Phytochemical screening and antibacterial activity of *Vernonia amygdalina* (bitter leaf) on some selected bacterial isolates

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**Abstract**

Plants with medicinal value produce certain chemical elements known as phytochemicals that have antibacterial activity. The study was aimed at determining the antibacterial activity of *Vernonia amygdalina* against bacterial isolates using agar well diffusion method. In addition, the phytochemicals analysis of the extracts was also determined. The phytochemical analysis showed the presence of saponins, steroids, terpenoids, tannins, alkaloids, and flavonoids. The result of *Vernonia amygdalina* showed that the average zones of inhibitions observed against these bacterial ranges from 6-22mm. The highest zone is also exhibited against *E. coli* with average diameter of zone of inhibition of 22mm. At 100mg/ml concentration for *Salmonella*, the zone of inhibition was recorded to be 21mm while at 12.5mg/ml there was no inhibition. At 25mg/ml and 12.5mg/ml, against *Pseudomonas* there was no inhibition. In other to further confirm the activity of these plant extracts, the minimum inhibitory concentration and minimum bactericidal concentration was determined and the result showed that the extract exerted good antibacterial activity on all the test organisms at different concentration. The result of minimum inhibitory concentration ranges from 10 to 12.5mg/ml and that of MBC ranges from 5 to 20mg/ml. It is worthy to note that MBC values is greater than that of minimum inhibitory concentration. The study provides insight into the antibacterial activities of the plant extracts and its use in the treatment of bacterial infections.

**Keywords:** Antibacterial; Isolates; Phytochemicals; *Vernonia amygdalina*

**1. Introduction**

Medicinal plants are extremely beneficial to people’s and communities’ welfare. Plant content continues to be a valuable resource in the fight against serious diseases around the world. Traditional plants continue to play an important role in meeting basic health needs in developing countries [1]. Antimicrobial resistance leads to more sickness, higher healthcare costs, and deaths. As a result, new antimicrobial compounds must be discovered, which would likely work by mechanisms different from existing drugs. As a result, new antimicrobial agents derived from natural plant products are required to tackle the problems associated with drug-resistant microorganisms. Our own mismanagement of antibiotics has resulted in a recent outbreak of multi-drug resistance as an emergent pathogen. As a result, researchers are looking for more efficient plant-based materials with the aim of finding potentially useful active ingredients that can be used as a source and blueprint for the development of new antimicrobial drugs [2-3]. Herbal medicine is abundant in our rich flora, is inexpensive, and has the potential to introduce new paradigms into modern medicine [4]. Many plants produce compounds that are beneficial to the health of humans and other animals. Plant extracts are being
researched all over the world, especially in developing countries, as effective and less expensive alternative sources of medication.

*Vernonia amygdalina* is a perennial herb in the Asteraceae family that is referred to as "bitter leaf" in English, "Oriwo" in Edo, "Ewuro" in Yoruba, "Shuwaka" in Hausa, and "Olubu" in Igbo. It is used as a vegetable in African soups or the aqueous extracts may be drunk to treat different illnesses [5]. After crushing and thoroughly washing the leaves to eliminate the bitterness, the leaves are consumed [6]. *Vernonia amygdalina* is a tropical shrub that can reach a height of 3 meters in the African tropics and other parts of Africa, especially Nigeria, Cameroon, and Zimbabwe. The species is native to tropical Africa, but it can be found in the wild or in cultivation in Sub-Saharan Africa. The bitterness is thought to be due to factors such as the presence of alkaloids, saponins, tannins, and glycosides, which have been found in bitter leaf by various authors.

However, almost every aspect of the plant is pharmacologically useful; both the root and the leaves are used in phytomedicine to treat a variety of ailments, including fever, hiccups, kidney disease, and stomach pain [7]. *V. amygdalina* has been found to have a variety of culinary and medicinal properties, some of which have bacteriostatic and bacteriocidal effects on bacteria [8]. The plant has antipyretic, analgesic, antidiabetic, anthelmintic, anti-inflammatory, antibacterial, antiparasitic, astringent, diaphoretic, anticancer, and purgative activities in folk medicine [5-10]. Extracts from the plant have also been stated to have anthelmintic and antimalarial properties, as well as antitumourigenic properties [11]. In addition, other studies have shown that the leaf extract has a hypoglycemic and hypolipidemic effect in laboratory animals.

