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M Gopu
Department of Chemistry, Madras Christian College (Autonomous), Tambaram, Chennai, Tamil Nadu, India

M Gurulakshmi
Department of Chemistry, Madras Christian College (Autonomous), Tambaram, Chennai, Tamil Nadu, India

D Estherlydia
Food Chemistry and Food Processing, Department of Chemistry, Loyola College (Autonomous), Chennai, Tamil Nadu, India

Correspondence
M Gurulakshmi
Department of Chemistry, Madras Christian College (Autonomous), Tambaram, Chennai, Tamil Nadu, India

Screening of Nanoparticles blended *Couroupita guianensis* for its antibacterial activity against gram positive and gram negative bacterial pathogens

M Gopu, M Gurulakshmi and D Estherlydia

Abstract
Phytochemical analysis, fluorescent character and antibacterial activity of *Couroupita guianensis* flower extract (petals and stamens) were investigated. The crude extract showed the presence of alkaloids, carbohydrate Flavonoid, Terpenoid and Gum and Mucilage. The solvents used for the extraction of flower were water and methanol. Further, the antibacterial activity of titania nanoparticles were compared with titania nanoparticles blended *Couroupita guianensis* extract. The in-vitro antibacterial activity was tested by well diffusion method against four bacterial pathogens: Escherichia coli, Staphylococcus aureus, Staphylococcus mutans and Klebsiella pneumonia. The antibacterial results showed methanol extracts of *Couroupita guianensis* for both petal and stamen showed maximum zone of inhibition – MIC against *S. aureus* and *E. coli*, respectively. The methanol extract of *Couroupita guianensis* blended with TiO_2 showed an enhanced phytochemical extract of petal and stamen. MIC was tested at various concentrations for both petals and stamen extracts blended with TiO_2. The methanol extract of *Couroupita guianensis* blended with TiO_2 with crude petal and stamen showed higher MIC% against *E. coli*. Thus based on the inhibitory concentration, the two extract blended with nanoparticles has potent antibacterial activity and can be used for treating drugs resistant human pathogens.

Keywords: *Couroupita guianensis*, extract, Titania, nanoparticles, blended

Introduction
Medicinal plants represent a rich source of antibiotic, antifungal, antiseptic and analgesic qualities (Nelson EK, 1937) [1]. Traditional medicinal usage is a common practice in developed and developing countries at a primary healthcare level. The discovery of new antimicrobial compounds from microorganism, animals and plants has been reported by many researchers. *Couroupita guianensis* belongs to the family *L. Ecythidaceae*, commonly known as cannon ball tree, and *"Nagalingam Pushpam in Tamil"* is found throughout India in plains. It bears large showy flowers almost through the year. The flowers are orange, scarlet and pink in colour. The leaves of this plant have been used in the treatment of skin disease. The flower and bark of *Couroupita guianensis* is used to treat hypertension, tumours, pain and inflammatory process (Regina and Umarajan, 2012; Morankar *et al*, 2013 and Tahira *et al*, 2017) [2, 3, 4]. The flowers are used to cure cold, intestinal gas formation in stomach and also for treating diarrhoea. The fragrance of flowers is used for curing asthma. The phytochemical, antioxidant and antimicrobial analysis of methanolic extract from the petals and stamens of the flowers is the rich source of saponins, quercetin and tryplanthrin (Sumathi, S and Anuradha, R, 2017) [5].
Metal oxide nanoparticles exhibit excellent biocide action against Gram positive and Gram negative bacteria. Photocatalytically active semiconductor absorbs light and photo-stimulate redox reactions on its surface producing reactive oxygen species. Titanium dioxide is one of the most promising semiconductor photcatalysts, known for its chemical stability and optical competency. (G. Li and X.S. Zhao, 2006) [6]. It has been used extensively for killing different groups of microorganisms including bacteria, fungi and viruses, because it has high photoreactivity, broad-spectrum antibiosis and chemical stability (Nadtochenko *et al*, 2006; Rincón, A.G. and Pulgarin, C., 2004, Yu, J. C. *et al*., 2003, Hur, J.S. andKoh, Y., 2002, Maness *et al*, 1999, and Chen, X. and Mao, S. S., 2007) [7-12]. Even though, there are numerous reports on the antibacterial activity of the nanomaterials, in particular employing semiconductors like TiO_2 and ZnO, the researchers were trying to improve the antibacterial activity by modifying these materials (Chae Sujin, 2017; Sze-Mun Lam, 2017; Gergely F. Samua, 2017 and Kebadiretse Lefatshea, 2017) [13-16].
The structural difference between the gram positive and gram negative bacteria account for the ability of an antimicrobial agent.

Some agents are active on both and are often referred to as broad spectrum agents. With this in view, the plant extract is tested for searching a potential source for new type of antibiotics for treating bacterial diseases. The folk-lore medicinal facts make the present work to investigate the antibacterial activity of $TiO_2$and ZnOblended with the plant extract. Hence, an attempt has been made to study the antibacterial activity of these nanoparticles and nanoparticles blended *Couroupita guianensis* extract against gram positive and gram negative bacterial pathogens.

Materials and Methods

Collection and identification of plant materials

The medicinal plant *Couroupita guianensis* was collected from Madras Christian College, Tambaram, Chennai, India during the month of mid May. The taxonomic position of the plants were identified and authenticatedaccording to the flora of madras presidency (Gamble JS, 1935) [17] and the flora of Tamil Nadu Carnatic (Mathew KM, 1981-1984) [18]. Petals and stamens of the flower were collected in a large quantity, washed thrice with distilled water and dried in an oven at 45°C for 24 h for extraction purposes.

