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Antibacterial activity of methanolic and aqueous extract of *Azadirachta indica* against *Xanthomonas citri* and *Xanthomonas campestris*

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ABSTRACT

Xanthomonas citri and *X. campestris* are two important plant pathogenic bacteria, severely affects different types of crops like citrus, beans, mustard, cotton etc worldwide. In our lab effective antibacterial activity was observed by the use of methanolic as well as aqueous extract of *A. indica* against *X. citri* and *X. campestris* at different volume of concentrations (20 µl, 40 µl, 60 µl, 80 µl and 100 µl) by using Agar well diffusion method. Each part of *A. indica* contains various pharmacological active substances against many diseases. The highest inhibitory activity was observed in methanolic leaf extract of *A. indica* against *X. citri* (18.33±0.46 mm) at 100 µl (100 µg/ µl) of concentration. Hence, the result of *A. indica* (leaves) indicated that it may be used for treating plant diseases as natural bactericides.

Key words: *Azadirachta indica*, *Xanthomonas citri*, *Xanthomonas campestris*, Antibacterial activity, methanol, aqueous

ABBREVIATIONS- mm (milli metre), µl (micro litre), ZOI (zone of inhibition), hrs (hours), % (percent), gm (gram), ℃ (degree Celsius), MHA (Muller-Hinton Agar), DMSO (Dimethyl sulphoxide), min (minute)

INTRODUCTION-

A number of plant pathogens like *Pseudomonas, Xanthomonas, Xylella, Xylophillus, Acidovorax, Agrobacterium, Erwinia, Pantoea, Ralstonia, Burkholderia, Clavibacter, Streptomyces, Spiroplasma* and

Phytoplasma (Ellis *et al*., 2008) cause various infections in plants throughout the world. Amongst them, several species of *Xanthomonas* are divesting plant pathogenic bacteria, which cause diseases in citrus, beans, grapes, cotton, mustard, rice and several other crops. Among the *Xanthomonas* species, *Xanthomonas citri* and *Xanthomonas campestris*, cause extreme loss to crops thus causing intense monetary misfortune for farmers around the world. Citrus crop is one of the most important crops of farmers worldwide, not because it is a vital branch of the economy of numerous countries but also play a key role as a machine for generating jobs (Mattos and Carlos, 2019). This incident happens, in addition to their utilization in nature, citrus fruits are also consumed in different ways such as juices and sweets (Rampersaud and Valim, 2017). In the last decade, numerous diseases have been accountable for causing different losses to the citrus industry such as citrus veriagated chlorosis (CVC), huanglongbing or greening (HLB), sudden citrus death (SCD) and citrus canker (Lopes, 2019; Sun *et al*., 2019; Matsumura *et al*., 2017). The excessive use of synthetic pesticides hampers the fertility of soil and cause deleterious effect to the environment. Recently, researchers have paid attention to the use of medicinal plants against plant and human pathogens. Plants are being utilized by human being from ancient time to cure the illnesses and for other therapeutic purposes. Undoubtedly, medicinal plants have raised their significance all over the world. Recently, researchers have paid interest in medicinal plants because of their extensive beneficial properties like low toxicity, pharmacological activities and economic viability (Auddy *et al*., 2003). The antimicrobial and antioxidant activity of restorative plants depends upon the phytochemicals like flavonoids, tannins, glycosides, terpenoids, anthraquinones and steroids (Calixto, 2000). *Azadirachta indica*, commonly known as Neem, is a perennial, fast-growing tree that can reach the height of 15–20 meters. It is native to South Asia and most of Indian sub-continent (Govindachari, 1993). Isomeldenin, nimbin, nimbinene, 6-desacetyllnimbinene, nimbandiol, immobile, nimocinol, quercetin, and beta-sitosterol, tetracyclic triterpenoids zafaral and meliacin anhydride were examined in the leaf extract of neem (Tiwary, 1985; Siddiqui *et al*., 2004). The certainty of active components of neem has not been fully examined (Mukherjee *et al*., 1996). Presence of azadirachtin H and azadirachtin I was also observed in neem seeds (Govindachari *et al*., 1992). Hence, the main aim of this study is to evaluate the antibacterial efficacy of *A. indica* against *X. citri* and *X. campestris*.

