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Antimicrobial studies of bioactive components from *P. americana* in comparison with TiO_2 nanoparticles

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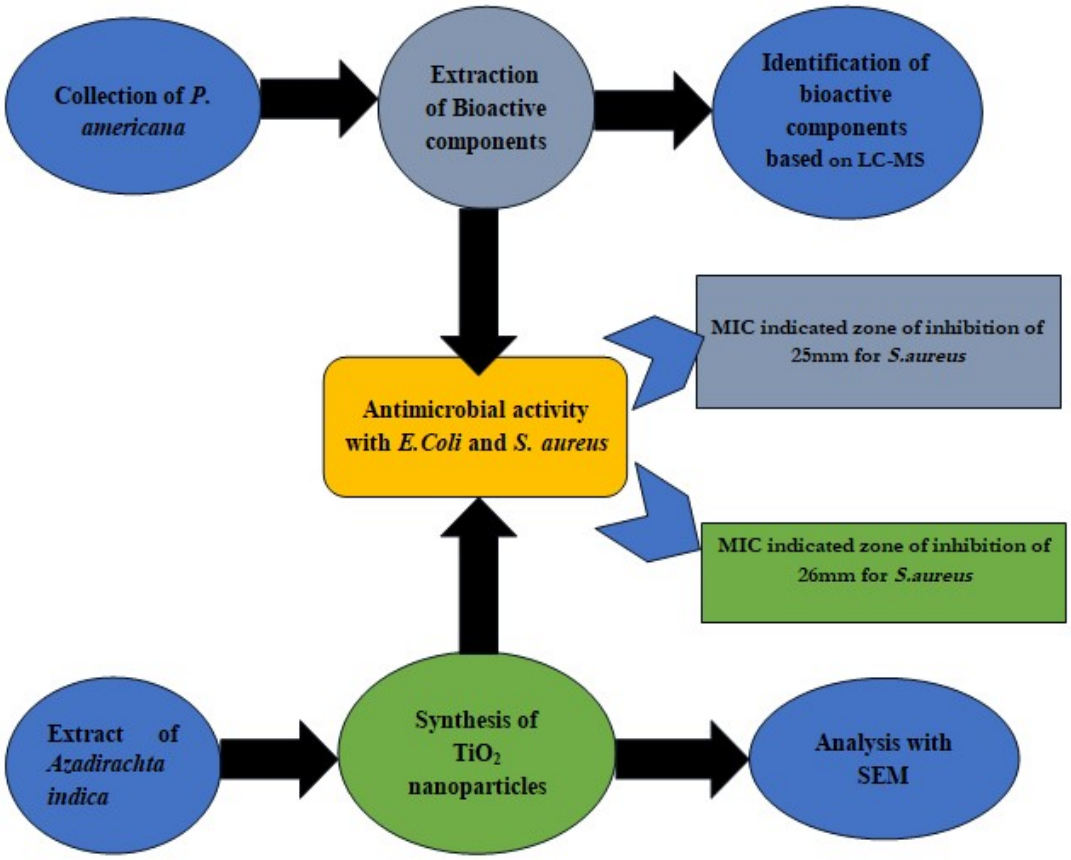
Graphical abstract



Abstract

Background: Antibiotic resistance emerged as a life-threatening concern for public health worldwide, posing challenges in the treatment of bacterial infections. The development of alternative strategies and preserving the effectiveness of existing antibiotics as improved efficacy in combating bacterial infection, however, continuous bacterial resistance to a present antibiotic emerged as a new threat. It demands new approach for developing novel antibiotic molecules. In the last few decades, nanotechnology has received great attention for the development of effective antimicrobial and antibiofilm. In the present work, cockroach gut microbiome is considered as an alternative approach to know its antibiotic activity along with titanium dioxide (TiO_2), which has the potential as antibacterial agents based on the photocatalytic properties, chemical firmness, and less cost as the insect based antimicrobial activity are emerging as new potential pharmaceuticals.

