Effects of Hot and Cold Leaves Extracts of *Azadirachta Indica* on *Xanthomonas Campestris*

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ABSTRACT

There is a worldwide interest in searching for safe and effective novel antibacterial compounds of plant origin for the control of plant pathogenic bacteria which is responsible for the great impact on the growth and productivity of agricultural crops. In this study, the effects of hot and cold leaf extracts of Azadirachta indica on Xanthomonas campestris was carried out. The organism was isolated from symptomatic pepper fruits and leaves using Yeast Dextrose Chalk Agar (YDCA). The isolate was characterized and identified using its colonial description, Gram staining and biochemical characteristics. The phytochemical constituents of the leaf extract of Azadirachta indica were determined quantitatively using spectrophotometeric method. The antibacterial activity of the leaf extracts was carried out using agar-well diffusion method. Tube dilution method was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using double-fold serial dilution at concentrations 31.25mg/ml to 500mg/ml. The phytochemical analysis of Azadirachta indica leaf extracts revealed the presence of tannins, flavonoid, alkaloids, steroids, cardiac glycosides, phenolics and saponins. The results of the present study revealed the pronounced activities of the leaf extracts against the tested organism, which differed significantly ($p < 0.05$) from ciprofloxacin. The activities of hot n-hexane (8.33mm), ethanolic (7.00mm), petroleum-ether (6.33mm) and aqueous (6.00mm) leaf extracts differed significantly ($p < 0.05$) from activities of cold ethanolic (22.67mm), n-hexane (20.67mm), petroleum-ether (19.33mm) and aqueous (8.33mm) leaf extracts. The results of the MICs and MBCs of the leaf extracts of Azadirachta indica showed that the cold extracts exhibited inhibitory and cidal activities against the organisms. This study recommends that cold extraction using organic solvents is suitable for studying the antibacterial activity of Azadirachta indica and Azadirachta indica leaf extracts could serve as alternative pesticide to plant diseases caused by Xanthomonas campestris.

Key words: Antibacterial, *Azadirachta indica, Xanthomonas campestris,* Phytopathogenicity, extracts

INTRODUCTION

Phytopathogenic bacteria are those bacteria that cause plant disease (Jackson, 2009). Many plant pathogenic bacteria spend most of their parasitic life in a nutrient limited environment that is guided by plant defenses known as apoplast (Jackson, 2009). Plant diseases are a major problem worldwide for agriculture with bacterial plant pathogens making major contributions. Many bacterial plant pathogens produce toxins or enzymes which are potent and directly harm plant cells and contribute significantly to disease and symptom development such as chlorosis and necrosis (Muhldorfer and Shafer, 2011). According to Reberto (2008), among 7,100 classified bacteria, roughly 150 species causes disease to plants and obtain their nutrients from this plant. Bacteria diseases of plants are most frequent and severe in tropical and subtropical countries where warm and humid conditions are ideal for bacterial growth and huge losses are recorded annually in these countries.

Studies have shown that most of these phytopathogenic bacteria cause diseases in man and these poses serious threat to public. In recent years, multiple drug resistance in human pathogenic bacteria has been developed due to indiscriminate use of commercial antibiotics in handling these diseases caused by phytopathogenic bacteria in man. The use of synthetic chemicals to control phytopathogenic bacteria is also difficult due to scarcity of the chemical agents, toxicity associated with the use of these agents, high cost of the agents, resistance of the some bacteria to these agents and inadequate control agents associated with the use of these agents .Over the last centuries, intensive efforts have been made to discover clinically useful antibiotics and effective, safe, biodegradable pesticides form natural origin (Ahmed, Mehmood, Z. and Mohammad, 1998; Perumalsamy and Ignacimuthu, 2000).

The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines, mostly from plant for their primary health care needs. Plants are found to be an effective reservoir for bioactive molecules and can provide valuable source for discovery of natural pesticides and natural

antibiotics. Plants have an almost limitless ability to synthesize secondary metabolites which serve as plant defense mechanism against predation by insects, herbivores and microorganisms mostly bacteria such as *Xanthomonas* spp. which is one of the predominant bacteria specie associated with plant disease (Umamaheswani, Shree and Aparma, 2008).

