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POTENTIAL OF AZADIRACHTIN-D FRACTION AGAINST BACTERIAL LEAF BLIGHT DISEASE IN RICE CAUSED BY *XANTHOMONAS ORYZAE* PV. *ORYZAE*

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Abstract

In the present study organic extracts (Chloroform) of twenty four plants were tested through anti-bacterial susceptibility test against specific bacterium *X. oryzae* causing Bacterial Leaf Blight (BLB) disease in rice. *Azarditacha indica* extract showed high efficacy against Xoo bacterium forming larger inhibition zone (18.5 mm) with activity index (0.64).Further extract of *Azarditacha indica* fractionated, most active fraction were tested using bio autography agar overlay method against test bacterium, isolate, purify and characterized the most promising fraction and it's supposed to be 1-Tigloyl-3-acetyl-1, 1-hydroxymeliacarpin (Azadirachtin-D).

Introduction

Azarditacha indica (neem) belongs to a family Meliaceae. It grow semi-tropical or tropical area of world, Neem native to Indian Subcontinent, its fast growing tree, possess secondary metabolite against number of microorganism (Dua *et al.,* 2009). Thousands of years before it seed, leaves, bark used as insecticidal , anti-bacterial, antiviral, anti-fungal, anti-cancer and anti-oxidant, Neem also used for purification of blood, its leaf extract showed high efficacy against skin diseases, its bark have characteristic for healing wounds. Centuries ago plant extracts were used for curing humans, animals and plant diseases, leaf extracts of *Bradyrlizobium japonicum* and *Azadirachta indica* showed high effectiveness against chick pea nematode and root knot nematode. The medicinal value of the plants is due to the presence of some chemical substances in the plant tissue, the most important being alkoliod, flavaniod, terpeniod and steroids, which showed bioactivity against the pathogenic organism. The common procedure for obtaining natural products involves drying, grinding and extracting plant material with organic solvents. Once pure natural product is isolated attention is focused on the elucidation of structures (Kraun, 2008). The chemical constituents of plants and their effect on the treatment of bacterial, fungal and viral diseases play miraculous role in science. A great deal of work is done in this field (Asia *et al.,* 2004; Adeoye & Oyedapo, 2004).

Xanthomonas oryzae Pv. *oryzae* is casual organism producing bacterial Leaf Blight in rice, and responsible for huge loss of grain. It has been reported from all continents of the world, except Europe (Alim, 1967; Ou, 1985). BLB observed bacterial leaf blight as early as 1884-1885. The disease increased in 1950 throughout Japan, being especially prevalent in Kyushu Island. The occurrence of BLB was then reported from many neighboring countries of Japan, Indonesia (Reitsma & Schure 1950), Taiwan (Hashioka, 1969 and Mainland China (Xi *et al.,* 2012). Bacterial leaf blight (BLB) was first time reported in Pakistan in 1977 (Mew & Majid, 1977).

Akhter *et al*., (2003) has made survey in rice zone of Pakistan and assessed that incidence of Bacterial Leaf Blight of rice increase in recent years in Pakistan especial in kaller belt which is very famous for cultivation of high quality rice.

Haque *et al.,* (1994) reported that leaf extracts of *Bradyrlizobium japonicum* and *Azarditacha indica* showed high effectiveness against Cicer arietinum nematode and root knot nematode. Dua *et al*., (2009) suggested that *Azardirachta indica* is a cheaper and readily available approach in the control of plant disease e.g., only 2.66% of *A. niger* on ground nut appeared after treatment. Leaf extract of Neem (2%) reported to be most effective for increasing seed germination upto 76%, followed by Thiran and Dithane. Extract of *Azardirachta indica* also showed highest efficacy against different pathogen (Alouani *et al.*, 2009). Realizing the negative side effects of chemical pesticides on environment and human health and the competition of world market to get high quality and disease free products, the present study was planned to develop safe ecofriendly system to screen out different plant species extracts for the control of BLB.

Methodology

Organic solvent extract: The plant material were ground to fine powder and extracted with chloroform (1:5 w/v) in sox let's extraction apparatus.

