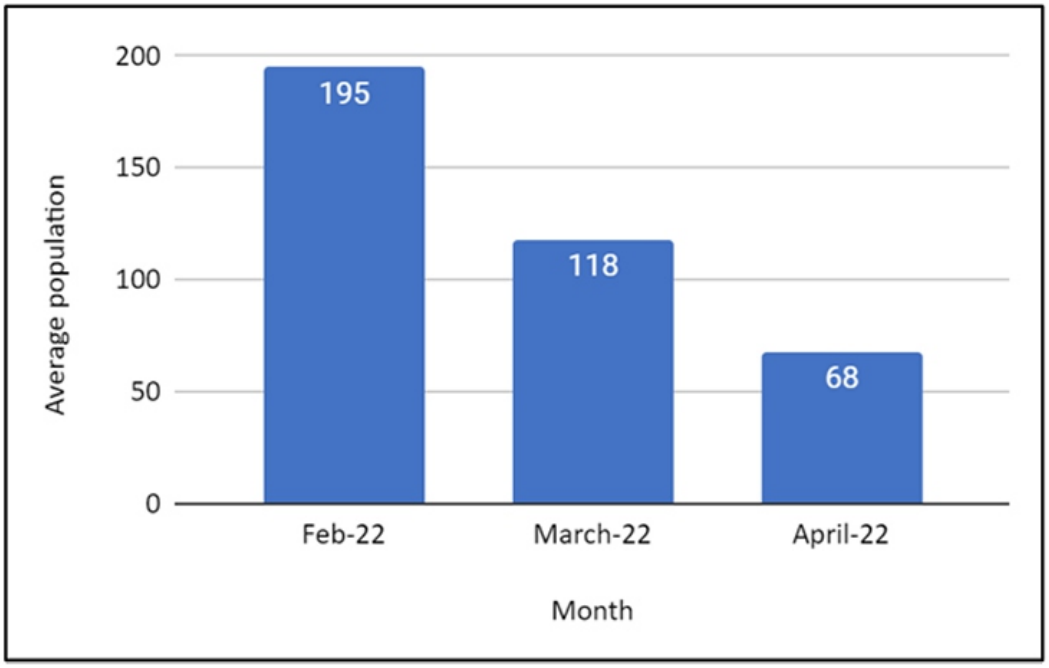

Figure 5. Average Total Count

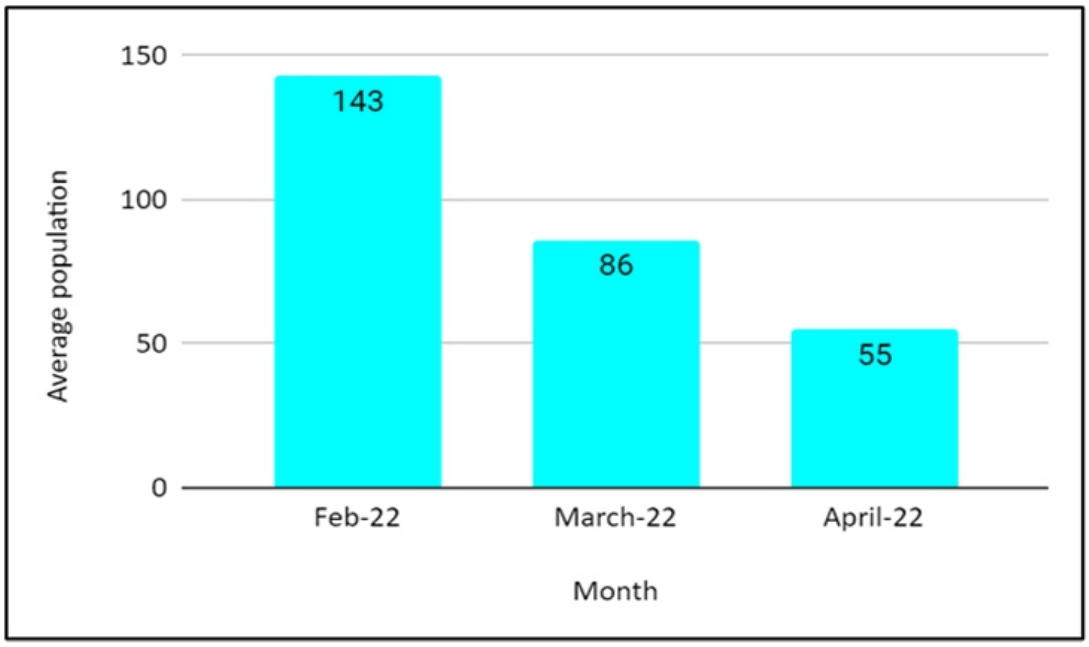

Figure 6. Month Wise Average of Waders

As shown in figure 7, the average population of other birds also was highest in February, which was followed by March and then April. Average population of waders dominated the average population of other birds. This is because the study area habitat has a major portion of wetland and marshland habitat which is more suitable for waders as compared to other birds.

As shown in figure 8, the average population of migratory species was highest in the month of February.

The average population decreased drastically in the months of March and April. Migratory birds started departing from India as the summer months of March- April approach. Month Wise average of resident birds as shown in figure 9 was highest in February followed by March, then followed by April. This may indicate that resident birds may have moved to shady areas to protect themselves from the heat.

As per the latest IUCN status, two near threatened species Black-headed Ibis and Painted Stork were seen during all three months of the study. One vulnerable species Greater Spotted Eagle was seen in February, while two vulnerable species Greater Spotted Eagle and Woolly necked Stork were seen in March. Other species found belonged to the least concerned category. The highest number of Least Concern species were found in the month of March, followed by February, then followed by April (refer figure 10).

The data revealed that the small patch of Bhopar Gaon wetlands provides asylum to a good diversity of birds. The local people are aware of the rich biodiversity of their locality. However, efforts to protect the same are not taken. Since the study area is close to Ganesh Idol immersion and Cremation ground, solid waste items like garlands, plastic cutlery, decoration articles and other plastic materials often find their way to water causing water pollution. Anthropogenic activities like water pollution, waste dumping and disturbance caused by small-scale fishing negatively affect the birds by impacting their foraging activities and nesting sites. Habitat destructions due to large scale of deforestation and land reclamation for the purpose of construction activities are key factors which pose a threat to the avifaunal population. If conservation efforts are not taken at the earliest then there is a high chance of loss of diversity in the study area.

