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In vitro antimicrobial and antioxidant studies of active principle hexacosylidenecyclohexane extracted from bark of *Madhuca longifolia* L

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Abstract
Plants are benefactor of humankind. The enormous increase in multiresistant strains irrespective of potent antibiotics has resulted in an exploration of new plant based products. Free radicals produced in the body are responsible for oxidation of biomolecules and increases the risk of chronic disorders. Antioxidants are the compounds which gives protection against cell damage. Active principle hexacosylidenecyclohexane isolated from the bark of *Madhuca longifolia* L. is used in the present antimicrobial and antioxidant studies. The antimicrobial activity of active principle was evaluated against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* by disk diffusion method and Minimum inhibitory concentration assay. The antioxidant activity of crude extract was tested by Superoxide dismutase assay and active principle was evaluated by DPPH and TRPA assay. The results obtained from the present work proved active principle have strong antimicrobial and antioxidant activity and can be used in herbal formulations.

Keywords: *Madhuca longifolia* L, hexacosylidenecyclohexane, antimicrobial activity, minimum inhibition concentration, antioxidant assay

1. Introduction
Nature has been a rich source of medicinally valuable plant**s** for long period of years since the beginning of man [1]. Plants are considered as an alternate source of medicine and are considered as "Drug House" in the future. They have played a significant role in improving the quality of human life and maintaining human health for thousands of years. For a long period of time, plants have been a very good source of herbal products for maintaining human health, especially in the last decade, with more intensive work for natural therapies [2]. Different plant parts such as rhizomes, bulbs, roots, leaves, fruits, latex, resins, seeds, flowers, bulbs were used in preparing formulations against various infections [3].
The excessive use of chemical antibiotics provide a way for resistance among different microbial species, hence there is an urgent need for replacement of chemical antibiotics with natural remedies because of their non-toxic nature and lesser side effects [4]. Even though hundreds of plant species and phytochemicals have been tested for antimicrobial properties, the vast majority of them have not yet been evaluated [5].
In the human body, endogenous metabolic processes or exogenous chemicals results in the formation of free radicals or highly reactive oxygen species (ROS). These are capable of oxidizing biomolecules like DNA, nucleic acids, proteins and lipids and causes different degenerative diseases like emphysema, atherosclerosis, arthritis, neurological disorders, cancer and cirrhosis [6, 7]. Antioxidants are the compounds which readily attack free radicals and thus reduce the risk of these disorders. Different parts of plants (root, leaf, flower, fruit, stem, bark) have been used successfully to treat many diseases. Their antioxidants affect many physiological processes in the body, thus protecting against free radicals. Derivation of compounds from plants with antioxidant activity would be a valuable tool for medicinal research.

2.2 *Madhuca longifolia* L.
Madhuca longifolia is highly regarded as a universal remedy in the ayurvedic medicine. It is a large evergreen tree distributed in India, Nepal and Sri Lanka [8]. *Madhuca* commonly called butternut or mahua tree, 17m high with a large top [9]. It belongs to the family Sapotaceae. It has a remarkable place in tribal culture. The bark is yellowish grey to dark brown red in colour and milky inside.

The bark is suggested for phlegm and in rheumatism bark flakes are mildly heated and tied on joints. The bark is a good remedy for snake-bite poisoning itches, swelling and fractures [10]. The present research work mainly aims to compile data generated through the antimicrobial and antioxidant activity on bioactive principle- hexacosylidenecyclohexane from bark of *M. longifolia.*

2. Materials and Methods

2.1 Antimicrobial activity-Agar-well diffusion method:

Antimicrobial screening of the active principle using methanol solvent was carried out by well diffusion method against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Candida albicans*. Soyabean casein digest agar (Bacteria) and potato dextrose agar (PDA) plates were prepared as per manufacturer instructions. Cell suspensions prepared with the above organisms were grown on specific broth and incubated in aerobic chamber for 24h at 35 °C Cell suspensions of all the cultures were adjusted to 1.2 × 10^5cells/ml. The different concentrations of active principle (20 µl), Ciprofloxacin and Itraconazole were loaded on 5mm well on agar plates. The plates were incubated at 35 °C for 24-48h. After the incubation, zones of inhibition formed around the well were measured with transparent ruler in millimetre.

