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Research Article

Antibacterial Activity and Phytochemical Analysis of Aqueous Leaf Extract of Azadirachta Indica on Bacteria Isolated From Cooked Foods (Rice and Beans) Among Street Hawkers

Odeh AP[1]*, Zainab AB[2] and Usman AA[3]

[1]*Nutrition International, PHCDA Dutse, Jigawa State*

[2]*Department of Microbiology, Sokoto State University*

[3]*Department of Microbiology, Usmanu Danfodio University Sokoto State*

***Corresponding author:** Odeh AP, Nutrition International, PHCDA Dutse, Jigawa State

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Abstract

The present study was carried out to screen the Phytocompounds and as well evaluate the antibacterial activity of the aqueous leaf extracts of the neem tree (Azadirachta indica) on bacteria isolated from prepared spoilt rice and beans. Aqueous extract of leaves of A. indica (Neem) were tested against Staphylococcus gallinarum, Bacillus lentus, Clostridium sordellii, Bacillus firmus and Clostridium botulinum that were isolated from the spoilt cooked rice and Beans. The Neem plants were collected fresh from UDUSOK/permanent site at the Biological garden and transferred to herbarium for authentication before allowing to dry. The activity of the extracts was studied and determined at different concentrations of 100mg/ml, 150g/ml, 200mg/ml and 250mg/ml on the bacteria using agar well-diffusion method. The susceptibility of the bacteria to the extracts was determined by measuring the diameter of inhibition zones formed around the plates. The test analysis showed different zones of inhibition that increased with the increase of the plant extracts concentration on the test organisms. In this study, B. lentus and C. sordellii were susceptible to the extracts at certain concentrations and S. gallinarum were susceptible to the extracts at all concentration while, B. firmus and C. botulinum were resistance to the effect of the extracts at all concentrations used as such there was no zone of inhibitions observed. The results showed that the effectiveness of the extracts was dependent on the concentration used thus the increase of extract concentration increase with the zone of inhibition.

Keywords: Aqueous Extracts; Susceptible; Zone of Inhibitions; Concentration

Introduction

Plants provide an alternative strategy in search for new drugs, there is a rich abundance of plants reputed in traditional medicine to possess protective and therapeutic properties [1]. Bacterial resistance to antibiotics represents a serious problem for clinicians and pharmaceutical industry. Therefore great efforts are being made to reverse this trend, of which one of them is the widespread screening of medicinal plants from the traditional system of medicine hoping to get some newer, safer, and more effective agents that can be used to fight infectious diseases Natarajan et al. [2]. Azadirachta indica is one of such medicinal plants belonging to the Meliaceae family (Mahogany family) and is indigenous to southern Asia. Azadirachta indica, commonly known as neem, has attracted worldwide prominence in recent years, owing to its wide range of medicinal properties and neem has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds have been isolated from different parts of neem. All parts of the neem tree leaves, flowers, seeds, fruits, roots and bark have been used traditionally for the treatment of inflammation, infections, fever, skin diseases and dental disorders. The medicinal utilities have been described especially for neem leaf Biswas et al. [3]. Neem leaf and its constituents have been demonstrated to exhibit

immunomodulatory,anti-inflammatory,antihyper-glycaemic,anti-ulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties Talwar et al. [4].

The Neem Azadirachta indica A. Juss [5] is an evergreen robust tree, belonging to the family Meliaceae. It is mostly found in the tropics and sub-tropical region of the world (Africa and Asia). It has medium to large size and have brown to dark grey bark and a dense rounded arrow of pinnate leaves Ogbuewu [6]. The plant has an extensive deep root system which is responsible for their survival in arid and semi-arid area of the world. The chemical constituent of the neem plant is a blend of 3 to 4 actively related compounds, with over 20 lesser ones. Presently over 250 compounds has been identified. The important classes include triterpenoids and limuloids: saladucin, valassin, meliacin, NimbinNimbicin, geducin, Azadirachtin etc. (Koul et al. [7,8]; Uko and Kamal [9]; Lale [10]). Accordingly, all parts of this plant are useful and have been used in treatment of diseases ranging from teeth decay, ulcers, swollen liver, malaria, dysentery, diarrhoea etc. (Ogbuewu, [6]; Allameh et al. [11]; Mossini et al. [12]). Aqueous of Neem leaf extract has a good therapeutic potential as anti-hyper glycaemic agent in IDDM and NIDDM Sonia and Srinivasan [13]. (Abu et al. [14]) suggests that anti-inflammatory effect of Neem extract is less than that produced by dexamethasone. Neem leaves has antibacterial properties and could be used for controlling airborne bacterial contamination in the residential premise, (Saseed et al. [15]; El-Mahmood et al. [16]). The trends in the development of resistance by Microorganism particularly bacteria to modern medicine is of immediate concern, as this makes the disease to be of imminent danger to the populace. Thus, the need arises to determine the phytocompounds present in the Neem tree so as to determine the antibacterial activity on the bacteria associated with spoilt foods.