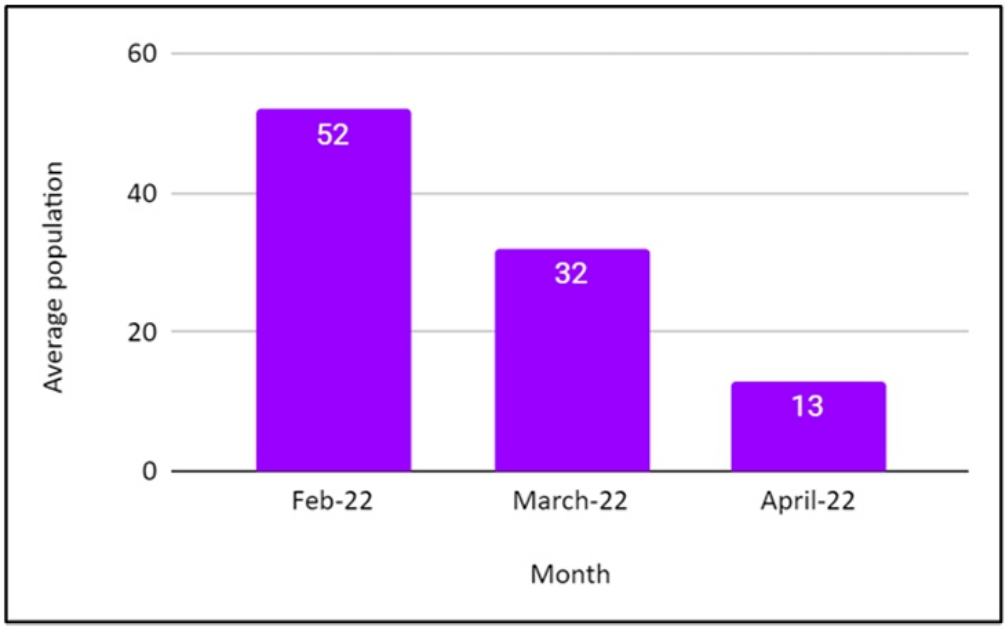

Figure 7. Month Wise Average of Other Birds

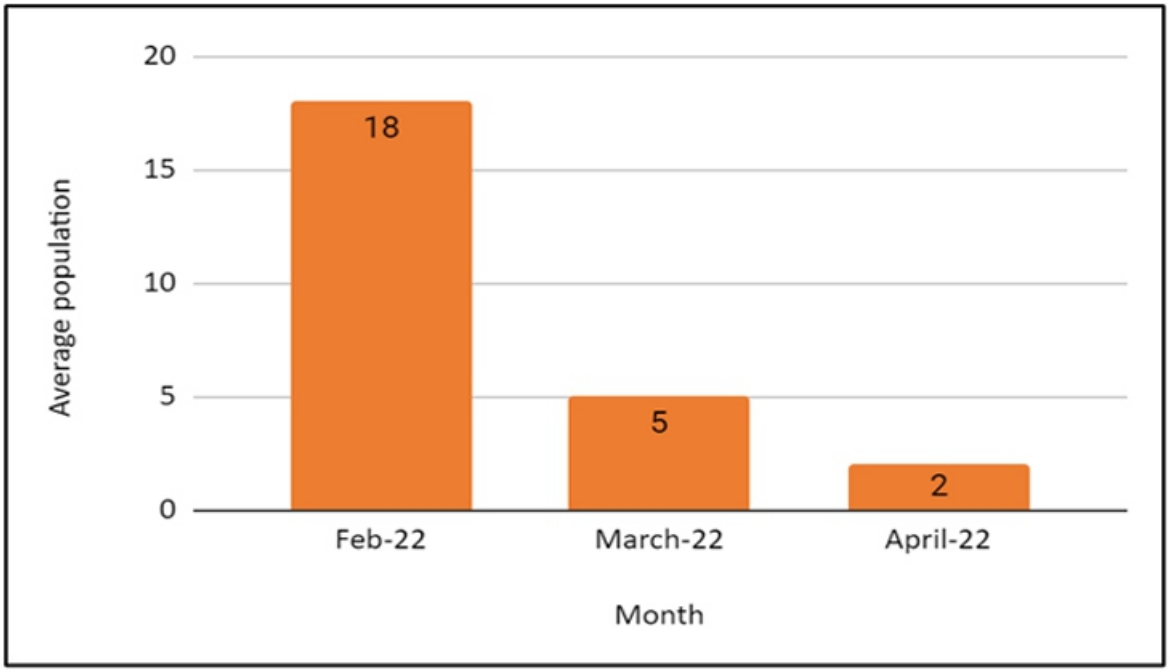

Figure 8. Month Wise Average of Migratory Birds

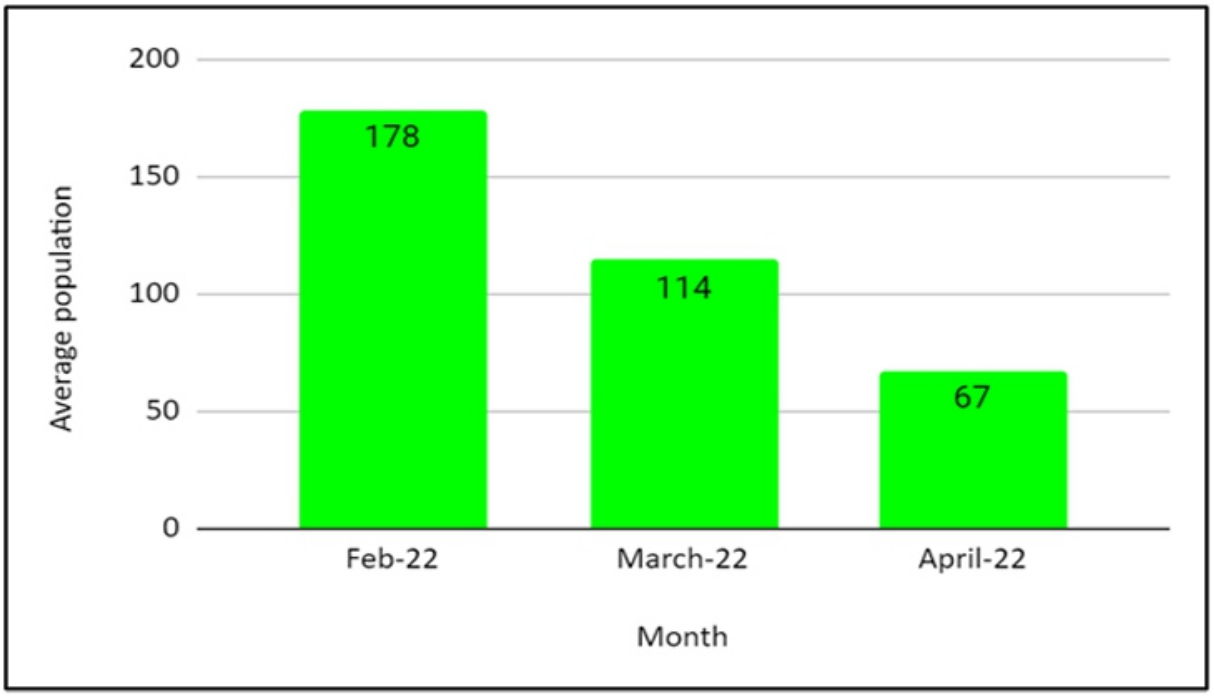

Figure 9. Month Wise Average of Resident Birds

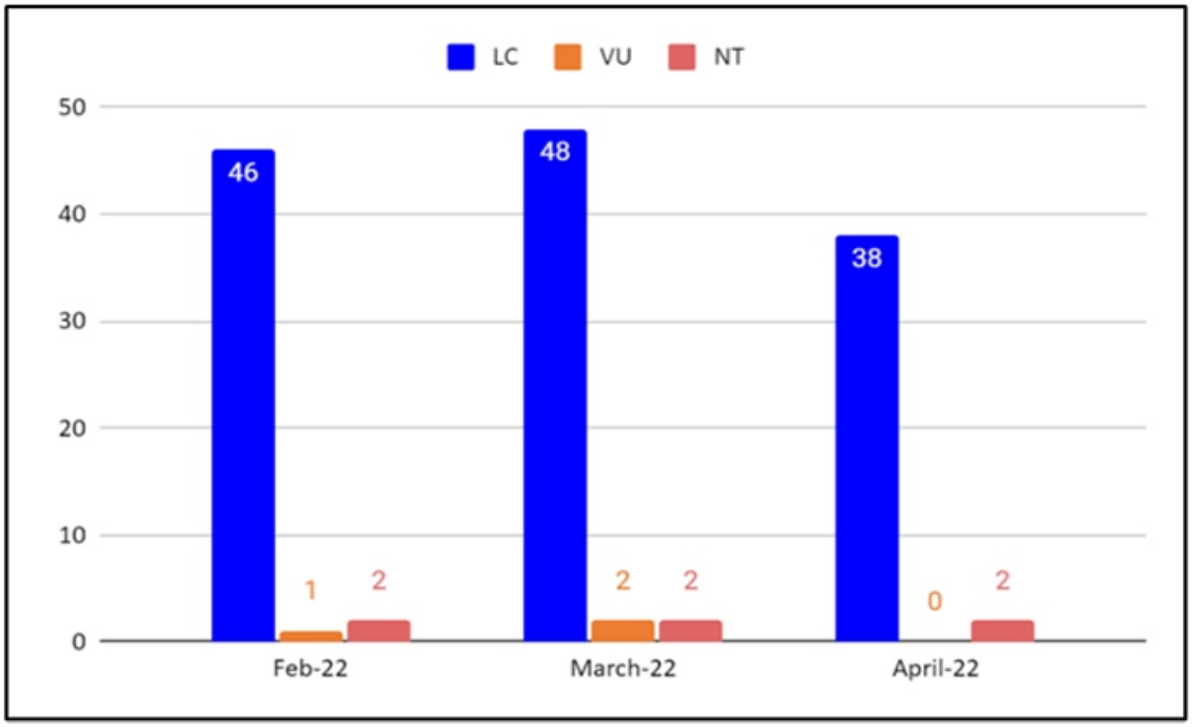

Figure 10. Species seen as per IUCN status (LC- Least Concerned, VU- Vulnerable, NT- Near Threatened)

4. Bird Checklist

Table 1. List of species along with their scientific names, IUCN status, Resident status and Population Trend

Sr No	Common Name	Scientific Name	Family	IUCN Status	Resident Status	Population Trend
1	Garganey	*Spatula querquedula*	Anatidae	LC	M	↓
2	Northern Pintail	*Anas acuta*	Anatidae	LC	M	↓
3	Northern Shoveler	*Spatula clypeata*	Anatidae	LC	M	↓
4	Indian spot billed Duck	*Anas poecilorhyncha*	Anatidae	LC	R	↓
5	Asian Openbill	*Anastomus oscitans*	Ciconiidae	LC	R	Unknown
6	Painted Stork	*Mycteria leucocephala*	Ciconiidae	NT	R	↓
7	Woolly-necked Stork	*Ciconia episcopus*	Ciconiidae	VU	R	↓
8	Ashy-crowned Sparrow Lark	*Eremopterix griseus*	Alaudidae	LC	R	=
9	Asian Pied Starling	*Gracupica contra*	Sturnidae	LC	R	↑
10	Rosy Starling	*Pastor roseus*	Sturnidae	LC	M	Unknown
11	Common Myna	*Acridotheres tristis*	Sturnidae	LC	R	↑
12	Barn Swallow	*Hirundo rustica*	Hirundinidae	LC	M	↓
13	Wire-tailed Swallow	*Hirundo rustica*	Hirundinidae	LC	R	↑

14	Black Drongo	*Dicrurus macrocercus*	Dicruridae	LC	R	Unknown
15	Black Kite	*Dicrurus macrocercus*	Accipitridae	LC	R	=
16	Eurasian Marsh Harrier	*Circus aeruginosus*	Accipitridae	LC	R	=
17	Greater Spotted Eagle	*Clanga clanga*	Accipitridae	VU	M	↓
18	Shikra	*Accipiter badius*	Accipitridae	LC	R	=
19	Black-crowned Night-Heron	*Nycticorax nycticorax*	Ardeidae	LC	R	↓
20	Cattle Egret	*Bubulcus ibis*	Ardeidae	LC	R	↑
21	Gray Heron	*Ardea cinerea*	Ardeidae	LC	R	Unknown
22	Great Egret	*Ardea alba*	Ardeidae	LC	R	Unknown
23	Indian Pond Heron	*Ardeola grayii*	Ardeidae	LC	R	Unknown
24	Intermediate Egret	*Ardea intermedia*	Ardeidae	LC	R	↓
25	Little Egret	*Egretta garzetta*	Ardeidae	LC	R	↑
26	Purple Heron	*Ardea purpurea*	Ardeidae	LC	R	↓
27	Black-headed Ibis	*Threskiornis melanocephalus*	Threskiornithidae	NT	R	↓
28	Eurasian Spoonbill	*Platalea leucorodia*	Threskiornithidae	LC	R	Unknown
29	Glossy Ibis	*Plegadis falcinellus*	Threskiornithidae	LC	R	↓
30	Black-winged Stilt	*Himantopus himantopu*	Recurvirostridae	LC	R	↑
31	Bluethroat	*Luscinia svecica*	Muscicapidae	LC	M	=
32	Indian Robin	*Acridotheres fuscus*	Muscicapidae	LC	R	=