2.2 Determination of MIC by micro broth dilution technique as per NCCLS method [11]:

Cell suspensions were prepared with bacterial cultures grown on Trypticose soya broth and fungal culture on potato dextrose broth were incubated in aerobic chamber for 24h at 35°C. The cell suspensions of all the culture were adjusted to 1.2 x 10^5cells/ml. Different concentrations of the active principle (1000, 500, 250,125, 62.5, 31.25 and 15.62 µg/ml) were prepared in broth. 90 µl active principle of different test concentration with 10 µl inoculum were placed in 96 well plate. Similarly, 90 µl standard drug of different test concentration with 10 µl inoculum were placed in 96 well plate. 90 µl broth without drug with 10 µl inoculums placed in 96 well plate as control. The plates were then incubated at 37 ºC at 24-48 h. After incubation optical density at 600nm is measured in Tecan plate reader. Minimum concentrations of drug and active principle giving 50% inhibition of OD as compared with control were determined.

2.3 DPPH Antioxidant Assay

A rapid, simple and inexpensive method to measure antioxidant capacity of plants involves the use of the free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity.

DPPH [1,1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple colour. Antioxidant compound present in plants reduces DPPH to 1,1-diphenyl-2-picryl hydrazine, colourless compound which is measured at an absorbance of 510 nm.

The free radical scavenging activity of the methanolic extract of *M. longifolia* bark was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. 75µl of solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the extract solutions at different concentrations. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 510 nm using a semi-autoanalyzer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage scavenging activity at different concentrations was determined and the IC_{50} value of the fractions was compared with that of quercetin.

The IC_{50} value was defined as the concentration in (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%. Percentage of inhibition (I %) was calculated in the following formula:

Calculating percentage growth inhibition:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control-OD of Sample})}{\text{OD of Control}} \times 100$$

2.4 Statistical evaluation

- Half maximal Inhibitory concentration (IC_{50}) is the concentration of the substance required to inhibit a biological process such as an enzyme, cell, cell receptor or microorganism by half.
- IC_{50} value was calculated using Graph Prism software version 5.0 by non linear regression analysis of % inhibition recorded for different concentrations of test substances/standard.
- For compounds showing <50% inhibition, IC_{50} value is not calculated.
- The relative activity of the sample can be determined by comparing the IC_{50} value of sample with standard.

1. Higher the IC_{50} value, lower will be the relative activity in comparison to standard and vice-versa.

2.5 Total reducing power assay Fe (III) to Fe (II).

Principle

The Reducing power was determined by the method prescribed by Oyaizu [12] is based on the substances which have reduction potential, reacts with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700nm, it is possible to

Potassium ferricyanade + Ferric chloride ⟹ Potassium ferrocyanide + ferrous chloride

determine the concentration of Fe^{3+} ion. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants. The measured reducing capacity provides a very useful 'total' antioxidant concentration, without measurement and summation of the concentration of all antioxidants involved.

The sample in 400µl of methanol at various concentrations were mixed with - Potassium ferricyanide $K_3[Fe(CN)_6]$ (1 ml, 1%) and Potassium dihydrogen phosphate and Dipotassium hydrogen phosphate $NaH_2PO_4Na_2HPO_4$ buffer (1 ml, 0.2 $molL^{-1}$, pH 6.6). Following incubated at 50ºC for 30 minutes trichloroacetic acid (1 ml, 10%) was added and the mixtures were centrifuged at 3000 rpm for 10 min. Finally, the supernatant fractions (1 ml) were mixed with distilled water (1 ml) and $FeCl_3$ (170µl, 0.1%) and the mixture was incubated at 50 ºC for 30min. The absorbance of the resulting solution was measured at 700 nm.

Reducing power was expressed in relation to the reducing power of ascorbic acid, as a positive control Ascorbate Equivalent Antioxidant Capacity, AEAC (mg AAE/g DE) (A = 0.0059x c(Ascorbic acid) + 0.121, R2 = 0.9995).

Increased absorbance of the reaction mixture indicates stronger reducing power.

