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Bactericidal activity of Flavonoids isolated from *Muntingia calabura*

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ABSTRACT

The investigation was carried out for the isolation and characterization of the compounds from heart wood of root and root bark of *Muntingia calabura.* We have isolated six compounds; three from each extract were identified as flavonoids. The bactericidal activity of these compounds found significant against tested bacterial strains (gram-positive and gram-negative). Among the tested compounds, 8-methoxy, 3',5'7'-trihydroxyflavone and 3,5,7-trihydroxyflavone (Galangin) showed paramount activity against Methicillin-resistant *Staphylococcus aureus* (MRSA). The results were compared with known standards gentamycin sulphate and cefixime.

Key-words: Bactericidal, Broth dilution method, Flavonoids, MRSA, *Muntingia calabura*

INTRODUCTION

Flavonoids are polyphenolic secondary metabolites, ubiquitously found in nature. Over 4,000 flavonoids have been identified from different sources. The potential therapeutic applications of these metabolites have been considerable interest in recent years [1-4]. Antibacterial resistance a "ticking time bomb" of public heath, serious threatening issue, whenever a simple infection turns to fatal and if tomorrow it extends its current course could become even worst. Majority of the plant metabolites in drug discovery has come from the diverse structures of the medicinal plants. These are often perceived as immense drug-likeness and more biological friendliness and making them good candidates in drug development [5-9].

M. calabura is native to Southern Mexico and Central America, distributed all over the tropical regions of the world and especially, In India. *M. calabura* crude extracts for the treatment of various human disorders requires a proper scientific evaluation and documentary reports by Sridhar [10]; Zakaria *et al.* [11] of active principle responsible. Several researchers round the globe have been isolated and identified the compounds of this plant as flavonoids [12-14]. Till the date, only few of these compounds have been evaluated for its therapeutic properties and still there are several compounds stand remained for scientific evidence based utilization [15,16]. Thus, the compounds isolated in current study have been further determined for their attributed biological activity. With regard microbial resistance and plant derived drugs, the current investigation has been documented about bactericidal activity of flavonoids isolated from heart wood root and root bark of *M. calabura*.

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MATERIALS AND METHODS

Plant Material- Heart wood of root and root bark of *M. calabura* was collected from College premises of Chaitanya Degree and Postgraduate College (Autonomous), Hanamkonnda, Warangal District, Telangana, India. The authenticity of the plant was carried out by Prof. V.S. Raju, Taxonomist, Plant systems laboratory, Department of Botany, Kakatiya University, Warangal, India (Voucher number: Dep/B/KU/WGL/MC-014/2013). The plant material was chopped into smaller fragments, dried under shade and grinded in homogenizer to coarse powder.

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Chemicals used- All the chemicals required for media preparation and bactericidal assay were purchased from Hi-media chemical laboratories, Mumbai, India and for analytical grade.

Extraction and separation of compounds- The plant material was finely powdered (500 g) and extracted with chloroform in a soxhlet apparatus. The extract was concentrated under reduced pressure. The resultant gummy product was further used for separation of compounds by column chromatography.

Bacterial Strains and their Growth- "Gram-positive" strains MRSA NCTC 13616, *Bacillus subtilis* ATCC 6633, *Bacillus cereus*, ATCC 14579 and "Gram-negative" strains *Klebsiella pneumoniae* ATCC 43816*, Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 13315 were procured from American type culture collection, USA. MRSA was purchased from culture collections, UK. All bacterial strain stored at -80^{o}C were streaked on Luria-Bertani (LB) agar plates (Hi-media Laboratories, Mumbai, India) and incubated at 37^{o}C for 20 to 24 h. A few isolated colonies were harvested from each plate and suspended in 5 ml of LB broth contained in a 15 ml of sterile plastic tube. The tube was capped tightly and incubated with gentle shaking (140 rpm) at 37^{o}C for 20 h.

Preparation of bacteria for bactericidal assay- Broth culture (1 ml) of test organisms was added separately to a 1.9 ml eppendorf tube, bacterial sedimentation was achieved by centrifugation at 12,000 rpm for 30 sec. The pellet was re-suspended using 1 ml of sterile PBS by gentle aspiration in and out of a transfer pipette. The optical density (OD) of the pellet was determined at 620 nm in spectrophotometer. The OD at 620 of the sample was adjusted approximately to 0.8 to 0.9 by the addition of PBS. Ten microliters of the diluted sample was subjected for serial dilution with PBS so that these dilutions would produce approximately 1,500 to 2,000 bacteria per 50ml sample. The ODs of the samples results in 60 to 200 CFU/mL.