33	Oriental Magpie Robin	*Copsychus saularis*	Muscicapidae	LC	R	=
34	Siberian Stonechat	*Saxicola maurus*	Muscicapidae	LC	R	Unknown
35	Citrine Wagtail	*Motacilla citreola*	Motacillidae	LC	M	↑
36	Western Yellow Wagtail	*Motacilla flava*	Motacillidae	LC	M	↓
37	Paddyfield Pipit	*Anthus rufulus*	Motacillidae	LC	R	=
38	Common Kingfisher	*Alcedo atthis*	Alcedinidae	LC	R	Unknown
39	White-throated Kingfisher	*Halcyon smyrnensis*	Alcedinidae	LC	R	Unknown
40	Common Sandpiper	*Actitis hypoleucos*	Scolopacidae	LC	M	↓

Table 1 continued

41	Common Snipe	*Gallinago gallinago*	Scolopacidae	LC	M	↓
42	Marsh Sandpiper	*Tringa stagnatilis*	Scolopacidae	LC	M	↓
43	Wood Sandpiper	*Tringa glareola*	Scolopacidae	LC	M	=
44	Gray-headed Swamphen	*Porphyrio porphyrio*	Rallidae	LC	R	Unknown
45	White-breasted Waterhen	*Amaurornis phoenicurus*	Rallidae	LC	R	Unknown
46	Greater Coucal	*Centropus sinensis*	Cuculidae	LC	R	=
47	Green Bee-eater	*Merops orientalis*	Meropidae	LC	R	↑
48	Indian Cormorant	*Phalacrocorax fuscicollis*	Phalacrocoracidae	LC	R	Unknown
49	Little Cormorant	*Microcarbo niger*	Phalacrocoracidae	LC	R	Unknown
50	Indian Golden-Oriole	*Oriolus kundoo*	Oriolidae	LC	R	Unknown
51	Little Ringed Plover	*Charadrius dubius*	Charadriidae	LC	R	↓

52	Red wattled lapwing	*Vanellus indicus*	Charadriidae	LC	R	Unknown
53	Long tailed Shrike	*Lanius schach*	Laniidae	LC	R	Unknown
54	Red Avadavat	*Amandava amandava*	Estrildidae	LC	R	=
55	Red-vented Bulbul	*Pycnonotus cafer*	Pycnonotidae	LC	R	↑
56	Spotted Dove	*Streptopelia chinensis*	Columbidae	LC	R	↑

IUCN Status: LC: Least Concerned, VU: Vulnerable, NT: Near Threatened
Population Trend: ↑: Increasing, ↓: Decreasing, =: Stable

5. Conclusions

Throughout the study site, a total of 56 bird species were found. This is a strong indication that the Bhopar Gaon Wetland is biologically productive and has the capacity to house a variety of bird species. The diversity of birds appears to be supported by the soil, water, climate, number of people walking, and other factors. Loss of habitat is by far the biggest hazard to birds. Major concerns to birds include deforestation, the draining of wetlands, the planting of non-native plants, the loss of land to urban development, and intensive agriculture. The ongoing construction activities and deforestation for the same might play a key role in negatively affecting the diversity of the present avian species. Further research is necessary to comprehend the seasonal variation of avifauna.

