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PURIFICATION AND BIOASSAYS OF BIOACTIVE FRACTION FROM *CURCUMA LONGA* AGAINST *XANTHOMONAS ORYZAE* PV.*ORYZAE CAUSING* BLB DISEASE IN RICE

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Abstract

In the present study hot water diffusates of different plant species (100gm /100ml) were screened for testing antibacterial activity using hole plate diffusion method against most aggressive isolates of *Xanthomonas oryzae* Pv.*oryzae* (Xoo 105). Eight plant species *Citrus limon,Linum usitatissimum, Mangifera indica Phyllanthus emblica, Prunus domestica,Tamarindus indica,Terminalia arjuna and Curcuma longa* exhibited maximum inhibitory action against test bacterium, among them *Curcuma longa* member of Zingiberaceae family showed maximum antibacterial activity , forming inhibition zone of 28.45mm in diameter, showing activity index 0.98 compared with streptomycin drug. Extract of *Curcuma longa* isolated from rhizome were supposed to be Curcumin.

Introduction

Rice (*Oryza sativa* L.) is a staple food for 27 billion people world wide (Salim *et al.*, 2003). It a member of the grass family (Poaceae) and is largely grown in the zone of monsoon, tropical and sub tropical regions of the world (Ezuka & Kaku, 2000). About 90% of the world rice is grown in the Asian continent. The rice crop is susceptible to more than 40 diseases which is a main factor for its low yield. Among bacterial diseases, Bacterial Leaf Blight is the most devastating disease in the world particularly in South East Asian countries where million tones of grain loss is reported annually due to BlB. The antibacterial activities of different plant extracts against plant disease have been observed in rice (Leksomboon *et al.*, 2001), wheat (Ayoub & Niazi, 2001), brinjal (Zarina *et al.*, 2003), peanut and soyabean (Suberu, 2004),

Curcuma longa a member of the family Zingiberaceae is distributed throughout tropical and subtropical region of the world, posses antimicrobial activity, anti insecticidal properties .as well as used as spices and dye. This paper deals with the isolation and purification of bio active compound of *Curcuma longa* and their chemical formula and structure were elucidated as new tools for the management of BLB disease of rice. Further investigations are needed on effective control of Bacterial Leaf Blight by these bioactive compounds with respect to their concentrations and development into biopesticides.

Materials and Methods

Extraction of hot water diffusates: Hot water diffusates were prepared from dried plant parts ground in powder form. 100gm of this powder was soaked in 100 ml hot water for 24h and filtered through three layers of cheese cloth.

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1. Plate assay

Antibacterial susceptibility testing: Crude hot water diffusate was used for testing antibacterial activity through hole plate diffusion method (Hweitt & Vincent, 1989). The activity index was calculated by using formula: with streptomycin drug 1gm/ml. Activity index = Inhibition zone of test sample/ Inhibition zone of the standard

2. Detached leaf assay: The most promising hot water diffusate extracts was used against most aggressive isolate Xoo 105 and most susceptible rice variety of Basmati 385.

Protective/curative methods: The young leaves of rice variety Basmati 385 were dipped for 5-10 min in different concentrations (50, 20, 5 gm/ml) of the above mentioned plant extracts and inoculated with bacterial suspension (10^{-8} cfu/ml) using pin prick method. Three leaves were kept in glass Petri plates on three layers of water saturated blotting paper. Three plates of each treatment and control were incubated at 28^{o} C for 24 h under illumination. The lesion length was measured in. In protective method the plant extracts are applied before inoculation and in curative method the plant extracts are applied after inoculation of test bacterium.

3. Glass house assay

Protective /curative methods: The leaves of 60 to 70 days old rice plants were inoculated with suspension of most aggressive isolate After two days of inoculation hot water diffusates were sprayed with hand sprayer and covered with polythene bags. After 24 h, the bags were removed and the lesion length was measured in cm after 14 to 22 days. In protective method the plant extracts are applied before inoculation and in curative method the plant extracts are applied after inoculation of test bacterium

Field trials: Field trials for testing efficacy of crude plant extracts against test bacterium *Xanthomonas oryzae* Pv. *oryzae* were conducted at fields of NARC (National Agriculture Research Centre) Islamabad, Pakistan.