MATERIAL AND METHODS-

Collection and authentication of medicinal plants

Different parts of plant like leaves and stems were collected from the campus of Jiwaji University, Gwalior (M.P.) India, and were authenticated by the experts of School of Studies in Botany, Jiwaji University, Gwalior, (M.P).

Preparation of plant extracts and Percent Yield

Fresh leaf and stem parts from selected plants were collected and washed 2-3 times with running tap water and allowed to shade dry at room temperature. After the process of natural drying, the dried plant materials were powdered using the clean pestle mortar. The powder was kept in separate air tight containers and stored in a dry place at room temperature. Plant materials (powder) were extracted in the solvent like methanol and double distilled water by using the method of Harborne, (1984) and Roopashree *et al*., (2008).

Fifteen gm of dried powdered plant materials were extracted in soxhlet apparatus using solvent methanol and double distilled water. The extraction was done for 48 hrs and after extraction the crude extract was evaporated at 40℃ on hot water bath. After evaporation process obtained extracts were weighed. Yield of extract was calculated by using the formula. The extracts were collected and stored at 4℃ in sterile air tight containers for further analysis.

$$Yield\ (\%) = \frac{W_1 \times 100}{W_2}$$

'W_1' denotes the weight of the extract after lyophilization of solvent and

'W_2'indicates the weight of the powdered material.

Antibacterial assay

Standard pure cultures i.e. *Xanthomonas citri* (ITCC No. BN 0001) and *Xanthomonas campesris* (ITCC No. BH 0001)**,** were procured from Indian Agriculture Research Institute, Pusa, New Dehli, India, and maintained by sub-culturing on Nutrient agar media (Hi Media) and Nutrient broth (Hi Media). The antibacterial activity of plant extracts was determined by agar well diffusion method (Magaldi and Mata-Essayag, 2004). Commercially available dehydrated MHA media (Hi Media) was used in antibacterial

study and prepared according to the manufacturer's directions. The leaf and stem extract of *M. arvensis* were dissolved in DMSO (Dimethyl Sulphoxide) in a concentration of 100 mg/ml (stock solution). In this method wells were made in MHA medium using sterile cork borer after the spreading of bacteria. The method is suitable for organisms, which grow rapidly at 35-37℃ in 24 hrs. The previously inoculated bacterial strain was spread on MHA. After few minutes five wells were made in each Petri plate and loaded with different concentration (20, 40, 60, 80 and 100 µl). Plates were incubated at 37℃ for 24 hrs. After the diffusion of extracts, Petri plates were left at room temperature for about 30 min and then incubated at 37℃ for 24 hrs. The diameter of zone of inhibition of bacterial growth around each well was measured and the susceptibility was determined by using Hi-media zone scale. Experiments were carried out in triplicates.

STATICALLY ANALYSIS-

In this study the results were expressed as the mean± standard deviation and to check the significance of data, One way ANOVA was used at the level of 0.05 ($p < 0.05$).

RESULTS-

The leaf and stem extracts of *A. indica* were carried out with both methanol and aqueous solvents. The highest percent yield was recorded in methanol leaf and stem extract of *A. indica* i.e. 6.8% and 6.74% respectively and 7.06% and 7.1% was noted in aqueous extracts (Table 1).

The antibacterial activity of methanolic and aqueous extract of *A. indica* was investigated using agar well diffusion method against *X.citri* and *X.campestris* at different level of concentrations like 20 µl, 40 µl, 60 µl, 80 µl and 100 µl.

At 100 µl of concentration the methanol extract of leaf and stem of *A. indica* exhibited the highest ZOI 18.66±0.46 mm and 11.66±0.60 mm against *X. citri* and at 80 µl of concentration the ZOI was 13±0.60 mm and 11.1±0.46mm (Table 2). 11.84±0.46mm and 10±0.46mm was recorded at 60 µl of concentration. At 40 µl of concentration the ZOI of leaf and stem were 8.02±0.03mm and 7.99±0.30 mm whereas minimum ZOI was showed in *A. indica* (leaf and stem) at 20 µl of concentration against *X. citri* was 7.02±0.04 mm and 2.96±0.07mm (Fig. 1).