Preparation of compound stocks and their dilutions- 10,000 mg of each isolated compound was dissolved in one liter of PBS. Further, 1 mL of this solution was diluted in 9 mL of PBS to generate 1000 mg/L stock. This stock was used for serial dilutions to produce the concentrations ranging from 0.1–200 mg/mL.

Cefixime and Gentamycin sulphate are used as positive control (10 µg/L). Solubility was achieved by adding few drops of saturated $NaHCO_3$. The dilution of the compounds was achieved by dissolving 1 mg of compound in 1L of WFI (water for Injection). 1 mL of this dilution was dissolved in 9 mL of WFI to produce 10 µg/L concentrations and used in the study.

Bactericidal assay- The assay was conducted to assess the bactericidal activity of the isolated compounds through microtiter plate described previously. The assay reaction mixture consisted of PBS (50 mM sodium phosphate, 150 mM NaCl [pH 7.0]) test compounds at various concentrations and the bacterial strains were prepared in sterile 96-well microtiter plates (Nunc, Inc). The wells were filled with 100 µl diluted test compounds in PBS and 50 µl of the diluted bacterial strains and incubated with gentle shaking (140 rpm) at 37^{o}C for various incubation periods [0 (baseline), 2, 4, 8, 12, and 24 h) (time-kill studies)] 24 h. Subsequently, positive and negative controls, was prepared and screened. Following incubation, a 20 µl aliquot from each well was spotted at the top of a square plate containing Nutrient agar medium. The plate was labeled and tapped gently to facilitate the movement of the liquid. There were approximately 200 cells in the spotted (20 µl) sample. Plates were placed uncovered in biohood until the sample liquid dried (ca. 10 min) and incubated overnight at 37^{o}C. CFU of test organisms were visible after 18 to 24 h and was counted. The experiments were performed in duplicate, and CFU for each streak were enumerated with a colony counter.

The control value to determine the percentage of bacteria killed per well. The percentage of the bacteria killed was plotted graphically, and the percentage of the test compound resulting decrease in the number of CFU at each dilution of test compounds was compared with the average of positive number of CFU (BA_{50}) was determined.

RESULTS

Characterization of isolated compounds- We had been isolated and identified five compounds as flavones and one compound as Chalcone and characterized by spectral data (^{1}HNMR, ^{13}C and Mass) as 8-hydroxy,7,3',4',5'-tetramethoxy flavone (Fig. 1); 8,4'-Dihydroxy,7,3',5'-trimethoxyflavone (Fig. 2); 8-

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methoxy,3′,5′7′-trihydroxyflavone (Fig. 3); 3,5,7-trihydroxyflavone (Galangin)(Fig. 4); 5,8-dihydroxy,6,7,4′-trimethoxy flavones (Fig. 5); 6,4′-dihydroxy, 3′-propen chalcone (Fig. 6 & Table 1). Based on mass spectra the compounds possess the molecular weight and molecular formula *m/z* 358.34202, $C_{19}O_7H_{18}$ (Fig. 1); *m/z* 344.31544, $C_{18}O_7H_{16}$ (Fig. 2); *m/z* 300.26288, $C_{21}H_{20}O_4$ (Fig. 3); *m/z* 270.2369, $C_{21}H_{25}O_4$ (Fig. 4); *m/z* 344.31544, $C_{18}H_{16}O_7$ (Fig. 5); *m/z* 280.109396, $C_{18}H_{16}O_3$ (Fig. 6) respectively. The structures of the isolated compounds were depicted in Fig. 1 to Fig. 6.