For nursery raising the seeds of rice variety Basmati 385 and Super Basmati were soaked ($100g/m^2$) overnight and sown during the first week of June.The seeds were spread on seed bed covered with dried plant material (wheat or rice straw) and kept moist by adding water. After one month (in the first week of July) the seedlings were removed from the nursery and transplanted in the field.

Preparation of bacterial inoculum: The cultures of the most aggressive isolate were prepared streaking a loop full of each isolate in the middle of nutrient agar plates and inoculated at 28^{o}C. The bacterium was washed from plate surface after 24h with 5ml of SDW. The inoculum was serially diluted and adjusted to a concentration of 10^{8}cfu ml^{-1}.
The hot water diffusate of *Curcum longa* (1000gm/100ml), was prepared by method as mentioned earlier

Inoculation/treatment: Sixty to seventy days old rice plants were inoculated with most aggressive isolates of *Xanthomonas oryzae*, using clipping method of inoculation. The curative and protective methods of application of plant extracts were performed .The percentage disease incidence was calculated by using formula:

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

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

Statistical analysis: The results of the measurement were subjected to ANOVA and significance at 5% level was tested by Duncan's multiple range test.

Phyto-chemical studies

Isolation and extraction of chemical compounds: The dried plant material was dipped 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 three extracts of methanol, ethyl acetate and chloroform were concentrated on vacuum rotary evaporator.

Purification of chemical compounds by column chromatography thin layer chromatography (TLC): The collected fractions were purified on glass column and preparative TLC plates.

Characterization of purified chemical compounds: Four spectroscopic tests Ultraviolet (UV) Spectroscopy ,Infra Red (IR) Spectroscopy, Mass Spectroscopy of compound Nuclear Magnetic Resonance (NMR), were carried out at Research Institute of Chemistry, International for Chemicals Sciences University of Karachi, Pakistan.

Bioautography assay: Bioautography assay was used to detect the active compounds followed by Hostettmann ,(1999)

Results and Discussion

The hot water diffusates of different plant species (100gm /100ml) were used for testing antibacterial activity using hole plate diffusion method against most aggressive isolate of (Xoo 105). Eight plant species viz., *Citrus limon,Linum usitatissimum, , Mangifera indica Phyllanthus emblica, Prunus domestica, , Tamarindus indica,, Terminalia arjuna,Curcuma longa* exhibited maximum inhibitory action against test bacterium with streptomycin drug, *Curcuma longa* showed (0.98) maximum activity index value (Fig. 1). In hot water extracts the bio active compounds easily dissolve in hot water which deeply deposit in skin, pulp and seeds and cannot easily diffuse out in cold water extracts. The efficacy of hot water extracts is checked by measuring inhibition zone diameter. Such observations are also supported by Okigobo and Nmeka (2005).