Azadirachta indica belongs to the family Meliaceae and has a long history of use in folkmedecine as treatment against various ailments. Reports on pharmacological and biological properties are numerous (Sonia and Snnivasan, 1999). The hypoglycemic actions of the leaves stem bark and seeds have been articulated in a review by Biswas, Ishita, Ranajit, and Uday, (2002). Ebong, Atangwo, Eyong and Egbung, (2008) indicate recently in their studies, the relative antidiabetic efficacy of this plant extracts when combined with *Vernonia amygdalia*. Despite these, there is little or no detailed scientific report on the use of this plant extracts as natural pesticide, which will transform the field of agriculture, providing more tools and resources for reducing plant diseases. The study was undertaken to determine bactericidal effects of hot and cold leaf extracts of *Azadirachta indica* on *Xanthomonas campestris*.

MATERIALS AND METHODS

Collection of Leaves Samples: Leaf samples were collected from *Azadirachta indica* plant growing at Our Saviour's hostel at Anambra State University, Uli Town, Ihiala Local Government Area, Nigeria

Figure 1: Leaves of *Azadirachta indica* plant in its natural environment

Preparation of Leaves for Extraction: The leaves were washed and dried under shade at room temperature for fourteen days. The dried leaves were ground into powder using electric blender. It is then weighed and kept ready for extraction of active ingredients (Iheukwumere, Uba, and Ubajekwu, 2012).

Extraction Procedures

Cold extraction: Extraction was done in two methods using both hot and cold extraction method. Cold extraction was done by weighing out 20g portion of the leaves into 200ml of the extracting solvents with shaking periodically for three days. The resulting extracts were subsequently filtered using Whatman NO I filter paper. The extracts were then evaporated to dryness at room temperature in a steady air current (Iheukwumere *et al*. 2012).

Hot extraction: Hot extraction was done by weighing out 20g of the powdered dried leaves and then the leaves were wrapped with a thick filter paper and then loaded into a thimble. Then 200ml extraction solvent to be used is take into a distillation flask and is then equipped into a condenser. The solvent is heated to reflux. The solvent vapour travels up to distillation and floods into the chamber housing the thimble of sold. The condenser ensures that

any solvent vapor cools and drops back down into the chamber housing the sample. The chamber containing the 20g powdered dried leaves are slowly filled with warm solvent. Some of the desired compound will then dissolve in the warm solvent when the soxhlet chamber is almost full; the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times; over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After extraction, the solvent is removed by evaporation thus yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble and is usually discarded. The solvent was then exposed to dryness at room temperature in a steady air current (Iheukwumere *et al*. 2012).

Determination of Extractive Value: The concentrations of Ethanol, n-hexane, petroleum ether and aqueous leaf extracts of both hot and cold extracts was determined by evaporating dish of known weight in an oven to dryness. The dish containing the residues was allowed to cool and then weighed. The weight of the residue was obtained by subtracting the weight of the empty by dish from the weight of the dish and residue. This process was repeated in duplicate.

Maintenance of Test Organism: The isolated test organisms were used for the antibacterial sensitivity testing. Prior to use, the organisms were subcultured on nutrient agar slant at 37^0C for 24 h.

Antibacterial Assay: This was carried out using broth cultures of the test organisms, a modified method of Atata, (2003). Each labeled medium plate was uniformly inoculated with the test organism using pour plating method. A sterile cork borer of 5mm diameter was used to bore holes on the inculcated plate, then 0.5ml of various concentrations of the extract were dropped into each labeled well. After that, the plates were incubated at 28^0C for 24 h. Antibacterial activity was determined by measuring the diameter zone of inhibition (mm) produced after incubation. 500mg/ml of ciprofloxacin was used as control.

Determination of Minimum Inhibitory Concentration (MIC): In determination of MIC, various concentrations of the extract both hot and cold were determined using double-fold serial dilution each dilution was assayed against the tests bacteria using the tube dilution method. Here 1.0ml of the test organism was added into each dilution of the extracts and incubated at 28^0C for 24 h concentrations able to inhibit any visible bacterial growth was recorded as the MIC (Iheukwumere *et al*. 2012).

Determination of Minimum Bactericidal Concentration (MBC): Here equal volume of various concentration of these tubes that did not produce any growth from the MIC were sub-cultured on fresh sterile plate containing Muller Hinton agar and incubated at 28^0C for 24 h. The highest dilution that yielded no single bacterial colony was recorded as the MBC (Iheukwumere *et al*. 2012).