Table 1: 1HNMR data of the isolated compounds from heart wood of root and root bark of *M. calabura*

	Compound-5			Compound-6	
	1H(δ in ppm)	**13 C (δ n ppm)**		**1H(δ in ppm)**	**13 C (δ n ppm)**
			1	-	120.03
2	-	163.01	2	7.60(S)	130.2
3	6.53 (S)	102	3	-	132.78
4	-	184.01	4	6.68(d)	130.42
5	12.69(-OH, S)	151.6	5	6.72(d)	123.45
6	4.02(OCH3, S)	130.01, 56.6(OCH3)	6	5.22(OH, S)	160.69
7	3.96(OCH3, S)	158.06, 5 6.2(OCH3)	7	-	170.2
8	8.61(OH, S)	128.3	A	7.43(d)	126.45
9	-	139.9	B	7.01(d)	147.32
10	-	108.6	1’	-	128.63
1’	-	125.8	2’,6’	7.70(d)	131.62
2’,6’	7.91(d, J=8.7Hz)	129.3	3’,5’	7.72(d)	117.02
3’,5’	7.01(d, J= 8.7 Hz)	114.01	4’	5.52(OH, S)	162.03
4’	3.91(OCH3, S)	161.6, 55.6(OCH3)	1’’	4.58(d)	132.04
			2’’	4.48(M)	124.72
			3’’	1.29(d)	19.23

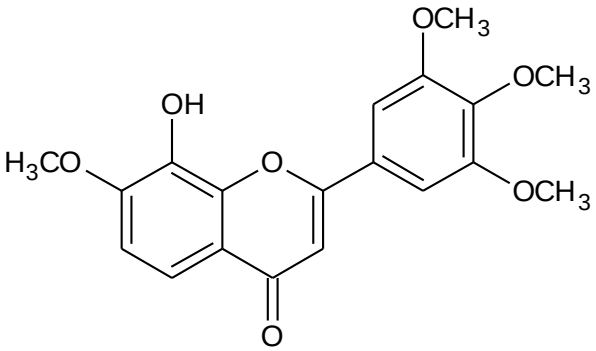

Fig. 1: 8- hydroxy-7,3′,4′,5′-tetramethoxy flavone

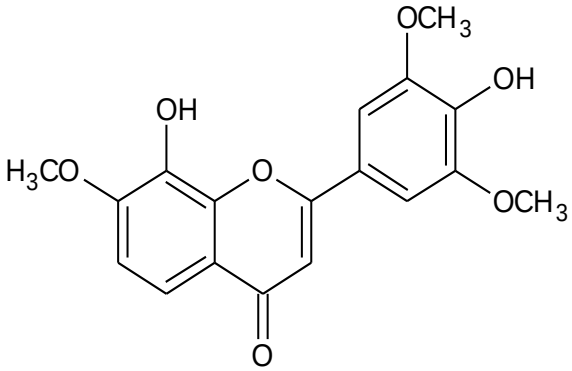

Fig. 2: 8,4′- Dihydroxyl-7,3′,5′-trimethoxyflavone

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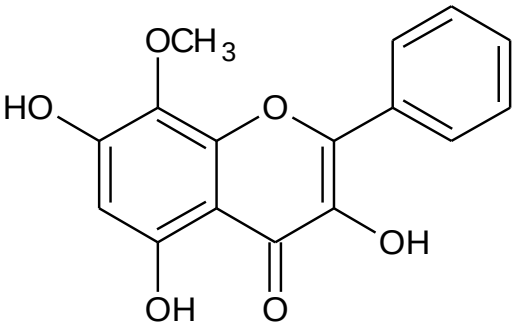

Fig. 3: 8-methoxy-3',5'7'-trihydroxyflavone

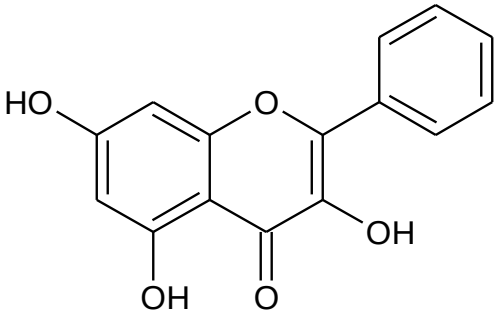

Fig. 4: 3,5,7-trihydroxyflavone (galangn)

