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BIO-AUTOGRAPHY: AN EFFICIENT METHOD TO CHECK THE IN VITRO ANTIMICROBIAL ACTIVITY OF *AEGLE MARMELOS* AGAINST ENTERIC PATHOGENS

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ABSTRACT

Aegle marmelos Correa (Rutaceae) is an indigenous plant of India. It is used in traditional medicine for a variety of purposes, in treating gastrointestinal disorders, especially diarrhea caused by enteric organisms. The great future potential of this plant has created a need for an antimicrobial assay, which can effectively confirm its antimicrobial activity. The objective of this study was to identify and confirm the compounds in the methanolic extract of fruit pulp of Aegle marmelos having antimicrobial effect against multi drug resistant clinical pathogens isolated from stool samples, by the bio-autography technique. The results obtained indicated that the compound with Rf value 0.71 and 0.75 respectively showed antibacterial activity in the methanolic extract of fruit pulp of Aegle marmelos against Salmonella typhi B330. Further, on purification and crystallization, the results could be validated. These two compounds, to the best of our knowledge, have shown antimicrobial activity for the first time by this method. Hence the method has potential in determining the efficacy of medicinal plants against other clinical pathogens as well.

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KEY WORDS

Traditional; diarrhea; enteric; assay; multidrug resistant

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[I] INTRODUCTION

In Asia, Africa and Latin America, gastrointestinal disorders are the leading cause of childhood mortality, resulting in 4,600,000 to 6,000,000 deaths each year. At the same time, diarrhoea is a common adverse effect of antibiotic treatment [1], which results from growth disruption of normal micro flora of the gut. The World Health Organization [2] attributes 3.5 millions deaths a year to diarrhea, with 80% of these deaths occurring in children under the age of five years and most often occurring in children between six months and three years of age. In Indian children, diarrhoea is the most common ailment (73%), and *Aegle marmelos* has been often used to cure diarrhoea and dysentery [3].

L. Correa (Rutaceae] known as "Bael" is an important indigenous fruit of India [4], and has great mythological and religious significance. It is called "shivadume", the tree of Lord Shiva. It is a large deciduous tropical tree, found all over India in Sub-Himalayan forests, Bengal, Central and South India and also in Burma. Since ancient times, its leaves and fruits are offered to Lord Shiva and Parvathi [5]. Ayurvedic physicians in India use almost all of its parts in many indigenous plant preparations. Extracts of *Aegle marmelos* are associated with various medicinal properties [6]. The unripe fruit is used in diarroheal and intestinal conditions and contains marmelosin (furocoumarin) as a chief constituent. It also contains carbohydrate, pectin, volatile oils and tannins and possesses astringent, digestive and stomachic actions and is useful in chronic diarroheas [7].

Due to its high pectin content, it is useful in diarrhea and dysentery and its volatile oils and tannins possess astringent properties [8-9]. In some studies, the unripe fruit showed activity against some intestinal parasites [10-11]. The plant has also shown to have antibacterial and antifungal activity [12-13]. The aqueous extract of its seed is hypoglycemic [14]. The ripe fruit has been shown to be effective against Ranikhet Disease virus, human Coxsackie virus B1-B6 [15] and in chronic dysentery and gastrointestinal diseases [16-18]. Its biological actions included antimicrobial, anthelminthic and antifilarial activities [19]. Aqueous extract of *Aegle marmelos* enhanced the susceptibility of beta-lactam resistant *Shigella flexneri* and *Shigella dysenteriae* towards beta-lactam antibiotics by altering porin channels [20]. As per Charaka (1500 BC], no tree has been in fact, longer or better known or appreciated by people of India than the Bael [21].

Aegle marmelos has also been widely investigated for its phytochemical constituents. The root, stem and leaves have been shown to contain tannins, alkaloids, sterols, coumarin, phenyl ethyl cinnamides and aromatic components [22, 23].

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Aegelin, marmelosine, marmelin, o-methyl hayordinol, alloimperatorin methyl ester,o-isopentanyl hayordinol and linoleic acid have been identified as its secondary metabolites [24-26] . The dry pulp of the fruit contains chiefly mucilage pectin like substance. Umbelliferone, psoralen and eugenol have been isolated from the dried fruit pulp and quantified by HPTLC [27]. Antimicrobial activity has also been shown in the seed oil against bacteria and fungi [28]. Analgesic, hypoglycemic, antioxidant and hepatoprotective effect of fruits and leaves has also been reported [29-31]. We report here, the results obtained using bioautography to detect antimicrobial compounds in a fruit pulp of *Aegle marmelos*, a plant used widely in traditional medicine for the treatment of diverse infectious diseases.

