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Full Length Research Article

EFFECT OF NEEM (*AZADIRACHTA INDICA* A. JUSS) LEAF EXTRACT ON THE GROWTH OF *ASPERGILLUS* SPECIES ISOLATED FROM FOLIAR DISEASES OF RICE (*ORYZEA SATIVA*)

[1]Keta J.N., [2]Suberu H.A, [3]Shehu K., [4]Yahayya U., [5]Mohammad N.K. and [6]Gudu G.B.

[1&4 - 6]Department of Biological Sciences, Kebbi State University of Science and Technology, Aliero
[2]Department of Biological Sciences, Federal University Lokoja, Kogi State
[3]Department of Biological Sciences, Federal University, Birnin Kebbi

*Corresponding Author Email Address Ketajb@gmail.com

ABSTRACT
Rice blast is probably the most frequent and most destructive rice disease caused by fungi. This study was carried out to determine effect of Neem (*Azadirachta indica* A. Juss) leaf extract on the growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans* and *Aspergillus fumigatus* isolated from foliar diseases of rice. The fungal isolated from foliar part of rice were revealed as *Aspergillus* spp out of 30 samples collected after a disease field survey. Ethanol and aqueous extracts of Neem leaf were tested against the fungal isolates. The efficacy of the extracts was studied and determined by applying in different concentrations of 5, 10 and 15ml/L. Effect of the extracts was determined by measuring radial growth (mm) of the isolated fungi on potato dextrose agar in petri-dishes. Resulted obtained shown that statistically, the ethanolic extract was more effective than aqueous extract in all cases. However, the effectiveness of the extracts was dependent on the concentration used. The phytochemical analysis of revealed the present of alkaloids, saponins, tannins, phenols, flavonoids, and terpenoids while anthraquinone, phalabatonine and steroids were not detected. Therefore, uses of *Azadirachta indica* could be recommended to control the foliar diseases (*Aspergillus* spp) of rice which help to increase the grain yield of that crop.

Keywords: Neem leaf, Oryzea sativa, Aspergillus spp, and Phytochemical

INTRODUCTION
Rice (*Oryza sativa* L.) is one of the most cultivated crops in the world (Habib *et al.,* 2012). *Oryza sativa* is important in the diet of the world population as it directly provides 20% of human calorie intake (Zeigler and Barclay, 2008). It is the main staple food crop in many developing countries and consumed globally after wheat and maize (Mwashasha *et al*., 2013). Rice cultivation is well suited to countries and regions with low labor costs and high rainfall, as it is labor intensive to cultivate and requires ample water (International Rice Research Institute, 2009). However, rice can be grown practically anywhere, even on a steep hill or mountain area with the use of water controlling terrace systems. Although its parent species are native to Asia and certain parts of Africa, centuries of trade and exportation have made it commonplace in many cultures worldwide (International Rice Research Institute, 2013). The traditional method for cultivating rice is flooding the field or after setting the young seedlings (Jianguo *et al.,* 2003). This simple method requires sound planning and servicing of the water damming and channeling, but reduces the growth of less robust weed and pest plants that have no submerged growth state, and deters vermin. While flooding is not mandatory for the cultivation of rice, all other methods of irrigation require higher effort in weed and pest control during growth periods and a different approach for fertilizing the soil (Jianguo *et al.,* 2003).
Rice blast, caused by a fungus, causes lesions to form on leaves, stems, peduncles, panicles, seeds and even roots (Howard and Valent, 1996). Blast has been observed on rice initial symptoms are white to gray green lesions or spots with darker borders produced on all parts of the shoot, while older lesions are elliptical or spindle shaped and whitish to gray with necrotic borders. Lesions may enlarge and coalesce to kill the entire leaf. Symptoms are observed on all above ground parts of the plant (Couch *et al*., 2005). Lesions can be seen on the leaf collar, culm, culm nodes, and panicle neck node. Intermodal infection of the culm occurs in a banded pattern. Nodal infection causes the culm to break at the infected node (rotten neck) (Couch and khon, 2002). It also affects reproduction by causing the host to produce fewer seeds. This is caused by the disease preventing maturation of the actual grain (Scardaci, 2003).
Neem (*Azadirachta indica*) commonly called 'India Lilac' or 'Margosa' and *Dogon yaro* in Hausa, belongs to the family Meliaceae, subfamily Meloideae and tribe Melieae (Tomoko *et al*., 2002). Neem and its products have been widely reported to control insect pests (Ascher, 1993); Schmutterer and Ascher, 1995). Plant bacterial diseases (Abbasi *et al*., 2003). Plant parasitic nematodes Muller and Gooch (1982), Akhtar and Mahmood (1995). Plant fungal diseases Vir and Sharma (1985), Amadioha (2000), Dubey *et al*., (2009). As well as a potential agricultural fertilizer (Gajalakshmi and Abbasi, 2004). Moreover, in ayurveda, unani and homeopathic medicine almost every part of this tree including seeds, leaves, roots, bark, trunk and branches have multiple uses (Subapriya and Nagini, 2005). Neem as a bio-control agent is used for centuries in Asia as a potential antifungal agent (Chaturvedi *et al*., 2003). Several studies have pointed out the potential of neem tree (*A. indica*) to control plant pathogenic fungi that could be listed it as top fungicide and harmless biocontrol agent Abbasi *et al*., (2003), Akhtar and Mahmood (1995), Amadioha (2000), Dubey *et al*., (2009). Disease of rice plant caused by fungal species remains endemic to rice farmers in Birnin Kebbi due to lack of information on disease prevalence. Therefore, it's necessary to isolate and identify fungal species on the foliar part of rice plant and then source a possible control in the leaf extract of *Azadirachta indica* A. Juss.