Antibacterial susceptibility testing: Organic plant extracts were used for analyzing anti-bacterial activity through antibacterial susceptibility test by applying whole plate diffusion method (Parekh & Chanda, 2006). The bacterium was maintained on nutrient glucose agar (NGA) by mixing nutrient agar + 1% glucose. The plates were prepared by pouring 20 mL NGA in 90 mm diameter petriplates and bacterial suspension was spread on agar with glass spreader. Five cavities were made on the agar with sterile cork borer of 7mm diameter and each cavity was filled with extract dimethyl sulphooxide (DMSO) The treatment was replicated five times by keeping the incubation time (48h) and temperature (28^oC) constant. The effect of each extracts was measured as inhibition zone in mm with the following scale:

The antibacterial activity of most effective plant diffusate was checked by comparing it with streptomycin drug 1gm/mL. The activity index (AI) was calculated by using formula:

IA = Inhibition zone of test sample/ Inhibition zone of the standard

Phytochemical studies: Phytochemical studies of the most effective plants were processed for further chemical investigation. The dried plant material was soaked in commercial methanol. After ten days, the mixture was filtered, the filtrate extract labeled as methanol extract. The residue was again dipped in ethyl acetate for ten days and then filtered, the filtrate labeled as ethyl acetate extract. The residue was again dipped in chloroform for ten days and then filtered, the filtrate was labeled as chloroform extract. The vacuum rotary evaporator was used to concentrate the extracts of methanol, ethyl acetate and chloroform (Fig. 1).

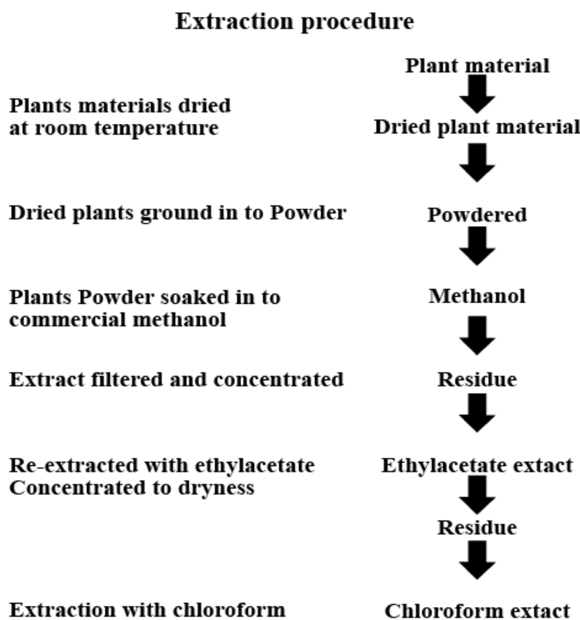

Fig. 1. Extraction of plant extracts through different solvents.

Column chromatography: The column chromatography technique was used to fractionate the plant crude extract. The crude extracts were subjected to glass column (1inch in diameter), packed with silica gel HF 256 (70-230 mesh size). The column was eluted with different solvent systems prepared by analyzing the nature of extract. The fractions were collected after small interval of time in glass test tubes (Furniss *et al.,* 1989).

Thin layer chromatography (TLC): The preparative TLC plate technique was used for further purification of promising fractions. The plates were made by silica gel (60 meshes 254) dissolved in methanol and coated on glass plates (20×20 inches) in smooth and thin layer. The plates were dried in oven at 60°C for 10 minute. The different solvent system was used for elucidation of fractions in TLC tank (Furniss *et al*., 1989).

Characterization of purified chemical compounds

Spectral studies of compound

1. Ultraviolet (UV) spectroscopy: Ultraviolet spectroscopy was carried out using Beckman model 25 at Quaid-i-Azam University, Islamabad, Pakistan.

2. Infra-Red (IR) spectroscopy: For IR spectroscopic results, Jasco A-302 (Japan) was usedat Quaid-i-University, Islamabad, Pakistan.

