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Bactericidal Effect of Cinnamomum verum Bark And Nigella sativa Seeds Extracts Towards Food Spoilage Pathogens

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1. ABSTRACT: This research paper is about the Antibacterial potential of *Cinnamomum* bark and *Nigella* sativa seeds extract was screened against the bacterial isolates isolated from spoiled tomato and lemon using serial dilution and plating. Various types of selective growth media like EMB agar media, cetrimide agar media and mannitol salt agar media for the isolation of pure cultures from spoiled tomato and lemon were used. The selective growth of the organisms on selective growth media was observed, various biochemical tests like lactose fermentation tests, IMViC tests and catalase tests were performed for detection of bacterial isolates and various phytochemical tests were performed to detect the presence of phytochemical components present in *Cinnamomum* bark and *Nigella* sativa seeds extracts. These phytochemical tests helped in identifying the phytochemicals present in the extracts and showed positive results. The antibacterial activity of these phytochemicals present in the extracts were identified by Agar well diffusion method by adding few drops of extract to the plates containing bacterial isolates and the phytochemicals have shown antibacterial property by forming the zone of inhibition around the bacterial colonies by inhibiting the growth of bacteria indicating the antimicrobial activity of cinnamon bark and Nigella seeds extracts. The effect of extracts on isolate T1 was more than isolates T2 and L1. The Cinnamon bark extract showed maximum inhibitory effect than Nigella seeds extracts .

KEYWORDS: Cinnamomum verum bark extract, nigella sativa seeds extract, acetone extraction, phytochemical tests, biochemical tests,antimicrobial activity, agar well diffusion method, zone of inhibition.

2. INTRODUCTION: The use of plant bark, seeds and leaf derivatives with medicinal properties have been increasingly grown in recent years for the treatment and cure for many diseases. The plant medicine which is a natural form called Ayurvedic medicine helped in curing many infectious diseases. Microbiologists,ethnopharmacologists,botanists and plant chemists have been experimenting with the effect of phytochemicals in prevention of various antibacterial,antifungal and antiviral diseases.

2.1 Role of medicinal plants in treating diseases: Medicinal plants have been playing a significant role in treating many infectious diseases which account for significant cause of mortality and morbidity worldwide, despite the great progress made in microbiology and in the control of various microorganisms,sporadic incidents caused by drug resistant microorganisms are causing major threat to the society. For over 100 years various chemical compounds extracted from medicinal plants have served as models for production of many clinically proven drugs and now are again re-emerging as antimicrobial agents. The main reason for the renaissance is the decrease in the new antibacterial drugs in pharmaceuticals and increase in antimicrobial resistance and more need for newly emerging pathogens. Recent tests have proven that thousands of plant species have been tested against hundreds of bacterial strains in vitro and most of the plants and plant extracts are active against a wide range of gram-negative and gram-positive pathogenic bacteria.

2.2 In this research work the main source of medicinal herbs that were used as a source of Phytochemicals with antibacterial properties are Cinnamomum verum and nigella sativa seeds extracts. Cinnamomum verum not only as a culinary flavoring item, it is also a wonderful medicinal product used in treating various antibacterial,antifungal diseases due to its essential oils and phytochemical composition like trans-cinnamaldehyde,eugenol,linalool,campor and various other phytochemicals have been tested with active effect on treating and curing wide range of respiratory, digestive and gynecological disease and infections. Cinnamomum verum is of two types Cinnamomum zeylanicum and Cinnamomum cassia

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are mainly native to Srilanka, India and west asian countries.

2.3 Nigella sativa is among one of the widely used effective medicinal plants which helps in curing and treating various diseases and has rich history and religious background. Nowadays it has emerged as a miracle herb. It is native to southern europe, north africa and southwest asia .Among Muslims, it is considered as one of the greatest forms of healing medicine available due to it being mentioned that black seed is the remedy for all diseases except death in one of the Prophetic hadith. It is also recommended for use on a regular basis in Tibb-e-Nabawi (Prophetic Medicine). *N. sativa* has been extensively studied for its biological activities and therapeutic potential and shown to possess a wide spectrum of activities *viz.* as diuretic, antihypertensive, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, anthelmintics, analgesics and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective and antioxidant properties with great pharmacological potential.

