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Research Article

ANTIMYCOBACTERIUM ACTIVITY OF COUMARINS FROM FRUIT PULP OF *AEGLE MARMELOS* (L.) CORREA

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ABSTRACT
Phytochemical investigation of n-butanol fraction of acetone extract of *Aegle marmelos* fruit has afforded four compounds coumarins marmelosin (1), marmin (2) and xanthotoxol (3) and flavonoid kaempferol 3-*O*-rhamnoside, afzelin (4). All the isolated compounds were evaluated for their antimycobacterium activity against *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis*. Compounds **1** and **2** exhibited antimycobacterium activity against *M. tuberculosis* H37Ra with an IC_{50} 12.46 µg/mL and 4.31 µg/mL respectively whereas, at 100 µg/mL, 62.5% and 82.4% growth inhibition of *M. bovis* was observed respectively. Compounds **1** and **2** were also evaluated against two gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis* and one gram negative bacteria *Escherichia coli*. Compound **1** at 100 µg/mL, showed growth inhibition, 64.6% and 74.9% of *E. coli* and *B. subtilis* respectively.

Keywords: *Aegle Marmelos, Rutaceae, Bael, Antimycobacterium, Antibacterial, Coumarins, Flavonoid*

INTRODUCTION
Medicinal plants play a major role in ancient Traditional Systems of Medicine such as Ayurveda, Siddha, Unani, Traditional Chinese Medicine, Kampo etc. These plants have potential therapeutic utilities and are used for the treatment of different diseases. *Aegles marmelos* (L.) Correa, commonly known as Bael belonging to family Rutaceae, is widely grown in India (Purohit and Vyas, 2004). This plant has been studied quite extensively from pharmacological point of view (Sekar *et al.*, 2011).
It is reported to exhibit wide range of pharmacological activities which include, antifungal, antibacterial, antiviral, antimalarial, antiinflammatory, anticancer, antidiabetic, hyperlipidaemic, antioxidant, antiulcer, radioprotective, antiscorbutic, astringent, coolant and laxative (Bansal and Bansal, 2011; Sharma *et al.*, 2011).
This literature indicates potential of this medicinal plant for the treatment of previously unexplored diseases. A number of studies on phytochemical analysis of different parts of this plant suggest presence of a number of chemical compounds with wide structural diversity like marmelosin, alloimperatorin, marmelide, marmin, umbelliferone, isoimperatorin, isopimpinellin, skimmin, marmesin, marmesinin, β-sitosterol, tannic acid and fatty acids (Farooq, 2005; Bansal and Bansal, 2011; Sharma *et al.*, 2007; Sharma *et al.*, 2011).
Tuberculosis (TB) continues to be a deadly disease, despite the availability of treatment (Dashti *et al.*, 2014; Global Tuberculosis Report, 2014; Supplement: Global Tuberculosis Report, 2014). The emergence of drug resistant TB and an insufficient global drug pipeline justify continued efforts toward the development of new drugs with a new mode of action and novel structures. There is currently a re-emerging interest in natural products as a source of lead compounds for drug discovery efforts, particularly in the area of antibacterials. We previously reported that many secondary metabolites of plant origin exhibited promising anti TB activity. These includes labdane, cembrane, phyllocladane diterpenes and flavonoid glycoside from the family Lamiaceae (Kulkarni *et al.*, 2013; Kulkarni and Joshi, 2013; Chinchansure *et al.*, 2015; Bharadwaj *et al.*, 2015).
In continuation to our previous work, this study was carried out to isolate major bioactive compounds from the fruits of *A. marmelos* with new biological activity. Four compounds **1 - 4** belonging to the coumarin and flavonoid classes were isolated and evaluated for antimycobacterium activity. There isolation, characterization and anti TB activity reported herein.

MATERIALS AND METHODS

General Experimental Procedures

The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance Ultra Shield NMR instrument (^{1}H: 400 MHz, ^{13}C: 100 MHz). Chemical shifts are reported in parts per million (δ). Residual solvent peaks in respective deuterated solvents were used as internal reference, 7.28 and 77.00 (central peak) for $CDCl_3$, 3.31 and 49 (central peak) for CD_3OD, 2.05 and 29.84 (central peak) for $(CD_3)_2CO$. LCESIMS were recorded with Waters Acquity LCMS instrument. Melting points were measured using Buchi B-540 melting point apparatus. All solvents used were distilled prior to use. Column chromatography was performed using silica gel procured from Thomas Baker, Ltd., Mumbai, India and preparative thin-layer chromatography (TLC) was carried out using TLC plates supplied by Merck Ltd. (Whitehouse Station, NJ, USA). MTT, kenamycin, ampicilin and rifampicin were purchased from Sigma Aldrich, USA. *Mycobacterium tuberculosis* H37Ra (ATCC No. 25177) and *M. bovis* BCG (ATCC 35734) were obtained from AstraZeneca, India. *Staphylococcus aureus* (NCIM2079), *Bacillus subtilis* (NCIM2010) and *Escherichia coli* (NCIM2803) were purchased from National Collection of Industrial Microorganisms, CSIR-NCL, Pune.

