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MEDICINAL PLANTS - A POTENT ANTIBACTERIAL SOURCE AGAINST BACTERIAL LEAF BLIGHT (BLB) OF RICE

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Abstract

The antibacterial potential of indigenous medicinal plants as alternative chemical pesticides for controlling bacterial leaf blight (BLB) of rice was investigated. Twenty-five different species of medicinal plants were collected from various sites in Pakistan. Decoctions of all medicinal plant species were screened by the disc plate diffusion method for testing the susceptibility of an aggressive isolate of *Xanthomonas oryzae* pv. *oryzae* (Xoo 105). Out of twenty five medicinal plants, *Thuja orientalis* (cone + leaves), *Azadirachta indica* (seeds + fruits), *Amomum subulatum* (fruits), *Terminalia chebula* (fruits), *Terminalia bellirica* (fruits), *Anethum graveolens* (fruits) and *Ferula assa-foetida* (fruits) decoctions showed significant activity. The efficacy of decoctions from six promising plants were further tested through detached leaf, glasshouse and field assays. A decoction of *Terminalia chebula* demonstrated the highest effectiveness in terms of regulating BLB in the plants both under laboratory and field conditions. Bioactive fractions of *Terminalia chebula* were purified, characterized and tentatively identified as allegic acid.

Introduction

Rice is one of the major cereals used all over the world (Salim *et al.,* 2003).Tropical and sub-tropical regions of the world are the major rice-producers, with 90% of production occurring in Asia (Ezuka & Kaku, 2000). This important crop suffers from 40 different microbial diseases, with bacterial leaf blight (BLB) the most devastating and harmful. The *Xanthomonas oryzae* pv. *oryzae* strain has been the most serious in South East Asia, particularly since the widespread cultivation of dwarf high-yielding cultivars . It has caused huge yield losses during recent years. In Japan the yield losses reported ranged between 20-30%, occasionally increasing up to 50% (Ou, 1985).

Bacterial leaf blight was first reported in Pakistan in 1977 (Mew & Majid, 1977). It is a very serious disease, causing millions of tons of grain loss annually. In Pakistan the incidence of BLB has increased in recent years, especially in Kaller belt, which is famous for producing high quality rice (Akhtar e*t al*., 2003).

Bacterial leaf blight can be effectively managed by using Bordeaux mixture, a copper mixture and a copper mercury mixture. An application of copper-oxychloride and streptomycin completely inhibited of bacterial growth in one study (Hori, 1973). A streptomycin mixture was tested in India for disinfection of rice seeds and it proved to be effective (Srivastava, 1972) BLB lesions in rice were reduced by using bleaching powder with 30% chlorine (2 kg/ha) to disinfect rice seeds (Chand *et al.*, 1979).

However, excessive chemical use has a detrimental effect on the environment, farmers' and consumers' health, and also causes severe foliar damage to the standing crop and harms beneficial predators and parasitoids (Susan *et al.,* 2001; Brown *et al*., 1990; Keifer *et al*., 1997). Certain plants have been known for their medicinal and antimicrobial properties since ancient times. Their products can offer advantages because they are relatively safe and easily biodegradable. Biologically active compounds that effectively control various pests and pathogens are known from approximately 2400 plant species (Saleem, 1988). The antibacterial activities of different plant extracts against plants disease have been previously investigated (Okigbo & Nmeka, 2005; Suberu, 2004; Leksomboon *et al.,* 2001; Aktar *et al*., 1997).

Plant extracts are screened to detect secondary metabolites with biological activities, including antimicrobial activity. Chromatography techniques, including thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and column chromatography, are used to identify and characterize secondary metabolites. Alkaloids are the most important active chemicals found in plant extracts. Alkaloids largely occur as common salts in plants.

Many secondary metabolites demonstrate high efficacy against microbes. Saponified and un-saponified fractions of brown and green seaweed were found to be very effective against *Xanthomonas oryzae* pv. *oryzae*, (Arun Kumar & Rengasamy, 2000). Essential oils are also known to possess antimicrobial activity and antioxidant properties. Thymol, carvacrol, *p*-cymene and *y*-terpinene (36.5%, 29.8%, 10.0%, 6.3%, respectively), isolated from essential oil of *Thymus spathulifolium*, gave positive results (Sokmen *et al*., 2004). Phenolic and flavanoid compounds separated from other constituents of crude extracts of plants have also been successfully tested for their antioxidant and antimicrobial activity (Mothana & Lindequist, 2005).

This study screened decoctions from various plants for their effectiveness in controlling bacterial leaf blight of rice. The most promising bioactive compounds were further purified and characterized and may serve as new tools for controlling BLB of rice.

Materials and Methods

The present research work was carried out at Plant Bacteriology lab, Crop Diseases Research Programme (CDRP), Institute for Plant and Environmental Protection (IPEP), National Agriculture Research Center (NARC), Islamabad, Pakistan in collaboration with the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

Source of bacterial inoculum

Sample collection: A comprehensive survey of various agro-ecological zones in Pakistan was conducted for the collection of leaf samples of rice suspected to be infected with bacterial leaf blight. The samples were used for isolation and characterization of bacterium. The recovered isolates and most aggressive isolate of *Xanthomonas oryzae* pv. *oryzae* (Xoo 105) were used for testing antibacterial activity.