Biodiversity studies are generally done in large habitats, and small habitat patches are usually overlooked. Small patches, however, are also crucial for preserving biodiversity and the ecosystem as a whole. There are limited studies on the interior parts of Thane district. Since no documented studies are available on Bhopar, this study can be used as a baseline to understand the condition of the birds in the Bhopar Gaon Wetlands and how development activities affect them. A conservation plan should be undertaken focusing on protection of habitats to keep the avifaunal diversity intact. Educating locals about the importance of biodiversity present in their locality, afforestation, limiting land reclamation for construction are some of the steps that could be taken to maintain the diversity present in the study area.

To summarize

56 bird species (including resident as well as migratory) have been identified in the study area.
Bhopar wetlands has all the potential of being converted into an avian biodiversity park if preserved.
Very limited studies are available of the said area
Anthropogenic activities need to be controlled to protect the avian biodiversity here.
This study may provide baseline data for future research in this area.

Future Perspective

For the present study, further work could involve an annual study that could cover seasonal variation and a more comprehensive data generation of the area. Community surveys could also be initiated to understand the local knowledge and thought process of the locals with respect to the biodiversity of the area. In the long term, the area could be developed as a biodiversity park or a protected bird area.

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Determination of Activities of Eucalyptus Leaves Oil and Multiple Extracts on Growth Inhibition of Gram

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A B S T R A C T

Background of the research: Eucalyptus is a genus and a member of Myrtaceae family. More than 700 various species are available for this genus. In ancient time, it was widely used for treatment of diseases caused by microbes and bacteria. As it has a good ability to suppress and kill microorganisms, it is widely used in formulation of cosmetics. It was proved by many researchers that its oil contains 1,8-cineole which is used in pulmonary infection treatments. Eucalyptus also showed activities such as antioxidant, antiseptic, anti-inflammatory, herbicidal, insecticidal, anthelmintics. Purpose: The main purpose of this study is to confirm the antimicrobial activity of various concentrations of eucalyptus oil and its extracts in different solvent systems according to the polarity of solvents. As activity against of microbes depends on the presence of antioxidants and less studies were present on antimicrobial effects for selected microbes. Methodologies: Eucalyptus leaves were collected, dried under shades for 48 hours then eucalyptus oil was extracted with Clevenger apparatus by fixing with condenser. The process of extraction was completed with soxhlation process with multiple solvents according to polarity. Then phytochemical evaluation is done for confirmation of antioxidants. Free Radical Scavenging Activity DPPH Test, ABTS RadicalScavenging Assay, Tannin, Flavonoids, and total Anthocyanin content were determined for good activity. Antimicrobial activity was determined by Disc-Diffusion Assay after calculating the MIC with agar dilution method. Results: The results of using the extract on microbes were found as expected. Eucalyptus oil and extract both were found enriched with antimicrobial properties. These showed good effects against positive, negative bacteria as well as for Fungi. As high antioxidant activity was detected in ethanol extract. Antimicrobial activity results showed that K. pneumoniae seems to be the most sensitive bacteria among both grams (positive and negative bacteria's). The MIC in gram-positive bacteria for S. aureus found 2.85 mg/ml, best in gram negative is K. pneumoniae that is 2.05 mg/ml and for Fungi best MIC is 3.10 mg/ml. Conclusion: These research findings lead us to conclude that eucalyptus essential oil and various extracts, mainly the ethanolic extract could be considered as a potential alternative for synthetic bactericides. It can be used in the pharmaceutical industry for the prevention of pathogenesis caused by microorganisms and free radicals.

Keywords Antioxidants, DPPH Test, ABTS Test, Phytochemicals, Bacteria, MIC

1. INTRODUCTION

The Eucalyptus is a part of Myrtaceae family and comprises about 800 species [1, 27]. Approximately 305 species of this genus contain volatile oils in their leaves, although less than twenty of these species have ever been used commercially to produce oil that is essential and rich with cineole derivative (1,8 cineole) which can be used in cosmeceuticals and pharmaceuticals [2]. Extract of eucalyptus leaves contains antioxidants which has also been certified as food preservative or additive [3].

It is known from years that essential oils originate from natural sources. Essential oils and extracts contain potential use as substitute medications for curing of various diseases and infections [4, 5]. Many studies have pointed those essential oils obtained from eucalyptus species show antimicrobial effects against various microbes. Eucalyptus globulus [6, 7], Eucalyptus camaldulensis, Eucalyptus tereticornis, and Eucalyptus citriodora [6] have all been the subject of several research. There are only some investigations present for activity measurement against pathogenic & bacteria of food spoilage [8, 9]. Essential oils originated from eucalyptus leaves contain the active constituent 1, 8-cineole which is widely used for the inhalation therapy in pulmonary infections [10, 28]. The essential oil consists of oxygenated monoterpenes & sesquiterpenes respectively 53.6% & 14.2%, monoterpene & sesquiterpene hydrocarbons 12.2% & 4.9% respectively and some other components that were also present might equal to 14.7% of total components [11].

From ancient time, eucalyptus tree has proved its importance via providing positive results against microbes. Eucalyptus leaves and its extracts are being used to treat, heal wounds. Eucalyptus leaves have also been used to treat fungal infections too. Multiple activities are shown by eucalyptus leaves extracts like antiseptic, antiinflammatory and antioxidant [12]. As various studies show antimicrobial activities of Eucalyptus leaves essential oil and its constituents show besides antimicrobial activity, the essential oil and its constituents also work as pesticide [13], activity as an insecticide [14,15], actions as anthelmintics [16], anti-tumour activity [17] and antileeching activity [18].

After taking awareness of all properties of eucalyptus oil and extract, this project was conducted with following objectives:

1. Eucalyptus oil extraction was done with distilled water and Clevenger apparatus.
2. To Extract out eucalyptus leaves using Hexane,Petroleum ether, Acetone, methanol, and water.
3. Phytochemical screening of different phytoconstituents present in leaves extract.
4. Antioxidant Activity Determination.
5. Minimum Inhibitory Concentration (MIC) of separated essential oil was calculated by Antimicrobial Activity against selected bacteria.

2. Experimental Materials and Methods

2.1. Chemicals

The chemicals and reagents, were such as sodium nitroprusside, sulphanilamide, naphthyl ethylene diamine dihydrochloride, methanol, and phosphoric acid. The selected reagents were analytical reagent grade purchased from R. S. Enterprises Jaipur.