Extraction of plant materials

Petals and stamen were taken separately for extraction procedure and grounded in a mortar and pestle under aseptic condition. The solvent extraction was done by soaking 20 g of dried petal and stamen powder, taken in two separate beakers, with each 100 mL methanol. The beaker was covered tightly using aluminium foil to avoid evaporation. It is stored for 24 h at room temperature. The extract was then filtered using Whatmann filter paper. The collected filtrate was kept in a water bath at 65°C for evaporation of solvent. The extracts were concentrated to dryness and used for further phytochemical, fluorescent and antimicrobial study.

Preparation of the nanoparticles

Titanium dioxide and Zinc dioxide were commercially obtained and both the nanoparticles were suspended in Ethanol in the ratio 100 mg /mL (w/v).The prepared nanoparticles blended with the flower extract and separately were screened for its antibacterial activity respectively.

Preparation of culture broth

Bacterial samples respectively were inoculated in the sterile nutrient broth prepared obtained from Himedia Mumbai LOT no. 0000132645 and incubated for the overnight at 37 ^oC. The bacteria employed for the study include, gram positive: *S.aureus* NCIM 2079, *S.mutants* NCIM 2611 and gram negative: *E.coli* NCIM 2065, *K.pneumoniae* NCIM 2957.

Qualitative phytochemical studies

Qualitative phytochemical analysis were done by using the procedures of Criddle W.H and Ellis G.P. Terpenoids, Flavonoids, Alkaloids, Carbohydrate, Proteins, Phenols and Tannins were qualitatively analyzed. (Criddle W.H and Ellis G.P., 1990) [19]. Fluorescent assay were also performed for the extracts in different solvents.

Assay of antibacterial activity

Testing the antibacterial activity – Mueller Hinton-Method:

Mueller Hinton agar (MHA) was used to check the antibacterial activity by well diffusion method. Autoclaved medium was poured into petri plates in the laminar air flow hood. On cooling the medium within petri plates the microorganism from 24 h old broth were spread then wells were made on the petri plates with the help of borer of diameter 6-8 mm. Three wells were made on entire surface of medium; one is for control to the test organism and remaining for different concentration of the sample. Now increasing volume (µL) of sample preparations was poured in the first well and subsequently for the other well. These plates were incubated for 24 - 48 h and the diameter of zone of inhibition (ZOI) was measured with the help of scale. The freshly prepared inoculum was swabbed all over the surface of the Mueller Hinton agar (MHA) plate using sterile cotton swab. Four wells of 6mm diameter were bored in the medium with the help of sterile cork-borer having 6mm diameter and were labeled properly and fifty micro-liters of the working suspension/solution of different medicinal plant extract and same volume of extraction solvent for control was filled in the wells with the help of micropipette. Plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h and measured using scale and mean were recorded after incubation. The plates were observed for zone of inhibition.

Well diffusion method

The Kirby-Bauer and Stokes' methods are usually employed for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the National Clinical Control Laboratory Service (NCCLS). The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here. Interpretative criteria of NCCLS are developed based on international collaborative studies and well correlated with MIC's and the results have corroborated with clinical data. Based on study results NCCLS interpretative criteria are revised frequently. NCCLS is approved by FDA-USA and recommended by WHO.

Calculation

$$\%\ \text{ofGrowthInhibition} = \frac{(DC - DT)}{DT} \times 100$$

Where, DC = colony diameter of control and DT = colony diameter of treated plates

Reading plates and interpreting results

1. After 16 to 18 h of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the well. Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light. If blood was added to the agar base (as with streptococci), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed.
2. The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited

growth, is ignored. However, weltered colonies growing within a clear zone of inhibition should be sub-cultured, re-identified, and retested.

3. The zone of inhibition was observed and the inhibitory zone was measured using Zone Inhibitory scale (HIMEDIA) and the values noted in mm.

Table 1: Preparation of the samples.

Bacteria	Sample taken for antibacterial activity							
	Petal	**Stamen**	**TiO_2**	**ZnO_2**	**Stamen-TiO_2**	**Stamen-ZnO_2**	**Petal-TiO_2**	**Petal-ZnO_2**
S. aureus	Each sample was assayed with 4 different concentrations and each antibacterial agent was screened for its antibacterial activity with 2 Grampositive and 2 Gramnegative bacteria. All the interpretations were recorded respective of its MICcorresponding with its inhibition zone and bacteria used.							
S. mutans								
E. coli								
K. pneumoniae								

Fig 1: *Couroupita guianensis*-Flower and Dried powder - stamen and petals.

Results and Discussion

Table 2: Valuating the moisture content.

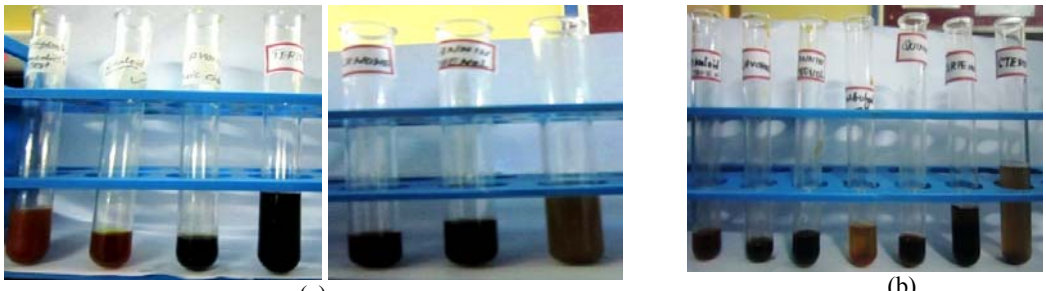
Sample	Initial weight (g)	Dried Weight(g)	Moisture content (%)
Petal	610	72.9	88.04
Stamen	560	66	88.21

Extract preparation

Fig 2: Crude petals and stamens along with nanoparticles.