Plant Material

Fruits of *A. marmelos* were collected from Adilabad on March, 2013 in full flowering season. Plant was identified by Dr. Swati P. Joshi, CSIR-NCL, Pune.

Extraction and Isolation

Fruits (1.9 kg) were crushed and extracted with acetone (6 L × 3 × 14 h) at room temperature. The acetone solubles were filtered and concentrated under reduced pressure to yield a greenish extract (237.4 gm, 12.59 % based on fruit weight). The extract, at room temperature, was treated with petroleum ether (300 mL x 3) to obtain defatted acetone extract (230.0 gm). This defatted extract was partitioned in n-butanol and water, 300 mL each. The n-butanol soluble fraction, after evaporation on rotary evaporator yielded yellowish solid (194 gm). This n-butanol soluble fraction, 100 g, was separated by column chromatography (CC) using acetone gradient in petroleum ether from 10 to 50 % as an eluent to collect 60 fractions. Fractions with similar TLC pattern were combined to get 19 fractions AM1-AM19.

Fraction AM4 (3.0 g) was subjected to CC using acetone gradient in petroleum ether from 25 to 50% as an eluent to collect 36 fractions (AM4-1 to AM4-36). Fractions AM4-17 to AM4-36 were combined from which compound **1** (1.0 g) was obtained by crystallization in acetone. Fractions AM8 and AM9 were combined from which compound **3** (20 mg) was obtained as white crystals. Fractions AM11 to AM14 were combined and subjected to CC using acetone gradient in petroleum ether from 30 to 50% as an eluent to collect 26 fractions (AM11-14-1 to AM11-14-26). Fractions AM11-14-9 to AM11-14-17 were combined from which compound **2** (100 mg) was isolated by crystallization. Fraction AM18 (2.5 g) was subjected to CC using methanol gradient in chloroform from 10 to 15% as an eluent to collect 12 fractions (AM18-1 to AM18-12). Fraction AM18-6 was purified by preparative TLC using 15% Methanol: chloroform as a developing system to isolate compound **4** (15 mg).

RESULTS AND DISCUSSION

Compound **1** (Figure 1) was isolated as white crystals. The molecular formula was determined as $C_{16}H_{14}O_4$ based on the observation of a pseudomolecular peak $(M + Na)^+$ at 293.26 (Calcd for $C_{16}H_{14}O_4$, 270.08) indicating ten indices of hydrogen deficiency. It was identified as a marmelosin or imperatorin by comparing its observed and literature NMR data and supported by LCESIMS data (Razavi *et al.*, 2010; Marumoto and Miyazawa, 2010). Compound **2** (Figure 1) was isolated as white crystals. The molecular formula was determined as $C_{19}H_{24}O_5$ based on the observation of a pseudomolecular peak $(M)^+$ at 332.15 (Calcd for $C_{19}H_{24}O_5$, 332.15) indicating eight indices of hydrogen deficiency. It was identified as a marmin by comparing its observed and literature NMR data and supported by LCESIMS data (Chatterjje and Bhattacharya, 1959). Compound **3** (Figure 1) was isolated as white needles. The molecular formula was determined as $C_{11}H_6O_4$ based on the observation of a pseudomolecular peak $(M)^+$ at 202.16 (Calcd for $C_{11}H_6O_4$, 202.02) indicating nine indices of hydrogen deficiency.