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Extraction method: Decoctions were prepared from dried plant parts was collected from Islamabad and dried under shade or purchased from the local market. The dried plants material was ground into powder form. Powder (50 g) and 100 ml of distilled water were placed in a conical flask, autoclaved for 15 min and filtered through 3 layers of cheese cloth. Extraction methodology was adopted from Rahber-Bhatti, (1986).

1. Lab assay

a. Hole plate diffusion assay: The Hewitt and Vincent, (1989) method was adopted for evaluating the plant decoctions' activity against the bacterium. The antibacterial activities of the most effective plant decoctions were checked by comparing them to streptomycin (1 gm/ml). The activity index was calculated by using formula:

$$\text{Activity index} = \frac{\text{Inhibition zone of test sample}}{\text{Inhibition zone of the standard}}$$

b. Evaluation of potential plant decoctions through detached leaf assay

1. Protective method: The young leaves of rice variety Basmati 385 were dipped 5-10 min in different concentrations (50, 20, 5 g/ml) of plant decoction and inoculated with Xoo 105 suspension (10^8 cfu/ml) using the pin prick method. Three leaves were kept in glass petri plates on three layers of water-saturated blotting paper. The control leaves were dipped in sterile distilled water and then inoculated with the most aggressive isolate. Three plates of each treatment and a control were incubated at 28°C for 24 h under illumination. The lesion length was measured in cm.

2. Curative method: The leaves of rice variety Basmati 385 were inoculated with a suspension (10^8cfu/ml of the most aggressive isolate (*Xanthomonas oryzae* pv. *oryzae*, Xoo 105). Plant decoctions of different concentrations (50, 20, 10, 0 g/100 ml) were applied on leaves placed in glass petri plates. The control leaves were dipped in sterile distilled water. The plates were sealed with adhesive tape and incubated at 28°C.

3. Glass house assay: For assessment of antibacterial activity under glass house conditions, a glass house assay was performed. Plant decoctions (50 g/100 ml) were applied to 60-70-day-old rice plants that were inoculated using the clipping method (Kauffman *et al*., 1973). The plants were inoculated with a suspension of Xoo 105(10^8 cfu/ml). Protective/curative effects were assessed by measuring the length of lesions in cm after 14 to 22 days.

2. Field trial: Field trials for testing the efficacy of plant decoctions against *Xanthomonas oryzae* pv. *oryzae* were conducted at National Agriculture Research Centre (NARC), Islamabad, Pakistan.

a. Field nursery: Seeds of rice varieties Basmati 385 and Super Basmati were soaked (100 g/m^2) overnight and sown in the field nursery during the first week of June, 2004. The seeds were spread on seed beds covered with dried plant material (wheat or rice straw) and kept moist. After one month (in the first week of July) the seedlings were removed from the nursery and transplanted into the field.

b. Preparation of bacterial inoculum: The cultures of the most aggressive isolate were prepared by streaking a loop full of each isolate in the middle of nutrient agar plates, which were then inoculated at 28°C. The bacterium was washed from the plate surface after 24 h with 5 ml of sterile saline water. The inoculum was serially diluted and adjusted to a concentration of 10^8 cfu/ml.

c. Preperation of decoctions: Decoctions plants were prepared from dried plant parts ground into powder form. Fifty grams of powder were added to 100 ml of distilled water in a conical flask, autoclaved for 15 min and filtered through three layers of cheese cloth.

d. Inoculation/treatment: Sixty- to seventy-day-old rice plants were inoculated with an aggressive isolate of *Xanthomonas oryzae* using the clipping method of inoculation. The curative and protective methods of application of plant decoctions were performed as mentioned earlier in the glass house assay. The percentage of disease incidence was calculated by using formula:

$$\text{Percent disease incidence} = \frac{\text{Total lesion length of the test sample}}{\text{Total leaf length of the test sample}} \text{ X } 100$$

$$\text{Percent control} = \% \text{ Disease incidence} - 100$$

Phytochemical studies: The plant decoctions that were most effective in terms of controlling BLB were selected for further phytochemical investigation.

Extraction procedure: Dried plant material (500 g/l) was extracted in chloroform, ethyl acetate and methanol; these extracts were concentrated by using a vacuum rotary evaporator.

Fractioning chemical compounds: The fractionation of crude extracts followed the method of Furniss *et al.*, (1989).

Characterization

Spectral studies of compounds: Mass spectrum and NMR spectrum were recorded at H. E. J. Research Institute of Chemistry, International for Chemical Sciences at the University of Karachi, Pakistan.

Bioautographic agar overlay method: The bioautography assay of Rahalison *et al.* (1991) was used for testing bioactivity of fractions against *Xanthmonas oryzae*.