Table 3: Quantity of extract.

Sample	Quantity for extract(g)	Methanol (mL)	Duration (h)	Empty weight(g)	Crude weight(g)	Total extract weight(g)	Yield %	Concentration for assay (mg/ml)
Petal	20	100	24	86.13	88.42	2.29	11.45	0.152
Stamen	20	100	24	113.07	117.83	4.76	23.8	0.317

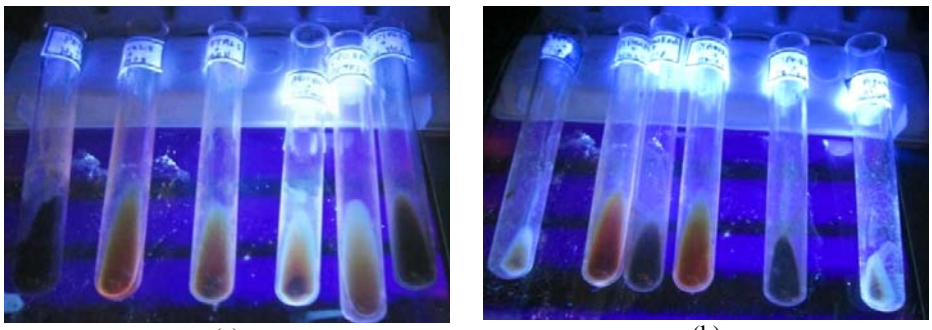


(a) (b)

Fig 3: Phytochemical Screening – (a) Petal and (b) Stamen

Table 4: interpretation of phytochemical- petal and stamen

Test	petal	Stamen
Extract+Distilled water	+	+
extract+1N HCl	+	+
extract+1N NaOH	+	+
extract+50% HNO_3	+	+
extract +H_2SO_4	+	+
Extract+ Methanol	+	+

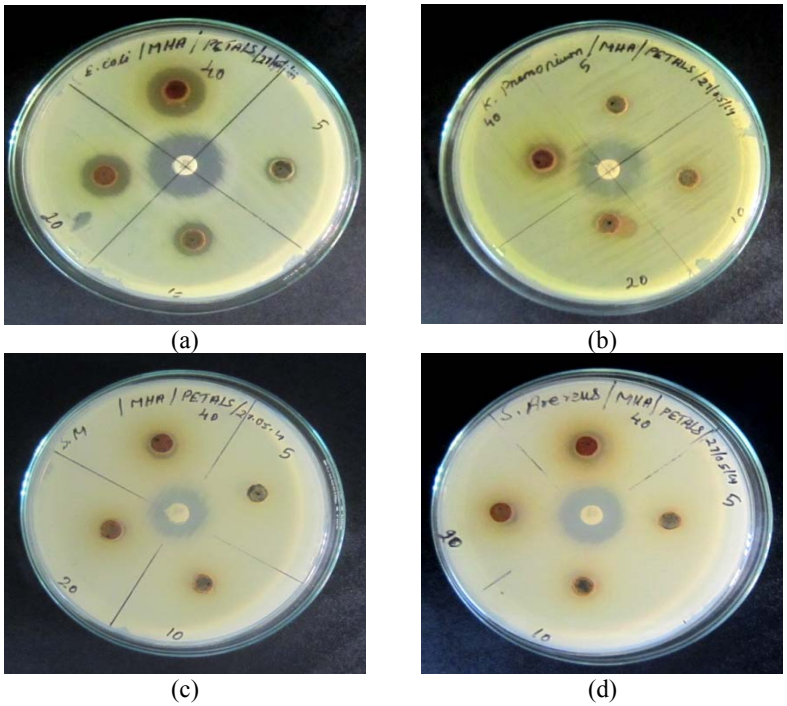


(a) (b)

Fig 4: Fluorescence test (a) petals and (b) stamens

Table 5: Interpretation of fluorescent assay.

Test	Petal	Stamen
Protein	-	-
Carbohydrate	+	+
Alkaloids	+	+
Flavonoid	+	+
Terpenoid	+	+
Tannin	-	+
Phenol	-	+
Gum and Mucilage	+	+



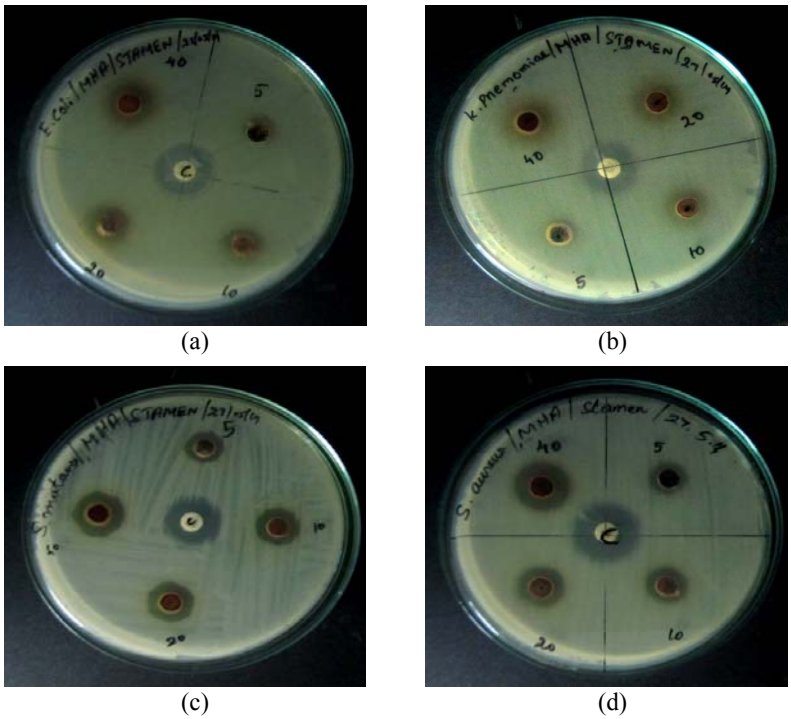
(a) (b)