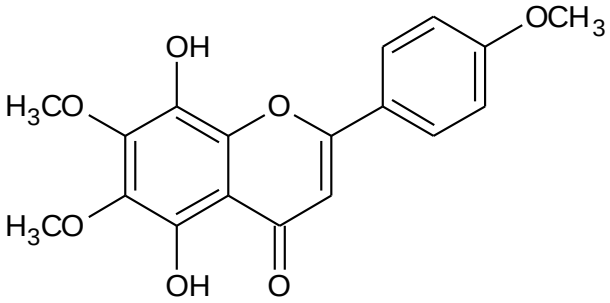

Fig. 5: 5,8-dihydroxy-6,7,4'trimethoxy flavones

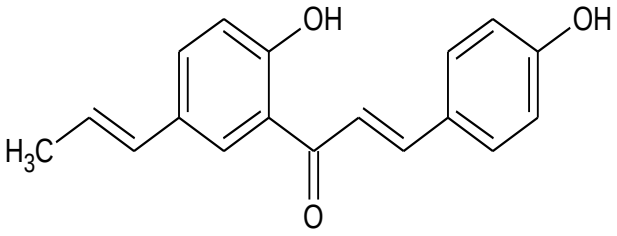

Fig. 6: 6,4' dihydroxy 3' propen chalcone

Bactericidal assay

8-hydroxy,7,3', 4',5'-tetramethoxy flavone (Compound-1)- The susceptibility nature of test strains against compound 8-hydroxy,7,3',4',5'-tetramethoxy flavone was found in concentration dependent manner and exhibited significant bactericidal activity against *B. cereus*, *B. subtilis*, *E. coli* and *P. vulgaris* with 79,73, 66 and 62 bactericidal percentages respectively. *K. pneumoniae* found slightly resistant and noticed 50% death percentage (Table 2).

4'-dihydroxy,7,3',5'-trimethoxy flavone (Compound-2)- 4'-Dihydroxy,7,3',5'-trimethoxy flavone was found very active against MRSA and *B. subtilis*, *E. coli*, *K. pneumonia*e and *P. vulgaris*. The bactericidal rates were found at 1.0 mg/mL is 71, 70, 71, 52 and 53 respectively. *B. cereus* exhibited average susceptibility with 44 bactericidal death rates at 0.7 mg/mL (Fig. 3 & Table 2).

8-methoxy,3',5'7'-trihydroxyflavone(Compound-3)- 8-methoxy,3',5'7'- trihydroxy flavone noticed highest bactericidal activity comparing to other compounds. The highest bactericidal percentages 94, 89, 77, 96 and 80 are noted at 1.0 mg/mL against MRSA, *B. subtilis, B. cereus, E. coli* and *P. vulgaris* respectively. The BA_{50} of this compound against MRSA was found <1mg/mL (Table 2).

3,5,7-trihydroxyflavone or Galangin (Compound-4)- Basing on the results the bactericidal activity of the compound was found against both Gram positive and Gram negative strains. MRSA was showed high susceptibility nature at all concentrations tested. The high bactericidal percentage 97 was observed at 1.0 mg/mL. Galangin at 1.0 mg/mL showed moderate activity against *B. subtilis* and *B. cereus* with 50 and 48 death percentages respectively. On the other hand, among Gram negative strains, *P. vulgaris* exhibited highest susceptibility 64% at 1.0 mg/mL (Table 2).

5,8-dihydroxy,6,7,4'-trimethoxyflavones (Compound-5)- Bactericidal efficacy of this compound was found effective against MRSA, *B. cereus, K. pneumoniae, B. subtilis, E. coli* and *P. vulgaris* noticed moderate activity (Table 2).

6,4'-dihydroxy,3'-propen chalcone (Compound-6)- This compound is most active against MRSA and *E. coli*. The bactericidal percentage 85 and 76 were recorded against *E. coli* and MRSA respectively. Other bacterial strains were exhibited moderate susceptibility (Table 2).

Effect of incubation period on bactericidal activity- To determine the sensitivity of the bacterial strains, we have performed time-kill studied, where the bacterial strains were incubated at different incubation time periods with test compounds. During the study, we have

noticed that for most of the tested bacterial species, the susceptibility was initiated after 4 hrs and some bacterial strains viz., MRSA, *E. coli*, and *B. subtilis* showed susceptibility with 2 hrs of incubation. The bactericidal activity of some compounds was found high for first 16–12 hrs and followed by plateau in activity during the next 12–24 hrs. However, the MRSA count was started to decrease after 2 hrs of incubation and the count was significantly reduced during 8–12 hrs of incubation.