2.4 Phytochemicals are the plant derivatives which are produced through primary and secondary metabolism by plants and have biological activity as they help plant hosts in proper growth and also in fighting against competitors,predators and pathogens as a defence mechanism. Phytochemicals are generally classified into two major categories,they are carotenoids and polyphenols which includes phenolic acids such as flavonoids and stilbenes/ligands and others like terpenoids, coumarins, catechins and epicatechins etc are also present.

2.5 Food spoilage is the most common problem and is a process where food becomes unsuitable for the consumer to ingest.This is mainly caused by undercooking, precleaning of the food materials prior to the cooking, improper sterilization and preservation techniques. The most common type of food spoilage is caused by microbial contamination where the microbes break down the food into few acids and other waste metabolites are released which are toxic in nature leading to foodborne illness.

2.6 Antibacterial activity of *Cinnamomum verum* bark and *Nigella sativa* seeds extracts

The bark and seed extracts from cinnamomum verum and nigella sativa have many phytochemicals which are responsible for antimicrobial activity. This mainly helped in production of various antibacterial drugs. This extraction showed antibacterial activity by forming the zone of inhibition on bacterial colonies. The major phytochemicals that are present in the extract are alkaloids, cinnamic acid, phenols, coumarins, etc . They are responsible for bacterial growth inhibition by destroying various metabolic activities of bacteria. These extracts are being used as disease treating agents, mainly in treating diseases like gastrointestinal disorders, inflammation, urinary tract infections, digestion problems, etc. The antibacterial property of the extracts is mainly screened by measuring the zone of inhibition caused by phytochemicals present in the extracts by inhibiting the bacterial growth. These phytochemicals have shown inhibitory properties on bacteria but are not toxic to humans when consumed .

Objectives:

- ➢ To extract cinnamomum verum bark and nigella sativa seed extractions by acetone extraction method
- ➢ To screen the phytochemicals
- ➢ To isolate bacteria from spoiled tomato and lemon
- ➢ To perform biochemical tests on isolates
- ➢ To detect antibacterial activity of the extracts against bacterial isolates

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3. Materials And Methods:

3.1 Acetone extraction of *Cinnamomum verum* bark and *Nigella sativa* seeds extracts.

Take fresh cinnamon bark and nigella seeds and crush them into fine powder separately. Three flasks were taken and 10g of each powder were added to two conical flasks separately.Now 10g of mixed powder of cinnamon bark and nigella seeds were added to the third flask. 100ml of water is added to each flask.The contents were boiled properly at 100 °C for 10-15 minutes. Using funnel and whatman filter paper the contents were filtered.The filtrate obtained was placed in cold centrifugal tubes and centrifugation is carried at 10,000 rpm for 5-10 minutes.The supernatant is discarded and the palette is retained, 20ml of acetone is added to the palette and is preserved in refrigerator. (Kristin and Merlin, 2013)

3.2. Phytochemical analysis :

3.2.1. Wagner's reagent :

To 1 ml of sample 2-3 drops of Wagner's reagent was added and observed for the formation of reddish brown ppt.

3.2.2. Alkaline reagent :

Test for flavonoids, take 20% NaoH. To 1ml of sample 3ml of NaOH was added and observed for yellow colour and it becomes colourless when 0.5 N dil.HCl was added

3.2.3. Aqueous Ferric chloride reagent:

Test for phenols, 5% of aqueous Ferric chloride was added to 100ml of water. To 1ml of sample 5-6 drops of aq.ferric chloride was added and observed for the formation of deep blue or black colour.

3.2.4. Ninhydrin reagent:

Test for amino acids, 1% solution of Ninhydrin was added to 20ml acetone.To 1ml of sample 5-6 drops of Ninhydrin reagent was added and observed for the formation of blue colour.

3.2.5.Alcoholic Ferric chloride reagent:

Test for tannins, 10% of Ferric chloride was added to 100ml of absolute alcohol solution.To 1ml of sample 10% alcohol ferric chloride solution was added and observed for the formation of blue or green colour.