(c) (d)

Fig 5: Antibacterial activity - crude petals
(a) *E. coli, (b) K. pneumoniae* (c) *S. mutans, (d) S. aureus*

Table 6: Inhibition Zone –MIC-% - Petals

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**5**	**10**	**20**	**40**		
Petals	*E. coli*	17	10	11	13	16	12.5	36.00
	S. aureus	19	10	12	14	16	13	46.15
	S. mutans	18	11	13	14	16	13.5	33.33
	K. pnemoniae	17	No zone	No zone	No zone	No zone	0	0.00

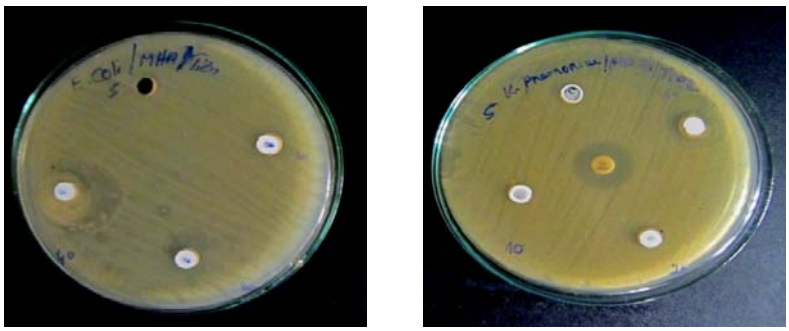


(a) (b) (c) (d)

Fig 6: Antibacterial activity - crude stamen
(a) *E. coli, (b) K. pneumoniae*(c) *S. mutans, (d) S. aureus*

Table 7: Inhibition Zone –MIC-% - Stamen

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**5**	**10**	**20**	**40**		
Stamens	*E. coli*	25	10	12	14	17	13.25	88.68
	S. aureus	21	10	10	15	18	13.25	58.49
	S. mutans	19	10	14	19	22	16.25	16.92
	K. pneumoniae	24	21	22	26	26	23.75	1.05



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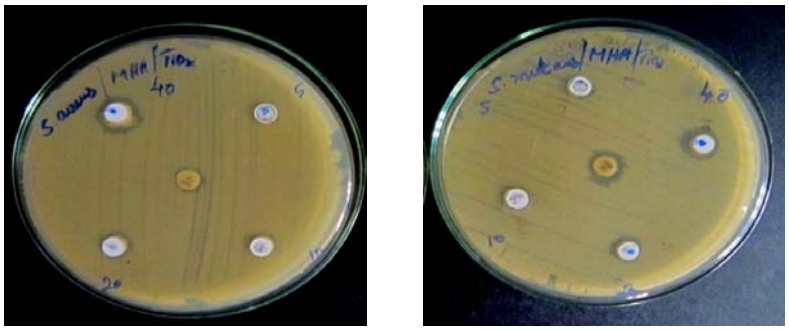
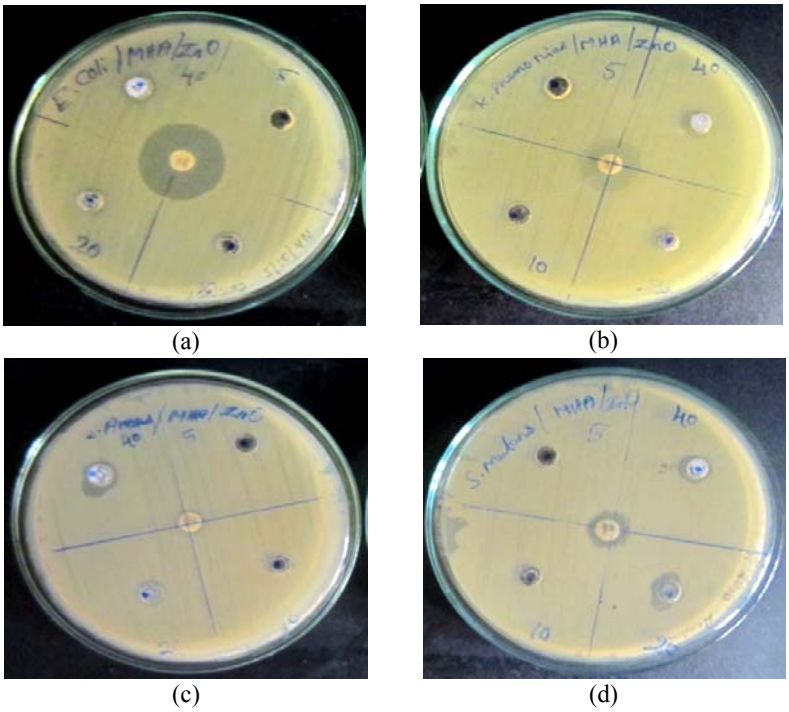


c) (d)

Fig 7: Antibacterial activity – titanium dioxide
(a) *E. coli, (b) K. pneumoniae*(c) *S. mutans, (d) S. aureus*

Table 8: Inhibition Zone –MIC-% - TiO_2

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**5**	**10**	**20**	**40**		
TiO_2	*E. coli*	0	0	0	0	0	0	0
	S. aureus	10	0	10	10	13	8.25	21.21
	S. mutans	12	10	10	10	12	10.5	14.29
	K. pnemoniae	16	10	10	10	20	12.5	28.00