Table 2: Bactericidal activity (BA_{50}) of isolated compounds and standards against tested bacterial strains

	MRSA	*B. subtilis*	*B. cereus*	*E. coli*	*K. pneumoniae*	*P. vulgaris*
Compound-1	0.8	>0.7	>0.7	>0.6	>0.7	>0.6
Compound-2	>1.0	>1.0	>0.7	>0.6	>0.7	>0.6
Compound-3	<0.4	<0.4	0.6	>0.3	<1.0	>0.3
Compound-4	>0.5	<0.8	0.8	<0.7	<0.8	<0.7
Compound-5	0.5	>0.5	>0.6	>0.6	0.7	>0.6
Compound-6	>0.6	>0.7	>0.6	>0.4	>0.8	>0.7
Gentamycin	<0.8	<0.6	0.5	<0.7	>0.9	0.8
Cefixime	>0.6	<0.5	<0.4	<0.7	<0.7	0.6

DISCUSSION

Isolated compounds, five flavonoids and one structurally sub-set of flavonoids chalcone (Fig. 1 to Fig. 6) were previously reported. However, bactericidal activity of these compounds is documented here for the first time. In accordance to the results obtained 8-methoxy,3′,5′ 7′-trihydroxyflavone (Fig. 3), 3,5,7-trihydroxyflavone (Fig. 4), 5,8-dihydroxy,6,7,4′-trimethoxy flavones (Fig. 5) exhibited significant BA_{50} values against the bacterial strains tested especially, MRSA. Whereas, 8-hydroxy,7,3′,4′,5′-tetramethoxy flavone (Fig. 1), 4′-Dihydroxy,7,3′,5′-trimethoxyflavone (Fig. 2), 6, 4′-dihydroxy, 3′-propen chalcone (Fig. 6) recorded average inhibition effect on all strains used in the study. The high susceptibility nature of MRSA might be attributed to dihydroxylation of A ring at 5^{th} and 7^{th} positions on of 8- methoxy,3′,5′7′-trihydroxyflavone (Fig. 3), 3,5,7-trihydroxyflavone (Fig. 4) [17]. Inhibition of H^+-ATPase-mediated proton pumping could also establish the higher activity of these compounds against MRSA [18]. In addition, the ability of 3,5, 7-trihydroxyflavone (Fig. 4) to induce the damage of cytoplasmic membrane and subsequent loss of potassium supported the destruction pathway of MRSA [19]. By Keen observations on destruction pathways of MRSA we also noticed that isolated compounds are capable of interference with energy metabolism for the inhibition of oxygen consumption by MRSA [20]. On the other hand, the susceptibility nature of Gram negative strains *E. coli*, *K. pneumoniae* and *P. vulgaris* was also found significant. This might be due to inhibition of DNA replication enzyme DNA gyrase [21]. Generally, flavonoids exhibit biological activity by the inhibition of eukaryotic enzymes [1]. In addition, the ability of disruption and denaturation of cell wall proteins by flavonoids add more value for bactericidal activity of the isolated compounds [18]. It was reported that anti-bacterial activity assay of flavones and chalcones isolated from leaf extracts of *M. calabura* possess significant activity [22]. As flavonoids are nonpolar and exhibit poor diffusion in agar gels [23], antibacterial assays of flavonoids that relay on agar diffusion is not suggestible. Therefore, we studied using broth micro-dilution method, which was more suitable for determining the bactericidal activity.

CONCLUSIONS

The nontoxic nature of flavonoids and their attributed biological activities for prevention and treatment of wide

range of pathologies is drastically gained immense importance round the globe. These are ubiquitous in plant kingdom and many of these are prescribed as traditional medicine for thousands of years. In the current investigation, we have investigated and given a detailed report on structural aspects and its bactericidal activity of flavonoids isolated from *M. calabura*. However, the study of flavonoids is perplexing because of their molecular heterogeneity. In conclusion, we initially suggested that the therapeutic strategies of flavonoids are an epitome for development of effective future drugs against variety of bacterial infections so many bacterial gets resistant to various antibiotics. Owing to excessive usage of antibiotics the bacteria converted into superbugs. Extendable research needed to discover novel flavonoids against bacterial superbug and replaces the outmoded antibiotics. In this context there is a need to develop research programmes on flavonoids against various pathogens to develop human health and reduces the usage of antibiotics.

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CONTRIBUTION OF AUTHORS

All authors were contributed equally for accomplishing this work.

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