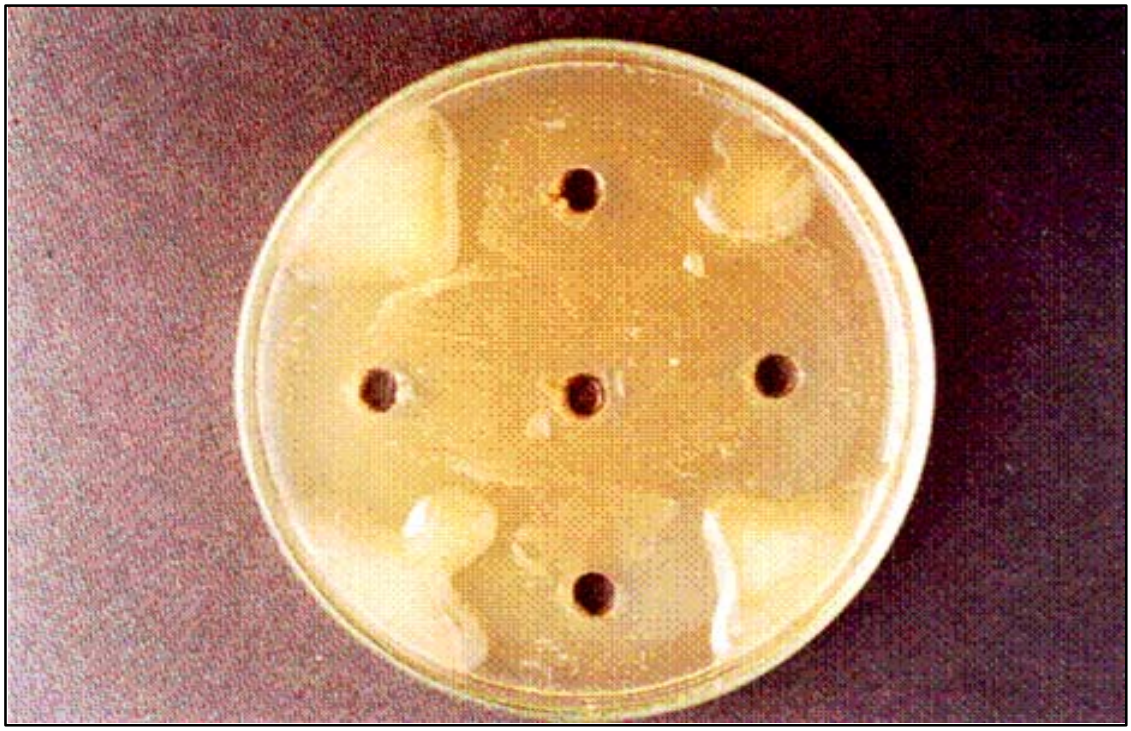


Fig. 1. Inhibition zones with hot water diffusates of *Curcuma longa* against *X. oryzae*

At 5% level of significance, efficacy of 8 hot water diffusates were tested through detached leaves assay,Potted plant assay and field assy using both protective and curative method. The most promising concentration was found to be 50 gm /100ml *Curcuma longa* showed high effectiveness to control BLB disease producing small lesion length 4.00 cm in detached leaf assay, 3.25cm in potted plant assay and 90.20% percentage disease control, as compared to control showing 23 % disease control in field assay (Table 1). The potential of plant extracts for controlling citrus canker through glass house assay has also been observed by Leksomboon *et al.,* (2001), who reported that *Tamarindus indica* extract effectively inhibits the citrus canker disease in lime. (Kagale *et al*., 2004).

Isolation and spectral studies of compound *Curcuma longa* Rj (2): The rhizome of *Curcuma longa* Vern domestica 1 kg was sucessively extractd with methonal,ethyl alcohol,chloroformTwelve fractions C1 to C12 were collected and tested antibacterial activity against *X. oryzae* Fraction C showed ,maximum activity against *Xanthomonas oryzae* and further purified on TLC plates using solvent system petroleum ether:ethylacetate:chlorofom (6:4:2) resulted a pure orange gummy compound Rj (2) with 200 mg yield ,UV(λ) 262 nm (0.908),Rf value 0.29

Infra red (IR) spectra showed the absorption band at 3440 cm^{-1} (OH stretching), 2880 cm^{1} (CH stretching), 1635 cm^{-1} (c=o stretching), 1605 cm^{-1} (C=C stretching) (Table 2).