Statistical analysis: Data were analyzed using ANOVA and significance at 5% level was tested with Duncan's Multiple Range Test (DMRT), using SAS/STAT software.

Results and Discussion

The antibacterial activity of 25 plant decoctions (50 g/100 ml) against *Xanthomonas oryzae* were assessed (Table 1). Only seven plant decoctions (*Thuja orientalis*, *Azadirachta indica*, *Terminalia chebula*, *T. bellirica*, *Anethum graveolens*, *Amomum subulatum* and *Ferula assa-foetida*) were found to have appreciable antibacterial activity. The best results were shown by *Terminalia chebula*, which showed a large inhibition zone (28.8 mm) with a high activity index value (0.98) (Table 1, Fig. 1).

Table 1. Controlling of *Xanthomonas oryzae* pv. *oryzae* with decoctions from parts of different plant species.

S. No.	English name	Latin name	Plant parts used	Mean I.Z. (mm)	A.I.
1.	Rai	*Brassica juncea* (L.) Czern	Seeds	12	0.42
2.	Night shade	*Solanum nigrum* L.	Seeds	7.2	0.2
3.	Cardamom	*Amomum subulatum* Roxb.	Fruits	22.16	0.75
4.	Tamarind	*Tamarindus indica* L.	Fruits	12	0.4
5.	Myrabolan	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Fruits	16.5	0.56
6.	Big myrabolan	*Terminalia bellirica* (Gaertn.) Roxb.	Fruits	22.5	0.77
7.	Black myrabolan	*Terminalia chebula* Retz.	Fruits	28.8	0.98
8.	Caraway	*Carum carvi* L.	Fruits	0	0
9.	Dill	*Anethum graveolens* L.	Fruits	22.8	0.78
10.	Black cumin	*Nigella sativa* L.	Seeds	0	0
11.	Basil	*Ocimum basilicum* L.	Seeds	0	0
12.	Neem	*Azadirchta indica* A.Juss.	Fruits	22.5	0.77
13.	Fennel	*Foeniculum vulgare* Mill.	fruits	0	0
14.	Turmeric	*Curcuma longa* L.	Rhizome	19	0.65
15.	Indian gooseberry	*Phyllanthus emblica* L.	Fruits	12	0.4
16.	Garlic	*Allium sativum* L.	Bulb	16.00	0.61
17.	Lemon	*Citrus* x *limon* (L.) Burm.f.	Fruits	15.00	0.51
18.	White cedar	*Thuja orientalis* L.	Cone	25.00	0.85
19.	Eucalyptus	*Eucalyptus globulus* Labill.	Leaves	0.00	0.00
20.	Sweet wood	*Glycyrrhiza glabra* L.	Stem	0.00	0.00
21.	Hemp	*Cannabis sativa* L.	Leaves	0.00	0.00
22.	Asafoetida	*Ferula assa-foetida* L.	Root	22.00	0.76
23.	Sesame	*Sesamum indicum* L.	Seeds	8	0.28
24.	Clove	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Fruits	0.00	0.00
25.	White mustard	*Sisymbrium irio* L.	Seeds	0.00	0.00

A.I. = Activity index, I.Z. = Inhibition Zone, H: highly effective (29 to 22 mm), I: Intermediately effective (22 to 14 mm), L: Less effective (14 to 8 mm), N: Not effective (0 mm), A.I. = Activity index

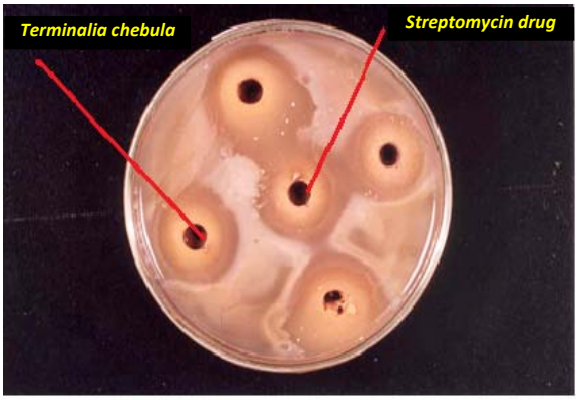


Fig. 1. Inhibition zone in culture of *X. oryzae* from decoction of *T. chebula*.

The plant decoction method adopted from Rahber-Bhatti, (1986) is an efficient method for extraction of plants' bioactive compounds. The active bio-compounds which are tightly bound and not easily extractable in cold water and hot water diffusates are easily released under high temperature boiling. This high temperature also sterilizes the extract, hence minimizing the chances of contamination. Similar work was done by Aktar *et al.*, (1997) to test plant decoctions against citrus canker disease of lime.