Materials and Methods

The fresh leaves of the Azadirachta indica plant, was obtained from permanent site of Usmanu Danfodiyo University Sokoto. The plant leaves were taken to Herbarium section in the Biological sciences Department of Usmanu Danfodiyo University Sokoto and were authenticated. It was later transferred to the Microbiology Laboratory of Usmanu Danfodiyo University Sokoto for further analysis. The Fresh leaves were sorted to remove the dead ones, and wash without squeezing to remove debris and dust particles. A large quantity of the leaves were sun-dried for four days. The dried leaves were mill to get a course powder used for the extraction. 24g of the powder was soaked in 250ml of distilled water and the mixture allowed to stand for 24 hours after which muslin cloth was used to filter the plant residues and the filtrate obtained was further purified by filtration through Whatman No 1 filter paper and then screened for its Phytochemical components, the filtrate was then evaporated to obtain the extract (Atata et al. [17]). cooked food (rice and beans,) was kept in a container at room temperature for about 3 days so as to allow spoilage, the sample was homogenize by putting into a sterilized universal bottle containing distilled water and was blended. Serial dilution of the sample was done to obtain 7 dilutions factor (101 to 107), by diluting 1ml in 9 mls of sterile peptone water, first from stock culture, then from subsequent dilutions. Exactly 0.1 ml of 106 and 107 dilution were then inoculated on Nutrient agar using spread plate technique. The petridishes were incubated at 37°C for 24 hours. After which the colonial characteristics noted and each colonial morphology subcultured to obtain pure isolates.

Gram Staining

A drop of distilled water was placed on the center of a clean glass slide. From the subcultures, a portion from each colony type was picked and emulsified into the drop of water to form a smear using a sterile wire loop. The smear was allowed to dry and was heat fixed. The fixed smear was flooded with Crystal Violet Stain and allowed to stand for 60 seconds and was washed with distilled water. Lugol's iodine was added and allowed to stand for another 60 seconds and washed with distilled water. This was stained with Acetone for 30 seconds before washing with water. Finally the smear was decolorized with Safranin and rinsed with distilled water. This was done for all the colonies observed. The slide were then allowed to dry and viewed microscopically for morphological characterization of the bacteria colonies using oil immersion objectives lenses (Cheesbrough [18]).

Biochemical Characterization

Catalase Test: A small portion of the bacteria subculture cell was picked using a wire loop and introduced into a drop of hydrogen peroxide on a glass slide. The present of catalyst was observe by which resulting air bubbles indicate a positive catalase while the absent of bubbles indicate negative catalase (Oyeleke and Manga [19]).

Citrate Utilization Test: An inoculum of the isolate was inoculated into the agar and incubated at 37°C for 48hrs (Cheesbrough [18]). A ready-to-use dehydrated Simmons Citrate Agar was used to prepare slopes of Simmons citrate medium in bijou bottle, according to standard. A sterile wire loop was used to inoculate the slope with a saline suspension of the test of the test organism by streaking over the entered surface of the slant (i.e in a zigzag manner to cover the surface) and then stabbing deep into butt and incubated for 24 to 48hrs. The media was observed for color change (Cheesbrough [18]).

Motility Test: A small portion of the isolate was stab into triple sugar iron (TSI) medium and it was incubated at 370 c for 24 hours. Motility was observed by the spread of the organisms outward of the stabbed area (Oyeleke and Manga [19]).

Indole Production Test : A small portion of the isolate growth was inoculated in 5ml of sterile peptone water enriched with 1% tryptophan and was incubated at 37°C for 24-48 hours in ambient air and 0.5ml of kovocs reagent was added and shacked gently. In the positive test indole, the presence of red layer at the surface of the test tube indicate positive, while yellow layer at the surface of the test tube indicate negative e.g Klebsiella pneumonia (Oyeleke and Manga [19]).