The nuclear magnetic resonance ^{1}HNMR spectrum of the compound Rj(2) 1-(2,6-dihydroxy-3-methoxy-4-(2-(pentyloxy) ethoxy) phenyl)-11-(2,4-dihydroxy-5-methoxy phenyl)-3,9-dihydroxy undeca-7,10-diene-5-one was carried out at 500m/z,($CDCL^{3}$).One siglet appear at δ 7.2 indicating methoxy group at aromatic ring. A doublet appeared at δ7.3 with coupling constant (J=1.0H/z) showing para coupling. A doublet of double appear at δ7.10 and δ7.45 (coupling constant j=7.28h/Z, J=15.7 H/Z showing aromatic proton. A doublet appeared at δ6.80 with coupling constant (J=7.28 H/Z) indicating olefinic proton. Two triplet appeared at δ3.92 and 4.06 with same coupling constant (J=6.8 H/Z). Two protons on adjacent carbon atoms.

Table 1. Protective and curative effects of different dosages of hot water diffusate against BLB disease lesion on different assays.

S. No.	Treatment	Plate assay		Detached leaf assay					Glass house assay				Field assay	
													%	% Disease
		IZ (mm)	A.I	Methods	50	20	10	0	50	20	10	0	incidence	control
1.	*Citrus limon*	25.00	0.85	Protective	6.73pqrst	9.43mnop	15.40hij	22.37ab	9.33opqr	11.46lmnop	19.46bcde	20.36abcd		
				Curative	4.36tu	5.60qrstu	12.43kl	22.93abcd	8.50qrs	11.46lmnop	17.4efg	18.7cdef		
				Mean					8.91i	11.47gh	18.47d	19.53bcd		
2.	*Linum usitatissimum*	23.00	0.78	Protective	13.6Jk	16.43fghi	19.93abcd	22.03ab	3.70vw	9.40opqr	12.23bcdef	20.40abcd		
				Curative	9.26mnop	10.50lmno	18.53cdef	20.50abcd	3.10vw	10.16nopqr	14.43hijk	19.13bcdef		
				Mean	11.43fg	13.47e	19.23c	21.27ab	3.40l	9.78hi	16.83e	19.77bcd		
3.	*Mangifera indica*	27.00	0.93	Protective	5.16qrstu	8.06opq	15.43fghi	21.57ab	5.33tuvw	13.33ijklm	19.36bcde	21.03abc		
				Curative	3.43u	8.03opq	17.0efgh	20.00abc	3.83vw	8.53qrs	18.80cdef	20.23abcd		
				Mean	4.30k	8.05h	16.47d	20.78a	4.58kl	10.93gh	19.08bcd	20.68b		
4.	*Phyllanthus emblica*	28.00	0.96	Protective	6.9pqrst	8.80nop	15.53ghij	21.57ab	3.60vw	11.16lmnop	15.56ghi	19.60bcde		
				Curative	4.33tu	6.60pqrst	11.30klmn	22.03ab	4.10vw	8.93pqrs	13.30ijklm	21.16abc		
				Mean	5.61ijk	7.70oh	13.42e	21.80ab	3.85kl	10.05hi	14.43f	20.38bc		
5.	*Prunus domestica*	25.30	0.86	Protective	8.10opq	11.20klmn	13.90ijk	22.7a	3.73vw	12.40jklmn	18.03def	21.66abcd		
				Curative	5.3qrstu	9.13mnop	13.80e	22.33ab	4.43uvw	11.25gh	16.68e	22.18a		
6.	*Tamarindus indica*	28.00	0.96	Protective	4.06tu	8.90nop	13.2ij	22.3a	7.63rst	12.53jklmn	18.03def	20.60abcd		
				Curative	4.60stu	7.83opqr	14.5b	22.6ab	5.46tuv	10.10nopq	15.33ghi	20.00abcde		
				Mean	4.33k	8.36opqrs	11.03g	20.37bc	6.55j	11.30gh	16.68e	20.30bc		
7.	*Terminalia arjuna*	24.80	0.85	Protective	4.93rstu	7.50pqrst	13.83ijk	20.67abcd	4.53uvw	12.00klmn	16.7mnopq	21.6ab		
				Curative	3.00u	6.56pqrst	13.00jkl	21.27abc	3.60vw	10.83klmn	14.73fgh	19.36bcde		
				Mean	5.55jk	7.56pqrst	13.00jkl	21.27abc	4.06kl	11.42gh	15.72ef	20.52b		
8.	*Curcuma longa*	28.45	0.98	Protective	4.76stu	8.96mnop	11.90klm	20.07abcd	6.56stu	13.46ijkl	19.93bcde	19.40bcde	9.75	90.20%
				Curative	4.00tu	6.56pqrst	10.43lmno	19.50bcde	3.25l	11.70lmno	17.46cdef	18.76cdef		
				Mean	5.79d	8.75c	14.06b	20.23a	4.90rstu	12.58g	18.70cd	19.08bcd		

