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Screening of antibacterial tannin compound from mango (*Mangifera indica*) seed kernel extract against Methicillin resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

The anti MRSA properties of mango seed kernel ethanol extract (MKE) were investigated. The MKE was separated by reverse phase HPLC with acetonitrile linear gradient and also identified by Nuclear Magnetic Resonance (NMR), Mass Spectroscopy (MS) and Infrared (IR) for structural characterization of antimicrobial tannin compounds. It showed significant activity against Methicillin Resistant *Staphylococcus aureus* (MRSA) at the MIC of 0.03mg/ml. These results indicated that the active component of the MKE was a type of complex Tannin.

Introduction

The constant use of antibiotics in the hospital environment has selected bacterial populations that are resistant to many antibiotics. In particular, many strains of *Staphylococcus aureus* are increasing resistance to available antibacterial agents (Methicillin Resistant *Staphylococcus aureus* – MRSA) producing a serious problem in medical microbiology[1]. The most problematic clinically relevant pathogens at present is Methicillin-resistant *Staphylococcus aureus* ranks as one of the most difficult bacteria to treat in patients and eradicate from hospital environments.

Plants have been used for centuries to treat infectious diseases and present an obvious source of new antimicrobial compounds[2]. Many studies showed that natural antioxidants, as flavonoids and other phenolic phytochemicals, present in plants are associated with reduced chronic disease risk[3]. Mango (*Mangifera indica*) which belongs to the family Anacardiaceae, it grown naturally or cultivated mainly in tropical and subtropical regions and is one of the most popular edible fruits in the world. Mango seed kernel is promising as a source of fat, cocoa butter substitutes and other food substitutes[4].

Tannins are a large diverse group of complex polyphenolic compounds of medium to large molecular weight that are widely distributed among plants, often in bark, roots, outer layers of plant tissues, leaves, stems and seeds where they are ascribed a protective function. The principal chemical property of tannins is the ability to form strong complexes with proteins, starches and other macromolecules. The tannins are applied widely in medicine especially in Asian (Japanese and Chinese) natural healing, the tannin containing plant extracts are used as astringents against diarrhea[5], antiviral, antibacterial[6] and as anti-inflammatory, antiseptic and haemostatic pharmaceuticals[7].

Recently the tannin has attracted scientific interest, especially due to the increase incidence of deadly illnesses such as AIDS and various cancers. The search for new lead compounds for the development of novel pharmaceuticals has become increasingly important, especially as the biological action of tannin containing plant extracts. This paper focus on i) Isolation of active antiMRSA compound from *Mangifera indica* seed kernel extract ii) Structural identification of the active compound was carried out by bioassay guided fractionation (chromatography), purification (High Performance Liquid Chromatography -HPLC) and spectroscopic (Infrared -IR, Mass Spectroscopy -MS and Nuclear Magnetic Resonance -NMR) methods.

Materials and methods

Preparation of mango seed kernel extracts

Mango seeds were collected from local fruit processing units at Namakkal District, Tamilnadu, India. The seeds were washed and air dried and the kernels were removed manually from seeds. The kernels were chopped and dried at 50°C. The dried material was ground into a fine powder. Absolute ethanol, methanol and acetone were added to the mango seed kernel powder at ratio of 2:1 (v/w) and kept 48hrs with gentle shaking at room temperature. Filtered extracts were dried using a rotary evaporator at 45°C and stored at 4°C for further use.

Screening of MRSA

Staphylococcus aureus listed in Table I were wound isolates (MRSA) isolated from Erode Government Hospital, Erode District, Tamilnadu. Antibiotic susceptibility was determined from the size of the inhibition zone according to the guidelines of the National Committee for Clinical Laboratory Standard[8]. The used strains were defined as MRSA based on the agar disc diffusion method for the determination of the antibacterial activity, Minimum Inhibition Concentration was tested using

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checkerboard assay method and occurrence of the *mec*A gene using PCR, which is described previously[9].

Extraction and Isolation of Active Fraction

Air dried and pulverized mango seed kernel (300g) was soaked in ethanol for overnight in dark condition. The solvent was removed by rotary evaporator and the extract was dried. 15 g of crude extract was used for column (40mm x 1000mm) chromatography. Silica gel (mesh 60-120) was used as column packing material. The column was eluted using a series of solvent systems: 100 % dichloromethane and followed Isopropyl alcohol/ ethyl acetate (2:8, 4:6, 6:4, 8:2, 10:0) finally 100% ethanol. Each series solvent was added 500 ml and fraction was collected 55 ml up to 61 fractions. Collected fractions were applied on TLC plate using methanol: ethyl acetate: water: acetic acid (3: 6: 0.5: 0.5) and visualized using UV 254 and 366 nm. Fractions which are having similar R_f value were mixed and dried using rotary evaporator. Then evaluated the antimicrobial activity of isolated compounds using checkerboard assay method.