Urease test: A sterile wire loop was used to incubate the organisms in slant medium in test tube contains area and the indicator phenol red by stabbing through the center of the medium. The test tube was incubated at 37°C for 24 hours. When the organisms produce urease enzyme, the color of the slant change from light to orange to magenta, it indicates positive while when the organisms produces urease the agar slant and butt remain light orange (medium retairs) original color (Oyeleke and Manga [19]).

Methyl Red Test (MR): A wire loop full of each isolate will be inoculated onto glucose phosphate peptone water medium and then incubate at 370 c for 48 hours. Few drops of methyl red will be added to the culture (Cheesebrough [18]).

Voges-Proskauer Test (VP): A wire loop full of each isolate was inoculated into Glucose Phosphate Peptone Medium and incubated at 37°C for 48 hours. 5 drops of 40% KOH was added by 15 drops, Naphtol in ethanol was added, shake and place in a sloppy position and examined for 30-60 minutes (Cheesebrough [18]).

Hydrogen Sulphide (H2S): Small portion of isolate was taken with straight wire loop to make a deep inoculation in the H2S medium. The tube was incubated at 37°C for 24 hours. The present of black precipitate in the medium indicate positive result while absent of precipitate indicate negative result (Ochei and Kolhatkar [20]).

Triple Sugar Iron (TSI): Triple sugar iron was prepared and with a sterile needle the culture from the solid medium was streaked on the surface of the slant and the butt was subbed three times and incubated at 37°C for 24 hours. Gas formation was determined by the appearance of one or several bubbles. In the butt, vigorous gas formations can result in cracks or it may be pushed from the bottom. TSI glucose fermenters were indicated by yellow color at the butt. If both the butt and slope were yellow, it indicated fermentation of lactose and sucrose. Fermentation of lactose and sucrose but not glucose was detected with red butt and yellow slope (Oyeleke and Manga [19]). Little portion of each isolate was stabbed in to triple sugar iron agar and incubated at 37°C for 24 hours. Motility was observed by the spread of the organisms outwards from the stabbed line (Ochei and Kolhatkar [20]). Little portion of each isolate was inoculated by streaking and stabbing into triple sugar iron and incubated at 37°C for 24 hours. Evolution of blackening on the medium indicated a positive test while absence of blackening indicated negative test result (Cheesbrough [18]).

Preparation of the McFarland Scale: 1% solution of sulfuric acid was prepared by adding 1ml of concentrated Sulfuric acid to 99ml of water and agitated. 1% of Bacl2 solution was prepared by dissolving 0.5g of anhydrous Bacl2 in 50ml of distilled water. They were then mixed in the following proportions 0.05/99.5ml, 0.1/9.9ml, 0.2/9.8ml, 0.3/9.7ml, 0.4/9.6ml and 0.5/9.5ml (1% Bacl2 / 1% H2SO4).the tubes were sealed tightly and stored in a dark place, the inoculum suspension was compared with the scale (Cheesbrough [18]).

Antibacterial Activity Assay: The determination of antibacterial activity assay was done using the agar diffusion method (Cheesbrough [18]). The bacteria were each inoculated into sterile nutrient agar and it was incubated at 37°C for 24 hours. Then the loop of inoculums was transferred into 5ml of nutrient broth and was incubated at 37°C for 6 hours. Ditch well of 6mm in diameter was made with a sterile cork borer and the inoculum of the test bacteria was spread on the solid plates with the aid of the sterile swab moistened with bacterial suspension. 100mg/ml, 150mg/ml, 200mg/ml and 250mg/ml of aqueous extract of Azadirachta indica were each poured in the wells respectively. The plates were incubated at 37°C for 24 hours, the presence or absence of zone of inhibition was observe in millimeters (mm), (Girish and Satish [21]).

Determination of Minimum Inhibitory Concentration (MIC): MIC of the extracts were carried out using the tube dilution technique described by (Cheesbrough [18]). A double fold sterile dilution was made using Muller-Hilton Broth. Equal volume of Muller-hilton broth and extracts were dispensed into sterile tubes. A quantity of 0.1ml of standardized inoculums was added to each test tube which was incubated aerobically at 37°C for 2hours. A tube with broth and inoculums served as organism control. A tube with broth and extracts served as extract control. The lowest concentration of the extracts which inhibited the microbial growth was recorded as the minimum inhibitory concentration (MIC).

Determination of Minimum Bactericidal Concentration (MBC): Sterile Muller-hilton agar plates were inoculated with samples from each of the test tube that showed visible growth from the MIC test. The plates were incubated for 24hours at temperature of 37°C. The lowest concentration of the extracts that yielded no was regarded as the minimum bactericidal concentration (MBC) (Cheesbrough [18]).