Methods: TiO2 nanoparticles from *Azadirachta indica* (Neem) leaves and the ethanol extracts of *Periplaneta americana* gut lysate, has been evaluated for its inhibitory activities against bacterial human pathogens; *Escherichia coli* and *Staphylococcus aureus*. Studies have indicated to be an appealing antimicrobial compound. It also has excellent antifungal and antimicrobial properties

Results: Studies indicated that the inhibition zones of TiO2 were 8mm and 12mm at minimum inhibitory concentration (MIC) of 10 µg/ml, for *Escherichia coli* and *Staphylococcus aureus.* Also, the ethanol extract of *Periplaneta americana* have shown the inhibitory zone of 1.5mm) at 0.9 µg/ml as MIC for *Staphylococcus aureus.* LC-MS indicted the presence of highly active biological compounds in the *Periplaneta americana* extract.

Conclusion: This study highlights the presence of bioactive compounds in the extract of *P. americana* lysate. Overall, the experimental work indicates the potential of biosynthesized titanium oxide and *P. americana* lysate extract as pharmaceutical agent against microbial infections.

Keywords: *P. americana*, titanium dioxide nanoparticles, bioactive extracts, *Staphylococcus aureus, Escherichia coli*, antibacterial, pharmaceutical agent

1 Introduction

Global public health is seriously threatened by the rise of infectious diseases, particularly in conjunction with the introduction of bacterial strains resistant to antibiotics. Nosocomial disease transmission is caused by the survival of these microorganisms on surfaces and in medical equipment. Antibiotics have been used for many years to treat infections that arise from hospital and community settings. Since humans have only been on earth for 10000 years, insects like cockroaches endured cataclysmic events and persisted for millions of years, demonstrating their capacity for environmental adaptation. These results confirm to seek for the possible antimicrobials in such species. Thus, research revealed that cockroaches has antibacterial properties. The rise of antimicrobial opposition pathogenic strains considered as one of the main issues all around the world. Of which wound contamination accounted as a typical hazardous worldwide medical issue bringing about 300,000 passing consistently. Deferred wound mending happens because of a few factors like age, persistent illnesses and disease with the pathogenic microorganisms. These variables increment the spread of contamination to the encompassing tissue and longer patients' hospitalization. Currently bacteria and other microbes are still developing strains resistant to the antimicrobial drugs now in use, new sources of antibiotics are urgently needed to stop this threat. The latest strategy against this problem is to use nanoparticles (NPs), which can create a useful nanostructure to convey the antimicrobial chemical and effectively target the bacterial community. Additionally, looking for new antibiotic compounds derived from uncommon natural resources could be helpful in the discovery of new medicines[1, 2].

Novel antibacterial agents are created as a result of developments in the field of nanobiotechnology. Given the distinct characteristics of nanoparticles, nano-sized

organic and inorganic particles such as zinc, copper, and iron metal oxides for biomedical research and antibacterial activity are being produced for eventual usage in medical practices. The antimicrobial activity of nanoparticles has been extensively investigated using human pathogenic microorganisms, including *Staphylococcus aureus* and *Escherichia coli (E. coli).* The size, stability, and concentration of nanoparticles in the growing media influence their bactericidal action. Certain interactions between the nanoparticles can prevent the growth of bacteria when they are growing in a media supplemented with the nanoparticles. Bacterial cells typically have a size in the micrometer range, and their outer cellular contents [1].

Metallic nanoparticles (NPs) have been concentrated as profoundly encouraging elective way to deal with treat wound disease. These NPs have a potential expansive range antimicrobial action and ready to repress an extensive variety of MDR microbes, which includes MRSA, *P.aeruginosa* and *E.coli* [2,3]. The antimicrobial activity of NPs is driven by several factors such as size, surface charge, shape and concentration[4]. Of which TiO_2 NPs are self-cleaning, non-poisonous, artificially stable exceptionally photograph receptive and have wide range anti-infection capacity. Presenting titanium oxide (TiO_2) NPs as antibacterial specialists are pushing for an original move toward the turn of events and improvement of progressive restorative techniques[5].