RESULTS

The results of the quantitative phytochemical analysis of the leaves extract of *Azadirachta indica* was shown in table 1. The result revealed the presence of alkaloids, saponins, tannins, flavonoids, phenolics, steroids, and cardiac glycosides. The phytochemicals may be responsible for the antibacterial activity of the leaf extracts show the characteristics and identity of *Xanthomonas campestris* isolated from pepper leaf using nutrient agar, Yeast Dextrose Chalk Agar and Phosphate Buffer Saline broth media. It was characterized and identified using the Grams reaction, colonial morphology and biochemical tests (table 2). The diameter zones of inhibition of hot and cold extracts of *Azadirachta indica* against *Xanthomonas campestris* are shown in table 3. The results revealed the pronounced activities of the extracts against the organisms. The inhibitory activities of the leaf extracts differed significantly ($p < 0.05$) from ciprofloxacin (control). The activity of n-hexane leaf extract was most, followed by ethanolic and petroleum ether leaf extracts, and aqueous leaf extract was the least for the hot leaf extracts. The activity of ethanolic leaf extract was the most, followed by n-hexane and petroleum- ether leaf extracts, and aqueous leaf extract was the least for the cold leaf extracts. The results of the present study revealed that the cold leaf extracts inhibited the test organism more than the hot leaf extracts, and the inhibitions of the cold extracts differed significant ($p < 0.05$) from that of the hot extracts. The Minimum Inhibitory Concentration (MICs) and Minimum Bactericidal Concentrations (MBCs) of hot and cold leaf extracts of *Azadirachta indica* on *Xanthomonas campestris* are shown in table 4. The results of hot leaf extracts showed no activity against the organism except n-hexane leaf extracts which exhibited slight inhibitory activity against the organism. The cold leaf extracts of *Azadirachta indica* showed pronounced

activities against the organism. The ethanolic, petroleum-ether and n-hexane leaf extracts exhibited similar inhibitory and bactericidal activities against the organism, and their activities differed significantly ($p < 0.05$) from that of ciprofloxacin.

Table 1: Phytochemical constituents of *Azadirachta indica* leaf extracts

Phytochemical	Amount (%)
Alkaloids	1.82
Saponins	1.88
Tannin	8.74
Flavonoid	0.78
Phenolics	0.14
Steroids	0.52
Cardiac glycosides	0.11

Table 2: Characteristics and identity of the test organism isolated

Parameter	*Xanthomonas campestris*
Appearance on agar plate	Smooth, yellow, shiny, mucoid on YDCA
Gram reaction Morphology	Straight rod
Catalase	+
H_2S	+
Urease	+
Oxidase	_
Motility	+

Key: YDCA: Yeast Dextrose Chalk Agar H_2S = Hydrogen sulfide + = Positive - = Negative

Table 3: Diameter zones of inhibitions (mm) of hot and cold extract of *Azadirachta indica* against *Xanthomonas campestris* using 5mm cork borer

Extract (500mg/ml)	*Xanthomonas campestris*	
	Hot extract	Cold extract
EEA	7.00 ± 1.06	22.67± 1.06
PEA	6.33 ± 0.47	19.33± 0.47
NEA	8.33± 0.47	20.67± 0.47
AEA	6.00± 0.82	8.33± 1.25
CPX (500mg/ml)	36.00± 0.82	36.00± 0.82

Key: EEA = Ethanolic extract of *Azadirachta indica;* PEA = Petroleum ether extract of *Azadirachta indica;* NEA = n-hexane extract of *Azadirachta indica;* AEA = Aqueous extract of *Azadirachta indica;* CPX = Ciprofloxacin

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of hot and cold extracts of *Azadirachta indica* on *Xanthomonas campestris*

Extract	Hot Extract		Cold extract	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
EEA	-	-	250	500
PEA	-	-	250	500
NEA	500	-	250	500
AEA	-	-	500	-
CPX	62.50	250	62.50	250

Key: EEA = Ethanolic extract of *Azadirachta indica*; PEA = Petroleum ether extract of *Azadirachta indica*; NEA = n-hexane extract of *Azadirachta indica*; AEA = Aqueous extract of *Azadirachta indica*; CPX = Ciprofloxacin