3. Mass spectroscopy of compound: For measurement of mass spectrum and major peaks, Jms 600 H was used at Research Institute of Chemistry International for Chemical Sciences, University of Karachi, Pakistan.

4. Nuclear magnetic resonance (NMR) spectroscopy of compounds: For NMR studies, 300 MHz and 500 MHz on Bruker AC-300, AM-500 nuclear magnetic resonance spectrometer at Research Institute of Chemistry, International for Chemicals Sciences University of Karachi, Pakistan was used.

Bio autography assay: Bio autography was used to detect the active compounds that were separated by column chromatography and TLC methods. The crude extracts fractionated on chromatograph were placed on sterile square covered glass dishes 9×9 cm. Ten milliliter of overlay media and 1mL of detecting strain (10^{-5}cell/mL) was evenly spreaded over the developed TLC plates (8×8 cm) with the help of glass spreader resulting in 1mm thick agar layer. The overlay plates were rapidly passing a low flame so that the air bubbles were removed. After solidification, the plates were incubated at 28°C for 18h. The bioautograms were sprayed with 1% triphenyl tetra zolium chloride, sprayed with 70% ethanol to stop bacterial growth. The antibacterial activity easily detected by white inhibition zones on a pinkish background (Hostettmann, 1999).

Results and Discussion

The antibacterial properties of organic solvents were investigated by antibacterial susceptibility test (Grewal *et al.*, 2012). All the crude extracts showed definite antibacterial activity, against most aggressive bacterial isolate (Xoo 105) of *Xanthomonas oryzae* pv. *oryzae*. The organic solvent (Chloroform) extracts of *Azadricha indica* was more active forming inhibition zone 18.5mm showed activity index 0.64 (Tables 1 and 2, Fig. 2). These findings are in line with the work of Parekh *et al.,* (2006).

Table 1. Scales for measurement of inhibition zone.

Symbol	Measurements	Reaction
H	29-22 mm	highly effective
I	22-14 mm	intermediately effective
L	14-18 mm	less effective
N	14-8 mm	Non effective

Isolation and spectral studies of compound *Azadirachata indica* Rj (1): The seed of *Azadirachata indica* was dried at room temperature plant material (1kg) was successively soaked for tendays in chloroform, ethyl acetate and methanol extracts and were concentrated at rotary evaporator under reduced pressure, extracts were tested against test bacterium, choloroform extract showed maximum antibacterial activity.

Table 2. Antibacterial activity of chloroform extrac of plant species against *X. oryzae*.

S.No.	Botanical name	Family	Plant part used	IZ mm	A.I	Standard drug streptomycin
1.	*Azadiricha indica*	Meliaceae	Seeds+fruits	18.5	0.64	29.16mm
2.	*Brassica compestris*	Asteraceae	seeds	8.5	0.29	…..
3.	*Detura stremonium*	Solanaceae	seeds	10.5	0.36	…..
4.	*Allium sativum*	Liliaceae	corn	12	0.14	…..
5.	*Phylanthus emblica*	Euphorbiaceae	fruits	15	0.52	…..
6.	*Terminalia arjuna*	Fabiaceae	fruits	13	0.45	…..
7.	*Terminelia chebule*	Fabiaceae	fruits	12	0.41	…..
8.	*Mengifere indice*	Anacardiaceae	fruits	16	0.55	…..
9.	*Punica grantum*	Punicaceae	Seeds+fruits	10	0.34	…..
10.	*Vemonia anthelmentica*	Brassicaceae	fruits	16	0.55	…..
11.	*Dodonee viscose*	Sapindaceae	fruits	805	0.29	…..
12.	*Thuja orientalis*	Pinaceae	fruits	18	0.62	…..
13.	*Zingiber officinalis*	Zingerbraceae	Rhizome	10.8	0.37	…..
14.	*Celotropis procere*	Asclepidaceae	fruits	8.5	0.29	…..
15.	*Nigella sativum*	Solanaceae	seeds	9	0.31	…..
16.	*Piper nigrum*	Piperaceae	seeds	7.8	0.27	…..
17.	*Anthum greveolens*	Umblliferae	fruits	9.5	0.33	…..
18.	*Amomum subuletum*	Zingerbraceae	Seeds+fruits	13	0.45	…..
19.	*Papver somnifere*	Paperveraceae	seeds	6.8	0.23	…..
20.	*Pegnum hermala*	Rutaceae	seeds	10.5	0.36	…..
21.	*Linum usitatissimum*	Linaceae	seeds	9.5	0.33	…..
22.	*Citrus limon*	Rutaceae	fruits	16.5	0.27	…..
23.	*Curcuma longa*	Zingerberaceae	Rhizome	10.8	0.37	…..
24.	*Ricinus communis*	Euphorbiaceae	Seeds+fruits	7.5	0.26	…..