Nanocrystalline titanium dioxide (TiO_2) has enormous potential as a self-cleaning and self-disinfecting material in various applications. Due to its high photoreactivity, broad-spectrum antibiosis, and chemical stability, TiO_2 has been widely employed to kill a variety of microorganisms, including fungi, viruses, and bacteria. When exposed to non-lethal ultraviolet (UV) radiation, TiO_2 NPs break down organic molecules by producing and continuously releasing superoxide ions and hydroxyl radicals. This process is particularly effective in preventing MRSA from growing. TiO_2 NPs' strong oxidizing ability can be utilized to combat bacteria and other organic materials [1].

Phytochemicals are used for many purposes such as drug discovery, anti-cancer and anti-microbial agents, but extracting them from plants and animals is quite laborious due to their bioavailability and time retention in the body, so studies have been conducted on other options such as animal and insect secretions, which may be useful for medical applications. Studies have shown that the phenolic compounds of the insect, *Blapsrynchidorae Fairmaire*, have anti-cancer activities *in vivo*. *P.americanana* extract has shown to have anti-cancer activity related to its enhancement of immune function. It also has favorable tissue repairing, antibacterial, anti-tumour and immune enhancing properties. It has a positive effect on the liver, promotes the growth of blood vessels, helps in the circulation of micro-circulation, and repairs the tissues, thus improving the immunity. Therefore, this study aims to analyze the anti-microbial properties of the bioactive components [6].

Periplaneta americana are the common insects found in areas of industries and residences. Though it is a persistent pest, *Periplaneta americana* have potential to disseminate human pathogens, thereby representing a public health risk [7]. They have evolved to external stimuli in order to defend themselves from microbial illnesses and exposure to contaminants since they reside in unsanitary and unclean settings and niches. It might have defense systems and that their gut microbiota produce molecules to fight against the invading pathogens. The lectin proteins, which recognize foreign or dangerous bacteria and trigger the innate immune response against infections. The intricate network of passageways in *Periplaneta americana* cavities, which is made up of antimicrobials that eliminate infections before they can spread, is another important element that may support cockroach immunity.

Y. Ma *et al* (2021) worked on the isolation and purification of the actinomycete WA 4-31 from the intestinal tract of *Periplaneta americana*.

Study also indicated its morphological characeristics and the phylogenetic analysis indicated it was homologous to *Gordonia terrae sp.* Bioactive components showed the antifungal activities against *Candida albicans*, *Aspergillus niger*, *A. fumigatus* and *Trichophyton rubrum*. Study indicated that the intestinal tract of Periplaneta americana is a potential source of active secondary metabolites [8].

Though phytocompounds were considered normally as the source of drug components against infectious diseases. Study have indicated the potentiality of insect-based drug discovery in the treatment of the infectious diseases. Cyclohexane extract of *Periplaneta americana* has the impact on the antibacterial and antifungal activities against the pathogens, besides the promising anticancer properties, Experimental studies have indicated the therapeutic properties against various pathogens. Extracts of *Periplaneta americana* have indicated the immune function at invivo condition. Also, it aids in tissue repairing, antibacterial and antitumor activities. Studies have also indicated that the extract of *Periplaneta americana L* ., protect the liver, promote blood vessel growth, tissue repair, improve microcirculation [9].

Chitosan, a polysaccharide and antibacterial compound present in the roaches' exoskeleton, may also be the source of the antimicrobial qualities of cockroach. It's interesting to note that the effects and activities of chitosan in cockroaches vary depending on the species of cockroaches meaning that the German and American cockroaches have different chitosan's antibacterial qualities. Reports also stated that the minimum inhibitory concentration (MIC) of chitosan against Gram-positive bacteria, including S. aureus and B. subtilis, was 2000 µg/ml. Chitosan can function as an antibacterial agent against these types of bacteria. The minimum inhibitory concentration (MIC) of chitosan against *E. coli* was found to be 1000 µg/ml, whereas the MIC against other Gram-negative bacteria was also shown to be significant [10]. Studies have indicated that the insects and its microbiomes are sources of antibiotics, that can be considered in medication. As an alternative to address the antimicrobial resistance to the human infections, attempts are made extract the gut bacteria from the *P. americana*. It also tests these bacteria's antagonistic activity against specific drug-resistant microbes [10]. Also potential approach would be the addition of nanoparticles to antibacterial drugs. Metal oxide nanoparticles (NPs) and their nanocomposites have shown remarkable antibacterial capabilities.