2.2. Collection of Plant Material

Eucalyptus leaves were detached from tree in April. Eucalyptus trees are growing in the road side earby area of Dr. K. N. Modi University, Newai, Rajasthan. Leaves of eucalyptus tree (as shown in figure 1) were placed at a dry place (non humid condition) for half a month. Leaves samples (specimens) were authentified at the Rajasthan University, Jaipur, Rajasthan. A voucher specimen for Eucalyptus Globulus was submitted to Botany Department, Rajasthan University, Jaipur, Rajasthan with book and receipt no. 343 and 34282 respectively, the authentication no. provided for Eucalyptus Globulus was RUBL21218

Figure 1. Eucalyptus leaves

2.3. Extraction

2.3.1. Extraction of Eucalyptus Oil

For extraction of eucalyptus oil, the leaves were air dried under a shade for 48 hours. After the leaves were sufficiently dried, some of them were ground to fine powder. Part of the leaves were halved and others left whole for investigation of the effect of leaf size in extraction. The ground leaves were stored in air-tight plastic bags and extraction done after 1 hr. A 100-gram portion of eucalyptus eaves were crushed and added into a clean round bottom flask and 500 ml of distilled water added. The round bottom flask together with its contents was placed in a heating mantle of equivalent volume that is using a 1 litre round bottom flask and would require either a 1 litre or 2 litre heating mantles. The round bottom flask was then connected to the other distillation apparatus: Clevenger apparatus and condenser (experimental assembly of Clevenger shown in figure 2). The water in the round bottom flask was heated to boiling. As soon as the boiling began, the temperature was regulated to a point where there was controlled boiling continuously for 3 hours [30]. The figures below illustrate the process.

Figure 2. Clevenger apparatus and eucalyptus oil collection in test tubes

The extracted essential oil (shown in figure 2) was firstly dried with sodium sulphate in anhydrous form and then kept in amber colour vials at 4°C for upcoming analysis.Placed Sodium Sulphate (Na2SO4) was removed before the use of the separated essential oil and the extracted yield was calculated using the formula given below:

Extracted yield = (Volume of Essential Oil × 100) / Dry Material [19].

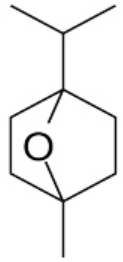

Figure 3. 1, 4 Cineol

Cineole, sometimes referred to as eucalyptol, is the primary component in eucalyptus oil. Moreover, the oil includes flavonoids and tannins, which have antioxidant and anti-inflammatory properties. Structure of cineole is shown in figure 3.

2.3.2. Preparation of Extracts from Different Solvent For extraction of eucalyptusleaves here I used 2 methods maceration and Soxhlation.

1) Extraction Using Maceration

Extraction process is used for the confirmation of various phytoconstituents present in the part of plant. Various solvents were used for the process of extraction. Some solvents were used according to their level of polarity. Their examples are ethanol, petroleum ether, methanol, hexane, acetone, and water. Extraction was processed by drying, crushing, and soaking plant part in a particular solvent one by one at a constant temperature and pressure. The quantity of solvents was the same 150 ml of each. This process is known as maceration. Soaking the extract that was filtered by Whatman no.1 paper which contained 110 mm pore size. This process wasrepeated with every solvent for the same quantity of crushed dried leaves of eucalyptus. As the procedure was repeated separately. After this process we got extract that was evaporated by using rotary evaporator by maintaining a temperature of 35 degrees centigrade. All the extracts were placed in amber colour vial at 4 degrees centigrade separately for further analysis [19].

2) Extraction by Soxhlet

Figure 4. Assembly set for extraction of eucalyptus extracts

This process also starts with the same procedure of drying crashing into small portions and soxhlation. Dried plant material was processed and crushed in small particles. About 70 grams of plant material or powdered material of eucalyptus leaves were used for this Soxhlet extraction process. The extraction assembly arranged in standard manner is shown in figure 4. The amount of solvent was 400 ml for the soxhlation placed at boiling temperature of specific solvent. As acetone requires 56 degree and methanol requires 67 degrees centigrade for boiling and evaporation. The reflux process gets continued for 48 hours. After that the extraction flask got removed and the extraction solvent was evaporated on rotary evaporator at a constant temperature. Before using rotary evaporator, the distillation flask got filtered by using filter paper. The rotary dryer dries the extract, which was used for further analysis. The non-soluble portion or thimble was discarded [29].

2.3.3. Qualitative Tests for Phytochemical Evaluation

The phytochemical tests were performed by using the procedures mentioned in various articles [25, 26]. Performed test results were shown in figure 5.

Figure 5. Various qualitative test for phytochemicals

1) Foam test

A test tube was taken for this test. 3 ml of distilled water was mixed with 2 milliliter of eucalyptus leaves extract then this mixture was shaken vigorously. By this shaking, foam formed on the top side of test tube which was stable and can be seen easily by naked eyes. That form formation confirms the presence of saponins in the extract.

2) Hansch test

A glass test tube was also used for this test. 2 ml of extract mixed with H2SO4 (1ml). H2SO4was added with the support of wall of the test tube. This process forms a brown coloured ring in the middle portion of the mixture. This confirmation test confirms carbohydrates present in the test extract.

3) Tannin Confirmatory test

1ml of the extracted solution was taken in a clean test tube following the addition of 1 ml of distilled water followed by 2 drops of FeCl3 solution addition. A deep blue colour was observed which confirms two types of tannins. First is gallic tannin which shows deep blue colour and green black color can be shown by catecholic tannins

4) Flavonoid test

2 mililiter of filtrate was filled in a clean test tube. Five to six drops of concentrated HCl were added. After that a few magnesium filings were added to it. The confirmation of flavonoids is shown by red colour appearance.

5) Phenol test

In two ml of extract in test tube a pinch of ferric chloride was added in the same test tube. Appearance of green colour indicates the presence of phenols in the selected sample for test.

6) Protein test

In a glass test tube of borosilicate 2 ml extract was taken, then HNO3 was added drop by drop in the mixture of test tube. Yellow colour comes and it confirms the presence of proteins.

7) Quinone test

2 ml of extracted sample was taken in test tube following the addition of few drops of concentrated. The presence of quinones was confirmed by the indication of red colour in test tube.

8) Fat confirmatory test

Fat can be confirmed by the spotting of oil on paper or filter paper. It is the easiest technique for fat confirmation. The extract was taken on a filter paper and no appearance of oil was found on it. This shows the negligence of fat in eucalyptus extract.

2.4. Free Radical Scavenging Activity DPPH Test

Free radical 1,1-diphenyl-2-picrylhydrazyl is also known as DPPH that is useful for Antioxidant scavenging activity. In this activity multiple sample dilutions were done for the extract or oil and then it used to mix with the 1,1-diphenyl-2-picrylhydrazyl solution. It will take at least 30 minutes to get stable or ready for test procedure. During this period, this mixture was placed in incubation at 25-30 egree centigrade and then absorbance was measured in UV spectrophotometer at wavelength 520 nanometre. In our laboratory (LMSP-UV1000B, LABMAN) spectrophotometer was used for wavelength detection. Firstly, blank procedure was carried out for measurement of baseline. Then the sample run was done as per the procedure and % inhibition was recorded. The % inhibition was calculated according to the following equation [31]:

$$\text{Percentage Inhibition} = \frac{\text{Blank} - \text{Sample}}{\text{Blank}} \times 100$$

IC50 was considered as antioxidant activity for sample or standard both. It can be defined as at least 50% was required of test material for DPPH concentration decrease in 50 %. In this test ascorbic acid was used as a standard. All tests were done three times (triplicates).