The highest ZOI i.e. 13±0.75 mm was recorded at 100 µl of concentration in leaf extract (methanolic) of *A. indica* against *X. campestris* and 12.55±0.90 mm was noted in stem. At 80 µl of concentration the ZOI was11.95±0.60 mm and 11±0.60 mm. Leaf and stem extracts showed 10.02±0.60

mm and 8.66±0.60 mm ZOI at 60 µl of concentration. Minimum ZOI was recorded at 40 µl and 20 µl of concentrations viz.7.95±0.06 mm, 8.66±0.46 mm, 4.55±0.075 mm and 2.02±0.04 mm respectively. In this study the values of *A. indica* (leaf and stem) at 60 µl, 80 µl and 100 µl of concentrations were significant at the level of 0.05 ($p < 0.05$) against *X. citri* and *X. campestris*.

Table 1. Percentage Yield of *Azadirachta indica* (Leaf and Stem) using methanol and aqueous solvent.

Plant part used	Extract	Extraction Yield (%)
Leaf	Methanol	6.8
Stem	Methanol	6.74
Leaf	Aqueous	7.06
Stem	Aqueous	7.1

Table 2. Comparative antibacterial activity of methanolic extract of *A. indica* against *X. citri* and *X. campestris*

S.No.	Volume of Concentration (µl)	Plant part	Zone of Inhibition (mm)	
			X. citri	*X. campestris*
1.	20	Leaf	7.02±0.04*	4.55±0.075*
		Stem	2.96±0.07*	2.02±0.04*
2.	40	Leaf	8.02±0.03*	7.95±0.06*
		Stem	7.99±0.30*	7.66±0.46*
3.	60	Leaf	11.84±0.46	10.02±0.60
		Stem	10±0.46	8.66±0.60
4.	80	Leaf	13±0.60	11.95±0.60
		Stem	11±0.46	11±0.60
5.	100	Leaf	18.33±0.46	13±0.75
		Stem	11.66±0.60	12.55±0.90

Each presented values were expressed as mean±SD. Mean of triplicate analysis (n=3).Values with symbol * at different concentrations in the table were not considered to be statistically significant at the level of 0.05($p < 0.05$) and rest of the values were significant at the level of 0.05($p < 0.05$). NI = No Inhibition