3.2.6. Salkowski's test reagent :

Test for terpenoids, to 20ml of chloroform 40ml of conc. H_2 SO_4 was added. To 1ml of sample a 0.5ml of chloroform and 3-5 drops of conc.H_2 SO_4 were added and observed for reddish brown ppt.

3.2.7.Quinones test reagent : Test for Quinones,conc.HCl. To 1ml of sample 0.5 ml of conc.HCl was added and observed for the formation yellow ppt

3.2.8. Coumarins reagent:

Test for coumarins, 10% NaOH. To 1ml of sample 1.5ml of 10% NaOH was added and observed for the yellow colour ppt.

3.2.9. Foam test :

Test for saponins, distilled water. To 1ml of sample 2ml of distilled water was added and shaken thoroughly and observed for the foam formation which remains for 10-15 min.(Asim Khan, Irena, 2017)

3.3. Isolation of bacteria from spoiled Tomato and Lemon

3.3.1. Serial dilution and plating

A ripened spoiled tomato and lemon were taken and 1ml of juice from the spoiled parts of the lemon and tomato were taken. 1ml of juice extract was serially diluted. The nutrient agar plates are prepared and 0.1ml of sample from the dilution rates of , 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} are platted by spread plate technique. Now the plates are incubated at 37 °C for 24-48 hrs and observed for the bacterial growth.

3.3.2. Isolation of pure cultures on selective growth media

The colonies from the mixed cultures were taken and cultured on selective growth media.

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The selective growth media like EMB agar (eosin methylene blue), Blood agar, Cetrimide agar, Mannitol salt agar are prepared for the isolation of few bacterial pure isolates

3.3.3. Gram staining of isolated colonies

A loopful of culture from the isolated colonies were taken and gram staining was performed to identify the gram nature of the isolated colony using crystal violet as primary stain, iodine as mordant and safranin as secondary stain using a microscope under 100x lens.

3.4. Biochemical tests

3.4.1. Lactose fermentation test

A loopful of inoculum from pure culture were taken into a sterile test tube containing phenol red lactose broth and was inoculated and is incubated at 37 °C for 24-48 hrs

3.4.2. IMVIC test

3.4.2.1. Indole test

A sterile test tube containing 5-6ml of tryptophan broth was taken and an inoculum from 18-24 hrs old culture was taken and inoculated. This is incubated at 37 °C for 24-34 hrs to this 0.5ml Kovacs reagent was added and observed for the results

3.4.2.2. Methyl red test

A pure culture of 24-48 hrs old was taken and inoculated on MRVP broth and was incubated at 37°C for 48 hrs with proper aeration. Now 5-6 drops of methyl red reagent was added and observed for the colour of the medium.

3.4.2.3.Voges-Proskauer test

A loopful of pure culture was taken and added to Voges-Proskauer broth and alpha naphthol and potassium hydroxide were added to the broth and observed for the colour change in the medium.

3.4.2.4. Citrate utilisation test

A loopful of pure culture was taken and inoculated on sodium citrate medium and bromothymol blue as a pH indicator and ammonium salt; it serves as a sole source for carbon and is incubated and observed for the colour change in the medium.

3.4.3. Catalase test

A clean glass slide was taken and loopful of culture was taken and was spread on the sterile slide. A drop of hydrogen peroxide was added and observed for the formation of the bubbles.

3.5. Antimicrobial activity

The screening for the antimicrobial activity was done by Agar well diffusion method. Nutrient agar plates are prepared and the organism i.e the inoculum from the test samples are taken and are spread on the agar by spread plate method. A sterile tip is taken and the wells are created on the agar plate accordingly depending upon the number of extracts that need to be placed. Now the acetone extracts that are prepared before are taken and acetone is taken as control. Take 0.5ml of acetone as a control and load it into the well carefully. Now take 0.5ml of cinnamon bark extract and load it into the other well. 0.5ml of Nigella seeds extract is taken and is loaded into the other well carefully. Now take 0.5ml of mixed extract of both cinnamon bark and nigella seeds and load it in another well carefully. The same process is repeated depending upon the number of bacterial isolates that are present. Now the plates are incubated carefully at 37°C for 24-48 hrs and observed for the zone of inhibition and measured.