The effectiveness of decoctions from these seven plants was also tested through a detached leaf assay on rice variety Basmati 385 against the aggressive *Xanthmonas oryzae* isolate Xoo 105. The protective treatment (before inoculation) was found to be significantly more effective than the curative treatment (after inoculation). The most effective plant decoction was found to be *Terminalia chebula*; rice plants treated with it developed the smallest lesions (mean of 4.18 cm) (Table 2, Fig. 2). The other 6 species had longer mean lesions: *Amomum subulatum* 4.78 cm, *T. bellirica* 5.45 cm, *Ferula assa-foetida* 5.54 cm, *Thuja orientalis* 5.80 cm, *Anethum graveolens* 6.65 cm, *Azadirachta indica* 6.90 cm.

The same seven plant decoctions were also tested through a glass house assay on attached leaves of rice variety Basmati 385 against isolate Xoo 105. The decoction of *Terminalia chebula* showed maximum efficacy against bacterial leaf blight in the glass house assay, with small lesions for protective (mean of 3.76 cm) and for curative (mean of 6.46 cm) treatments.

Table 2. Protective and curative effects of different dosages of plant decoctions against BLB.

S. No.	Treatment	Plate assay		Detached leaf assay					Glass house assay				Field assay	
		I.Z. (mm)	A.I	Bacterial blight lesion length (cm) / Isolates									Incidence % (cm)	% Disease control
				Methods			10	0	50gm/ ml	20	10	0		
1.	*Amomum subulatum*	22.16	0.75	Protective	4.2	8.56	11.5	24	5.93	10.5	16.37	25.67	9.03	76.75
				Curative	5.36	9.36	11.37	25.1	6.76	12.5	16.67	17.67	11.36	71.60
				Mean	4.78ij	8.9g	11.68	24.5a	6.35gh	11.50cdefg	16.25bcdeg	21.67abc	Control-30	23
2.	*Anethum graveolens*	22.8	0.78	Protective	6.16	8.46	14.43	12.97	6.06	12.5	15.9	20.63		
				Curative	7.13	8.9	12.7	13.9	7.6	14.17	17.83	20.6		
				Mean	6.65h	8.68g	13.3b	13.43d	6.83fgh	13.33bcdefgh	16.8bcdef	20.62abcd		
3.	*Azadirachta indica*	22.5	0.77	Protective	6.66	13.17	17.8	23.87	6.46	11.53	17	21.43		
				Curative	7.13	13.1	18.86	25.13	6.56	11.2	19.5	20.13		
				Mean	6.90h	13.13de	18.30b	24.50a	6.51gh	11.37cdefgh	18.25abcde	20.78abcd		
4.	*Terminalia bellirica*	22	0.75	Protective	5.16	8.83	16	24.87	6.53	13.73	17.43	21.13		
				Curative	5.73	9.03	18.5	25.77	10.5	14.63	18.3	22.13		
				Mean	5.45hij	8.93g	17.25b	25.32a	8.51efgh	11.18bcdefgh	17.87abcde	21.63abc		
5.	*Thuja orientalis*	25	0.85	Protective	5.93	7.33	12.97	25.1	4.4	11.6	14.6	19.07	11	71.25
				Curative	5.66	10.13	13.9	26.6	7.6	10.23	16.47	19.03	25.36	74.64
				Mean	5.80hi	8.73g	13.43d	25.85a	6.7fgh	10.92defgh	15.53bcdefg	19.05abcd	Control-30	
6.	*Terminalia chebula*	28.8	0.98	Protective	3.56	6	11	25.1	3.76	12.23	13.9	21.5	6.7	83.25
				Curative	4.8	7.1	12.7	25.57	6.46	11.73	17.57	20.83	14.6	63.50
				Mean	4.18j	6.60h	11.85fi	25.33a	5.11h	27.33a	15.73bccdefg	21.17abcd	Control-30	
7.	*Ferula assa-foetida*	22.25	0.76	Protective	5.16	8.83	16	24-87	4.56	12.5	18.3	23.7		
				Curative	5.73	9.03	18.5	25.77	8.83	12.67	19.03	21.33		
				Mean	5.45hij	8.93g	17.25b	25.32a	6.7fgh	12.58bcdefgh	18.67abcde	22.52ab		

IZ= Inhibition zone (in mm), A.I. = Activity index; Activity index = Inhibition zone of the test sample/inhibition zone of the standard
Percentage disease incidence = Total lesion length of test sample/leaf length; % Disease control = % Disease incidence -100; Total leaf length = 40 cm

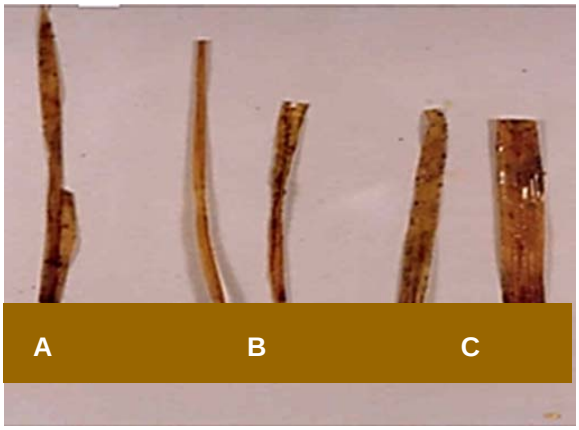


Fig. 2. Effects of decoctions on BLB lesions through detached leaf assay. A= *Terminalia chebula*, B= *Amomum subulatum*, C= *Thuja orientalis*

The 3 plant decoctions (*Terminalia chebula, Amomum subulatum, Thuja orientalis*) that showed maximum efficacy against the bacterium on the agar plate, detached leaf and glass house assays were selected for a field assay. The decoction of *Terminalia chebula* showed the maximum inhibition of BLB with a percentage disease control of 83.25%, compared to the control which showed 23% disease control. In field assays the curative method of extract application gave the best results (Table 2, Figs. 2, 3).