IZ= Inhibition Zone (in mm), A.I= Activity Index

Activity Index = Inhibition Zone of the test sample/Inhibition zone of the standard

Standard drug streptomycin =29mm

Percentage Disease incidence = Total lesion length of test sample/ leaf length

% Disease Control = % Disease incidence -100

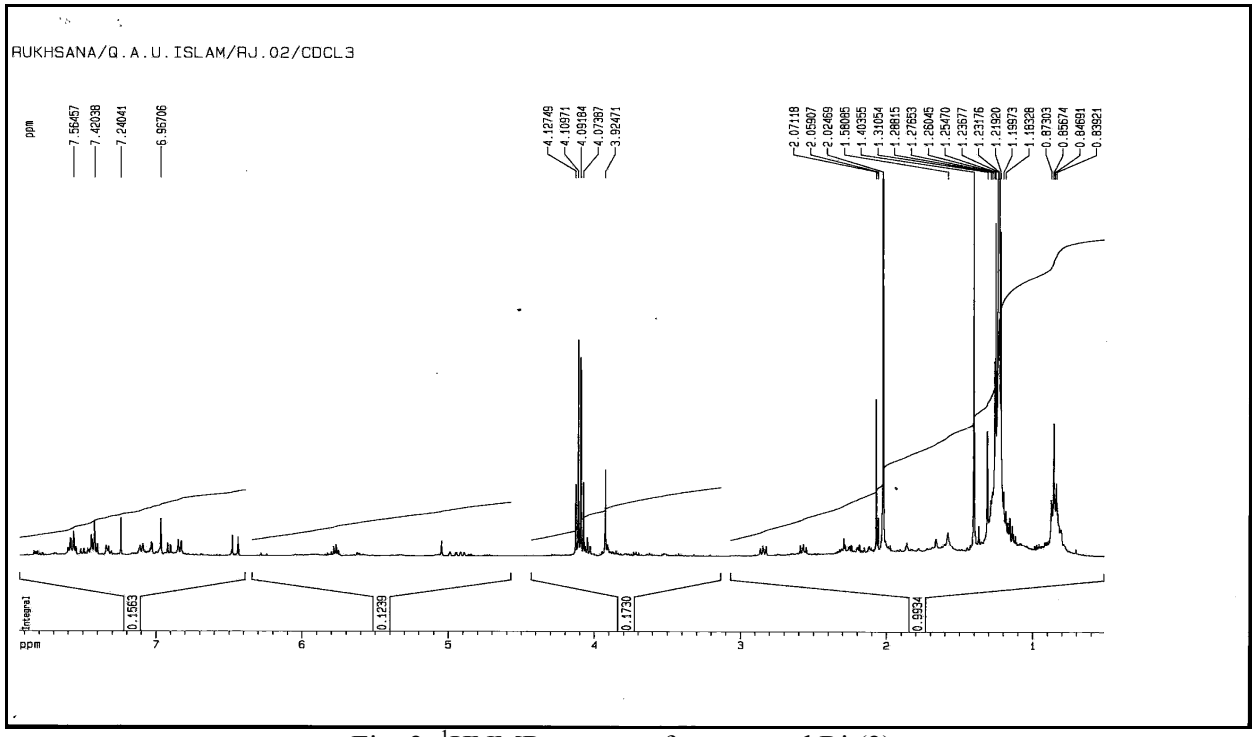


Fig. 2. [1]HNMR spectra of compound Rj (2).