4. Results:

4.1 Acetone extraction of Cinnamon bark and Nigella sativa seeds extraction The powder of Cinnamon bark and Nigella seeds was made into solution and boiled. The obtained filtrate was taken and subjected to centrifugation (Figure 1,2,3). To the resultant palette 20ml of acetone was added and Cinnamon bark and Nigella seed extracts were extracted. Results were found to be similar to (Kristin and John, 2003) who also noted the similar results with acetone extraction method.

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Figure:1.Boiling the extracts

Figure:2. Cooling down the extracts

Figure:3. Extraction of the filtrate

4.2. Phytochemical analysis

The presence of phytochemicals in the extracts of *Cinnamomum verum* bark and *Nigella sativa* seeds have been screened using various phytochemical reagents. The Cinnamon bark extract showed positive results (Figure 4) for the presence of Alkaloids, Phenols, Saponins, Tannins and Coumarins.The Nigella seed extract showed positive results (Figure 5) for the presence of Alkaloids , Phenols, Amino acids, Terpenoids and Quinones (Table 2). Results were found to be similar to Mohammad and Anne, 2014, who also noted similar results when they performed phytochemical tests on plant extracts.

Table 4. 2. Screening of phytochemicals present in Cinnamon bark and Nigella extract

	Name of the test	**Cinnamon bark Extract**	**Nigella seeds Extract**
1	Test for Alkaloids	+	+
2	Test for Flavonoids	-	-
3	Test for Phenols	+	+
4	Test for Saponins	+	+
5	Test for Amino acids	-	-
6	Test for Tannins	+	+
7	Test for Terpenoids	-	-
8	Test for Phlobatannins	-	+
9	Test for Quinones	-	-
10	Test for Coumarins	+	-

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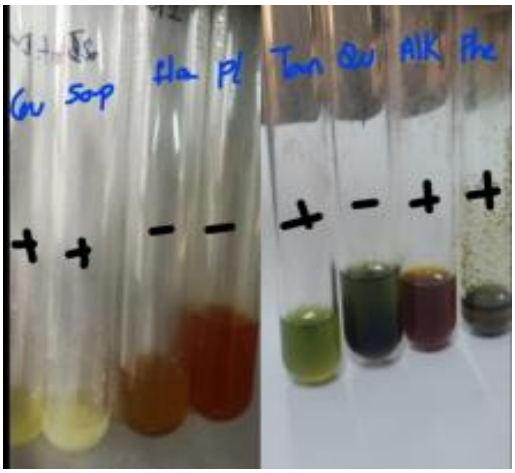

Fig 4: Phytochemical tests of cinnamomum bark extract

Fig 5: Phytochemical tests of Nigella sativa seeds extract

4.3. Isolation of Bacteria from spoiled Tomato and Lemon

4.3.1 Serial dilution and plating

The bacterial isolates from spoiled tomato and lemon were isolated by serial dilution and culturing them on nutrient agar plates by spread plate method and mixed colonies were isolated after 24 hrs of incubation at 37°C.

Different mixed cultures of bacteria were grown on the nutrient agar plates and the colony morphology was clear on 10^{-3} dilution plates (Figure 6,7). Similar results were found by, (Amir Mohammad and Shabir, 2001) who also noted similar bacterial growth when spoiled pineapple extract was inoculated on nutrient agar.