The decoction showed maximum efficacy during the glass house assay, perhaps due to controlled environment and incubation conditions that are not possible in field assays. The potential of plant extracts for controlling citrus canker, tested through glass house assays, has been reported by Leksomboon *et al*., (2001), who found that *Tamarindus indica* extract effectively inhibited citrus canker disease in lime. Kagale *et al*., (2004) reported that extracts of *Datura metel* effectively reduced the incidence of bacterial leaf blight under glass house conditions when a foliar treatment was applied before inoculation.

Isolation and spectral compound

Extracts (ethyl acetate, chloroform and methanol) from *Terminalia chebula* were tested for antibacterial activity against *Xanthomonas oryzae*. Methanolic extracts showed maximum activity against the bacterium. Hexane:ethyl acetate:chloroform (5:3:3) afforded the best separation. Five fractions (T1-T5) were collected; fraction T3 showed maximum activity against *X. oryzae* and was further purified on TLC plates using a solvent system of hexane:ethyl acetate (10:5). Pure compound Rj (4) was isolated in the form of a white liquid with 40 mg yield, UV (272) nm (1.54), Rf value 0.91. Infrared (IR) spectra showed bands at the following frequencies: 3400, 2910, 1730, 1470, 1120 nm, suggesting the presence of OH stretching, CH stretching, COOR, C=C stretching, and C-H (cyclic) functional groups (Table 3, Fig. 6).

The HNMR spectrums 500 MHz CDCl of the compound Rj (4) indicate the presence of a three-spin system in the molecule. One singlet appeared at d7.51, indicating the presence of hydrogen for one monomer (benzylic), while another singlet appeared at d7.68, indicating the presence of hydrogen for another monomer (Table 4, Fig. 5).

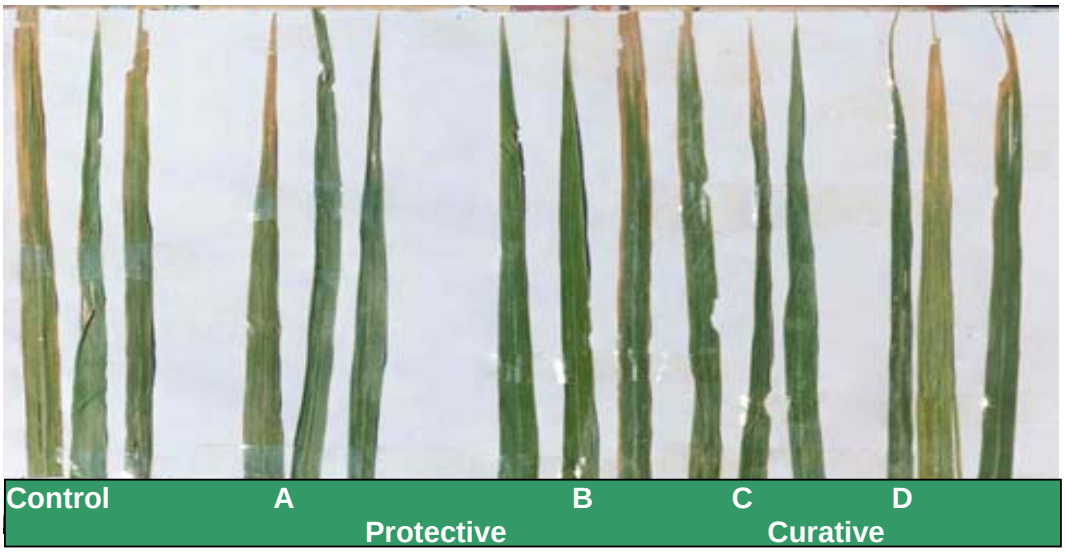


Fig. 3. Effects of *Terminalia chebula* decoctions on BLB lesion through field assay. Control, A-B. Protective treatment, C-D. Curative treatment.

Table 3. IR spectra of the compound Rj (4).

No.	Functional group	Absorption (nm)
1.	OH	3400
2.	CH	2910
3.	COOR	1730
4.	C=C	1470
5.	C-H (cyclic)	1120

Table 4. ^{1}HNMR spectra of the compound Rj (4).