High performance liquid chromatography (HPLC)

Fractionation of the *Mangifera indica* extract was performed by HPLC to identify active compounds. A isocratic HPLC (Shimadzu HPLC Class VP series) with one LC-10 AT VP, pump (Shimadzu), variable wavelength UV-Visible Detector SPD-10A VP, (Shimadzu), and reverse phase gemini 5u C18 110A, Phenomenex column (250 X 4.60mm) was used. The mobile phase components acetonitrile:water:aceticacid (60:40:0.5) were filtered through 0.2 micron membrane filter before use, and pumped from the solvent reservoir at a flow rate of 0.5ml/min, which yielded column backup, pressure of 180-200 kgf/cm2. The column was maintained at 27°C. 20µl of *Mangifera indica* seed fractions were injected using syringe (Bonaduz schweiz, Hamilton).

NMR, GC and IR

^{1}H and ^{13}C NMR experiments were performed on a Bruker advance DPX300 spectrometer operating at 300 and 75 MHz respectively. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants given in hertz. Molecular Mass (MS) was determined by a gas chromatography/mass spectrometry-SHIMADZU GC-17-A QP 5000, under the following conditions. Column DP-5, oven temperature 70ºC, initial holding time 1 min, rate of increase 10ºC/min, final temperature 250ºC, final holding time 5 min, column temperature 70ºC, injector temperature 250ºC, interface temperature 300ºC, MS-40-4000 and detector 1.2kv, library (MS. WILLY 139 library) search of spectra for elucidation of molecular structures of compounds. Infrared Spectrum (IR) spectra were recorded on a NICOLET 360 FT-IR spectrophotometer.

Results and discussion

Antibacterial assay

The mango seed kernel extracts showed good antimicrobial activities against MRSA strains determined by the disc diffusion method. Ethanol extract of *Mangifera indica* seed produced larger inhibition zone (18mm) than methanol and acetone compared with methicillin disc. No inhibition zone was observed in 5% DMSO. The ethanol extracts of *M. indica* presented MIC between 0.11 to 0.23 mg/ml against all the MRSA strains (Table I). In PCR analysis, all *S. aureus* strains were positive for the *mec*A gene in the molecular weight of 533bp.

Similarly, the ethanol extract of all fruit seeds tested against *Bacillus cereus*, *Salmonella typhi* and *Staphylococcus aureus* by using agar disc diffusion method. They observed that the mango seed, maprang's seeds and makham pom's seed extract exhibited high antimicrobial activities against *Bacillus cereus* and *Staphylococcus aureus*, while the mango seed showed highest potential activity against both species. The diameter of inhibition zone against *B. cereus* and *S. aureus* obtained from mango seed extract was 19 and 17 mm respectively[10].

Present results were agreed with Abdalla[11] who investigated that the mango seed kernel methanol extract using agar well diffusion assay against target entero pathogenic *E. coli*. A positive relationship was found between antimicrobial activity and the concentration of extract. Diameter of inhibition zone were 21, 19 and 18 mm for 6000, 4000 and 3000 ppm respectively and also agreed with Kabuki[12] found that the mango seed kernel methanol extract had a broad antimicrobial spectrum and was more active against gram positive than gram negative bacteria with a few exceptions.

Isolation of active compound

The ethanol seed extracts of *Mangifera indica* was fractionated by column chromatography on silica gel eluted with isopropyl alcohol and ethyl acetate. Totally 61 fractions were collected, which were pooled into eight fractions according to their physical similarity and R_f value. The antibacterial activities of these mixed fractions were tested by checkerboard method. The pooled fraction obtained from the isopropyl alcohol, ethyl acetate combination clearly showed that the most effective once were fractions 14-16 and the highest concentration of the antimicrobial compounds in the 40:60 isopropyl alcohol: ethyl acetate ratio and some compounds at a low *isopropyl alcohol* concentration (F_{5-13}). Also observed that the other fractions were less efficient in MIC. The F_{14-16} was considered as compound 1, it showed significant activity against MRSA with MIC of 0.03 mg/ml.

Structural elucidation

Ethanol seed extract of *Mangifera indica* was analyzed using reversed phase HPLC. Good resolution was achieved by acetonitrile: water: aceticacid (60:40:0.5) as mobile phase. Active pure fraction showed single peak visualized at the retention time of 5 min. Crude extract have several peaks indicates the presents of mixed compound (Fig I and Ia).