IZ = Inhibition zone (in mm), AI = Activity Index
Standard drug: Sreptomycin
Activity Index = Inhibition zone of the test sample inhibition zone of the standard drug

Fig. 2. Inhibition zones with decoction *Azadirachata indica* against *X. oryzae*.

Dried extract was subjected to TLC plate experiment in different solvent system and petroleum. ether: Ethyl acetate EtOAc: toulene (10:7:3) afforded best separation. Five fractions A1toA5 were collected and tested antibacterial activity against *Xanthomonas oryzae*, Fraction A1 found to be more effective.

TLC plate fraction (Activity Index) was further purified using solvent system (petroleum ether: toluene: ethyl acetate: (10:2:10), resulted a pure compound RJ (1) as semi liquid compound 40 mg yield, UV (λ) 267nm (0.953), Rf value 0.83.

Compound Rj(1) showed band under Infra-red IR spectrum , 3440cm^{-1} indicating (OH stretching) peak at 2910 cm^{-1} representing (CH stretching),while band of 1740 cm^{-1} indicating (COOR) functional group, band at 1600 cm^{-1} indicating (C=C stretching) ,while peak at 1250 cm^{-1} representing (C-O-C stretching (Table 3).

Table 3. IR Spectral data of compound Rj(1).

S. No.	Functional group	Absorption (cm^{-2})
1.	OH	3440
2.	CH	2910
3.	COOH	1740
4.	C=C	1600
5.	C-O-C	1250

The Nuclear magnetic resonance (1HNMR) spectrums 500 MHz $CDCl_3$, The three-spin system in the molecule indicate in compound Rj (1) two singlet at δ5.29 at H-23/24, while two doublet appeared at δ 5.30 with coupling constant (j=6.60H/z) indicating olefanic proton. Triplet appeared at two triplet at δ1.56 with coupling constant (j=6.52H/z) due to two proton at adjacent one triplet appeared at δ1.2 with coupling constant δ2.92 with coupling constant j=2.92 H/Z) indicating the presence of H-7 having two adjacent proton, while six singlet appeared at the peaks δ1.56, δ1.56, δ0.84, δ3.6, δ1.18, δ1.18 (Fig. 3; Table 4).