Fig 6. Bacterial isolates from spoiled tomato at the dilution rate of 10^{-3}

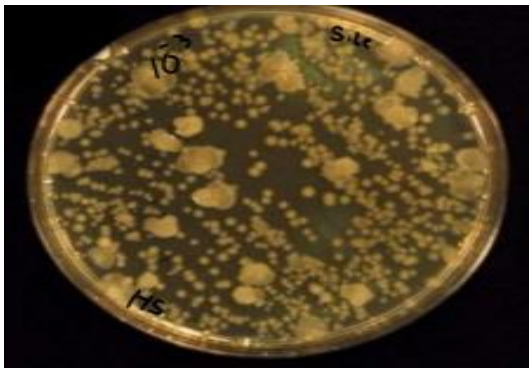

Figure 7. Bacterial isolates from spoiled lemon at the dilute rate of 10^{-3}

4.3.2. Isolation of pure colonies using selective growth media The colonies from the mixed cultures were taken and cultured on selective growth media and the Pure colonies were isolated using selective growth media like EMB (eosin methylene blue) for *E.coli* mannitol salt agar for *staphylococcus sp.* and cetrimide agar for pseudomonas sp. The isolate T1 which is from spoiled tomato showed metallic sheen colonies on eosin methylene blue agar. The isolate T2 from tomato showed green coloured colonies on

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cetrimide agar (Figure 8, 9 and 10). The isolate L1 from lemon showed yellow colonies of mannitol salt agar. Results were similar to(Rezaei and Hassan, 2014) who also observed similar growth of bacterial colonies on cetrimide agar.

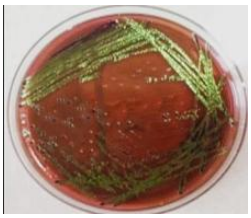

Figure 8. Isolate T1 on EMB agar from spoiled tomato

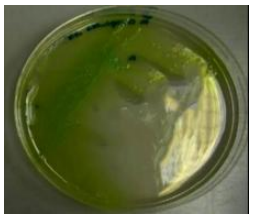

Figure 9. Isolate T2 on cetrimide agar from spoiled tomato

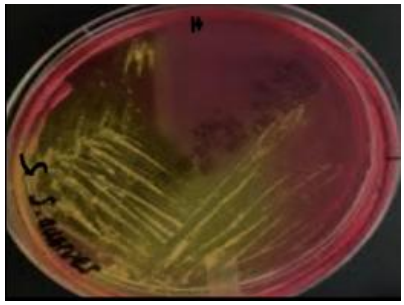

Figure 10. Isolate L1 on Mannitol salt agar from spoiled lemon

4.3.3. Gram nature of isolated pure cultures

Gram staining was performed and gram nature of the isolates were identified. The isolate T1 showed gram negative rods (Figure 11). The isolate T2 showed gram negative rods (Figure 12).The isolate L1 showed gram positive cocci in clusters (Figure 13). The isolate L2 showed gram positive cocci in bunches (Figure 15) when observed under a microscope of 100x lens (Table 4.3.3). Similar results were found by Ladislav and Irena, 2012, who also observed similar gram nature of bacterial isolates when gram staining was performed using crystal violet as primary stain and safranin as secondary stain.

Table 4.3.3 Gram nature of the isolates

S.No	Bacterial isolate	Gram nature
1.	Isolate T1	Negative rods
2.	Isolate T2	Negative rods
3.	Isolate L1	Positive cocci in clusters
4.	Isolate L2	Positive cocci in bunches

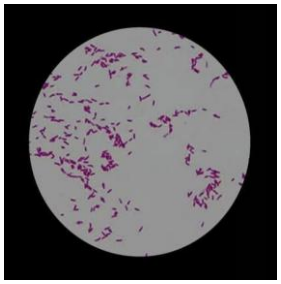

Figure 11. Isolate T1 gram negative rods

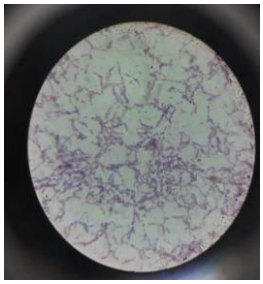

Figure 12. Isolate T2 gram negative rods

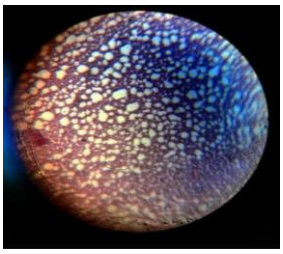

Figure 13. Isolate L1 positive cocci

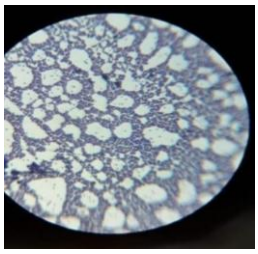

Figure 14. Isolate L2 positive cocci

4.4. Biochemical tests

Biochemical tests like lactose fermentation test, IMViC tests and catalase tests were performed on the isolated pure cultures and positive results were observed depending