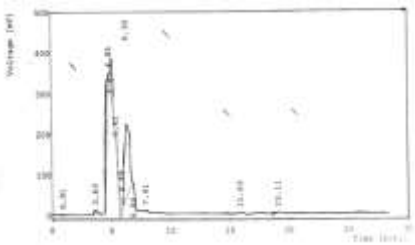


Fig. I HPLC profile of *M. indica* seed extract

In the year 2000, Schieber[13] characterized polyphenols from mango puree by HPLC with diode array and mass spectrometric detection. The predominate flavonol glycosides were Q3-galactoside, Q3- glucoside and Q3- arabinoside and also reported a gallotannin consisting of glucose and four gallic acid unit. The mango kernel tannin in gravimetric yield of 6.4% (w/w) compares favorably with a titrimetric yield and 4.5% (w/w) expressed as catechin equivalent for the same variety of mango kernel[14]. Different in varietal composition became apparent when the yield (4.5% w/w) and melting point (238ºC)

of gallotannin of African mango kernel were compared with those of Asian origin[15].

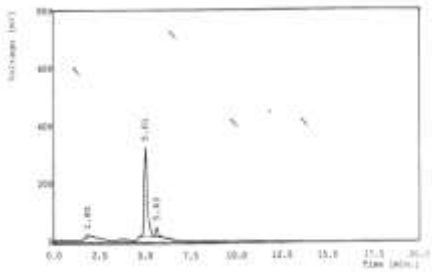
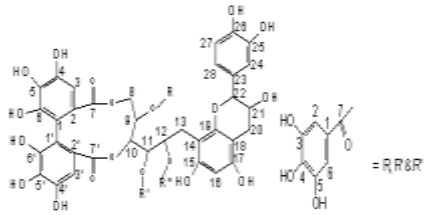


Fig. Ia HPLC profile of *M. indica* seed fraction 14-16

The infrared spectrum of compound 1 (F_{14-16}) showed at 3405 (OH), 3204 (ArH), 2900 (CH_2 , CH), 1713 (C=O), 1614 (C=C), 1538 (C=C), 1447 (C=O), 1353 (CH), 1231 (C-O), 1033 (C-O), 869 (C=CH), 761 (ArH) Cm^{-1} (Fig. II). The molecular ion in the mass spectrum of compound was found as m/z 575 $(m-653)^+$ (Data not shown). The structure of compound was elucidated by its ^1H and ^{13}C NMR spectrum (Table II, Fig. III and IIIa).

Structure of complex tannin



Complex tannin showed significant activity against MRSA. Small quantity of the purified complex tannin compound enabled to inhibit MRSA. The results indicated that the active compound was a type of complex tannin.

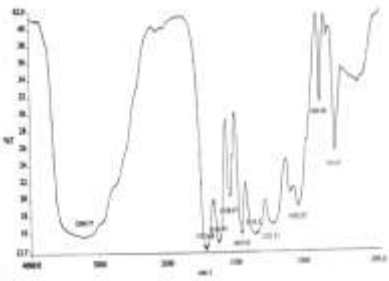


Fig II. FT-Infrared (IR) spectrum of Mangifera indica seed fraction 14-16

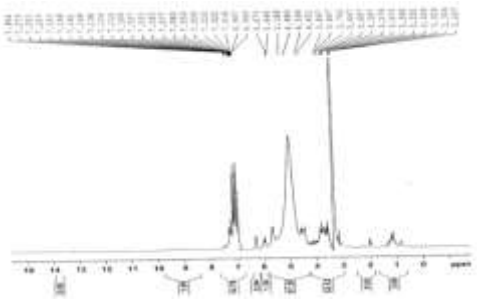


Fig.III ^1H Nuclear Magnetic Resonance (NMR) spectrum of *M. indica* seed fraction 14-16

Therefore the *Mangifera indica* seed kernel extract could be used against MRSA. The phytochemical analysis of the *M. indica* showed the presence of alkaloids, sponins and tannins. These compounds were known to be biologically active. Tannins have been found to form irreversible complexes with proline rich proteins resulting in the inhibition of the cell protein synthesis[16]. Apart from antimicrobial activity exhibited by tannins they also react with proteins to provide the typical tannins effect, medicinally, this is important for the treatment of inflamed or ulcerated tissues[17]. *Mangifera indica* contains tannins, bitter gum and resins[18].