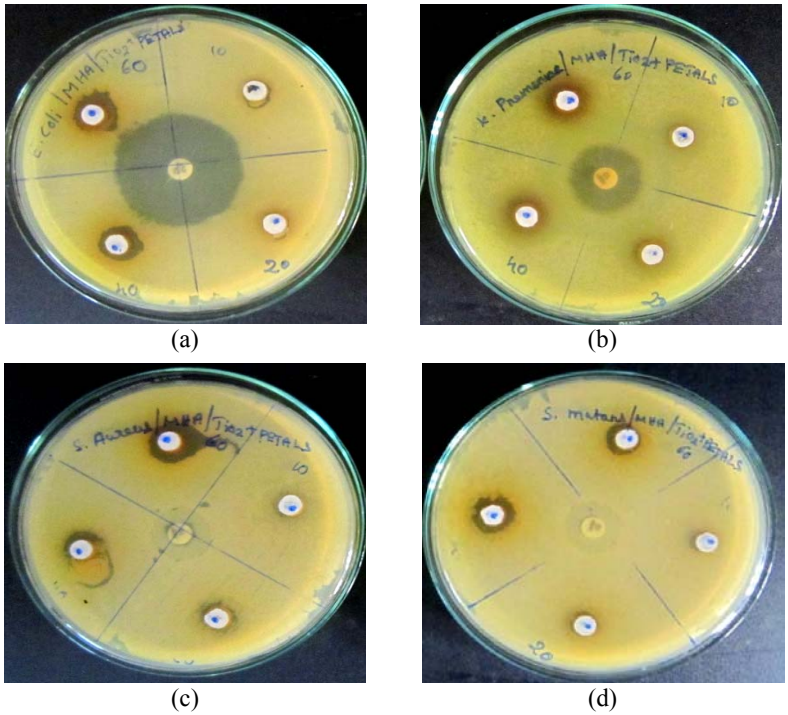
(a) (b)

(c) (d)

Fig 8: Antibacterial activity – zinc oxide
(a) *E. coli, (b) K. pneumoniae*(c) *S. mutans, (d) S. aureus*

Table 9: Inhibition Zone –MIC-% - ZnO

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**5**	**10**	**20**	**40**		
ZnO	*E. coli*	25	10	10	10	11	10.25	143.90
	S. aureus	8	0	0	0	10	2.5	220.00
	S. mutans	13	0	0	11	10	5.25	147.62
	K. pnemoniae	15	10	10	12	12	11	36.36

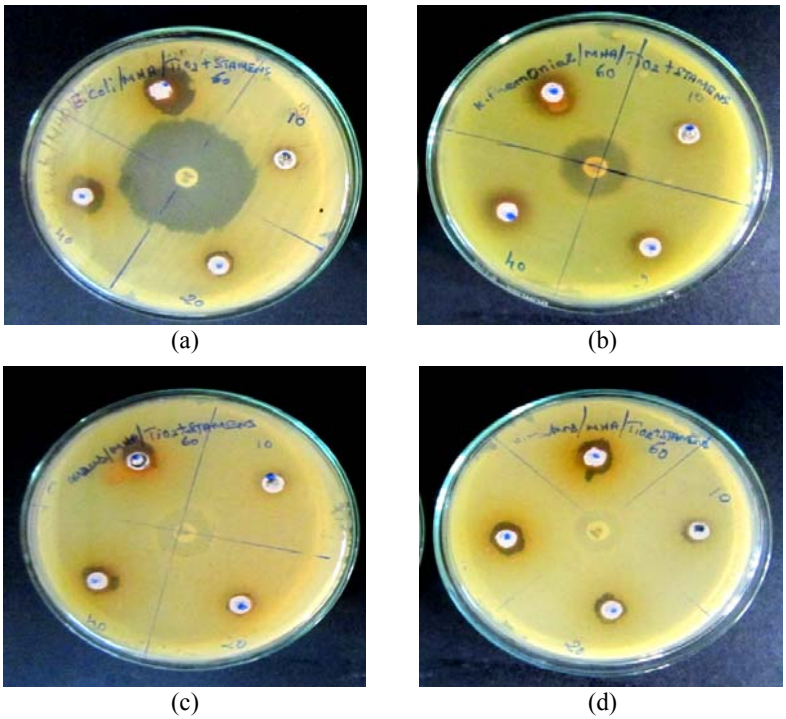


(a) (b) (c) (d)

Fig 9: Antibacterial activity – TiO_2 with crude petals
(a) *E. coli*, (b) *K. pneumoniae* (c) *S. mutans*, (d) *S. aureus*

Table 10: Inhibition Zone –MIC-% - TiO_2Petal.

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**5**	**10**	**20**	**40**		
ZnO	*E. coli*	34	7	7	12	14	10	240.00
	S. aureus	17	11	13	13	14	12.75	33.33
	S. mutans	16	10	12	13	11	11.5	39.13
	K. pnemoniae	21	16	17	20	20	18.25	15.07