Several studies were made on the antibacterial/ antibiotic activities of nanomaterials, due to the distinctive qualities, including strong photocatalytic activity, process efficiency, element abundance, simplicity in manufacture, chemical and photo stability, biocompatibility, non-toxicity, recyclability, and low cost [11]. TiO_2 nanoparticles showed non-genotoxic and non-mutagenic outcomes at the most extreme fixations. The consequences of this study could drives the eventual fate of the remedial methodologies against the safe pathogenic strains that cause wound contaminations [5].

The microbes close contact makes it possible for substrate exchange, metabolic product distribution, and toxin end product removal, all of which are necessary for the various species to coexist. Additionally, the design of biofilm communities can shield the bacteria inside them from the immune system, shear pressures, and antimicrobials[10, 12]. Studies also have indicated animals that live in areas where there are lots of germs are a rich supply of antimicrobials. *Periplaneta americana* and other species that live in contaminated environments come into contact with a variety of bacteria, including superbugs. Such organisms are able to resist the assault of superbugs and prevent sickness by creating antimicrobial compounds that have strong activity in the nervous system. Based on research into the *Periplaneta americana* brain's antimicrobial activity, new antibiotics may be developed as a result of research into antimicrobials from unconventional sources[13].

According to Thomas Bjarnsholt *et al.,* (2013) antibodies are utilized to break down the

presence, association and circulation of microorganisms and biofilms in constant diseases, in view of the distinguishing proof of bacterial totals and their networks to work on the conclusion of biofilm-related contaminations, where the old style microbiological culture techniques are unseemly, so sub-atomic tests and microscopy were applied to a wide assortment of tests from persistent human diseases[14,15]. Based on the literature, this work was aimed with the biosynthesis of titanium oxide nanoparticles using *Azadirachta indica* (neem) leaves, extraction of active components from *Periplaneta americana* and its characterization and antimicrobial studies of titanium oxide nanoparticles and bioactive components from *P. americana.*

2 Methodology

2.1 Synthesis of TiO_2 Nanoparticles

Healthy leaves *of Azadirachta indica* were collected from Acharya Institute of Technology, Karnataka, India. For antimicrobial activity, bacterial pathogens viz. *Stapylococcus aureus* and *E. coli* were obtained from ICAR-Indian Institute of Horticultural Research (IIHR), Karnataka, India.

The *Azadirachta indica* (Neem) leaves were properly washed with double distilled water to remove debris stuck to the surface. Leaves were allowed to dry in a tray dryer at a temperature of 60°C. These dried leaves were grinded into a powder using a lab grinder, and the powder was collected and stored. To make leaf extract, 20g of dried powder was mixed with 100ml of distilled water and was subjected to heating at 80°C for 60 minutes (Figure 1). The extract was filtered through No. 1 Whatman filter paper[16] to remove particulate matter. Resultant solution was stored at 4 °C for further use.

Green synthesis of TiO_2 nanoparticles was performed as described earlier by Krishnasamy et al. (2015) with modifications. 80ml of 5mM Titanium (IV) isopropoxide (TTIP) solution was mixed with the extract of *Azadirachta indica* in equal proportion in 1:1 ratio (v/v basis), it was then subjected to continuous stirring for 8h at room temperature (Figure 2). Then the nanoparticles were separated by centrifuging for 10 minutes at 9000 rpm (Figure 3). Finally, heat treatment was given at 60 °C for 1 h and stored for further use [16].

Figure 1. Plant extract of *Azadirachta indica* (Neem) leaves

Figure 2. Constant stirring of TTIP solution with the extract of Azadirachta indica in 1:1 ratio (v/v basis)

Characterization of nanoparticles

Synthesized titanium dioxide nanoparticles were characterized using SEM. Morphology and size and of the synthesized TiO_2 nanoparticles were analysed by field emission scanning electron microscope.