MATERIALS AND METHODS

Study area

Kebbi is a state in north western Nigeria with its capital at Birnin Kebbi. It has a total area of 36,800 km². The vegetation consists of short grass savanna that is drained southwestward by the Niger River. The state is located on longitude 12°27'13"N and latitude4°12'01"E (Traditional States of Nigeria, 2010). The rainy period in Birnin Kebbi is between April and October. The month of August has the highest magnitude of rainfall (Adelana *et al*., 2006). There is a wet season between May and September, with little rain in the remainder of the year. Mean annual rainfall is about 800mm. Average temperatures are about 26°C, ranging from 21°C in harmattan to 40°C between April and June (Physical Setting, 2010). Farming is the main occupation of the people especially in rural areas and the most important economic activity, with riverine floodplains producing cash crops; mainly grains, rice, sorghum, millet, peanuts (groundnuts), cowpeas, and onions.

Sample Collection

Random sampling method was adapted to obtained samples of rice plants (30) from each area Dukku, Makera and Kara in Birnin Kebbi L.G. A. A total of one hundred and fifty (30) samples were collected. Infected leaves of rice were cut with sterilized razor blade and placed in well labeled polythene bags and taken to mycology laboratory (Cheesbrough, 2000).

Preparation of Neem leaves for extraction

Fresh and uninfected leaves of *Azadirachta indica* (A.Juss) was collected from the University campus, Aliero (KSUSTA). The leaves were washed under running tap water and rinsed with distilled water and then shade dried for seven days at room temperature 2± 27°C. The dried leaves were grinded with pistil and mortar and a 25-mesh diameter sieve was used to obtain a fine powder.

Aqueous Extraction

The aqueous extract of plant was prepared by weighting 200g of the powder on a weighing balance and soaked in 500ml distilled water in one liter conical flask. It was stirred for 30 min and left for 48h. The extract was filtered through a fine cloth and again through filter paper (Whatman No, 1). The filtrate was poured in a round bottom flask and then concentrated by evaporation to dryness in a water bath at 60°C (Muyibi and Evison, 2000). The condensed extract was preserved in tightly corked labeled bottles and stored in a refrigerator at 4˚C until required. This was done according to the method adopted by Amin *et al.* (2009).

Ethanolic Extraction

100g air dried powders of neem leaves of were soaked in a container of 1000 ml of ethanol solvent. The mixtures was placed at room temperature2± 27°Cfor 24 h and was thoroughly shaking. Solution was filtered through muslin cloth and then re-filtered by passing through Whatman filter No. 1. The filtrate obtained was concentrated by complete evaporation of solvent in a water bath at 50°C. Solution was stored at 4°C after collecting in sterilized bottles until required (Rauha *et al.,* 2000).

Phytochemical Screening of the leaves' crude extract

The ethanol crude extract was used for the phytochemicals, by dissolving 5g in 40ml of distilled water to detect. The presence of Alkaloids, Resins, Saponins, Tannins, Phenol, Flavonoids, Terpenoids, Anthraquinones, Steroids and Phlobatannins were determined by using standard analytical procedures as described by Harborne (1998), Sofowora (1996); (1990) Trease and Evans (2002).