2.6 Superoxide dismutase activity

Crude extract was prepared by homogenization of frozen plant sample in buffer medium. 10 g of the sample was homogenized in 50 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM ascorbic acid and 0.5% (w/v) polyvinylpyrrolidone for 5 min at 4°C. The homogenate was filtered through three layers of cheesecloth and then the filtrate was centrifuged at 5,000 x g for 15 min, and the supernatant was collected.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition in photoreduction of nitrobluetetrazolium (NBT) by SOD enzyme. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µMNBT, 10 µM riboflavin and 100 µl of crude extract in a final volume of 3.0 ml. A control reaction was performed without crude extract. The SOD reaction was carried out by exposing the reaction mixture to white light for 15 min at room temperature. After 15 min incubation, absorbance was recorded at 560 nm using a spectrophotometer. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

3. Results and Discussion

3.1 Antimicrobial activity

Madhuca longifolia belongs to the family Sapotaceae and many of the trees belonging to genera *Madhuca* show good antimicrobial activity. The antimicrobial activity of *Madhuca* crude bark extract was tested against different microorganisms where most of the bacteria were found to be sensitive towards the extract. But in the present work antimicrobial activity is focused on active principle hexacosylidenecyclohexane isolated from bark of *M. longifolia.*

Active principle showed a zone of inhibition against *P. aeruginosa* was 16.16mm and *S. aureus* was 15.10 mm followed by *E. coli* was 8.46mm.Standard drug Ciprofloxacin showed 28.033mm for *P. aeruginosa*, 33.100mm for *S. aureus* followed by 12.166mm for *E. coli.*

Active principle tested against fungus *C. albicans* showed zone of inhibition of 11.13mm with respect to standard drug Itraconazole, which showed 10.26mm.Results obtained were tabulated in the following Table and Figures.

Table 1: Antimicrobial activity of active principle against microorganisms

Organisms	N	Mean in mm	Std. Deviation	Std. Error
P. aeruginosa	3	16.16	2.25	1.30
E. coli	3	8.46	1.45	0.84
S. aureus	3	15.1	2.35	1.35
C. albicans	3	11.13	1.60	0.92
Total	12	12.71	3.63	1.04

Organisms	N	Subset for alpha = 0.05	
		1	2
E. coli	3	8.46	
C. albicans	3	11.13	11.13
S. aureus	3		15.1
P. aeruginosa	3		16.16
Sig.		0.39	0.054

Means for groups in homogeneous subsets are displayed (Harmonic Mean Sample Size = 3.000).

3.1.1 Statistical analysis

Data were expressed as the mean ± SEM. The data were analyzed using one way analysis of variance (ANOVA) followed by T-test.

Fig 1: Inhibitory activity of test sample against *P. aeruginosa.* S-standard (Ciprofloxacin); Control (methanol); 1- Active principle (2000µg/well)

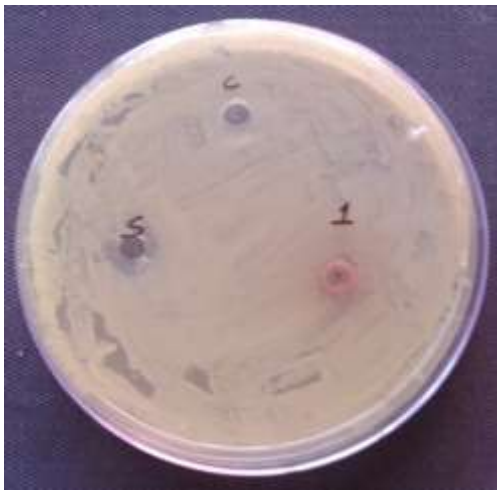

Fig 2: Inhibitory activity of test sample against *E.coli*. S-standard (Ciprofloxacin); Control (methanol); 1- Active principle (2000µg/well)

Fig 3: Inhibitory activity of test sample against *S. aureus.* S-standard

(Ciprofloxacin); Control (methanol); 1- Active principle (2000µg/well)

Fig 4: Inhibitory activity of test sample against *C. albicans.* S-standard (Itraconazole); Control (methanol); 1- Active principle (2000µg/well).

3.1.2 Determination of Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism after overnight incubation. MIC values obtained for active principle against *E. coli* 1000, *P. aeruginosa* and *S. aureus* was 250 and *C. albicans* was 500.MIC of Standard drug Ciprofloaxcin were 0.5 for *E. coli* and *S. aureus* and 0.25 for *P. aeruginosa*. MIC of standard fungal drug Itraconazole was 62.5ug/ml. Experimental values and test parameters were tabulated in the Table 6.

Table 2: Determination of Minimum inhibitory concentration (MIC).

No.	Sample Code	MIC (µg/mL)			
		E. coli	***P. aeruginosa***	***S. aureus***	***C. albicans***
1	Ciprofloxacin	0.5	0.25	0.5	-
2	Itraconazole	-	-	-	62.5
3	Active principle	1000	250	250	500

3.2 DPPH assay

The DPPH free radical scavenging assay was very useful in evaluating the antioxidant activity. This assay measures the free radical scavenging ability of active principle. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour typical for free DPPH radical decays and the absorbance change is measured at 510 nm. The antiradical activity of the plant extract was examined based on the scavenging effect of the stable DPPH free radical activity [13]. Reference standard compound being used was quercetin. The IC 50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity [16].