TiO_2 nanoparticles preparation

A stock solution was prepared by suspending the nanoparticles in methanol to yield a final conc. of 10 mg/mL. The stock solution was sonicated for 15 min and every assay was done within 1–2 h of sonication. The suspension was kept at 4 °C.

2.2 Extraction of bioactive component from *Periplaneta americana*

Collection of the cockroaches: Cockroaches (n = 20) were gathered utilizing snare catching and dynamic assortment strategies. A dichotomous key was utilized to order and distinguish the examples as *Periplaneta americana*.

Organ lysate of cockroach: Gathered cockroaches were immobilized at 4 °C for 15 min, and their legs and wings separated. The cockroaches were homogenized with two solvents (10g of cockroaches: 100ml of solvent) with pestle and mortar for the extraction. Samples were filtered, and the lysates were maintained at 20°C.

Antimicrobial assay

Antibiotic susceptibility test: The antibiotic susceptibility test was carried out by disc diffusion method. MH agar media was poured in 90 mm petri dishes and incubated at 37°C overnight to check sterility. The bacterial suspension was adjusted to an optical density OD at 600nm = 0.1 AU (106 CFU/ml). Bacterial suspension was inoculated on each plate by spread-plate method and then antibiotic sensitivity discs were placed on the surface of the medium. After that, TiO_2 NPs samples were added from two stocks into each disc and incubated for 24 h at 37 °C and the antibacterial sensitivity was measured as the zone of inhibition in mm diameter, for activation of TiO_2 UV light was applied within a time interval of 10 min reaching adjusted.

Briefly 4–5 well isolated colonies of the same morphological type were selected from an agar plate culture and transferred into a tube containing 5–6 mL of Nutrient broth medium. The broth culture was incubated at 37 °C for 24h. The turbidity of inoculum was compared with 0.5 McFarland standards containing 1–2 × 108 CFU/mL.

The antimicrobial activity of the TiO_2 nanoparticles and whole lysate was measured against human pathogenic bacteria; *E. coli* and *S. aureus* obtained from the Regional Centre for Microbiology and Biotechnology, Bangalore. In a sterile plate, 20 ml of the medium (using nutrient agar media to test bacteria) is added and left to solidify. *S. aureus* and *E. coli* inoculum were applied to the plates, and were incubated for testing human pathogenic bacteria for 24h at 37°C[17,18]. At the end of the incubation period, the inhibition zones were determined along the diameter of the plate and the mean was calculated. Determination of the Minimum Inhibitory Concentrations (MIC): Dilution method based on two-fold serial dilution starting with 1000 µg/ml was used to determine the Minimum Inhibitory Concentrations (MIC) of cockroach extract after testing against *E. coli* and *S. aureus*. Inhibition zones were measured on the plates after incubation, and the diameter of these zones was measured in millimetre. The lysate concentration that gave noticeable inhibition at the lowest level was determined to be the MIC[19].

3 Results and Discussion

Hydrothermally synthesized TiO_2 nanoparticles was characterized with SEM analysis (Figure 4). Agglomerates of smaller TiO_2 nanoparticles that are possibly electrostatically stabilized and may be mechanically disrupted. The TiO_2 nanoparticles particle exhibited a mean size of about 250 nm[20].

Figure 3. Synthesised TiO_2 Nanoparticles after centrifugation

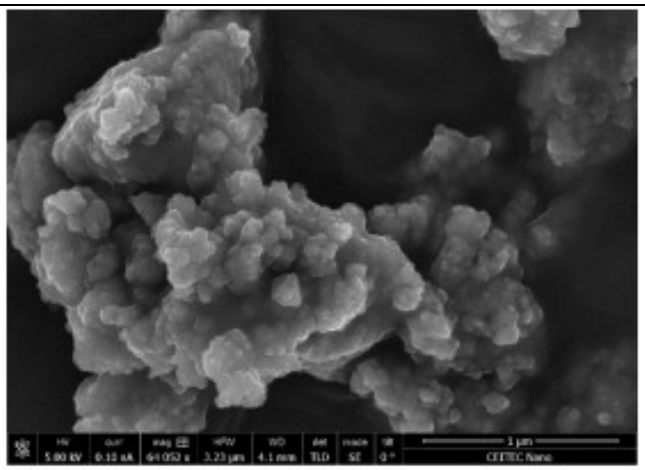


Figure 4. Nanoparticle image in Scanning Electron Microscopy (SEM)