Protons	Multiplicity	Chemical shift
H-1	S	7.68
H-2	S	7.51

EI/M 603 (2M-H) 515 (2.26%) 449 (2%) 109 (24%) 92 (25%) 80 (44%) 64 (3%): ^{1}HNMR data were further supported by EI-MS; mass spectra of compound Rj (4) showed a molecular ion peak at m/z 603, corresponding to the molecular formula C_{14} H_6 O_8.

Other major peaks were found to occur at m/z 515 (2.26%), 449 (2%), 109 (24%), 92 (25%), 80 (44%), 64 (3%). The peak 515 m/z showed the loss 88 m.u., indicating 2 CO molecules. Peak 449 m/z, 109 m/z, 92 m/z showed the loss of 66 m.u., 340 m.u., 17 m.u. peaks at 80 m/z and at 64 m/z indicate the loss of 12 m.u. (Fig. 4).

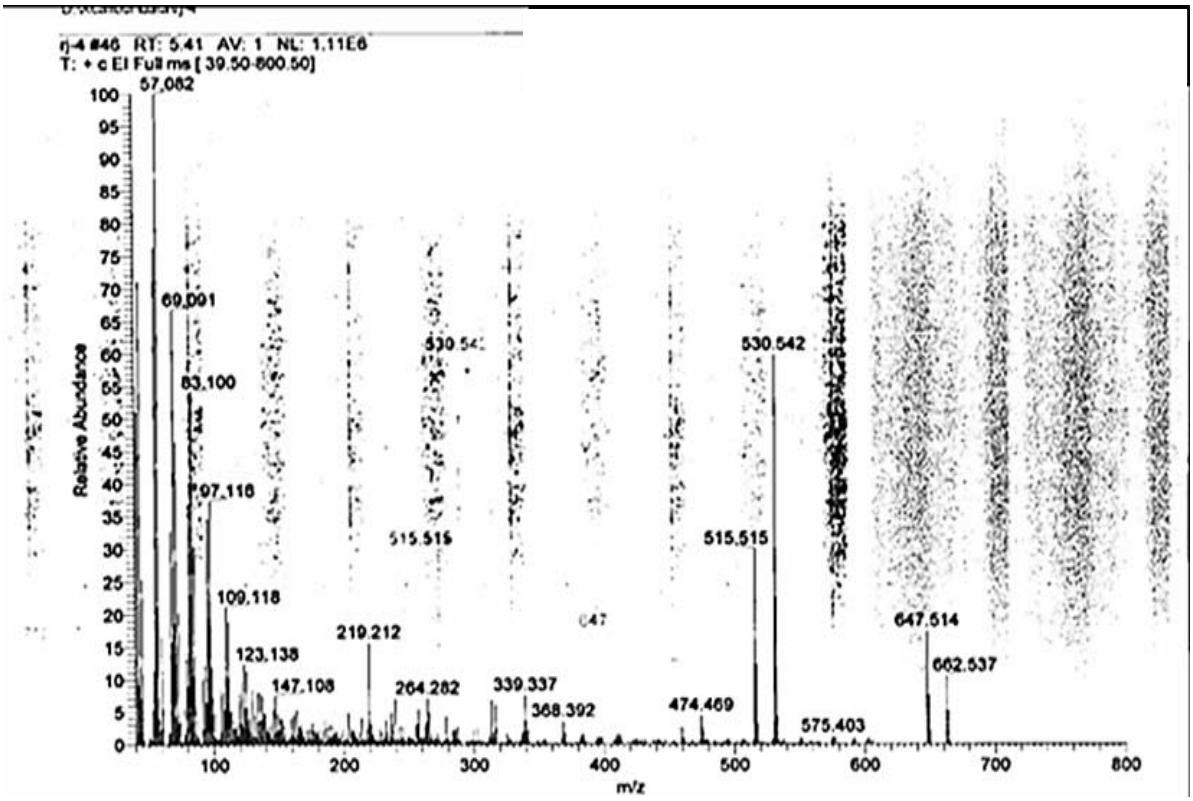


Fig. 4. Mass spectra of compound Rj (4).