2.5. ABTS Radical-Scavenging Assay

2,2'-azinobis-3-ethylbenzothiazoline6-sulphonate is also known as ABTS. This test is used for the determination of the activity of antioxidants. It is the interaction between antioxidants and radical ions which are pre-generated. 2,2'- azinobis-3-ethylbenzothiazoline6-sulphonate by aintaining 7.4 pH. In this 7 mM of solution of ABTS with 2.5 mM K2S2O8 potassium perchlorate. (ABTS is a mixture of 154 mM of sodium chloride, 5 mM solution of Na2HPO4, 5 mM solution of NaH 2PO4). This mixture is used to be stored in a darkish cubical at a maintained room temperature of 25-28 degree centigrade for 16-18 hours' prior use. Further dilutions were made by persulfate buffer solution. It was observed at a specific wavelength of 734nano meter by using UV spectrophotometer situated in central Instrumentation room with equipment ID: LMSPUV1000B from LABMAN). It has shown its absorbance of 0.72 ±0.02 units [34].

Every sample was repeated with the same procedure. As essential oil was diluted and mixed with ABTS solution which was freshly prepared with 900 µL concentration. This mixture was used after 6 minutes after mixing for detection of absorbance. The free radical scavenging was expressed by IC50 in milligram per ml, which represents the 50% of the scavenging of ABTS radicals can be done. This measurement was done three times for better efficacy. The solution of ascorbic acid was used as a standard for comparative analysis. Radical-Scavenging can be calculated by the above-mentioned equation of DPPH testing [19].

2.6. Total Amount of Phenolic Compounds

The multiple stored extracts are used for phenolic determination by fluka. Fluka is also known as Folin Ciocalteu reagent. Fluka can be prepared by mixing a specific amount of following ingredients [20]. Those are:

a. sodium tungstate: 10 grams

b. sodium molybdate: 2.5 grams

c. phosphoric acid: 5 ml

d. concentrated hydrochloric acid: 10 ml

e. lithium sulphate: 15 grams

f. 1 drop of bromine

g. Water: 75 ml water divided in two portions.

Separately mixed the poured in one beaker. Dissolve a) and b) in 70 ml of water. Then add 85% phosphoric acid in a quantity of 5 ml. After it d) added 10 ml. Reflux was done for 10 hours. The addition of e) and 5ml water was followed by 1 drop of bromine. This mixture was placed for reflux for 15 minutes. After refluxing, it was cooled at room temperature and volume made up to 100 mL with water. Some complexes were formed in solution that was of Hexavalent phosphomolybdic/ phosphotungstic acid [38]. All the aqueous extracts solutions were diluted and a specific volume of 0.1 ml was mixed with 0.5 ml freshly prepared fluka with 0.2 Normality. The prepared solution of these components was placed safely for five minutes at normal temperature of room. After 5 minutes 0.4 ml solution of NaHCO3 was added at 75 grams per ml. This mixture was placed in incubation for 60 minutes and then absorbance was measured at 765 nm against water in a LABMAN spectrophotometer (LMSP-UV1000B, LABMAN). After this process a standard calibration curve was plotted using gallic acid from 0 to 200 mg/ml [20,32].

2.7. Tannin Content Determination

Vanillin method was used for the determination of tannins. As proanthocyanins and Catechins both are reactive to vanillin so this method was preferred. Vanillin solvent can be known as a 1% of 7 M H2SO4. So, this reagent was mixed with 1 ml of each extracted sample in a glass test tube.

Then this mixture along with test tube was placed on ice bath. After this the incubation was done for 15 minutes at 25 degrees centigrade. After taking out from incubation, this was tested for absorbance at 500 nm in the same spectrophotometer. This procedure was repeated for multiple samples and calibration curve plotted [21,22].

2.8. Flavonoids Determination

Arvouet et al wrote an article for flavonoids determination. They have used the Dowd method for determinations. In this determination aluminium trichloride and methanol play an important role for evaluation. The extracted samples or solution was taken and 2 % methanolic mixture of aluminium trichloride solution was mixed with each other in an equal amount of 0.5 ml in a test tube. This was mixed properly and placed in cuvette for determination in UV Spectrophotometer at 415 nm against blank sample. This blank sample contains methanol 0.5 mL and extract 0.5 mL exempting the addition of AlCl3. C15H10O7 was used as a reference. The name of this substance is quercetin. The outcomes were given in terms of grammes of quercetin equivalents (QE) per kilogramme of dry mass. [33].

2.9. Determination of Anthocyanin Content

These can be determined by the pH difference method. In this method. In this an absorbance range can be used which would be 510 to 700 nm. A buffer solution was prepared by mixing and setting pH 1 and 4.5 respectively for (0.2 M of both HCL and KCl) and Sodium acetate and acetic acid at 1 M solutions. As incubation is required before taking absorbance. It was incubated for 15 minutes. The content of anthocyanin was calculated by using cyanidin-3-glucoside that has a molar extinction coefficient (ε) of 29,600 and an absorbance of [(A510 A700) pH 1.0 (A510 A700) pH4.5]. Results were given in milligrams of cyanidin-3-glucoside equivalent (C3GE) per kilogramme of dry mass [37].

2.10. Antimicrobial Activity

2.10.1. Microbial Strains

All microbial sample strains were collected from IARI, New Delhi. The sample extracted essential oil was separately tested on group of micro-organisms. Depending on the intended media volume, different amounts of the elements are used (Here, we will make for 1000 ml of media). Peptone (5g), NaCl (5g), Beef or yeast extract (3g), and agar (3g) were weighed separately (15g). 900 ml of autoclaved distilled water or deionized water was added to a conical flask. Then the media were prepared.

Gram positive (S. aureus, Bacillus subtilis), and three Gram negative (Pseudomonas aeruginosa, Escherchia coli, Klebseilla pneumoniae) bacteria were used. Two fungi (Aspergillus parasiticus, Mucor ramamnianus) were used for testing. Nutrient agar was used for the culture of bacteria at 37 degrees centigrade for 48 hours. Fungi were generated in isopiperitenol dehydrogenases 2 (ISPD2). Two species were selected, those were (A. parasiticus, M. ramamnianus). Those were cultured for 48 hou rs to 3 days

at 30 degrees centigrade. The selected microorganisms were placed at their required conditions of storage and those were regenerated before use for manipulations twice.

Trypticase-soy was used at 37 degree centigrade with distilled water. That distilled water got sterilized before use. The bacterial strength was fixed nearer to 4 x ao6 bacteria per ml. One after one growth cycles were grown from the stock culture. Different strength and concentrations of bacteria and fungi were used for the stock culture preparation. Swabbing was done for seven days for covering of fungi strains spores. For good dispersion of pores some contents were used like polysorbate 80, Sabouraud agar and distil water which sterilized before use. Multiple concentrations of inoculum were used for analysis on microbes for antimicrobial activity. Some quantity of bacteria was placed in prepared culture medium to categorise multiple changes in that. The suspension or culture, meticulously prepared to elicit a favorable response from microorganisms, serves as a conducive environment for testing different antimicrobial agents. The spore's concentration for fungi was counted by light microscopy.