The plant's young, succulent, and fresh leaves are commonly used in the treatment of diabetes, malaria, fever, constipation, and high blood pressure, as well as laxative. Many African herbalists and native doctors suggest its aqueous extract for a variety of ailments, including nausea, diabetes, loss of appetite, dysentery, and other gastrointestinal disorders, as well as sexually transmitted diseases and diabetes mellitus [4]. Microorganisms' tolerance to many commercially manufactured synthetic antimicrobial agents has been growing over time, necessitating the development of new antimicrobial agents. The need for alternative antibacterial agents has sparked interest in Pharmaceuticals Company evaluating extracts from plants considered to have medicinal value for the manufacture of herbal antibacterial agents. The majority of bacteria have shown the potential to establish resistance to some of the commercially available antibacterial agents. This has contributed to the search for new raw materials that can be used in the development of new antibacterial agents to combat the bacterial pathogens' rising resistance. The purpose of this study was to verify the veracity of the aforementioned conventional statements, as well as the protection of the leaf of this widely used plant (*Vernonia amygdalina*) and its antibacterial impact on the selected bacterial pathogens.

### 2. Material and methods

#### 2.1. Collection, identification and processing of Plant Materials

Fresh leaves of *Vernonia amygdalina* was purchased from Abubakar Rimi Market, SabonGari Kano and transported in a polythene bag to Microbiology Laboratory in the Department of Microbiology, Kano University of Science and Technology, Wudil, Kano State Nigeria. And was identified by a Botanist at the Department of Biology, Faculty of Science, Kano University of Science and Technology, Wudil, Kano. The leave of *Vernonia amygdalina* was washed with running water and dried under shade at room temperature for 3 weeks. The dried leaves were grinded into powder and stored until require for use.

#### 2.2. Preparation of Ethanol Extract Plant

Fifty grams (50g) of the powder of *Vernonia amygdalina* was extracted using maceration method with 250ml of ethanol for 72hours with shaking at regular intervals. The extracts were filtered through a sieve to remove debris, the filtrate was then filtered through filter paper. The final filtrates were evaporated to dryness using thermostat water bath. The crude Ethanol extract was packaged in sample bottles and store at room temperature until require for use [12-13].

#### 2.3. Phytochemical Screening of the Extract

Standard methods were used to detect the presence of plant constituents such as hormones, flavonoids, tannins, saponins, and terpenoids in *Vernonia amygdalina* ethanol extracts.
2.4. Test for steroids
One gram of the plant's ethanoic extract was combined with two milliliters of acetic anhydride and two milliliters of sulphuric acid. The appearance of steroids is shown by the appearance of blue or green colour [13].

2.5. Test for tannins
To 1 ml of extract, 2 ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins [14].

2.6. Test for alkaloids
To 2 ml of extract, 2 ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids [14].

2.7. Test for flavonoids
A few drops of dilute sodium hydroxide were applied to one milliliter of crude stock extract. The plant crude extract had an intense yellow color that turned colorless after a few drops of dilute acid was added, indicating the existence of flavonoids [13].

2.8. Test for saponins
5.0 ml of distilled water was mixed with aqueous crude plant extract in a test tube and it was mixed vigorously. The frothing was mixed with few drops of olive oil and mixed vigorously and the foam appearance showed the presence of saponins [15].

2.9. Test for terpenoids
2.0 ml of chloroform was added with the 5 ml aqueous plant extract and evaporated on the water path and then boiled with 3 ml of H₂SO₄ concentrated. A grey color formed which showed the entity of terpenoids [15].

2.10. Test Organisms
The organisms used for the experiment includes; *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli*. The type bacterial isolates of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Pseudomonas aeruginosa*, was obtained from the Microbiology Laboratory of Department of Microbiology, Kano University of Science and Technology, Wudil, Kano.

2.11. Resuscitation of Organisms
The experimental bacterial was resuscitated by sub-culturing on Nutrient agar plate, which was obtained from the Microbiology Laboratory of Kano University of Science and Technology Wudil, Kano state. Seven grams (7g) of nutrient agar were weighed into 250ml of distilled water and placed on a burner. After several minutes, it was removed from the burner and allowed to cool to room temperature. An autoclave was used to sterilize the nutrient agar at 121°C for 15 minutes. The nutrient agar was poured into Petri dishes and allowed to set before being used. On the dishes, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhi* were cultured and incubated for 24 hours at 37°C [13].