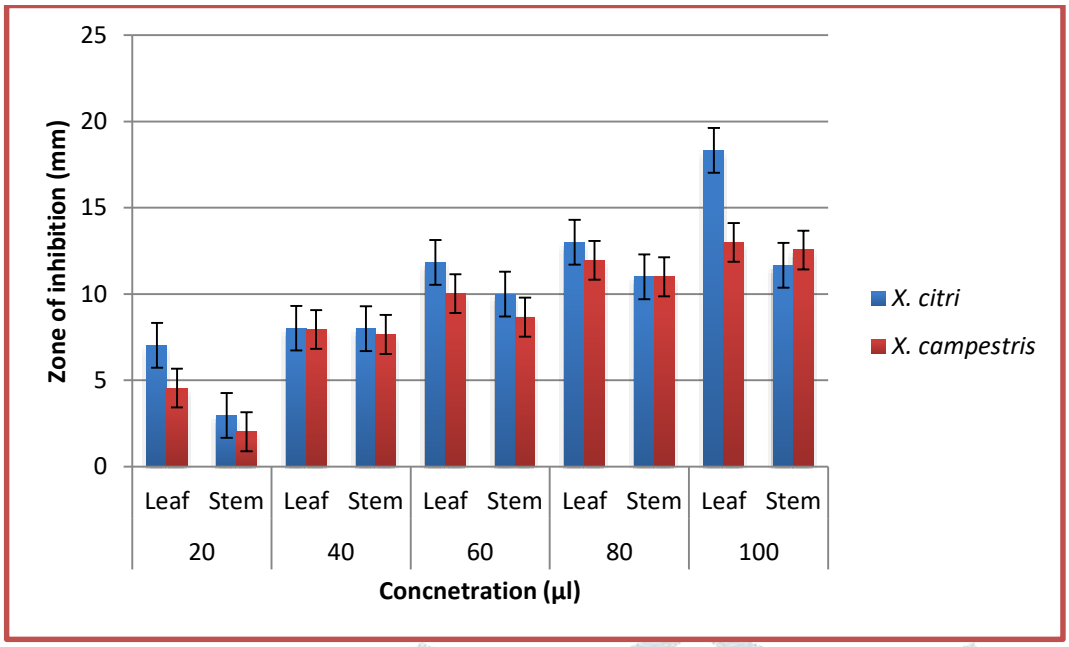

Fig. 1 Comparative antibacterial activity of methanolic extract of *A. indica* against *X. citri* and *X. campestris*

The leaf extract of *A. indica* showed highest ZOI i.e. 12±0.60 mm against *X.citri* at 100 µl of concentration and 8±0.60 mm at 80 µl. It was noted that the aqueous stem extract of *A. indica* did not show any antibacterial activity against *X.citri* at any level of concentrations.

At 100µl of concentration of *A. indica* (leaf and stem) exhibited 10±0.60 mm and 8±0.60 mm ZOI against *X. campestris* (Table 3 & Fig. 2). 9±0.60 mm, 8±0.60 mm, 6.66±0.75 and 6±0.60 was recorded at 80 µl, 60 µl, 40 µl and 20 µl concentrations of leaf extract of *A. indica* while stem did not show any activity. The values of aqueous leaf extract of *A. indica* were found significant, 0.05 ($p < 0.05$) at 100 µl of concentration against *X. citri* and *X. campestris*.

Table 3. Comparative antibacterial activity of aqueous extract of *A. indica* against *X. citri* and *X. campestris*

S.No.	Volume of Concentration (µl)	Plant part	Zone of Inhibition (mm)	
			X. citri	*X. campestris*
1.	20	Leaf	NI	6±0.60*
		Stem	NI	NI
2.	40	Leaf	NI	6.66±0.75*
		Stem	NI	NI
3.	60	Leaf	NI	8±0.60*
		Stem	NI	NI

4.	80	Leaf	8±0.60*	9±0.60*
		Stem	NI	NI
5.	100	Leaf	12±0.60	10±0.60
		Stem	NI	8±0.60*

Each presented values were expressed as mean±SD. Mean of triplicate analysis (n=3). Values with symbol * at different concentrations in the table were not considered to be statistically significant at the level of 0.05($p < 0.05$) and rest of the values were significant at the level of 0.05($p < 0.05$). NI = No Inhibition