[II] METHODS

2.1. Plant material

Aegle marmelos fruit was collected in and around Chandigarh. The identity was established with the help of Department of Botany, Panjab University, Chandigarh.

2.2. Preparation of plant extracts

Two types of extracts [methanolic and aqueous] were used. Extracts were prepared by stirring the plant material overnight at room temperature with a seven-fold amount of water/methanol. The suspension was cold centrifuged at 3000 rpm for 15-20 minutes and the supernatant collected. The suspension was filtered through a Whatman filter paper No. 1, concentrated to half of its original volume at room temperature, filter sterilized with 0.45 µm Millipore filters and stored in screw capped vials at –20^{0}C [32].

2.3. Microorganisms used

Clinical isolates of *Salmonella typhi* from patients with enteric diseases were procured from Govt. Medical College and Hospital, Chandigarh. The antibiotic resistance pattern was studied by the disc diffusion method using standard Octodiscs [Hi media, Pvt. Bombay, India]. The zone of inhibition was measured after incubation at 37^{0}C for 24 hrs. In addition, a standard strain of *Salmonella typhi* [MTCC-531] was procured from the Institute of Microbial Technology, Chandigarh.

2.4. Extraction and fractionation of components from the fruit pulp of A.marmelos

The fresh mashed fruit pulp [500gm] of *Aegle marmelos* was macerated two times with methanol [[1.5 L×24h, 1.5 L×24h]] at room temperature. Each time, the extract was filtered and solvent removed under vacuum in rotatory evaporator below 50^{0}C to obtain a syrupy residue. The residue was combined to get 10.2gm of methanolic extract **[Figure– 1]**.

2.5. Fractionation using column chromatography

The residue [10.2gm] was subjected to chromatography over silica gel [150 gm, 150- 200 mesh] packed in petroleum ether [60-80^{0}C]. The column was eluted using acetone and petroleum ether [60-80^{0}C] with increasing portions of acetone [petroleum ether, PE+10% acetone, PE+30% acetone, and PE+50% acetone, acetone]. Finally the column was eluted with acetone. A total of 10 fractions, of 250ml each were collected and pooled according to their similarity to analytical TLC plates and dried. The different fractions thus obtained were dehydrated and concentrated in a rotary evaporator at 40°C. The active fraction was selected for isolation and purification of the antimicrobial compound.

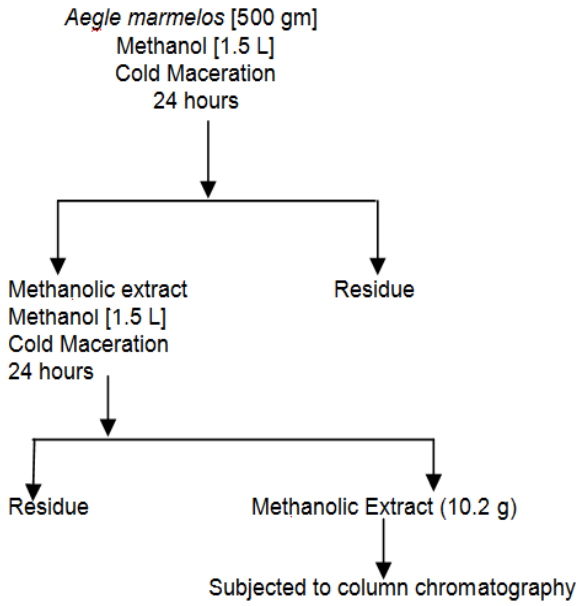

Fig: 1. Extraction and fractionation of *A. marmelos* fruit pulp

2.6. Isolation of an antimicrobial constituent (KVNK/1) by column chromatography

The fraction number F4, eluted in petroleum ether containing 10% acetone, and F5, eluted in petroleum ether containing 30% acetone, exhibited a major spot **[Table– 2]** with the same Rf value of 0.75 **[Figure–3]** and were pooled together. These fractions had also shown strong antimicrobial activity with zone of inhibition more than 15mm using the disc diffusion method against *Salmonella typhi* M531 and S. typhi B330.The solvent from the combined mixture of these two active fractions was evaporated in a rotary evaporator at reduced pressure to obtain cream colored powdery residue. The residue was washed with different solvents to remove the adhering pigmentary material. It was then crystallized from a 3:1 v/v mixture of chloroform and hexane to obtain white colored needles of a pure compound, designated KVNK/1. The compound was characterized using state-of-the-art techniques of spectroscopy and X-ray crystallography [33].