2.10.2. MIC Agar Dilution Assay

Agar dilution method was used for study of various microorganisms. A combination of Eucalyptus essential oil and methanol in equal quantity was taken. The sterile nutrient agar was mixed with tween 80 in 0.5 % concentration. Tween 80 was used for concentration making up 0.5-20 mg per ml. This mixture was used for mixing of essential oil. This mixture was filled immediately in petri dishes and waited for 30 minutes' solidification at room temperature. The prepared plates were spotted for bacteria and fungi strains. And microbial activity was recorded. For fungi positive reference Nystatin was used in 0.5-20 mg per ml concentration. For bacterial activity determination Ampicillin and Nalidixic acid were used in the same concentration. These plates were placed for 2 hours at four degree centigrade, after that bacteria were incubated at 37 degree centigrade and fungi plates were incubated at 30 degree centigrade. All the tests were performed in duplicated for good results [19-24].

2.10.3. Disc-Diffusion Assay

For various eucalyptus extracts this paper disc diffusion method was used [35]. Suspension was spreaded on the prepared plate of nutrient agar for the testing of microorganisms. The extract was diluted with methanol. After dilution of this mixture, it was poured on plates which were inoculated previously. Positive standard reference nalidixic acid with ampicillin was used for various microbial activity determination as planned. In this both bacteria and fungi were tested. Nystatin was also used in the same concentration as ampicillin and nalidixic acid. As a result, antifungal and anti-microbial activity was not found for methanol. Separate temperatures for fungi and bacteria were set for incubation. It was 30 degrees centigrade and 37 degrees centigrade respectively for 2 days and 2 nights or 48 hours.

After this process zone of inhibition was calculated and sensitivity for individual extract was measured. The criteria were decided according to the diameter of inhibition zone. If it comes smaller than 9 milimetre then it is considered as non-active or nonsensitive. If it is found in between 10-15 milimetre is known as good. For more sensitive activity it should come in 16-20 milimetre. If it founds a diameter more than 20 milimetre then it will be considered as extremely sensitive [36]. Note:

The concentration of Ampicillin and nalidixic acid was taken 40 µg/disc.

The diameter of disc was taken 9 milimetre.

The extract was diluted in methanol in concentration of 40 µL. It can be considered as 1/100 from the initial concentration.

3. Results

3.1. Extraction of Essential Oil

The extraction of essential oil from eucalyptus leaves is done with the help of Clevenger apparatus in the Department of pharmacognosy laboratory no 2 at Dr. K. N. Modi University, Newai, Rajasthan. After completion of the process, I found the yield of oil which is tabulated hereunder in table 1.

The percentage comparison of extracts is presented in figure 6

Table 1. Extraction % yield of essential oil

Samples	Yield (%)
Essential oil	2
Hexane	29.7
Petroleum ether	0.2
Acetone	4.8
Ethanol	8.3
Methanol	5.8
Water	4.8

3.2. Phytochemical Screening of Eucalyptus Extract

Extraction is performed by the Soxhlet with the help of various solvents according to polarity but according to cost and availability of solvents, comparison of methanol and acetonic extract is mentioned hereunder for better understanding.

The extract of eucalyptus leaves in methanol was tested for multiple phytoconstituents for the confirmation of presence of individual phytoconstituents i.e.; carbohydrates, tannins, saponins, flavonoids etc. In this extract proteins and fats were not confirmed by the test. So, those were considered as absent. Multiple articles were studied and they show various results in various extracts, different

all quinones, carbohydrates, tannic acid, phenols, & flavonoids as methanol used for extraction of leaves of eucalyptus [40]. I found proteins and fat absent in methanolic extracts of eucalyptus leaves. It is presented in the following table 2

Table 2. Phytoconstituent confirmation for eucalyptus leaves extract

S. No	Phytoconstituent	Eucalyptus leave extract in methanol	Eucalyptus leaves extract in Acetone
1	Quinones (Alkaloid)	P	A
2	Saponins	P	P
3	carbohydrates	P	P
4	Tannins	P	P
5	Phenols	P	P
6	Flavonoids	P	A
7	Proteins	A	A
8	Fat	A	A

As this table represents that maximum phytoconstituents are present in the methanolic extract as compared to acetonic extract of the same eucalyptus leaves. So, this xtract was used for antioxidative determination, phenolic determination & flavonoid determination of leaves extract of eucalyptus.

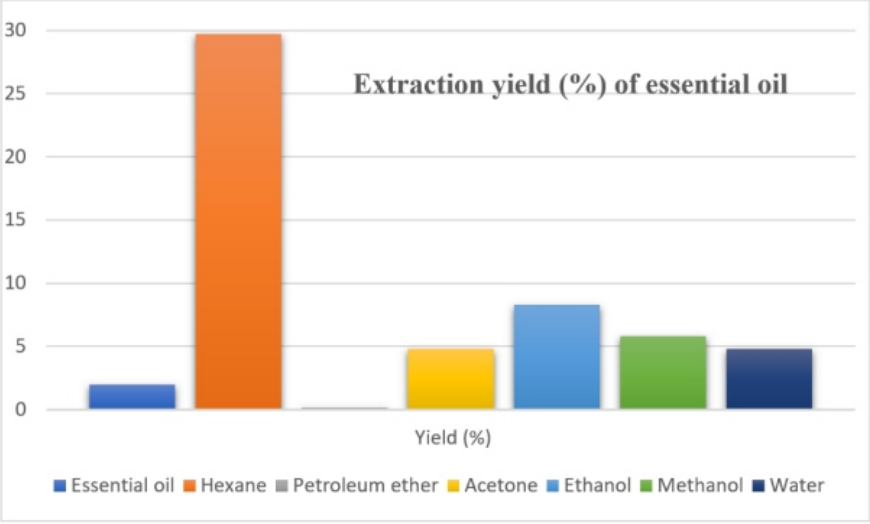

Figure 6. Extraction yield (%) of essential oil

3.3. Eucalyptus Leaves Extract and its Estimation for Phenolic Content

In this estimation methanolic extract of eucalyptus leaves was taken for the determination of phenolic content at an absorption range of 650 nano meter. This was compared against gallic acid solution. This phenolic phytoconstituent was measured in parts per million. Total phenolic content of 10 ppm methanolic eucalyptus extract was confirmed 10.82% and it is shown in figure 7 [39].

3.4. Eucalyptus Leaves Extract and its Estimation for Antioxidative Activity

This determination method is a well-known method with the name of DPPH testing assay. DPPH stands for a compound (2,2-diphenyl-1-picrylhydrazyl) which is used for this determination. Eucalyptus leave extract in methanol at 200 parts per million was confirmed 443.4 whereas ascorbic acid represented its antioxidative activity at 37.42. These data are presented by the chart plotted hereunder in figure 8 [15].

3.5. Eucalyptus Leaves Extract and its Estimation for Flavonoid Content

Total flavonoid content can be determined by the aluminium chloride method. All above mentioned methods are performed on spectrophotometer and absorption at specific wavelength is recorded. For this procedure I used Quercetin as a standard. Absorbance was written down at 415 nm. The total content in methanolic extract was confirmed 34.92% and it is represented in figure 9 [15].