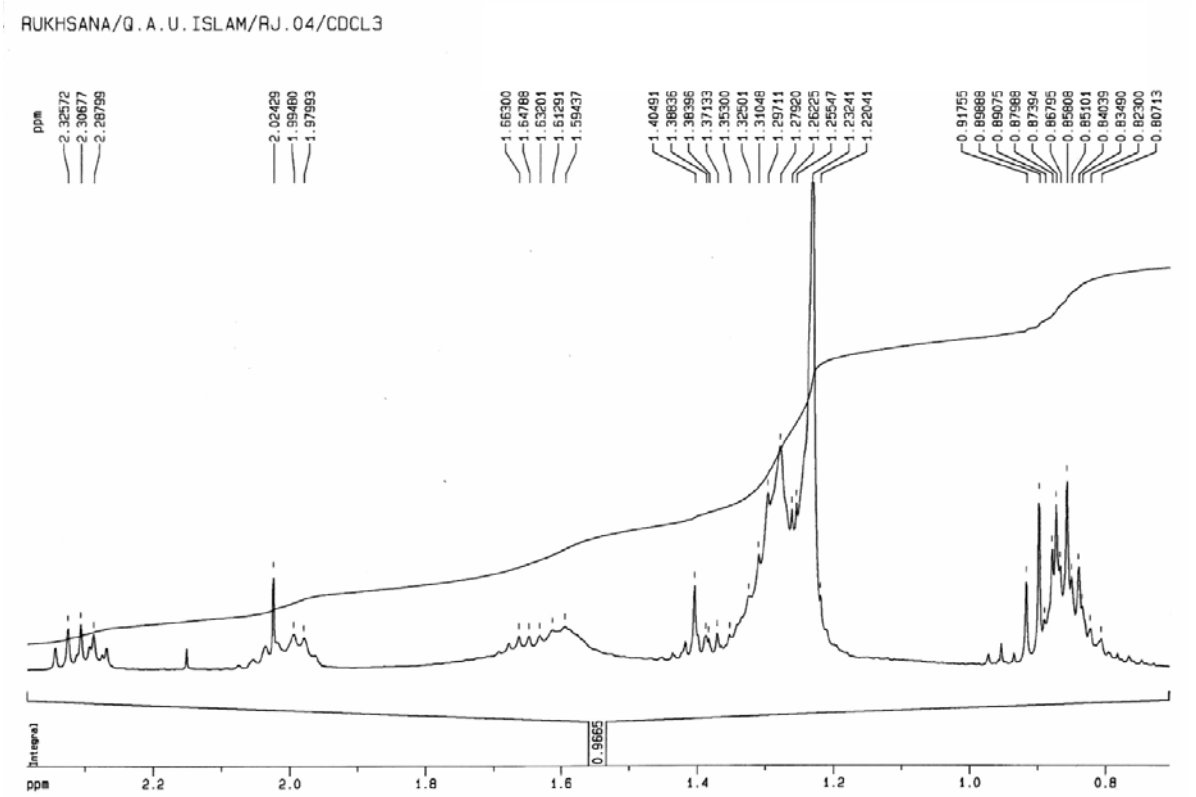


Fig. 5. [1]HNMR spectra of compound Rj (4).

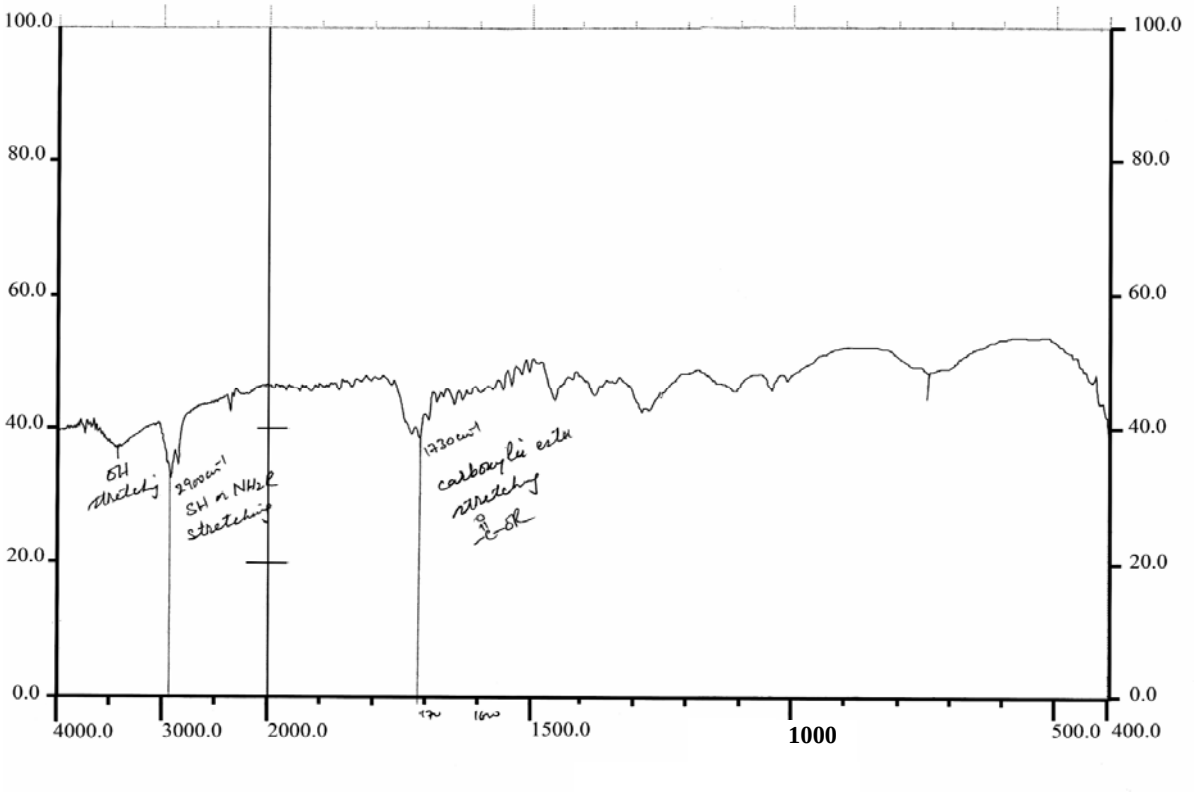
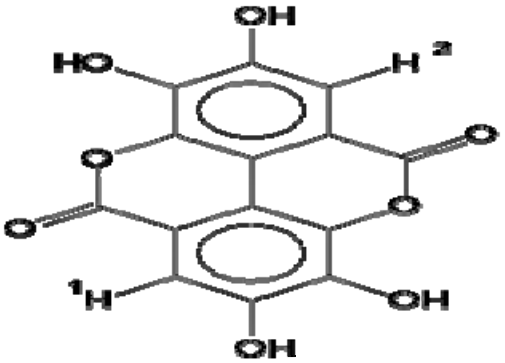