3.1 Extraction of bioactive component from *Periplaneta americana*

Obtained extract of *Periplaneta americana* were subjected to LC-MS studies. Few of the homologous compounds identified were derivatives of chromene, thiazine groups, pyrrole containing analogs, sulfonamides, furanones, flavanones that are known to possess antimicrobial properties[21].LC- MS analysis indicated the following components in the sample of *P. americana* extract (Table 1)

Table 1. Components of *P. americana* identified from LC-MS analysis

No.	Compounds	Formula
1	1-(S, S) -1,1-Bis(ethoxycarbonyl)-,2-bis-ptolylsulfinyl-1-ethanol	$C_{22}H_{26}O_7S_2$
2	1-Allyl(2-nitro-1-phenylethyl) sulfane	$C_{11}H_{13}NO_2S$
3	2–2-Ethyl-3-tertbutoxyaminoquinaz olin-4(3H)-one	$C_{14}H_{19}N_3O_2$
4	7-(Isopropoxy)-2,2,5-trimethylchromene	$C_{15}H_{20}O_2$
5	5-Ethyl-6,7-dihydr 5Hcyclopentapyrazine	$C_9H_{12}N_2$
6	Di-isopropyl 2-phenyl ethenyl phosphonate	$C_{14}H_{21}O_3P$
7	(E)-3-Benzylidene-5 methyldihydrofuran-2(3H)-one	$C_{12}H_{12}O_2$
8	2,5-Dihydroxy-40 -methoxy-flavanone	$C_{16}H_{14}O_5$
9	4,4-Dimethyl-3-oxacholest-5-en-7-one	$C_{28}H_{46}O_2$
10	5-Methyl-2-(thiophen-2-yl) pyridine	$C_{10}H_9NS$
11	(2S,5R, Rs)-2-Allyl-5-methyl-2(ptolylsulfinylmethyl) Tetrahydropyran	$C_{17}H_{22}O_2S$
12	(E)-3-Benzylidene-5 methyldihydrofuran-2(3H)-one	$C_{16}H_{16}N_2S$
13	2,7,12,17-Tetraethyl-3,8,13,18-tetramethyl-21H,23H-porphine copper	$C_{32}H_{36}N$
14	2-Methyl-6-(3,7,12-trihydroxy-10,13-dimethylhexadecahydro1Hcyclopenta[a]phenanthren-17-yl) heptanoic acid	$C_{27}H_{46}O$
15	2-Ethyl-3-tertbutoxyaminoquinazol in-4(3H)-one	$C_{14}H_{19}N_3O_2$
16	Dimethyl alpha-(N acetylamino)benzyl phosphonate	$C_{11}H_{16}NO_4P$
17	2-(Allylthio)-1-nitro-2-phenylethane $$ [1-(allylsulfanyl)-2-nitroethyl] benzene	$C_{11}H_{13}NO_2S$

Studies have indicated that *Periplaneta americana* has uncommon ability to resist environmental intimidations. In the treatment of arthritis and diabetics cockroach extracts are used. They produce varieties of immune induced molecules which possess antibacterial and antifungal actions [6].

The ability of the animal derived compounds with pharmacological and biological potentials are of emerging in the last few decades. *Periplaneta americana* is considered in this present study due its ability to resist environmental threats. Also, insect extracts are used in various disease treatments, such as arthritis and diabetics. Larval extracts of the insects are used the folklore medicine. Insects acts on the microbial infections by producing immune induced molecules indicating antibacterial and antifungal properties [22].