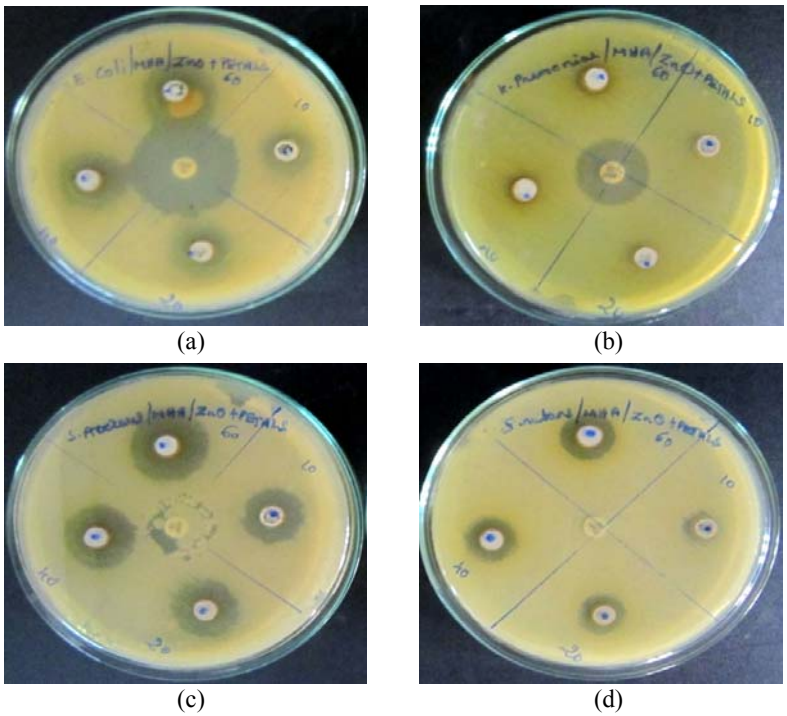


(a) (b) (c) (d)

Fig 10: Antibacterial activity –$TiO_2$with crude stamens
(a) *E. coli*, (b) *K. pneumoniae* (c) *S. mutans*, (d) *S. aureus*

Table 11: Inhibition Zone –MIC-% - TiO_2 with stamen

Zone measurement (mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**10**	**20**	**40**	**50**		
TiO2 with crude stamens	*E. coli*	38	8	10	10	12	10	280.00
	S. aureus	16	0	8	12	12	8	100.00
	S. mutans	14	8	10	12	13	10.75	30.23
	K. pnemoniae	19	10	12	14	16	13	46.15

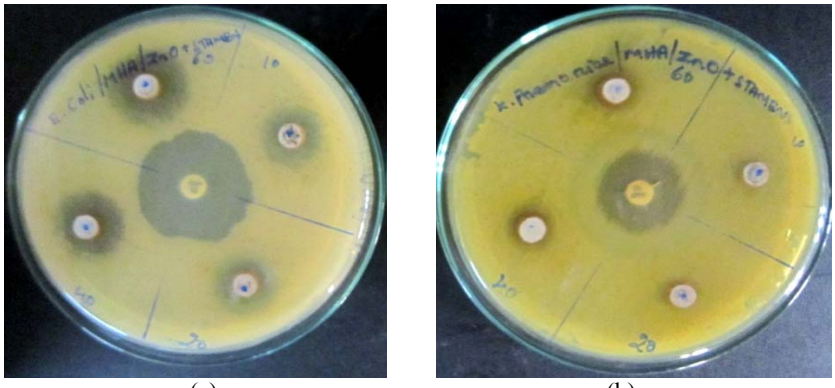


(a) (b) (c) (d)

Fig 11: Antibacterial activity – ZnO with crude petals
(a) *E. coli, (b) K. pneumoniae*(c) *S. mutans, (d) S. aureus*

Table 12: Inhibition Zone –MIC-% - ZnO with Petal

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**10**	**20**	**40**	**50**		
ZnO with crude petals	*E. coli*	31	14	20	21	22	19.25	61.04
	S. aureus	20	17	18	20	22	19.25	3.90
	S. mutans	16	13	14	16	17	15	6.67
	K. pnemoniae	21	0	0	8	10	4.5	366.67