2.7. Phytochemical screening

The methanolic extract and the purified compound were separately tested for the presence or absence of the various groups of phytoconstituents [34, 35].

2.8. Thin layer chromatography [TLC]

The methanolic extracts and the purified compound were subjected to thin-layer chromatography Plates [Kieselgel 60 F254, MERCK] were developed with Choloroform: Acetone: Formic acid [5:4:1] which

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separated the components into a wide range of Rf values. TLC plates were run in duplicate and one set was used as the reference chromatogram. Eluted TLC plates were observed under ambient lighting and illuminated with ultraviolet lamp at 254 nm and 366nm. The other set was used for bioautography.

2.9. *Bio-autography*

After development, the TLC chromatogram plates were dried to remove the solvent. Active extracts were evaluated through the adapted bioautography technique [36, 37]. Briefly, a bacterial suspension [24 hr old culture *Salmonella typhi* M531 & B330] was centrifuged at 3000rpm/min for 20 min. Bacteria were suspended in fresh Muller Hinton [0.6%] agar and the count adjusted to109 bacteria/ml. A fine layer [15 ml] of bacterial suspension was overlaid on the freshly run TLC plates [reference plate, extract and purified compound respectively]. The plates were incubated at 37C for 48h, in humid conditions [petri plates saturated with wet filter paper] and then sprayed with an aqueous solution of 2 mg/ml 2,3,5, - triphenyltetrazolium chloride [TTC, Sigma]. The plates were reincubated at 37C for 24 hours. The areas of inhibition were compared with the Rf value of the related spots on the reference TLC plate.

[III] RESULTS

3.1. Phytochemical screening

A variety of phytoconstituents were observed in the fruit pulp. However, the active fraction contained only furanocoumarins **[Table– 1]**.

3.2. Fingerprint profile and bioautography of the methanolic extract of A. marmelos

To obtain some information on the active components, plant fractions were analyzed by TLC on silica gel plates. The best solvent system out of all those tested, was chloroform : acetone : formic acid [5:4:1]. Besides this, n-hexane : dichloromethane : methanol [10:10:1.25] also gave good resolution and produced four bands under 366 nm ultraviolet light. The thin layer chomatography of the methanolic extract of *A. marmelos* showed values as given in **Table– 2**.

Table: 1. Phytochemical screening of *A. marmelos*

S. No	Class of Phytoconstituent	Methanolic extract	Purified fraction
1	Carbohydrates	+	-
2	Alkaloids	+	-
3	Proteins	+	-
4	Amino acids	+	-
5	Triterpenoids	-	-
6	Flavonoids	+	-
7	Tannins	+	-
8	Anthraquinone glycosides	-	-
9	Cardioglycosides	-	-
10	Saponins	+	-
11	Furanocoumarins	+	+

Present (+), Absent(-)

Table: 2. TLC fingerprint profile of methanolic extract of *Aegle marmelos*

Band no.	Rf value
A	0.083
B	0.167
C	0.57
D	0.61
E	0.71
F	0.75

The bioautographs revealed two distinct areas of bacterial growth inhibition in TLC plates **[Figure– 2]**. The panel A shows the chromatogram of methanolic extract under 366 nm ultraviolet light and the panel B shows the appearance of same chromatogram after treatment with bacterial inoculum, indicating the location of bacterial inhibition zone. These areas corresponded to fractions E [Rf 0.71] and F [Rf 0.75]. Thus, antimicrobial components were present in the methanolic extract of the fruit pulp.