DISCUSSION

Seeds and leaves serve as important microcosm for saprophytic and pathogenic microorganisms and pepper leaves and seeds are no exception to this. Bacteria invade and colonize pepper seeds at pre and post-harvest stages causing considerable loss in yield and quality (Lalitha, Raveesha, and Kiran, 2010). Use of plant extracts is found to be an effective way of controlling plant diseases pathogens over the use of synthetic chemicals which are not ecofriendly due to their toxicity and biomagnifications in food chain which is in line with what other researchers found out (Das 2003; Opara and Wokocha, 2008; Lalitha, *et al.,* 2010; Dixon, 2011; Dwivedi, Sheeraz, and Wagay, 2014). In view of this fact, the effect of different cold and hot solvent extracts of *Azadirachta indica* leaves against phytopathogenic *Xanthomonas campestris* was studied in this work and the data evaluated clearly revealed a pronounced activity of the extracts against the tested bacteria.

The phytochemical screening of *Azadirachta indica* leaves extract indicted the presence of Alkaloid, Saponin, Tannin, Flavonoids, Phenolics, Steroids and Cardiac glycoside which is in line with previous studies made by other researchers (De and Ifeoma, 2002; Biwas, *et al*., 2002; Natarajan, Veugopal and Menon, 2003; El Mahmood, Ogbonna, and Raji, 2010). The presence of these phytochemicals independently or in combination may be responsible for the extraordinary antimicrobial activity of the plant extracts as described by many researchers (Baez, De los Ryos, Crescente and Caserta, 1998; Satish, Raveesha, and Janardhana, 1999; Perumal Samy and Ignacimuthu, 2000; Umamaheswari and Chatterjee, 2008; Amtul, 2014). Some phytochemicals work by intercalating with DNA of the organism (example alkaloid), interferes with protein synthesis and disrupt cell membrane (example saponins) while others interfere signal transduction pathway, metabolic processes, damage metabolic and cellular enzymes, disrupt proton motive force, electron flow, coagulation of cell component and modulation of gene expression (Kotzekidou, Giannakidis and Boulamatsis, 2008).

The result of the diameter zones of inhibitions of the cold and hot leaf extracts of *Azadirachta indica* against *Xanthomonas campestris* clearly revealed that cold solvent extract showed more activity against *Xanthomonas campestris.* This could be because the potent phytochemicals are heat labile. Similar conclusion was drawn by different researchers (Scalbert, 1991; Hammer, Carson, and Riley, 1999; Iheukwumere, *et al*., 2012; Iheukwumere and Umedum 2013). Though aqueous extracts produced higher amount of extract but exhibited relatively lower activity than the ethanolic extract which was obtained in lower quantity. This indicates that the amount of yield did not always influence the inhibition of microbial growth but the active ingredients found in the extract play the major role. Similar observation was made by Iheukwumere, *et al*., (2012). The study further highlighted that cold ethanol was able to extract more of the phytochemical constituents because ethanol is an organic and polar solvent and most of the phytochemical constituents are organic and polar in nature. This observation suggested that the organic solvent extraction is suitable to verify the antibacterial properties of medicinal plants (Ali, Haider, Hanif, and Akhatar, 2001; Parekh, Nair, and Chanda, 2005; Corato, 2010; Iheukwumere, *et al*., 2012; Ucop, Vetti, M and Agus, 2013).

The results of the MIC and MBC of the cold and hot leaf extracts of *Azadirachta indica* showed that the hot solvent extracts showed no bactericidal activity against *Xanthomonas campestris* but the cold organic solvent extracts at the highest concentration (500mg/ml) showed bactericidal activity against *Xanthomonas campestris* compared to ciprofloxacin which exhibited the most pronounced activity at concentration (250mg/ml). This means that infections caused by *Xanthomonas campestris* could be managed effectively using the cold leaf extracts of the organic solvents. Continued further research involving *in vivo* assays will be needed to establish the relationship between the MICs and MBCs obtained within study and the effective dosage that should be administered in ethnomedical practice.

CONCLUSION

This study revealed the effects of different solvent extracts of *Azadirachta indica* leaves (Hot and cold) on *Xanthomonas campestris*. The cold leaves extract of ethanol, n-hexane and petroleum ether showed an activity against *Xanthomonas campestris* with cold ethanolic leaf extract showing the most pronounced activity. From this evidence, it is clear that the cold leaves extract of ethanol has tremendous inhibitory potential against *Xanthomonas campestris.*

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