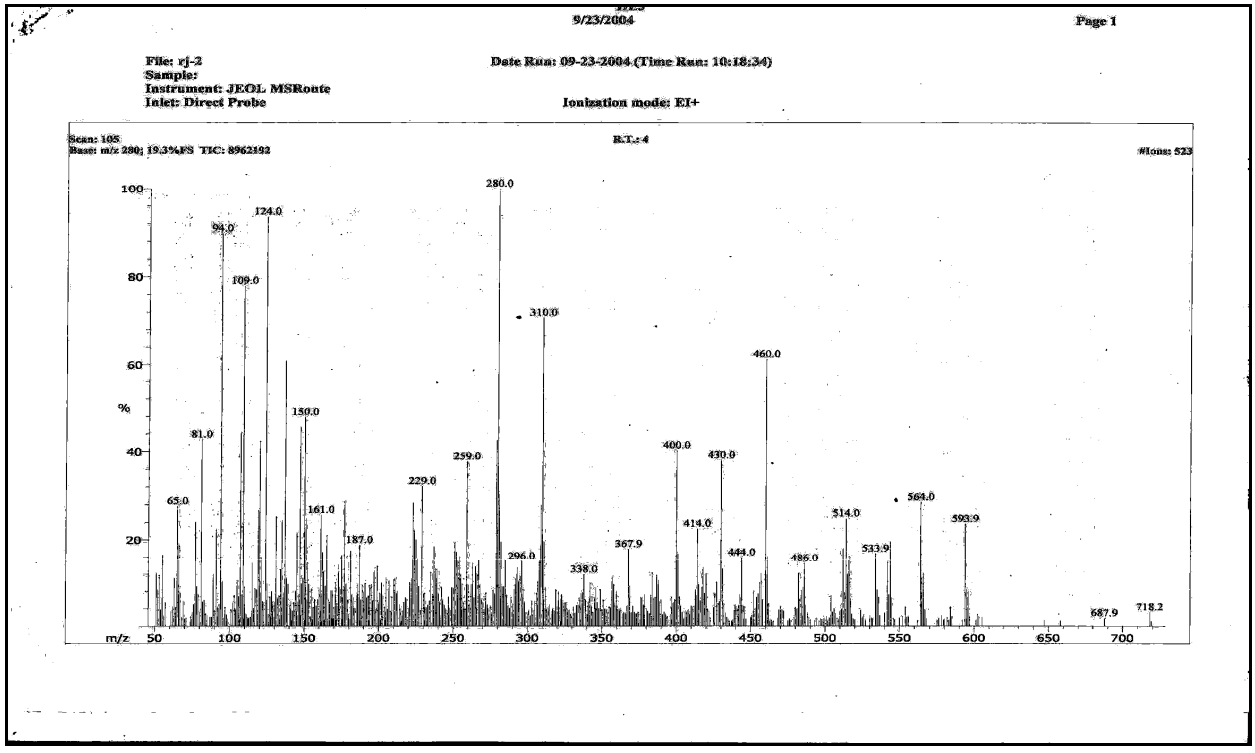


Fig. 3. Mass spectra of compound Rj (2).

Triplets appeared at δ2.05, δ2.02 and δ1.40 with coupling constant J=1.5H/Z, J=1.5H/Z and J=1.4 H/Z respectively, two proton an adjacent carbon, one triplet appeared at δ0.82 with coupli constant (j=1.2H/Z) indicate two proton at adjacent carbon atom (Fig. 2).

Table 2. IR spectral data of compound Rj (2) .

Sr. No.	Functional group	Absorption (cm^{-1})
1	OH	3440
2	CH	2880
3	C=O	1635
4	C=C	1605

Table 3. ^{1}HNMR spectral data of compound Rj (2).