Table: 3. Antimicrobial activity of different fractions of column chromatography [Disc diffusion assay]

S.No.	Fraction code	Eluant	Zone of inhibition [mm]	
			Aegle marmelos [M531]	*Aegle marmelos* [B330]
1	F1	Petroleum ether	_	_
2	F2	Petroleum ether	_	_
3	F3	Petroleum ether : acetone [90 :10]	_	_
4	F4	Petroleum ether : acetone [90 :10]	+++	+++
5	F5	Petroleum ether : acetone [70 : 30]	+++	+++
6	F6	Petroleum ether : acetone [70 : 30]	_	_
7	F7	Petroleum ether : acetone [50 : 50]	_	_
8	F8	Petroleum ether : acetone [50 : 50]	_	_
9	F9	Acetone	_	_
10	F10	Acetone	_	_

(+++) ≥ 15mm zone of inhibition; (-) No inhibition

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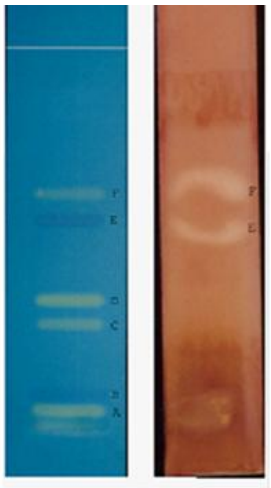

Fig: 2. A Fig: 2. B

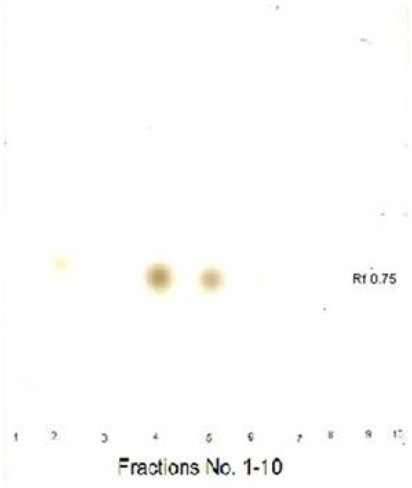

Fig: 3

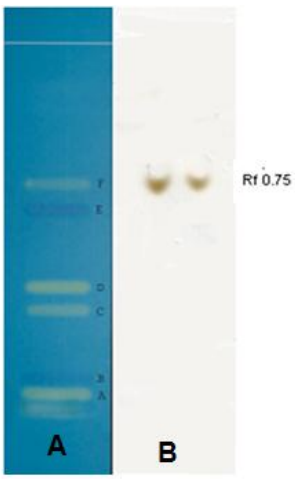

Fig: 4

Fig: 2. TLC and Bioautography of methanolic extract of *Aegle marmelos* against *S. typhi* B330. A) TLC chomatogram of *Aegle marmelos* extract under 366 nm ultraviolet light. **B)** Bioautography of methanolic extract of *Aegle marmelos* against *S. typhi* B330. **Fig: 3. TLC details of fractions 1-10 of methanolic extract of *A. marmelos*, Fig: 4. TLC fingerprint profile of the purified compound [KVNK/1] in comparison to the original extract. A)** Crude extract of *A. marmelos*; **B)** KVNK/1

3.3. *Fingerprint profile and bioautography of an antimicrobial constituent KVNK/1 from fractions of column chromatography*

The fraction number F4, eluted in petroleum ether containing 10% acetone, and F5, eluted in petroleum ether containing 30% acetone, exhibited a major spot **[Figure– 3]** with the same Rf value of 0.75 **[Figure– 3]** and were pooled together. These fractions had also shown strong antimicrobial activity with zone of inhibition ≥ 15mm in disc diffusion method against *Salmonella typhi* M531 and *S.typhi* B330 **[Table–3]**. After further purification by crystallization of the compound, it showed up at the same position [Rf value 0.75] in comparison with the original methanolic extract **[Figure– 4]**.

[V] DISCUSSION

Many drugs are being used as chemotherapeutic agents against various forms of gastrointestinal infections in the children. However, most of the antibiotics invariably have cell toxicity and resistance to the causative agents of these infections i.e. enteric organisms. In recent years, many natural compounds derived from plants or crude plant extracts have proven to exhibit a protective and therapeutic effect in a variety of ailments [38-40]. Plants have a long history of use in the treatment of gastrointestinal disorders [41]. The need to find a safe and highly effective cure for gastrointestinal disorder in children remains a major challenge for modern medicine. Flavones, isoflanones, catechins and tannins present in many plants have been shown to possess anti diarrhoeal potential. Further, some of the herbal medicines and their constituents have been reported to inhibit the ever-increasing number of resistant strains of *Salmonella typhi* [42]. Therefore, an attempt has been made to evaluate the antimicrobial activity of the fruit extract of *Aegle marmelos* which is commonly used in the Ayurvedic system of medicine for a variety of ailments.