Phytochemical Screening

Twenty grams (20g) of neem (Azadirachta indica) leaves was weight on weighing balance and transferred into beaker containing 200ml of distilled water, it was allowed to be soaked for exactly 24hrs in order to determine the presence of phytochemical compounds. The extracts were evaluated for the presence of alkaloids, balsams, flavonoids, anthraquinones, cardiac glycosides, tannis, steroids, saponins and Volatile Oil (Oyeleke and Manga [19]).

Test for Alkaloids: 2ml of extracts was stirred with 2ml of 10% aquences hydrochloric acid. 1ml was treated with few drops of Wanger' reagent and second 1ml was treated similarly with Mayers reagent. Precipitation was observed for the presence of alkaloids (Harbone et al. [22]).

Test for Volatile Oil: One ml of the extract was mixed with dilute HCL acid. The appearance of white precipitate indicates the presence of Volatile Oil (Evans and Gupta [23]).

Test for Tannins (ferric chloride test): 5% of Ferric chloride solution was added by drop by drop into 2ml of the extracts and immediate dark green color is an evidence for the presence of Tannis (Harbone et al. [22]).

Test for Steroids: 2ml of the extracts was dissolved in 2ml of chloroform and 2ml of Sulphuric acid were carefully poured

through the wall of the test tube, the lower layer was formed. A reddish brown color was observed for the presence of steroid (Harbone et al. [22]).

Test for Flavonoids: Three milligrams (3mg) aliquot of the filtrate and one milligram of ten percent (10%) NaOH (Sodium Hydroxide Solution) were added, when yellow colour is developed then it indicates the possible presence of Flavonoids (Oyeleke and Manga [19]).

Test for Saponins: Ten (10) ml of distilled water were added to 0.5cm^3 of the extracts, it was shacked vigorously with the test tube for 2minutes. The presence of frothing indicates Saponins presence (Oyeleke and Manga [19]).

Test for Cardiac Glycosides (Keiller killiani's test): Two ml of 3.5% ferric chloride solution were added and allowed to stand for one minute. One ml of concentrate H2SO4 was carefully pour dawn the wall of the tube so as to form lower layer. A reddish brawn ring when formed at the interface indicate the presence of cardiac glycoside (Oyeleke and Manga [19]).

Test for Anthraquinones: Two (2.0) ml of the aqueous extracts were mixed with 10ml of benzene, and five (5.0) ml of ammonia solution was added and shacked. Presence of pink, red or violet colour in the ammoniac (lower) phase indicates the presence of anthraquinones (Harbone et al. [22]).

Test for Balsams: The extracts were mixed with the 90% volume of ethanol. Two drop of alcoholic ferric chloride solution was added to the extracts. A dark green color indicates the presence of balsams (Oley et al. [24]).

Results and Discussions

In this study, five different bacteria were isolated with respect to the spoilt food although they have a considerable percentage occurences on the spoilt foods. The bacteria identified are B. lentus, B. firmus S. gallinarum C. sordellii and C. botulinum respectively. The occurrence of some of these organisms in the covered spoilt foods might be due to their ability to form spores. However the occurrence of this bacterial isolates is presented in Table 1. The antibacterial activity of aqueous leaf extracts of Azadirachta indica determined at concentrations of 100mg/ml, 150mg/ml, 200g/ml and 250mg/ml respectively is presented in Table 2. The antibacterial activity of the aqueous leaf extracts against Staphylococcus gallinarum was recorded at concentration of 100mg/ml (12mm), 150mg/ml (13mm), 200mg/ml (15mm) and 250mg/ml (19mm). The activity of the aqueous leaf extracts against Bacillus lentus and Clostridium sordellii was recorded at concentration of 200mg/ml (9mm) and 250mg/ml each. This indicates that from 100mg/ml as the concentration was increased, the rate of the antibacterial activity increases. Uwimbabazi et al. [25] evaluated the antibacterial activity of the aqueous and ethanolic extracts of leaf and bark of A. indica stating that "the bactericidal activity increases with the increase on the extract concentration", this is also same in the report of Esimone et al. [26], which says that extract of plants inhibit the growth of various microorganisms at different concentrations with significant differences in the diameter of inhibition zones. This disagrees with the report by Rizza et al., (2001) whom according to them there were no significant difference on the effect of different concentrations of the aqueous leaf extracts of A. indica. In this study the antibacterial activity of the aqueous extracts on B. firmus and C. botulinum on the specified concentrationswere 100mg/ml (0,00mm), 150mg/ml (0,00mm), 200mg/ml (0,00mm) and 250mg/ml (0,00mm) each which shows that they were resistant to the extracts at the concentrations used. This may be as a result of some phytocomponds that were in trace amount in the extracts. (Tayloe et al. [27]) active compounds may be present in an insufficient quantity in extracts to show activity with the concentration employed. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined respectively as to determine the minimum concentration of the extracts which is active against the test isolates as shown in Table 3 and Table 4, It was discovered that at concentration of 150mg/ml the MIC was noted on both B. lentus and C. sordellii. While, at 75mg/ml MBC of the extracts was noted on the bacteria. This shows that the leaf extracts kill the organisms at 150mg/ml but, at concentration below 150mg/ml it may only stop the growth but no killing activity. The antibacterial activity of the aqueous leaf extracts may be as a result of presence of phytocompounds found in the extracts, though some of the phytocompounds were found in trace amount as shown in Table 5. The phytocompounds include; Saponins, Flavonoids, Alkaloid, Volatile oil, Cardiac glycoside, Steroids, and Tannins, the presence of these phytocompounds suggests potential for the plant extracts as a source of phytomedicine (Bala [28]). The antibacterial activity is greatly influenced by the presence of Saponins and Flavonoids [29-52].