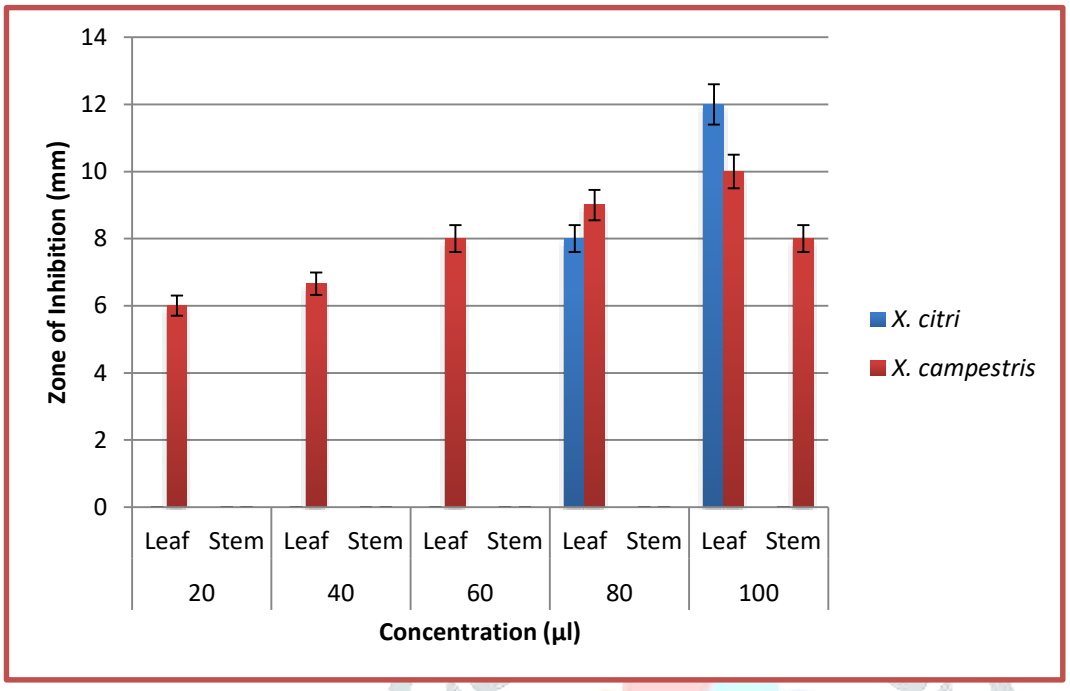

Fig. 2 Comparative antibacterial activity of aqueous extract of *A. indica* against *X. citri* and *X. campestris*

DISCUSSION-

In recent years microbial natural biocides have paid interest in order to avoid the unrestricted use of chemical pesticides in the environment (Baazeem *et al*., 2021). Among the plant pathogens, *Xanthomonas* is an awfully significant kind of phytopathogen, which causes the plant diseases all around the world. The hosts of this genus include at least 124 monocotyledonous and 268 dicotyledonous plants and cause different diseases in rice, mustard, cotton, citrus etc, which cause a big economic loss for farmer every year. Thus, pathovars of *Xanthomonas* are known to cause diseases on several vegetable and cash crops (Mandavia *et al*., 1999). This study is an initial step to develop the natural bactericides against *X. citri* and *X. campestris.* Jayashree and Londonkar, (2014) analysed the extractive value of different solvents like aqueous, methanol, petroleum ether and chloroform (14%, 10.7%, 6.1% and 3.8%) of *Feronia limonia*. Terblanche *et al*., (2017) evaluated the extraction yield of *Cotyledon orbiculata* in aqueous and methanol

solvent extract. Chandra, (2013) studied the antimicrobial activity of methanol and aqueous leaf extracts of *Lagerstroemia indica* and *Annona reticulata* against *Klebsiella pneumoniae, Staphylococcus auerus, Salmonella typhi, Proteus vulgaris* and *Pseudomonas aeruginosa*. The highest ZOI was observed in methanol extract as compared to aqueous extract. Orhue *et al*., (2014) worked on different solvent extracts of *Azadirachta indica* like aqueous, ethanol, 1% HCl, acetone and petroleum ether. The antibacterial potency of petroleum ether extract of leaf and bark of *A. indica* were found to be more active against the urinary tract bacterial isolates rather than other solvent extracts. Parashar *et al*., (2018) reported the antibacterial activity of leaf extracts of *Azadirachta indica* and *Lantana camara* against *Escherichia coli, Staphylococcus aureus, Pseudomonas aureginosa* and *Bacillus subtilis.* The combination of leaf extract of *A. indica* and *L. camara* showed maximum ZOI against the tested organisms. *X. citri* and *X. campestris* seriously affect different economically important crops and due to this the main aim of this study is to find out the strategies to curd the crop losses due to bacterial diseases.

CONCLUSION-

Different types of chemical pesticides or antibiotics are usually applied for the treatment of plant diseases, resulting environmental hazards and cause big economic loss for farmers throughout the world. The degradable character, low cost and safe for the environment, medicinal plant extracts will be serving as a good substitute of chemical pesticides. Hence, the aim of this study will develop natural pesticides which are ecofriendly. The comparison between the antibacterial efficacy of leaf and stem part of *A. indica* against *X. citri* and *X. campestris* we found the significant results in methanolic as well as aqueous extract of the selected plant because of the presence of active metabolites present in this plant parts. But among, the methanolic leaf extract of *A. indica* showed excellent inhibitory activity against *X. citri*. So, from this study it can be suggested that these plant parts could be used as a substitute for chemical pesticides and antibiotics.

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AUTHOR'S CONTRIBUTION-

AS and SP did experimental design work. AS conducted experiments. AS and SP analyzed the data. AS wrote the manuscript. All authors read and approved the Manuscript.

CONFLICT OF INTEREST-

The authors declare that there are no conflicts of interest.

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