Table 1: ^{1}H and ^{13}C NMR data for compounds 1 - 4

Position	1*		2*		3**		4***	
	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C
1	-	-	-	-	-	-	-	-
2	-	160.3	-	161.7	-	160.7	-	158.61
3	6.25 d (9.62)	113.0	6.24 d (9.64)	112.9	6.34 d (9.7)	114.9	-	135.84
4	7.72 d (9.62)	144.2	7.65 d (9.64)	143.8	8.1 d (9.7)	146.0	-	179.04
4a	-	116.1	-	112.5	-	117.3	-	104.40
5	7.29	114.1	7.37 d (8.3)	128.8	7.43 s	111.2	-	161.70
6	-	125.6	6.84 dd (8.3, 2.3)	113.4	-	126.6	6.36 s	101.57
7	-	148.1	-	162.1	-	146.4	-	162.9
8	-	131.1	6.82 d (2.3)	101.5	-	131.2	6.19 s	96.00
8a	-	143.4	-	155.8	-	140.7	-	162.95
9	7.62 d (1.83)	146.3	-	-	7.9 d (1.8)	148.09	-	-
10	6.74 d (1.83)	106.5	-	-	7.0 d (1.8)	107.8	-	-
11	4.90-4.92 (7.33)	69.8	-	-	-	-	-	-
12	5.32 t	119.5	-	-	-	-	-	-
13	-	139.3	-	-	-	-	-	-
14	1.64 s	25.5	-	-	-	-	-	-
15	1.63 s	17.9	-	-	-	-	-	-
1’	-	-	4.60 d (6.3)	65.4	-	-	-	122.74
2’	-	-	5.50 t (6.4)	118.8	-	-	7.79 d (8.72)	131.80
3’	-	-	-	142.2	-	-	6.96 d (8.72)	116.60
4’	-	-	2.16, 2.36 m	36.6	-	-		159.00
5’	-	-	1.47, 1.64 m	29.4	-	-	6.96 d (8.72)	116.60
6’	-	-	3.37 dd (1.8,10.6)	78.0	-	-	7.79 d (8.72)	131.80
7’	-	-	-	73.3	-	-		-
1’’	-	-	-	-	-	-	5.38 d (1.6)	103.50
2’’	-	-	-	-	-	-	4.49-3.58	72.12
3’’	-	-	-	-	-	-		72.00
4’’	-	-	-	-	-	-		73.23
5’’	-	-	-	-	-	-		71.92
6’’	-	-	-	-	-	-	0.91 d (5.6)	17.65
3’- Me	-	-	1.75	16.8	-	-	-	-
7’-Me	-	-	1.16	23.2	-	-	-	-
7’-Me	-	-	1.20	26.5	-	-	-	-

*: $CDCl_3$, **: Acetone d_6, ***: Methanol d_4, s: singlet, d: doublet, t: triplet, dd: doublet of doublet

It was identified as a xanthotoxol by comparing its observed and literature NMR data and supported by LCESIMS data (Marumoto and Miyazawa, 2010). Compound **4** (Figure 1) was isolated as a yellow gum. The molecular formula was determined as $C_{21}H_{20}O_{10}$ based on the observation of a pseudomolecular peak $(M+K)^+$ at 471.57 (Calcd for $C_{21}H_{20}O_{10}$, 432.12) indicating twelve indices of hydrogen deficiency. It was identified as a kaempferol 3-*O*-rhamnoside (afzelin) by comparing its observed and literature NMR data and supported by LCESIMS data (Hyun *et al.*, 2006; Cho *et al.*, 2014).

Compounds Characterization

Marmelosin (**1**): White crystals, mp: 96-97 ^{0}C, LCESIMS: *m/z* $(M + Na)^+$ at 293.72 (Calcd for $C_{16}H_{14}O_4$, 270.08), ^{1}H NMR (400MHz, δ_H, $CDCl_3$) and ^{13}C NMR (100MHz, δc, $CDCl_3$) as shown in Table 1.

Marmin (**2**): White crystals, mp: 126-128 ^{0}C, LCESIMS: *m/z* $(M + Na)^+$ at 355.34 (Calcd for $C_{19}H_{24}O_5$, 332.15), ^{1}H NMR (400MHz, δ_H, $CDCl_3$) and ^{13}C NMR (100MHz, δc, $CDCl_3$) as shown in Table 1.

Xanthotoxol (**3**): White needles, mp: 248-251 ^{0}C, LCESIMS: *m/z* $(M)^+$ at 225.72 (Calcd for $C_{11}H_6O_4$, 202.02), ^{1}H NMR (400MHz, δ_H, Acetone d6) and ^{13}C NMR (100MHz, δc, Acetone d6) as shown in Table 1.

Afzelin (**4**): Yellow gum, LCESIMS: *m/z* $(M+K)^+$ at 471.57 (Calcd for $C_{21}H_{20}O_{10}$, 432.12), ^{1}H NMR (400MHz, δ_H, Methanol d4) and ^{13}C NMR (100MHz, δc, Methanol d4) as shown in Table 1.