2.12. Confirmation of Test Organism
2.12.1. Gram staining reaction
On a clean grease-free glass slide, a thin smear film of the organism (a 24-hour old bacterial culture) was prepared, air-dried, and heat-fixed by moving it horizontally over the Bunsen flame. The dried smear was stained for 60 seconds with Crystal violet stain until being rinsed with tap water. The resulting smear was then stained for 60 seconds with Lugo’s iodine before being rinsed with tap water. The smear was decolorized with 95 percent ethanol before the crystal violet stain was removed from the slide. After rinsing with tap water, the slide was counter-stained with safranin for 1 minute until being rinsed with tap water. The prepared slide was dried before being studied under the microscope with an x40 objective lens and an x100 magnification (oil immersion objective lens). Gram positive organisms preserved the crystal violet stain (purple in color), while Gram negative organisms appeared pinkish or reddish [16].
2.12.2. Biochemical tests

Biochemical tests are confirmatory tests that are used to differentiate the species of microorganisms from their genera. The test is used to demonstrate the enzymes in a bacteria cell. The following were some of the biochemical tests carried out in the isolation of Enterobacteriaceae microorganisms.

2.12.3. Indole Test

This test is important in the identification of enterobacteriaceae to differentiate Gram negative rods, particularly Escherichia coli. Most strains of E. coli breakdown an amino acid tryptophan with the release of indole. The organism was grown in bijou bottle containing 5ml peptone water for 24hrs after which 3 drops of Kovac’s reagent was added and shaken gently. A positive reaction indicated by a red color formation on the surface [16].

2.12.4. Methyl Red (MR)

MR is a pH indicator to determine the ability of an organism to carries out mixed acid fermentation. The medium was inoculated and then incubated for at least 48 hours at 37°C. The pH of the culture was tested by adding a few drops of 0.04% methyl red (yellow at pH 6.2, red at pH 4.4). MR-positive organism the culture becomes red.

2.12.5. Voges-Proskauer test (VP test)

This test detects the ability of an organism to form acetone. A phosphate-buffered glucose peptone medium was inoculated with the test strain and incubated at 37°C for 2 days. 0.6 ml of an ethanolic solution of 5% nepthanol and 0.2 ml of 40% KOH solution were added to 1 ml of the culture. The tube or bottle was shaken vigorously and placed in a sloping position and examined after 30 and 60 minutes. Red color indicates positive test [16].

2.12.6. Citrate test

The citrate test was carried out in order to determine the ability of the isolates to utilize citrate as their sole source of carbon and ammonia as the only source of nitrogen. Simmon citrate agar was used for this test; the agar was prepared in test tubes and was inoculated with a 24-hours old culture of each of the isolates aseptically. This was then incubated at 37°C for 24-hours. A colour change from green to deep blue indicates positive reaction while the absence of a colour change indicates negative reaction.

2.13. Preparation of Extracts

2.13.1. Preparation of Vernonia amygdalina

Four grams (4g) of the extracts Vernonia amygdalina was measured into a test tube and 10ml of DMSO was added to concentrate [17]. This is done to enable the plant extract to dissolve properly due to its oil content. Aliquot 2ml of the above mixture was taken into a fresh test tube and 2ml of distilled water was added to dissolve it. 1ml was taken from the solution into another test tube. This was done to complete serial dilution for 4 test tubes containing (100, 50.25 and 12.5) mg/ml concentration respectively.


2.14.1. Preparation of Mueller Hinton Agar (MHA)

Nineteen grams (19g) of Mueller Hinton Agar was dissolved in 500ml of distilled water and was autoclaved at 121°C for 15 minutes and cooled to room temperature.

2.14.2. Microbiological Assay (Sensitivity Test)

The antibacterial activity was assessed using well plate method and was examined for the size of inhibitions. 0.1ml of Escherichia coli, Staphylococcus aureus, Pseudomona aeruginosa, and Salmonella typhi comparable with the 0.5 McFarland’s turbidity standards were inoculated on the Mueller Hinton Agar plate. Using 6mm sterile cork borer 4 holes were made asymetical, labeled I, II,III, IV with one in the middle which serve as control, 0.1ml of the different concentrations of the extracts were then introduced into the wells respectively for the individual extract. Gentamycine was use as the control. The extractions were allowed to diffuse for 10minutes and were incubated at 37°C for 24hours. The zones of inhibition of were measured in millimetre (mm).
2.14.3. Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the active extracts was determined by dividing the extraction nutrient broth to give concentration of 100, 50, 25, and 12.5mg/ml of sterile extract was added to the first tube containing 2ml of broth. The tube was shaken and 1ml transferred aseptically to the next tube containing the same quality of broth, until it reaches the concentration of 12.5mg/ml. The 0.5ml of the bacteria suspension of Mc farland's standard was inoculated into each test tube and was incubated at 37°C for 24 hours. The minimum inhibitory concentration tubes that show no visible growth when compared with control tubes.