Outstanding antimicrobial activity was demonstra ted by gut bacteria isolated from P. americana L., the American cockroach, against the majority of t ested MDR human pathogens.
It is clear that in order to address the issue of drug resistance, the isolated gut bacteria may be able t o manufacture unique substances (metabolites) th at might be employed as alternatives to the antimi crobial medications currently in use [23].

The abundance and unexplored potential of insects as a source of novel antimicrobial medications has lead to the exploring of the antibacterial properties of their diverse tissues. Living multicellular creatures tissue have the potential to be a rich source of nutrition of micro organisms, therefore the host organism must devise practical defenses against microbial digestion.

3.2. Antimicrobial studies

Antimicrobial activity of the synthesized TiO_2 nanoparticles:

Antimicrobial activity of the synthesized TiO_2 nanoparticles, bioactive components obtained from cockroach were checked by disk diffusion method (Figure 5,6). Its Minimum Inhibitory Concentration against E.coli and S.aureus were noted(Table 2,3).

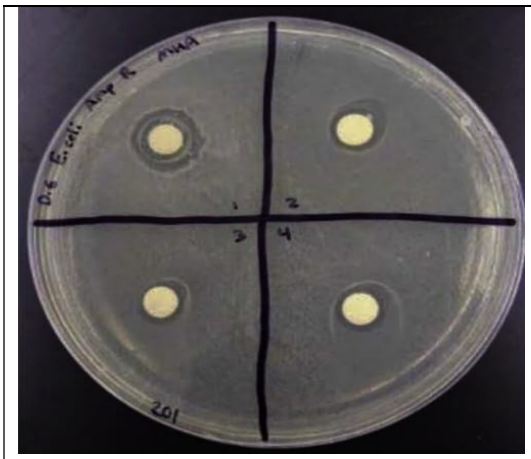


Figure.5. Antimicrobial test TiO_2 nanoparticles against *E.coli*

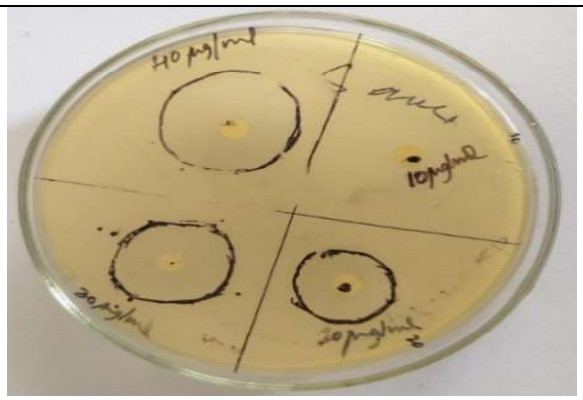


Figure.6 Antimicrobial test TiO_2 nanoparticles against *S. aureus*

Table 2. MIC of TiO_2 NPs against *E. coli*

TiO_2 NPs (µg/ml)	Zone of Inhibition (mm)
10	-
20	8
30	12
40	15

Table 3. MIC of TiO_2 NPs against *S. aureus*

TiO_2 NPs (µg/ml)	Zone of Inhibition (mm)
10	-
20	18
30	22
40	26

TiO_2 nanoparticles at the concentration of 40 µg/ml indicated the more zone of inhibition against *S.auerus* (26mm) compared to *E.coli* (15mm).

Antimicrobial activity of bioactive component from *P. americana*: Antimicrobial activity of the bioactive component form P.americana was checked against *E.coli* and *S.aureus,(*Figure 7,8) and its minimum inhibitory concentration were determined as 12 mm and 25mm (Table 4)