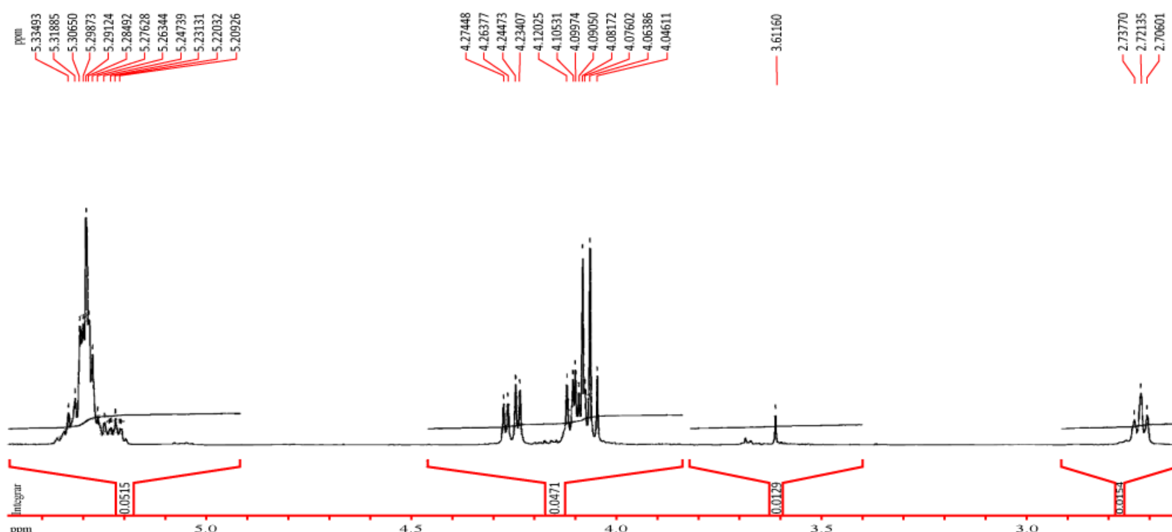

Fig. 3. ^{1}HNMR spectra of compound Rj(1).

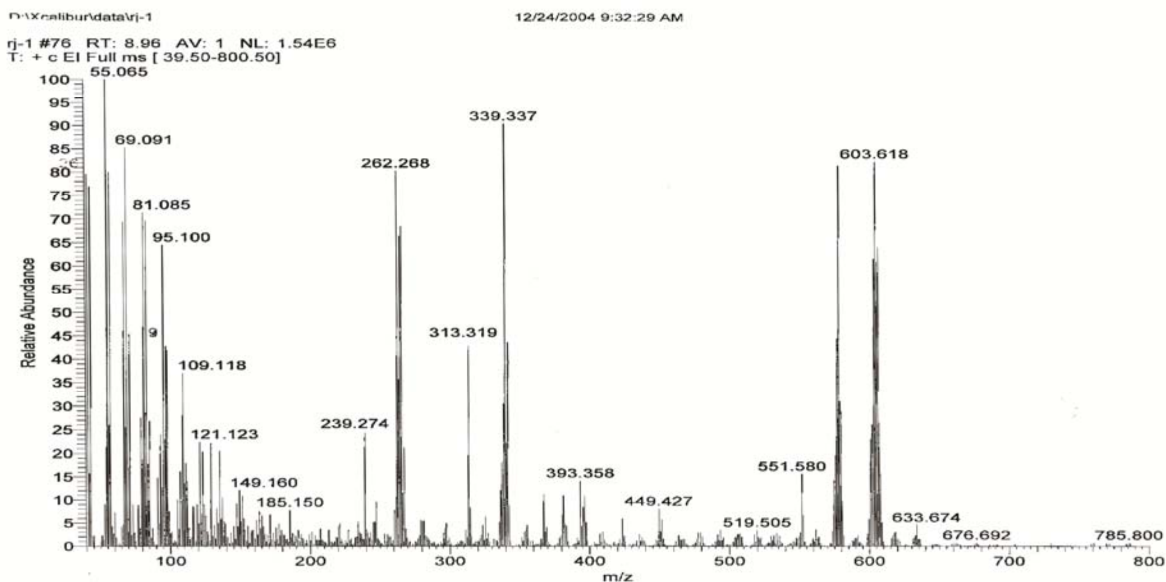

Fig. 4. Mass spectra of compound Rj(1).

EI-MS, mass spectrum of compound Rj (1) showed molecular ion peak at m/z 676, corresponding to molecular formula $C_{34}H_{40}O_{14}$, at m/z 633(5%), 617(3%), 604 (85%), 449 (8%), 393(14%), 339(92%), 313(45%), 83(69%), 55 (100%) were major peaks. The m/z55 were the basic peak.

The peak of 633m/z indicates the loss of 43 m.u of CH_3CO fragment. While peak at 617 m.u indicating the loss of CH_3COO m.u 59. Peak 604m/z shown the loss of 13 m.u showing the loss of CH. Peaks at 449m/z, 393m/z, 339m/z, 313m/z representing the loss of 155m.u, 56m.u, 54m.u, 26m.u, indicating the loss of $C_{12}O_4$,$O_{15.}$ While peak at 83m/z,55m/z showed the loss of 230 m.u, 28 m.u indicating the loss of C_2O_2, 2(C_2H_4) C_2H_4 (Fig. 4).