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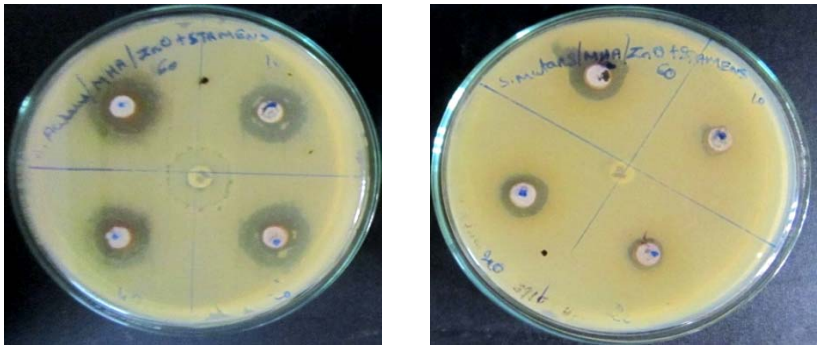
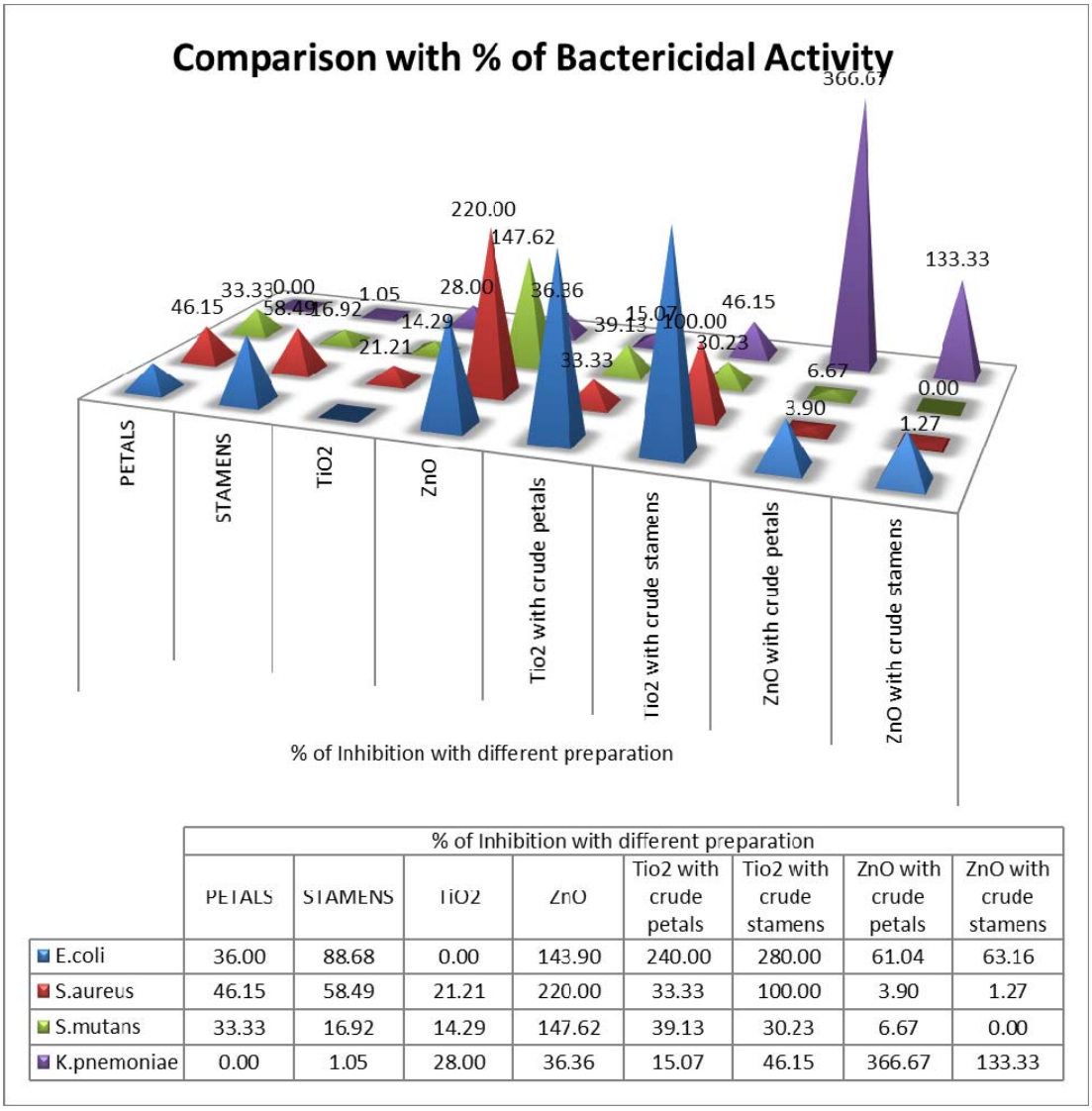


(c) (d)

Fig 12: Antibacterial activity – ZnO with stamens
(a) *E. coli, (b) K. pneumoniae*(c) *S. mutans, (d) S. aureus*

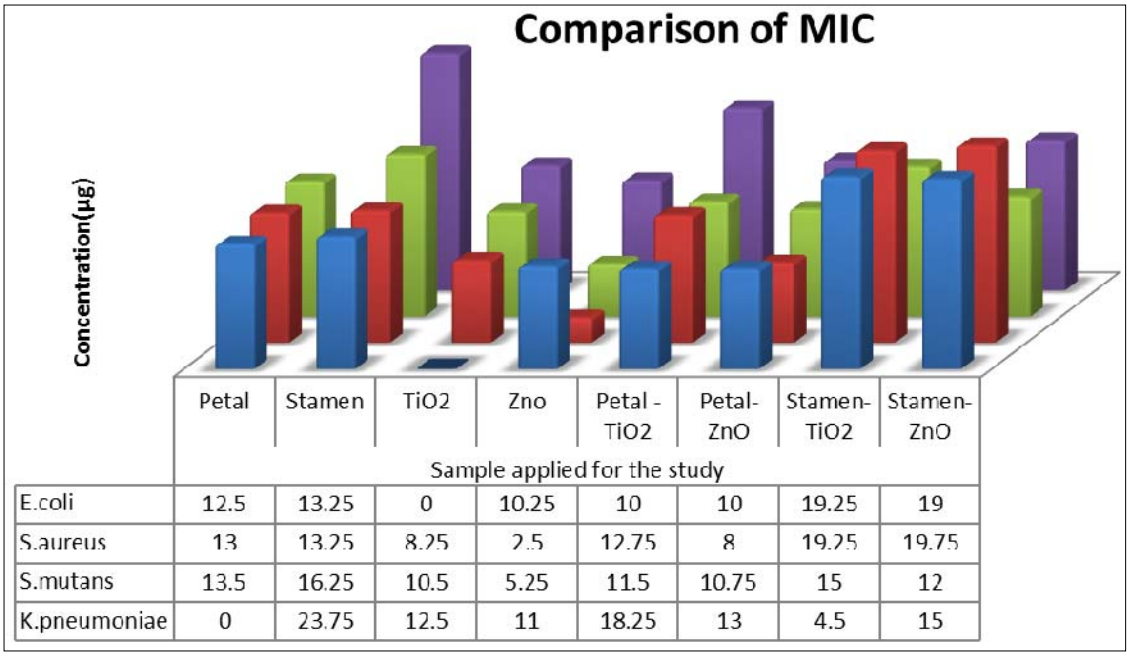
Table 13: Inhibition Zone –MIC-% - ZnO with stamen