Preparation of Fungal Culture Medium

The culture mediums used in this study were prepared in accordance to the manufacturer's instructions using standard aseptic technique (Cheesbrough, 2000).

Sterilization of Glassware

The glassware were washed thoroughly with detergent and sterilized using a hot air oven at 160°C for one hour. Then, the oven was allowed enough time to cool, to prevent, the glass from cracking and for ease of handling.

Inoculation and Incubation

The infected leaf of *Oryza* sativa probably containing lesions, were surface sterilized with 0.5% of sodium hypochlorite for five min, rinsed in running tap water, and in sterile distilled water and cut into 3×3mm size with sterilized knife. The pieces were separately inoculated on PDA media contained in petridishes (Nur.Izzati, 2013). The plates were incubated at room temperature 27±2ºC in the dark for 5 to 30 days for the associated fungi to culture. The plates were observed for colony growth and the colony types were noted and recorded by using the modification of Reza (2010), method. The pure culture was obtained by sub-culturing each different fungus that appeared by using needle mount method onto PDA medium and the Petridishes were incubated for 5 days at 27 ±2ºC. The inoculating needles were sterilized by flaming to red hot after each cut, to avoid the yield of mixed cultures. It was observed after 5 days of incubation and inoculation in the plates for pure colony growth.

Identification of Fungal Isolates

The fungi were identified using morphological and cultural characteristics type of fruiting structure and the spores produced. The technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. The slide were then mounted and observed with x10 and x40 objective lenses respectively (Cheesbrough, 2000).

Sensitivity Tests

Sensitivity of the isolates to neem leaf extract was determined *in vitro* (Dutta, 2001). Potato dextrose agar (PDA) medium was prepared with incorporated neem leaf extract at varying concentrations: 5ml/l, 10ml/l and 15ml/liter. The amended medium with the extracts in the Petri dishes were inoculated at the centre with 2mm inoculums disc of the pure cultures of the fungi raised on PDA. They were then incubated at 28±2°C for 7 days and the diameter of colony of the pathogen was measured in each. The medium with inoculum disc but without any extract served as positive control. The effect of various extracts obtained from neem leaf were subjected to one way analysis of variance (ANOVA) using statistical package for Social Science (SPSS) version 22.0. However values were expressed at mean± standard deviation (SD). Results were considered as significant when p values were less than 0.05 ($P<0.05$)

RESULTS

The results of phytochemical of aqueous and ethanolic extracts of neem tree were presented in (Table 1). Effect of *Azadirachta indica* leaf ethanolic and aqueous extracts on the radial growth of *Aspergillus* species isolates on the *Oryza sativa* were seen in (Table 2) respectively:

Table 1: Phytochemical analysis of Neem on aqueous and ethanolic extracts

Tested groups	Aqueous Extract	Ethanolic Extract
Alkaloids	++	+++
Saponins	+	++
Tannins	+	++
Phenols	+	++
Flavonoids	++	+++
Terpenoids	+	++
Anthraquinone	-	-
Phlabatonins	-	-
Steroids	-	-

Keys:
(+++) Indicates heavily present
(++) Indicates moderate of tested groups
(+) Indicates present
(-) indicates not detected

Table 2: Effect of *Azadirachta indica* leaf ethanolic and aqueous extracts on the radial growth of *Aspergillus* species isolates on the *Oryza sativa*

Concentration (ml/l)	Ethanolic extract	Aqueous extract
Radial mycelia growth of *A. flavus* (mm)		
5	7.00±1.00^{b}	29.00±1.00^{b}
10	5.00±0.00^{a}	30.33±0.57^{a}
15	2.67±0.57^{a}	30.33±0.57^{a}
Control	36.00±0.00	36.00±0.00
Radial mycelial growth of *A. fumigatus* (mm)		
5	8.66±0.57^{a}	30.33±0.57^{a}
10	4.33±0.57^{a}	29.66±0.57^{a}
15	1.33±0.57^{a}	28.66±0.57^{a}
Control	31.00±0.00	31.00±0.00
Radial mycelial growth of *A. nidulans* (mm)		
5	14.00±1.00^{b}	25.33±0.57^{a}
10	8.00±1.00^{b}	23.00±1.00^{b}
15	3.33±0.57^{a}	22.66±0.57^{a}
Control	31.00±0.00	31.00±0.00
Radial mycelial growth of *A. niger* (mm)		
5	6.00±1.00^{b}	18.00±1.00^{b}
10	3.66±0.57^{a}	15.00±1.00^{b}
15	1.33±0.57^{a}	13.66±1.15^{b}
Control	38.00±0.00	38.00±0.00