Proton	Multiplicity	Chemical shift	Coupling Constant (H_Z)
H-1	t	0.80	1.2
H-2	m	1.23	
H-3	m	1.23	
H-4	m	1.23	
H-5	t	1.40	1.40
H-6	t	2.02	1.5
H-7	t	2.05	1.5
H-8	t	7.30	
H-9	t	3.92	6.81
H-10	m	4.06	6.81
H-11	t	4.09	
H-12	d	4.04	6.92
H-13	d	4.84	2.20
H-14	d	6.45	15.7
H-15	d	6.80	15.7
H-16	dd	7.45	7.28
H-17	dd	7.10	15.7
H-18	d	6.80	15.7
H-19	d	7.40	1.0
H-20	s	7.20	
H-21	d	7.3	1.0
H-22	s	7.20	

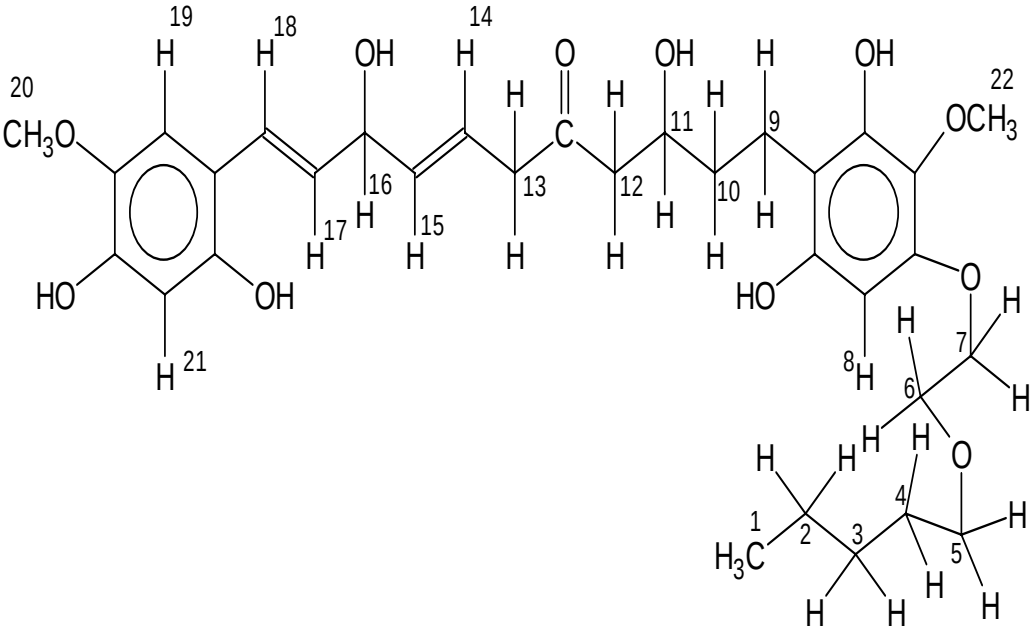
EI/MS

590 (M-3) 593 (23.6%) 534 (20%) 516 (19%) 356 (16%) 307 (17%)
280 (100%) 263 (18%) 153 (10%) 139 (5%) 121 (42%)

^{1}HNMR data was further supported by EI-MS, mass spectrum of compound Rj(2) showed molecular ion peak at m/z 687,other major peak were found to occur at m/z 593 (23.6%), 534 (20%), 516 (19%), 356(16%), 307(17%), 280 (100%), 363(18%), 153 (10%), 139 (5%), 121(42%).

The basic peak occurred at 593m/z showed the loss of 94 m.u from the molecular ion peak and represent the loss of $[n-3]^{+}$The peak at 534 m/z showed the loss 59 m.u ,while the peak at 516 m/z,356m/z,307m/z,280m/z showed the loss of 17,110,14,18 m.u (Fig. 3).

From the above findings the purposed structure of a compound was found to be



curcumin

In the present study, curcumin was isolated from *Curcma longa* rhizome. Similar compound has also been isolated by different scientists (Masuda *et al.,* 1992, Nakakyama *et al.,* 1993).

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