The active principle hexacosylidenecyclohexane showed dose dependent DPPH radical scavenging activity with IC_{50} value of 3.614µg/ml when compared to standard Quercetin withIC_{50} value of 5.787 µg/ml. The absorbance at 510nm by semiauto-analyzer were 0.97, 0.92, 0.88, 0.70, 0.55, 0.40 and 0.18 for standard quercetin and active principle showed 0.564, 0.426, 0.282, 0.177, 0.111 and 0.063. IC_{50} value obtained was 3.164 µg/ml and 5.787 µg/ml for quercetin indicated that active principle at higher concentration captured more free radicals formed by DPPH resulting into decrease in absorbance and increase in IC 50 value. Control reaction was carried out without the test sample. Values obtained and inhibition curve were tabulated in Table 3 and Figure 5 and 6.

Table 3: DPPH assay of standard compound-Quercetin

Standard	Concentration (µg/ml)	N	Mean Absorbance	Standard Deviation	Standard Error	% Inhibition	IC_{50}(µg/ml)
Control	0	3	0.93	0.040	0.023	0	3.645
Quercetin	0.35	3	0.88	0.040	0.023	5.24	
	0.61	3	0.84	0.035	0.020	9.3	
	1.25	3	0.65	0.040	0.023	27.41	
	2.5	3	0.52	0.036	0.020	42.97	
	5	3	0.36	0.035	0.020	58.6	
	10	3	0.16	0.032	0.018	81.43	
Total		21	0.62	0.276	0.060	224.95	

Table 4: DPPH assay of Active principle

Standard	Concentration (µg/ml)	N	Mean Absorbance	Standard Deviation	Standard Error	% Inhibition	IC_{50} (µg/ml)
Control	0	3	.933	.0404	.0233	0	
Active principle	3.125	3	.561	.0025	.0014	41.70	
	6.25	3	.423	.0030	.0017	55.97	
	12.5	3	.280	.0015	.0008	70.85	5.787
	25	3	.174	.0030	.0017	81.70	
	50	3	.109	.0020	.0011	88.53	
	100	3	.062	.0030	.0017	93.49	
Total		21	.363	.2916	.0636		

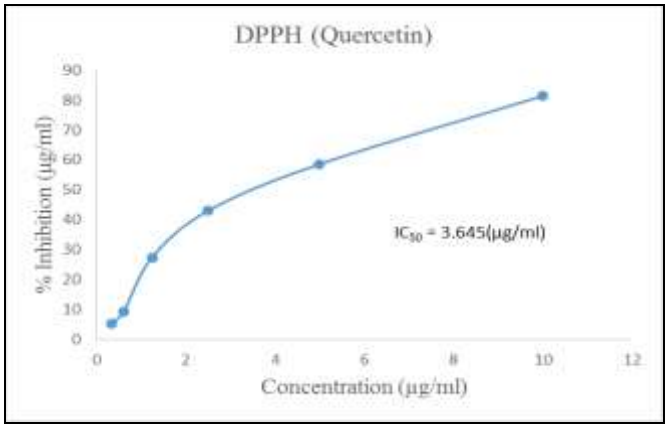

Fig 5: % inhibition curve of DPPH (Quercetin)

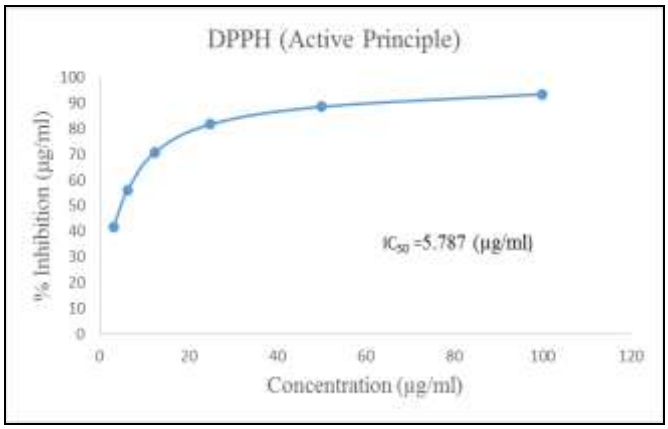

Fig 6: % inhibition of DPPH (Active Principle).