Means in a column with superscripts ($P<0.05$) are significantly different, the mean growth in the groups at means ± standard of 3 replicates

DISCUSSION

These research study had shown that the foliar part of rice (*Oryza sativa* L.) were infected with Aspergillus species. The efficacy of *Azadirachta indica* (A. Juss) Leaf ethanolic and aqueous extracts on the growth of fungal isolates from foliar part of rice (*Oryza sativa* L.) in various concentrations of (5ml, 10ml and 15ml). Ethanolic extracts of *Azadirachta indica* Leaf shows inhibited growth in almost concentrations of (5ml, 10ml and 15ml). This research shows that the ethanolic extracts of *Azadirachta indica* (A. Juss) Leaf are more effective than aqueous extract. This is also in conformity with the finding of Gabrial *et al.,* (2014). Another finding showed the antimicrobial role of aqueous extracts of neem cake in the inhibition of spore germination against three sporulating fungi such as *Cochliobolus lunata* and *Colletotrichum gloeosporioides* f. sp. *Mangiferae* (Anjali *et al*., 2013) and results of the study revealed that methanol and ethanol extract of *Azadirachta indica* showed growth inhibition against *Aspergillus flavus* species *Alternaria solani*, and *Cladosporium* (Shrivastava and Swarnkar, 2014) Aqueous extracts of various parts of neem such as neem oil and its chief principles have antifungal activities and have been reported by earlier investigators (Jabeen *et al.* 2013). Experiment was made to evaluate the efficacy of extracts of neem leaf on seed borne fungi *Aspergillus* and *Rhizopus* and results confirmed that growth of both the fungal species was significantly inhibited and controlled with both ethanolic and water extract. Furthermore, ethanolic extract of neem leaf are more effective than to aqueous extract for retarding the growth of both fungal species (Mondali *et al*., 2009). It also conformity with present research that *Aspergillus* and *Rhizopus* species was significantly inhibited and controlled with both ethanolic and water extract.

This research has shown that the potential used of crude and aqueous extracts of *Azadirachta indica* Leaves for controlling of fungal pathogens was due to the phyto-chemicals present in the extract and presence of these bioactive compounds in the *Azadirachta indica* Leaf extract is thought to be responsible for the antifungal activity. Numerous investigations have proved that medicinal plant contains diverse classes of bioactive compounds. The presence of the phytochemical constituents mainly alkaloids, flavonoids, tannins, and phenolic compounds has been reported to be most important compounds in many other medicinal plants (Jayasree *et al.,* 2014). Table 1: has shown preliminary phytochemical screening of aqueous and ethanolic extracts of *Azadirachta indica* leaf. In this study, *Azadirachta indica* Leaf, aqueous and ethanolic extracts, contain alkaloids, saponins, tannins, phenols, flavonoids and terpenoids. These results are consistent with findings of Ruchi *et al.* (2014) and Indra *et al*. (2012). The results of phytochemical analysis of aqueous leaf extract (ALE) and ethanolic Leaf extract (ELE) of Neem, results indicate the presence of many phyto-components in both the extracts. Alkaloids, saponins, tannins, phenols, flavonoids, terpenoids, glycosides, but absent of amino acid and terpenoids due to the reason that the extract was treated with chloroform unlike the other extracts was treated with ethanol. But, the result reported by Pandey *et al*. (2014) was also consistent with absence of terpenoids and presence of amino acid. However the result of SenthilKumar *et al*. (2013) consistent with numerous reported with presence of Alkaloids, saponins, tannins, phenols, flavonoids, terpenoids and amino acid respectively. The results of phytochemical in the present investigation showed that the neem leaf contains eight phytochemical components which agree with

the works of some researchers as (Mahapatra *et al.,* 2014; Abdullah *et al.,* 2011) with presence of alkaloids, tannin, glycoside, anthraquinones, reducing sugar, polyphenol, terpenoid and steroid. The presences of these phytochemicals are the reasons neem leaf ethanol extracts have antifungal and antimicrobial activity.

Conclusion

From the resulted obtained confirmed that growth of both the fungal species isolated was significantly inhibited and controlled with both ethanolic and aqueous extracts. Furthermore, ethanolic extract of neem leaf was more effective than to aqueous extract for retarding the growth of both fungal species.

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