2.14.4. Determination of Minimum Bacterial Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of the plant extract on the clinical bacterial isolates was carried out according to [18]. Briefly, 1ml bacterial cultured was pipette from the mixture obtained in the determination of Minimum Inhibitory Concentration (MIC) tubes which did not show any growth and were sub-cultured onto Mueller Hinton Agar and incubated at 37°C for 24 hours. Minimum Bactericidal Concentration (MBCs) was defined as the lowest concentration yielding negative subcultures or only one colony.

3. Results

The physical properties of Vernonia amygdalina are shown in Table 1 below. The table shows the weight of the leaf powder of Vernonia amygdalina to be 50g, the residue weight be 38.7g (75.6%) after dissolving it in 250ml of ethanol and evaporated using thermostat water bath and the color of the filtrate to be greenish in color.

Table 1 Properties of plant Extract and percentage yield

<table>
<thead>
<tr>
<th>S/No</th>
<th>Parameters</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weight of leaf powder</td>
<td>50g</td>
</tr>
<tr>
<td>2</td>
<td>Colour of filtrate</td>
<td>Green</td>
</tr>
<tr>
<td>3</td>
<td>Weight of residue</td>
<td>38.7g (75.6%)</td>
</tr>
</tbody>
</table>

Table 2 Phytochemical Screening of Vernonia amygdalina (bitter Leaves) Ethanol leaves Extract

<table>
<thead>
<tr>
<th>S/No</th>
<th>Phytochemicals</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Terpenoids</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3 Result of confirmation of test organisms of Gram staining and Biochemical test

<table>
<thead>
<tr>
<th>S/NO</th>
<th>Test organism</th>
<th>S. typhi</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gram staining</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Methyl red</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>VP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Citrate</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Catalase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Coagulase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Result of phytochemical screened was presented in Table 2 and revealed the presence of flavonoids, alkaloids, saponins, Tannins, glycosides, steroids, and Terpenoids. These phytochemical compounds exert antibacterial activity through various mechanisms. These secondary metabolites are known to be biologically active and therefore play significant roles in bioactivity of medicinal plants because the medicinal values of medicinal plant lies in these phytochemical compounds which produced a definite and specific action on the human body.

The results of Gram staining and biochemical studies on test species can be seen in Table 3. *Staphlococcus aureus* was found to be positive in Gram staining, while *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Escherichia coli* were found to be negative. In the Indole test, *E. coli* was the only organism that was positive; in the Methyl red test, both *E. coli* and *S. typhi* were positive; and in the Voges-Proskauer test, *S. aureus* was the only organism that was positive. *P. aeruginosa* and *S. typhi* were both positive in the citrate test, *S. typhi* was the only negative organism in the catalase test, and *S. typhi* and *S. aureus* were both positive in the coagulase test.

The Inhibition Effect of Ethanolic Leaf Extract of *V. Amygdalina* on Test Bacteria Isolates is shown in Table 4. The results revealed that the zones of inhibition recorded by the isolates are dependent on the type of bacteria and extract concentration. *E. coli* had the largest zone of inhibition (22mm) at 100mg/ml, while *P. aeruginosa* had the smallest zone of inhibition (15mm) at 100mg/ml. In all of the test species, there is no zone of inhibition at 12.5mg/ml.