Zone measurement(mm)							Average of inhibition	% of inhibition
	Bacteria	**Control**	**10**	**20**	**40**	**50**		
ZnO with crude stamens	*E. coli*	31	16	17	20	23	19	63.16
	S. aureus	20	18	19	20	22	19.75	1.27
	S. mutans	0	8	11	14	15	12	-100.00
	K. pnemoniae	35	15	14	15	16	15	133.33



	% of Inhibition with different preparation							
	PETALS	STAMENS	TiO2	ZnO	Tio2 with crude petals	Tio2 with crude stamens	ZnO with crude petals	ZnO with crude stamens
E.coli	36.00	88.68	0.00	143.90	240.00	280.00	61.04	63.16
S.aureus	46.15	58.49	21.21	220.00	33.33	100.00	3.90	1.27
S.mutans	33.33	16.92	14.29	147.62	39.13	30.23	6.67	0.00
K.pnemoniae	0.00	1.05	28.00	36.36	15.07	46.15	366.67	133.33

Fig 13: Graphical representation of Inhibition %



	Petal	Stamen	TiO2	Zno	Petal - TiO2	Petal-ZnO	Stamen-TiO2	Stamen-ZnO
	Sample applied for the study							
E.coli	12.5	13.25	0	10.25	10	10	19.25	19
S.aureus	13	13.25	8.25	2.5	12.75	8	19.25	19.75
S.mutans	13.5	16.25	10.5	5.25	11.5	10.75	15	12
K.pneumoniae	0	23.75	12.5	11	18.25	13	4.5	15

Fig 14: Graphical representation of MIC

Discussion

The objective of the research work put forth the exploitation of natural resources and a few waste for medicinal application. By preceding the study the usage of phytochemical present in food sources a seed, flower and crust of a vegetable been applied for the extraction of the plant phytochemical and its relative property over the bacteria to kill them. This application employs a simple solvent extraction procedure to retain the total phytoconstituents and analyzed for the presence of different compounds which includes phenols, alkaloids, terpenoids, flavanoids etc. These components play a role in inhibiting the growth of bacteria, this phenomenon said to be anti-bactericidal activity.

The bacteria employed for the study include,

a. *S.aureus* NCIM 2079
b. *S.mutants* NCIM 2611

} Gram positive

c. *E. coli* NCIM 2065
d. *K.pneumoniae* NCIM 2957

} Gram negative

The possibility of bactericidal activity was established with the phytocompounds analysis and the output reveals that the phytoconstituents extract of *C. guianensis* flower possess to be strong, good and average bactericidal activity respectively. The zone measurement dimension measured in mm was compared with concentration of phytocompounds required for the respective inhibiting property.

To enhance the activity by the application of nanoparticles – TiO_2 and ZnO was examined for its antibacterial activity which upon its concentration i.e. 100 mg/ml showed a positive effect of antibacterial activity with different concentration applied for the study which as an average provides a significant MIC.As a comparison ZnO showed a noteworthy antibacterial effect for all the bacteria applied whereas TiO_2 showed a positive inhibition only for *S. aureus, S. mutans, K. pneumoniae* rather *E. coli* was resistant towards TiO_2.

To make potent antibacterial effect both the extract and the Nanoparticels were blended in appropriate ratio and evaluated for its antibacterial property. By doing so significant outcome was noted in all the prepared combination of the Extract-NP. With the interpretation observed the extract –NP blended can be applied a potent drug delivery system in the bacterial infections which on further investigations show a complete profile of the study. As a laboratory trial it's proved a vital statistical data at which MIC the extract –NP will serve a good antibacterial agent.

With the utilization of these sources for the anti-bactericidal activity toward the bacteria where the water borne infection, urinary tract infection, respiratory infection etc., is prevalent can be cured by using these resources as therapeutic agent.

Summary and conclusion

It is concluded from this study that *C. guinensis* flower (petal and stamen) extracts possessed antibacterial activity. The antibacterial potential extracts of it may attribute to the presence of terpenoid, alkaloid, flavonoid, protein and carbohydrate etc. Based on the results, it was found methanalic extract gave more antibacterial activity towards the bacteria. The alkaloids present in the flower extract are expected to be the major reason for its antimicrobial potential against the clinical pathogens. Therefore it is necessary to exploit its maximum potential in the field of Medicinal and pharmaceutical sciences for novel and fruitful application and can be further related with other pharmacological activities.

Metal nanoparticles exert cytotoxicity depending on the charge at membrane surface. Gram positive cells are less prone to nanotoxic effects due to the presence of thicker peptidoglycan layer compared to Gram negative cells. Nanotoxicity may he attributed to electrostatic interaction between nanoparticles with membrane and their accumulation in cytoplasm. The inclusion of nanoparticles – TiO_2 and ZnO with the extract enhance the phytochemical extract of petal and stamen

Thus, the researchers to investigate the synergistic capacity of plants or other natural products, independent of the antimicrobial activity they have. Therefore the result of the present study seems to be promising and may enhance the

natural products uses, showing the potentiality of *C. guinensis*in the treatment of various infectious diseases caused by bacteria. Further studies on the chemical characteristics of extract and active components should be carried out for the plant and its antimicrobial property.

The possible activities of substances found in plant extracts on ribosome structure and bacterial enzymes inhibition appear to be related with synergism profile between plant extracts and the inhibitions of protein synthesis, however, the understanding of synergism mechanism is fundamental to development of pharmacological agents to treat diseases by various bacteria using medicinal plants.

Traditional indigenous medicine is limited to small tribal and geographical areas called "little traditions" are an excellent repository of knowledge about medicinal properties of botanical sources. Bioactive extracts should be standardized on the basis of phytochemical compounds. Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutically and industrially important compounds. It is imperative to initiate an urgent step for screening of plants for its secondary metabolites which provide as a novel drug.

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