From above, spectroscopic data purposed structure of a compound, it was to be 1-Tigloyl-3-acetyl-1, 1-hydroxymeliacarpin Azadirachtin D (Figs. 5, 6).

EI/MS: 676 (M) 633 (5%), 617 (3%), 604 (85%), 449 (8%), 393 (14%), 339 (92%), 313 (45%), 83 (69%).

Bio autographic assay: In the present work, the bioautographic method proved effective for detecting the active promising fractionated compound in crude plant extracts. 1-Tigloyl-3-acetyl-1, 1-hydroxymeliacarpin1-Tigloyl-3-acetyl-1 and 1-hydroxymeliacarpin were tested for antibacterial activity through direct bio autographic method. Isolated compound showed inhibition against tested bacterium forming dark red inhibition zones against blue background, proving them to be effective. Similar results also reported in literature (Hostettmann, 1999; Pacher *et al.*, 2001; Yang *et al.*, 2011; Hassan & Sadek, 2012).

Table 4. ^{1}HNMR spectral data of compound Rj (1).

Proton	Multipticity	Chemical shift	Coupling constant (H_2)
H-1	s	1.18	
H-2	s	1.18	
H-3	s	3.6	
H-4	s	0.84	
H-5	s	1.56	
H-6	s	1.56	
H-7	t	1.2	2.92
H-8	m	1.97	
H-9	m	1.97	
H-10	t	1.2	2.92
H-11	s	1.25	
H-12	s	1.25	
H-13	d	4.2	4.28
H-14	d	4.2	4.28
H-15	s	0.84	
H-16	s	1.25	
H-17	t	1.56	6.52
H-18	t	1.56	6.52
H-19	m	2.25	
H-20	m	2.25	
H-21	d	5.3	6.6
H-22	d	5.3	6.6
H-23	s	5.29	
H-24	s	5.29	

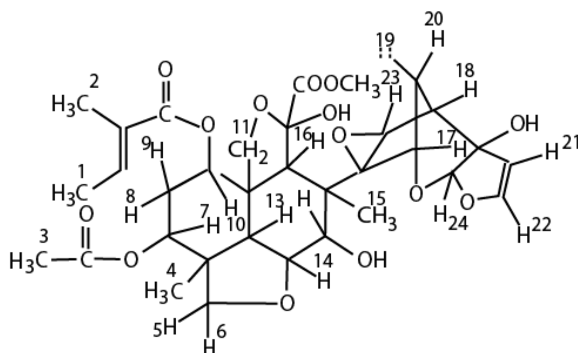

Fig. 5. 1Tigloyl-3-acetyl-1. 1-hydroxymeliacarpin (Azadirachtin D).

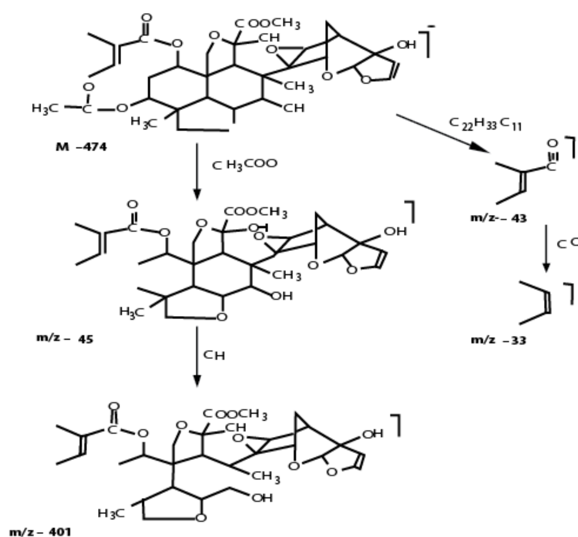

Fig. 6. Mass fragmentation pattern of compound Rj(1).

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