Table 1: Bacteria identified and their percentage frequency of occurences

Bacteria	No of occurences	% frequency of occurences
B lentus	2	8 7
B firmus	5	21 74
S gallinarum	3	13 04
C sordellii	2	8 69
C botulinum	11	47 83
Total	23	100

Table 2: Antibacterial activity of the extracts at different concentrations on the bacteria.

Bacteria	Concentrations(mg/ml)			
	100	150	200	250
	Diameter of inhibition zones(mm)			
B firmus	0	0	0	0
C botulinum	0	0	0	0
B lentus	0	0	9	10
C sordellii	0	0	10	12
S gallinarium	12	13	15	19

Table 3: Minimum Inhibitory Concentration (MIC) of an aqueous leaf extracts of Azadirachta indica on Bacillus lentus, Clostridium sordellii and Staphylococcus gallinarum.

S/N	Bacteria	Concentration (mg/ml)								MIC
		300	150	75	37.5	18.75	9.375	4.69	2.34	(mg/ml)
1	B lentus	-	-	+	+	+	+	+	+	150
2	C sordellii	-	-	+	+	+	+	+	+	150
3	S gallinarum	-	-	-	+	+	+	+	+	75

Key: - =No Growth; + =Growth Found.

Table 4: Minimum Bactericidal Concentration (MBC) of an aqueous leaf extracts of A. indica on S gallinarum, B lentus and C sordellii.

S/N	Bacteria	Concentration (mg/ml)			MIC
		300	150	75	(mg/ml)
1	S gallinarum	-	-	+	150
2	B lentus	-	-	+	150
3	C sordellii	-	-	+	150

Key: - =No Growth; + =Growth Found.

Table 5: Phytochemical analysis of the aqueous leaf extract of A. indica.

Phytocompounds	Results
Flavonoids	+++
Tannins	+
Saponins	+++
Cardiac glycosides	+
Steroids	++
Anthraquinone	ND
Alkaloids (Wagner and Meyers Test)	++
Volatile oil	ND
Balsams	+

Keys: + = Present in trace amount, ++= Present in moderate amount, +++= Present in high amount, ND= Not Detected

Conclusion

Antibacterial activity of the aqueous leaf extracts of A. indica on both B. lentus and C. sordellii was recorded at concentration of 200mg/ml and 250mg/ml each, and the activity of the extracts was also observed on S. gallinarum at concentration of 100 mg/ml, 150mg/ml, 200mg/ml, and 250mg/ml, while Bacillus firmus and Clostridium botulinum were resistant at all concentrations used. The extracts have minimum inhibitory concentration (MIC) of 150mg/ml on both B. lentus and C. sordellii.Having an MIC of 75mg/ml on S. gallinarum and MBC of 150mg/ml was noted on the bacteria. The A. indica leaves possess an antibacterial activity due to the presence of bioactive compounds found present even though, some of these compounds were found in trace amount. Thus, Further research is needed to investigate the activity of neem leaf extracts using other solvents to obtain more information of the

effectiveness of the plant leaf. It is important to isolate and separate the bioactive compounds responsible for this antibacterial activity using advance scientific techniques and Spoilt food should be discarded not consumed.

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