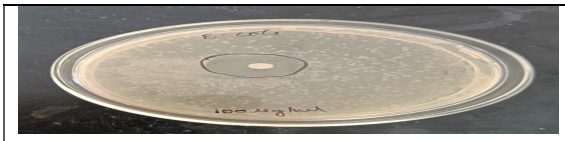


Figure 7. Antimicrobial assay of *E. coli*

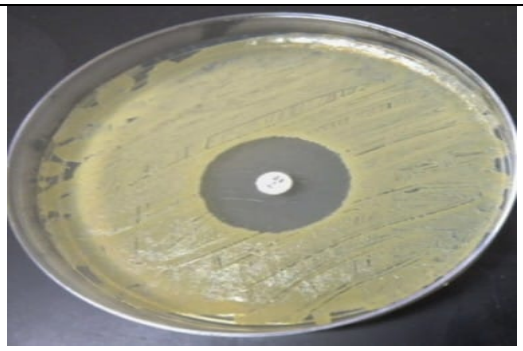


Figure 8. Antimicrobial assay of *S. aureus*

Table 4. Inhibitory effects of cockroach extracts against pathogenic bacteria.

Extract	*E. coli*	*S. aureus*
Cockroach extract	12mm	25mm

Of which antimicrobial activity against *S. aureus bacteria* showed more zone of inhibition. Further at different concentration of bioactive extract, zone of inhibition was noted and checked (Figure 9 and table 5).

Figure 9. Microbial Inhibition Concentration (MIC) of *S. aureus* at different concentration of bioactive component

Table 5. Minimal inhibitory concentration of organ lysate of *P. americana* on *S. aureus.*

No.	Concentration (µg/ml)	Inhibition zones (mm)
1	1000	27
2	500	25
3	250	25
4	125	22
5	62.5	18
6	31.25	15
7	15.6	13
8	7.8	11
9	3.9	10
10	1.9	9
11	0.9	8

The antimicrobial studies of TiO_2 and bioactive components extracted by cockroaches showing high activity on *S. aureus* bacteria (25mm). TiO_2 nanoparticles is thermally steady and biocompatible substance compound with high photocatalytic action and has shown a decent antimicrobial movement. The standard component were their morphology, gem nature and size. Additionally, one of the component of its activity is through the age of reactive oxygen species (ROS) on its surface during the course of photocatalysis when it presented to light of specific frequency. Study has also indicated that the increased antimicrobial activity is due to its photocatalytic nature[18].

The two main components of effective antibacterial action against microorganisms are TiO_2 concentration and NP size. The breakdown of bacterial outer membranes by reactive oxygen species (ROS), especially hydroxyl radicals (OH), is responsible for the antibacterial activity of TiO_2 NPs. This process causes phospholipid peroxidation and ultimately cell death.

Bioactive mixtures from *P. americana* results uncovered that the metabolites of these microbes could be utilized as substitutes to the generally

utilized anti-toxins to conquer the issue of multidrug-safe human microorganisms[21, 24].

Studies have shown cockroach extract has inhibition activity against gram positive and gram negative resistant pathogenic bacteria also, it has indicated antifungal activity against pathogenic fungi and yeast [6].

4. Conclusion

The antimicrobial studies of TiO_2 and bioactive components extracted by cockroach showing high activity on *S. aureus* bacteria. There is an extraordinary contest in tracking the advances against MDR microbes. Nanoparticles are broadly utilized as antibacterial specialists against a few MDR microorganisms. Subsequently, titanium oxide NPs can be a legitimate elective antibacterial specialist. The TiO2 NPs showed high viability as a solid antibacterial specialist towards the tested strains. Their antibacterial action against microbes was as per the following: MRSA (Gram-positive) > E. coli (Gram-negative). Customarily the phytocompounds were used as the wellspring of medication applicants against irresistible infections. nonetheless, the current work indicates the potential of insect-based drug action. The cyclohexane concentrate of the cockroach has shown promising antibacterial against drug-safe microorganisms. Additionally, a few future examinations are expected to screening the antimicrobial activities of gut-associated bacteria *P. americana*

Cockroaches are sources for a number of alternative medicines, according to studies. Additionally, several studies demonstrate the antibacterial efficacy of certain gut-associated cockroach bacterial species against drug-resistant pathogenic human microorganisms. The results are highly encouraging, particularly for the development of antimicrobial medicines. Based on the current findings, more clinical testing and chemical investigation of the nature of such antimicrobial activity must be carried out before the final pharmaceutical product is produced in order to achieve this goal.

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