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upon the type of isolates and its fermentation efficiency and its ability to utilise the substrate (Table 4.4). Isolate T1 showed positive test results for the indole test. Isolates T1, L1, L2 showed positive test results for MR test. Isolates L1 and L2 showed positive test results for the VP test. Isolates T2, L1, L2 showed positive test results for Citrate test. Isolates T1, T2, L1, L2 showed positive test results for Glucose and Catalase tests. Isolates T1, L1, and L2 showed positive test results for Lactose test.

Table 4.4 Biochemical tests

Biochemical Test	Isolate T1	Isolate T2	Isolate L1	Isolate L2
Indole test	+	-	-	-
MR test	+	-	+	+
VP test	-	-	+	+
Citrate test	-	+	+	+
Glucose	+	+	+	+
Catalase test	+	-	+	+
Lactose	+	+	+	+

4.5 Antimicrobial activity of Cinnamon bark extract and nigella sativa seed extract on bacterial isolates

The extractions from *Cinnamomum verum* bark and *Nigella sativa* seeds have shown antimicrobial activity on bacterial isolates T1, isolate T2 and ioslate L1 by forming the zone of inhibition around the colonies (Figure 15, 16 and 17) and the zone the zone of inhibition was measured. Isolate T1 was more sensitive towards Cinnamon bark and Nigella seed extracts. Isolate T2 was moderately sensitive towards Cinnamon bark and Nigella seed extracts. Isolate L1 was less sensitive towards Cinnamon bark and Nigella seed extracts (Table 4.5).Similar results were shown by (Nadeem and Rafiq Khan, 2013) when they observed zone of inhibition against their bacterial strains when Agar well diffusion method was performed.

Table 4.5 Antimicrobial activity by measuring the zone of inhibition

S.No	Extract	Bacterial isolate T1	Bacterial isolate T2	Bacterial isolate L1
1	Cinnamomum verum bark extract	6mm	4mm	2mm
2	Nigella sativa seeds extract	4mm	2mm	5mm
3	Mixed extract	5mm	3mm	2mm

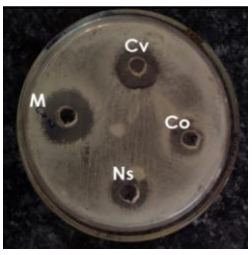

Figure 15 Antimicrobial activity on Isolate T1

Figure 16. Antimicrobial activity on Isolate T2

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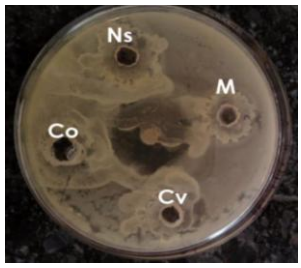

Figure 17. Antimicrobial activity on Isolate L1

5. CONCLUSION : The phytochemicals present in the *Cinnamomum verum* bark extract and *Nigella sativa* seeds extract were screened using various phytochemical tests and are identified. When the antimicrobial activity of these extracts against the bacterial isolates was performed using Agar well diffusion method it was observed that the zone of inhibition formed by cinnamomum verum bark extract is more than nigella sativa seeds extract. The *Cinnamomum verum* bark extract has better antibacterial properties than *Nigella sativa* seeds extract by forming a better zone of inhibition on bacterial colonies. The present study indicates that isolate T1 *E.coli* was more sensitive towards Cinnamon bark and Nigella seeds extract, isolate T2 *pseudomonas sp.* was moderately sensitive towards Cinnamon bark and Nigella seeds extracts, isolating T3 *Staphylococcus sp* . was less sensitive towards Cinnamon bark and Nigella seeds extracts.

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