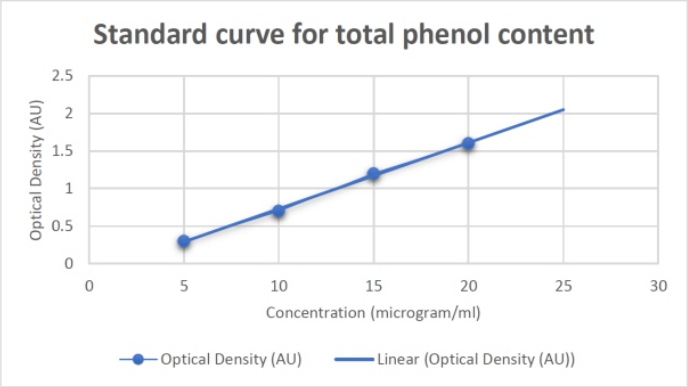

Figure 7. Plantation for total phenolic constituent

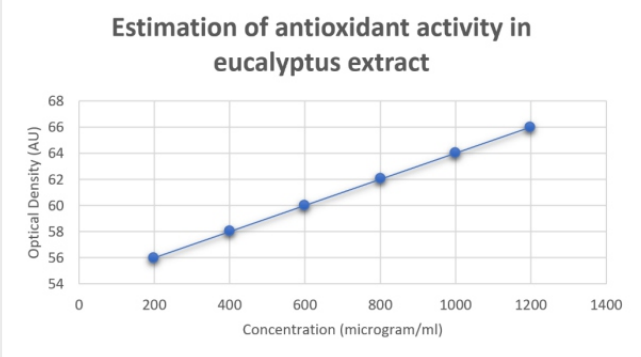

Figure 8. Estimation of antioxidant activity in eucalyptus extract by spectrophotometer

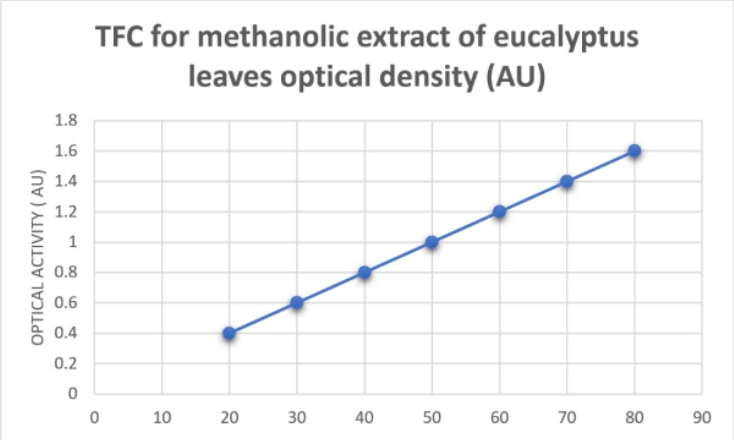

Figure 9. TFC in eucalyptus leaves methanolic extract TFC stands for total flavonoid content

3.6. Determinations of Antimicrobial Activity

The results of using the extract on microbes were found as expected. Eucalyptus oil and extract both were found enriched with antimicrobial properties. These showed good ffects against both types of bacteria as well as for Fungi. The detailed description is given below:

3.6.1. Minimum Inhibitory Concentration (MIC) of Essential Oil

Eucalyptus oil was evaluated for the MIC determination. MIC was determined by no microbial growth at a specific concentration of essential oil. In results I found the eucalyptus essential oil ability or potential to inhibit gram positive bacteria, especially S. Aureus. The minimum inhibitory concentration was deducted 2.85 milligram/ ml. IF we discuss about the gram-negative bacteria it comes to Klebseilla pneumoniae which was found the most sensitive for this essential oil. For Klebseilla pneumoniae minimum inhibitory concentration was deducted 2.05 milligram/ml. It also showed its response of inhibition against M. ramamnianus and the minimum inhibitory concentration was found 3.10 milligram/ ml.

Various reports and articles were studied before this evaluation and determination of activity against microbes. With respect to this study fruits of eucalyptus tree specifically from globulus species were also used for the extraction of oil and its activity was determined. These determinations for MIC resulted in a range from 0.06 to 1 milligram / milliliter for gram positive bacteria. When resulting for gram negative bacteria, results were found negative for P. aureginosa, E. Coli and K. pneumoniae. Other studies and results were used for evaluation and result comparison against various gram positive /negative bacteria [41-48]. For the comparison of those results I tried to explain it in an easier way of the activity of eucalyptus oil. It makes the bacterial cell wall permeable which was made up of lipids. So, this essential oil is responsible for

the cell death by losing metabolites and ions [48-50].

3.6.2. Minimum Inhibitory Concentration (MIC) of Extracts

According to the polarity of solvents, various extracts of eucalyptus leaves were prepared and the extracts were stored for further studies on different species of bacteria and fungi. Below table represents the study results which show the effectivity of extracts against various microbes. With this effectivity inhibition zone was measured. Methanol extract from all extracts (methanol, ethanol, petroleum ether, acetone, hexane, and water) was found highly efficient against microorganisms. Zone diameter was selected ranging from 11 to 20 mm

For positive bacteria, B. subtilis was considered as the most sensitive followed by S. aureus. Concerning Gram negative bacteria E. coli, was the most sensitive, the zone inhibition diameter was found 15 mm, followed by K. pneumoniae and P. aeruginosa. Aspergillus parasiticus was the most sensitive fungi, and showed good activity in all extracts, but M. ramamnianus was found resistant since we did not find inhibition zone with the extracts of acetone and petroleum ether. The water did not show any activity on any of the strain. For the conclusion gram positive bacteria were found sensitive for all extracts. Here I have not calculated the MIC because Disc diffusion method resulted in moderate activity toward samples. It is presented in the following table 3.

Results show that if we keep aside the morphological diversity of strains, every extract is showing its antimicrobial activity according to the phytoconstituent present in suitable concentration. For every activity I used disc diffusion method and that is resulting positively in aspect to anti-microbial activity. The result explains that as anthocyanins increases in the extract it impacts on growth of B. subtilis. I found as anthocyanins increases the zone of inhibition also increases.

Table 3. Eucalyptus leaves oil MIC

		Gram Positive		**Gram Negative**			**Fungi**	
Sr. No.	Samples	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. pneumoniae*	*A. parasiticus*	*M. ramamnianus*
1	Essential oil	3.75 ±0.11	2.85 ±0.10	3.74 ±0.10	3.90 ±0.12	2.05 ±0.06	3.74 ±0.10	3.10 ±0.10
2	Ampicillin	0.02 +0.00	0.02 +0.00	0.01 +0.00	0.02 +0.00	0.01 +0.00	0.02 +0.00	0.02 +0.00
3	Nalidixic Acid	0.02 ±0.00	0.02 ±0.00	0.01 ±0.00	0.02 ±0.00	0.01 ±0.00	0.02 ±0.00	0.02 ±0.00
4	Nystatin	0.02 ±0.00	0.09 ±0.00	0.02 ±0.00	0.02 ±0.00	0.02 ±0.00	0.01 ±0.00	0.02 ±0.00

4. Conclusions

Eucalyptus essential oil and its extracts showed good efficacy against multiple microbes and fungi. They may be used in pharmaceutical industry as flavouring agents [51,52]. In this work, good antioxidative activity was present in ethanolic extract (IC50= 12.6 + 0.7 milligram/ml) by (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay of ABTS as compared to methanolic extract. Antimicrobial ctivity results proved K. pneumoniae was found to have more sensitive bacteria among gram positive and gram negative. So, the MIC of best in gram-positive bacteria is (S. aureus 2.85 mg/ml), best in gram negative is K. pneumoniae (2.05 mg/ml). Fungi present best MIC is (3.10 milligram / milliliter). This research study took me to the conclusion about eucalyptus oil and extracts preferably ethanolic as a synthetic bactericide with the applications in pharmaceutical industry.

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