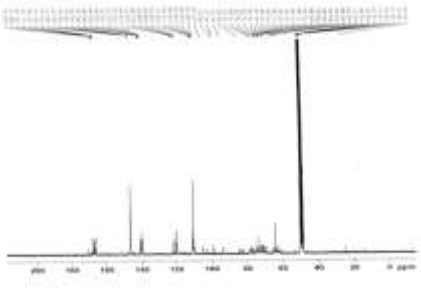


Fig.IIIa ^{13}C Nuclear Magnetic Resonance (NMR) spectrum of *M. indica* seed fraction 14-16

The mango seed kernel contained phenolic compounds as well as high levels of tocophenols, squalene and phytosterols in unsaponifiable matter of oil[11]. Our results agreed with Branen & Davidson[19] found that different phenolic compounds were inhibit the growth of several gram positive and gram negative bacteria. Total polyphenol content of the mango seed kernel ethanol extract (MKE) was 79.5% while total carbohydrate, nitrogen, ash and fat content were 21.7, 3.1, 1.6, and 0.5% respectively and the qualitative analysis showed that polyphenols are the major compound of the MKE. From the HPLC analysis there were two fractions with antimicrobial activity, both peaks has maximum absorbance at 275nm, which also indicated that existence of polyphenols[12]. Sakanake and Zhao[20,21] also reported that polyphenols have an antimicrobial activity. Thus, it can be concluded that a possible antiMRSA activity of the seed of *Mangifera indica* could be due to the tannin Compound. We believe that these findings will be helpful to many researches in the field of the evolution of antibacterial activities in plant seeds.

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Table: I. Antibacterial activity of mango seed kernel extract against MRSA

S. No.	Isolates	*mecA* gene	Class	Mean Zone of Inhibition (mm) *Mangifera indica* 30mg/ml E	M	A	Methicillin 5mcg	DMSO 5%	Minimum Inhibition Concentration (mg/ml) Ethanol Extract MIC	MBC	Isolated Compound MIC	MBC
1.	SaW1	+	MRSA	18	15	16	nil	nil	0.23	0.46	0.03	0.03
2	SaW2	+	MRSA	18	15	15	nil	nil	0.23	0.46	0.06	0.06
3.	SaW3	+	MRSA	18	15	16	nil	nil	0.23	0.23	0.06	0.06
4.	SaW4	+	MRSA	18	15	16	11	nil	0.11	0.23	0.03	0.06
5.	SaW5	+	MRSA	16	15	16	10	nil	0.23	0.46	0.06	0.06
6.	SaW6	+	MRSA	18	16	16	nil	nil	0.23	0.23	0.03	0.03
7.	SaW7	+	MRSA	16	14	14	nil	nil	0.23	0.46	0.06	0.06
8.	SaW8	+	MRSA	18	16	16	13	nil	0.23	0.46	0.06	0.06
9.	SaW9	+	MRSA	18	16	15	14	nil	0.23	0.46	0.06	0.06
10.	SaW10	+	MRSA	17	17	16	7	nil	0.23	0.46	0.06	0.06
11.	SaW11	+	MRSA	16	17	16	nil	nil	0.23	0.46	0.06	0.06
12.	SaW12	+	MRSA	18	16	16	7	nil	0.23	0.46	0.06	0.06

MRSA: Methicillin Resistant *Staphylococcus aureus*, SaW: *Staphylococcus aureus* Wound, +: Positive
E: Ethanol, M: Methanol, A: Acetone, -: no inhibition of the concentrated tested, DMSO: Dimethyl sulphoxide. MIC: Minimum Inhibition Concentration, MBC: Minimum Bacterial Concentration.

Table: II NMR spectral data of Complex tannin

Carbon	Delta, ppm	Hydrogen	Delta, ppm
C-1, & 1'	C-1, & 1' -	-	
C-2 & 2'	122.07	-	-
C-3 & 3'	110.28	H-3	6.96 (1H, m)
C-4 & 4'	146.32	-	
C-5 & 5'	140.10	-	
C-6 & 6'	146.32	-	
C-7	170.74	-	
C-8	64.59	H-8	4.98 (2H, m)
C-9	73.81	H-9	5.18 (1H, m)
C-10	71.83	H-10	5.18 (1H, m)
C-11	76.32	H-11	5.18 (1H, m)
C-12	77.88	H-12	4.98 (1H, m)
C-13	16.60	H-13	3.26 (2H, m)
C-14	105.22	-	-
C-15	146.59	-	-
C-16	93.91	H-16	5.64 (1H, m)
C-17	146.47	-	
C-18	99 .82	-	
C-19	146.44	-	
C-20	25.32	H-20	3.30 (2H, m)
C-21	69.51	H-21	4.56 (1H, m)
C-22	84.65	H-22	5.18 (1H, m)
C-23	139.69	-	
C-24	110.07	H-24	7.03 (1H, m)
C-25	146.32	-	
C-26	146.32	-	
C-27	111.07	H-27	7.05 (1H, m)
C-28	122.07	H-28	7.06 (1H, m)
R,R' & R" -1	122.07	-	-
R,R' & R" –2	109.34		6.96 (1H, m)
R,R' & R" –3	146.57	-	
R,R' & R" –4	140.88	-	-
R,R' & R" -5	146.57	-	-
R,R' & R" -6	109.34		6.96 (1H, m)
R,R' & R" –7	166.40-167.45	-	

m- multiplet