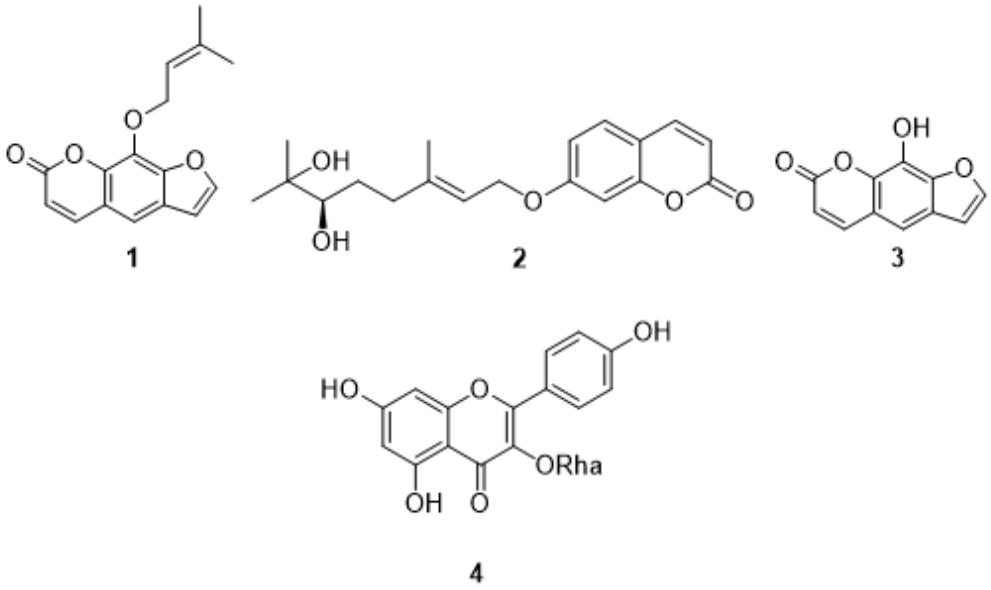

Figure 1: Compounds isolated from *Aegle marmelos* fruits

Antimycobacterium Assay

Mycobacterium tuberculosis H37Ra (ATCC No. 25177) and *M. bovis* BCG (ATCC 35734) strain were tested for their susceptibility to compounds **1** – **4** in active and dormant phases by using XRMA protocol. *M. tuberculosis* H37Ra and *M. bovis* were grown to logarithmic phase (O.D. 1.0) in a M. phlei medium. The stock culture was maintained at -70^{0}C and sub-cultured once in M. phlei medium before inoculation into experimental culture.

All experiments were performed in triplicates and IC_{50} and MIC values were calculated from their dose response curves (Table 2).

The MIC was defined as the lowest concentration of the anti-tubercular agents that prevented visible growth with respect to the growth control (Dzoyem *et al.*, 2013; Khan and Sarkar, 2008; Singh and Sarkar, 2011).

Compounds **1** and **2** at 100 µg/mL, showed growth inhibition of *M. bovis* (Table 2).

Table 2: Antimycobacterium activity of compound 1and 2

Compound	***M. tuberculosis* H37Ra**		***M. bovis***
	Dormant IC_{50} (µg/mL ± SD)	**Active IC_{50} (µg/mL ± SD)**	**Growth inhibition %**
1	11.9 ± 0.3	12.6 ±0.2	62.5
2	19.32 ±0.2	4.31 ±0.1	82.4
Rifampicin	0.018 ±0.005	0.0018 ±0.0004	0.0054 ±0.0003

Antibacterial Assay

Staphylococcus aureus, *Bacillus subtillis*, and *Escherichia coli* were grown to logarithmic phase (O.D. 1.0) in a Luria-Bertani (LB) medium. The stock culture was maintained in refrigerator and sub-cultured

once in LB medium before inoculation into experimental culture. Incubation time for *E. coli* was 8 Hrs. and for *S. aureus* and *B. subtillis* was 14 Hrs. Inhibitory effect was determined by using directly OD count. Compounds **1** and **2** showed growth inhibition of bacteria was shown in Table 3.

Table 3: Antibacterial activities of compound 1 and 2

Concentration (µg/mL)	Compound	Growth inhibition %		
		S. aureus	***B. subtillis***	***E. coli***
100	**1**	-	74.9	64.6
100	**2**	18.4	26.7	10.9
	Kenamycin	16 ± 0.1	0.25 ± 0.02	0.67 ± 0.05
	Ampicilin	0.12 ± 0.01	5.89 ± 0.1	0.41 ± 0.03

Conclusion

Phytochemical investigation of n-butanol fraction of acetone extract of *A. marmelos* fruits has afforded three coumarins, marmelosin (**1**), marmin (**2**) and xanthotoxol (**3**) and flavonoid afzelin (**4**). Compounds **1** and **2** exhibited antimycobacterium activity against *M. tuberculosis* H37Ra with an IC_{50} 12.46 µg/mL and 4.31 µg/mL respectively whereas, compounds **1** and **2**, at 100 µg/mL, showed 62.5% and 82.4% growth inhibition of *M. bovis* respectively. Compounds were also evaluated against two gram positive bacteria *S. aureus*, *B. subtilis* and gram negative bacteria *E. coli*. Compound **1**, at 100 µg/mL, showed 64.6% and 74.9% growth inhibition of *E. coli* and *B. subtilis* respectively. This study showed antimycobacterium activity of the major constituents from the fruits of this valuable medicinal plant. To the best of our knowledge, this is the first report of marmelosin (**1**) and marmin (**2**) for its evaluation against *M. tuberculosis* H37Ra with significant activity.

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