Fig. 6. IR spectra of compound Rj (4).

From the mass fragmentation of compound rj4 (fig-8) determin the formula of the compound **C_{14} H_6 O_8,** allegic acid. A 3,4,5-trihydrooxybenzoic acid, was isolated from the fruits of *Terminalia chebula*.(fig-7) A similar phytochemical study of *Terminalia arjuna* elucidated the structure of terminic acid, a new dihydroxytriterpene carboxylic acid isolated from its roots (Anjaneyulu & Prasd, 1982). The bioautographic method permits localization of antimicrobial active compounds that have been separated by TLC. In the present work, the method proved effective in detecting the active components in crude plant extracts. Allegic acid was tested for antibacterial activity through the direct bioautographic method. Isolated compounds showed inhibition against the tested bacterium, forming dark red inhibition zones against a blue background, proving them to be effective. This technique was outlined by Rahalison *et al.* (1991).



ig. 7. C_{14} H_6 O_8 (Allegic acid)

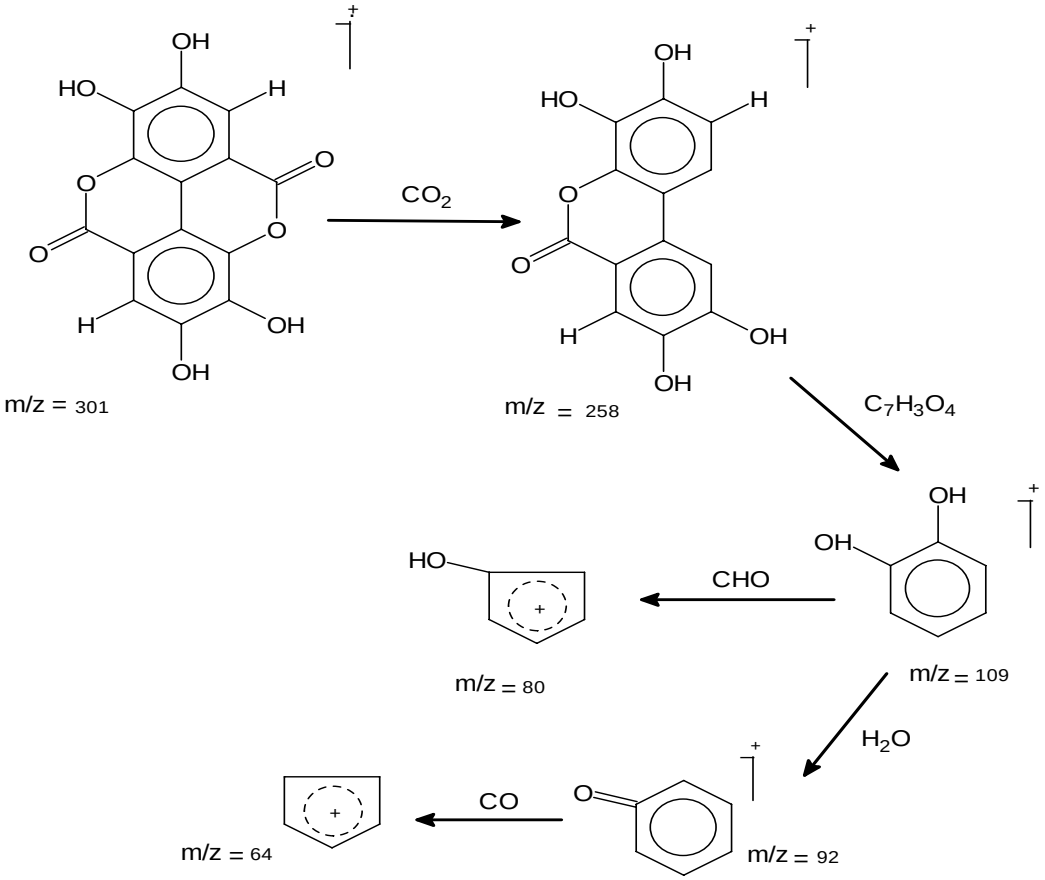


Fig. 8. Mass fragmentation pattern of compound Rj (4).

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