Table 4 Inhibition Effect of Ethanolic Leaf Extract of *V. amygdalina* on Test Bacteria Pathogen

<table>
<thead>
<tr>
<th>Sn</th>
<th>Test Organism</th>
<th>Ethanol Extract (mg/ml)</th>
<th>Gentamycin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 50 25 12.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>S. aureus</em></td>
<td>18 17 15 6 26</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em></td>
<td>22 19 17 6 32</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>S. typhi</em></td>
<td>21 17 13 6 28</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>P. aeruginosa</em></td>
<td>15 13 6 6 26</td>
<td></td>
</tr>
</tbody>
</table>

Key: - (No inhibition)

Table 5 Percentage of Resistance and Susceptibility of Bacterial isolates

<table>
<thead>
<tr>
<th>S/NO</th>
<th>Isolates</th>
<th>Resistance (%)</th>
<th>Susceptibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>S. aureus</em></td>
<td>5(41.7)</td>
<td>7(41.7)</td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em></td>
<td>1(8.3)</td>
<td>11(91.7)</td>
</tr>
<tr>
<td>3</td>
<td><em>P. aeruginosa</em></td>
<td>7(58.3)</td>
<td>5(41.7)</td>
</tr>
<tr>
<td>4</td>
<td><em>S. typhi</em></td>
<td>4(33.3)</td>
<td>8(66.7)</td>
</tr>
</tbody>
</table>

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Vernonia amygdalina* Ethanolic Extract are shown in Table 6. The MIC result ranges from 10 to 12.5 mg/ml, while the MBC result ranges from 5 to 20 mg/ml. This demonstrates that MBC has a higher value than MIC.

Table 6 MIC and MBC of Ethanolic Extract of *Vernonia amygdalina*

<table>
<thead>
<tr>
<th>S/NO</th>
<th>Test organism</th>
<th>MIC (mg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>S. typhi</em></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td><em>S. aureus</em></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td><em>E. coli</em></td>
<td>12.5</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td><em>P. aeruginosa</em></td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
4. Discussion

The leaf extract of V. amygdalina was obtained using ethanol as the solvent, and phytochemical analysis showed the presence of alkaloids, saponins, flavonoids, tannins, steroids, and terpenoids, as shown in the table above. According to [19], the presence of saponins, flavonoids, tannins, and anthraquinones in bitter leaf was found to have very potent antibacterial properties. Secondary metabolites of plants, such as tannins, reducing sugar, and saponins, as well as all other active principles of plants, have been found to be responsible for this extract’s antibacterial properties. Variations in resistance between Gram positive and Gram-negative bacteria may also be explained by differences in cell wall composition. Gram positive bacteria have a thick layer of peptidoglycan, whereas Gram negative bacteria have a thick layer of lipopolysaccharide. Antibacterial agents are differentially resistant to these layers. Table 3 shows the antibacterial effect of Vernonia amygdalina in comparison to antibiotics (Gentamycin). The extract exhibited good antibacterial activity against the entire test organism and are in concentration dependent i.e. the zone of inhibition increases as the concentration increases.

When compared to a regular antibiotic, all of the plant extracts had less efficacy against the test organism based on the diameter of the zone of inhibition (Gentamycin). Antibacterial activity monitoring revealed that all test organisms were susceptible to the antibiotic, with inhibition zones ranging from 20, 28, 20, and 20mm, respectively. The highest zone of inhibition observed against these bacteria range from 7-16mm, according to the Vernonia amygdalina results. The average diameter of inhibition for Samonella at 100mg/ml was measured to be 15mm, although there was no inhibition at 12.5mg/ml. There was no inhibition against Pseudomonas at 25mg/ml or 12.5mg/ml. This finding agrees in part with [20], who found that the extract is more active against Gram negative bacteria than Gram positive bacteria, and that the antibacterial activity is attributable to the existence of certain phytochemicals. The MIC and MBC were determined to further validate the efficacy of these plant extracts, and the results revealed that the extract had strong antibacterial activity against all of the test species at various concentrations. The MIC result varies from 25 to 50 mg/ml, while the MBC result ranges from 50 to 100 mg/ml. It’s worth noting that MBC values are higher than MIC values.

5. Conclusion

Finally, this research has shown that Vernonia amygdalina contains bioactive phytochemicals that are responsible for its antibacterial properties. More research is needed to allow the purification of unique bio-potential chemicals and their subsequent transformation into chemotherapeutic agents. The findings support the use of these plants’ extracts in folk medicine to cure a variety of infectious diseases caused by the test species. According to this report, people should make it a tradition to consume bitter leaf because it has a lot of medicinal benefit in the treatment of different human pathogens. It was discovered that the test species were susceptible to the plant extract, which has been shown to have therapeutic value; thus, purification of the extract is recommended in order to produce pure bioactive components for industrial use. Toxicity levels can also be monitored in mammalian susceptible organs. It is suggested that more testing be done to see how effective the extract is against other pathogens.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

References


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