Although, a lot of phytochemicals from this plant have been isolated, but to our knowledge, those responsible for antimicrobial activity have not been identified and characterized so far. Therefore this extract was analyzed to identify the bioactive compounds and to examine the future therapeutic potential of the isolated purified compound against clinical pathogenic strains. Our phytochemical analysis ascertained the presence of some potential phytochemical groups i.e., alkaloids, saponins, tannins, flavonoids and furanocoumarins. However, to our knowledge, the antimicrobial effect of coumarins of *A. marmelos* has not been reported so far. In the present phytochemical study, the purified compound KVNK/1 was found to be a furanocoumarin **[Table–1]**. Thus, in consistence with the previous reports that tannins, essential oils and saponins are responsible for antimicrobial activity, the present report has extended the list to furanocoumarins.

In our previous studies, we evaluated the activity of the aqueous and methanolic extract of the fruit pulp of *A.marmelos* against *S. typhi* by using the microdilution techniques [32]. Hence, the methanolic extract was used for further studies. The bioactivity of identified extracts was confirmed in the second stage and quantified by the microdilution and the bioautography assay.

Among the numerous in vitro methods for studying the antimicrobial activity of plant extracts, bioautography has found wide spread application, especially for the detection of new compounds in complex plant extracts. [43]. In this study,

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bioautography revealed promising antimicrobials in the extract of *Aegle marmelos*. These results confirmed the activity of methanolic extract of the fruit pulp of this plant detected in the disc diffusion assay **[Figure–2]**. These antimicrobials were highly active against *Salmonella typhi* B330 and M531.

In some earlier studies done, using the bioautography technique, the phenols and flavonoids were indicated as major active phytochemicals against methicillin Resistant Staphylococcus aureus [44]. Similarly, sterols and terpenes were isolated as antimicrobial components from the methanolic extract of *Eremophila duttonii* [45]. Volatile constituents of *Tambourissa eptophylla* [46] and alkaloids of *Bocconia arborea* have also shown antimicrobial activity against Gram negative and Gram positive bacteria as well as *Candida albicans* [47] using bioautography.

Antimicrobial activity has been shown in the leaves of *A.marmelos* [48]. Our study is in consistence with Raja et al [20] who studied the differential expression of ompC and ompF in multi-drug resistant *Shigella dysentriae* and *Shigella flexneri*. According to these authors, the aqueous extract of *Aegle marmelos* [AEAM] influenced susceptibility of beta lactam resistant Shigella towards beta lactam antibiotics by altering porin channels. They also suggested that AEAM along with beta lactam can be used for the treatment of multi-drug resistant Shigella. Similarly, Shirazi et al [49] studied the antienteric properties of *Salvia officinalis* [sage] extract. The tested extracts [0.1 - 0.05 g/l] exhibited the same effect as ampicillin and streptomycin against *S. typhi*. In another report, Yismaw et al [50] tested the in vitro antimicrobial activity of papaya seed extract against clinical isolates from wound, urine and stool samples and found it be effective with MIC value of 11.8 mg/ml against *S. typhi*.

In the present work, the antimicrobial properties of *A.marmelos* suggested its potential use in traditional med-icine for the treatment of gastrointestinal disorders caused by enteric organisms. As the extract showed very strong anti Salmonella activity, further studies were carried out to purify the antimicrobial compound/s and to characterize them.

Systematic downstream chromatographic analysis of the active methanol fraction done in our lab led to the isolation of white needle shaped crystalline compound [KVNK/1] identified as imperatorin through chemical characterization and spectral studies [33]. In the present work, the antimicrobial properties of *A.marmelos* suggest its potential usage in traditional medicine for the treatment of gastrointestinal disorders caused by enteric organisms. Further studies are being done in the laboratory to elucidate the mechanism of action of these compounds. In conclusion, bioautography proved to be an efficient method in extracting the active compounds.

[VI] CONCLUSION

Aegle marmelos has a great potential in terms of its numerous biological properties. We hereby present a reliable method which can be used to determine the efficacy of *Aegle marmelos* and other medicinal plants in producing antimicrobial substances effective against the clinical enteric pathogen *Salmonella typhi*.

FINANCIAL DISCLOSURE

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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