3.3 Total reducing power assay

Results are obtained as absorbance increases at 700nm and can be measured to test the amount of iron reduced and can be expressed as µmolar Fe^{2+} equivalents or relative to an antioxidant reference standard vitamin C. As shown in Figure 4.7 and 4.8, higher absorbance value indicates a stronger reducing power of the sample. Sample showed concentration-dependent reducing power. However, its reducing power at highest concentration at 1mg/ml is 59.02mg vitamin C/g. Overall, the results obtained suggested that the tested sample have good antioxidant activity.

Table 5: TRPA of Standard vitamin C

Compound Name	Conc. µg/ml	Abs
Control	0	0.12
Vitamin C	50	0.426
	100	0.695
	200	1.302

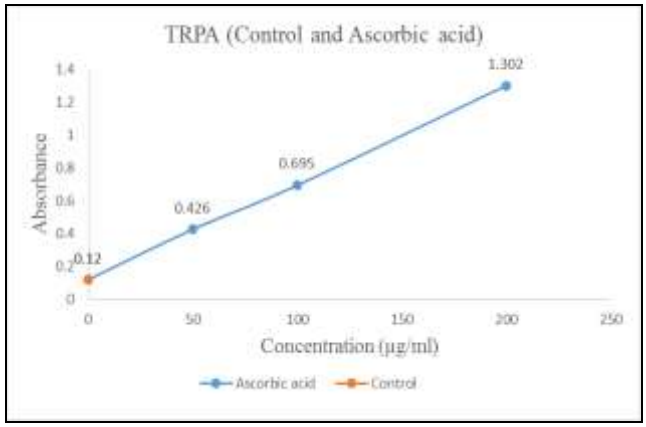

Fig 7: TRPA for both Control and Ascorbic acid

Table 6: TRPA

Compound Name	Conc. µg/ml	Abs	Std deviation	Conc.µg Vit-C
Active principle	100	0.2297	0.0424	21.73
	200	0.2230	0.7312 0.0422	20.40
	400	0.2367	0.0853 0.0395	23.13

3.4 Statistical analysis

Each test was performed in triplicate and the results were expressed as mean±standard deviation. The IC_{50} was determined with the multiple regression analysis. The software SPSS version 10.1 for Windows was used for statistical analysis.

3.5 Superoxide dismutase activity

One unit of SOD is defined as the amount of enzyme, which gave 50% inhibition of NBT reduction in one minute under standard assay conditions. SOD activity was then measured by the degree of inhibition of the reaction unit of enzyme providing 50% inhibition of NBT reduction. Results are expressed as U/ml. SOD enzyme isolated from the bark of *M. longifolia* was found to have specific activity of 1.33 units/ml.

Table 4.3: SOD activity of bark extract

Test solution	Absorbance	% inhibition	SOD units/100ml	SOD units/1ml
Control	0.90	0.00	-	-
Bark extract	0.84	6.67	0.13	1.33

The review of various ethno medical and traditional uses of bark and leaves of *M. longifolia* was very much helpful for exploration of medicinal properties [15]. Previous studies

shown that methanol extract of flowers, leaves, stem and stem bark of *M. longifolia* had been reported to have antimicrobial activity [16]. Present work also showed good antimicrobial activity with active principle. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants. From the results it can be stated that the *M. longifolia* possess hydrogen donating capabilities and acts as an antioxidant. The total antioxidant activity can be measured by the ferric reducing antioxidant power assay (FRAP). The flavonoids and phenolic acids are present in the medicinal plant exhibit strong antioxidant activity which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures, the absorbance increases the antioxidant activity increases. In the present study, active principle showed good reducing ability. The super oxide dismutase in bark extract showed the capability of conversion of superoxide radical into hydrogen peroxide and molecular oxygen. Active principle showed positive results for both antimicrobial and antioxidant activities. The emergence of multi-drug resistant strains resulted in the discovery of novel and efficient antimicrobial agents of plant origin [17]. Plant species that have been tested for antioxidant activities which have also shown to possess antidiabetic properties [18].

4. Conclusion

Active principle hexacosylidenecyclohexane extracted from the bark of *Madhuca longifolia* L. have potential antimicrobial and antioxidant activity. Hence, the obtained active principle can be used to replace synthetic antimicrobial and antioxidant agents. The herbal based phytomedicine have large therapeutic applications since they can have fewer side effects when compared with synthetic antimicrobials and antioxidants.

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