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Research Article

AZIMA TETRACANTHA LAM. AGAINST CAUSATIVE AGENTS IN DIABETIC FOOT INFECTIONS

K. NATARAJAN*, T. RASHID, CK. SHUHAIB, V. BALU

Department of Pharmaceutical Biotechnology, Email: nata14@rediffmail.com

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ABSTRACT

Objective: *Azima tetracantha* Lam. (Salvadoraceae), a widely growing herb has been used in the traditional medicine for treating many ailments. The objective of the present study was to evaluate the antimicrobial activity of aqueous extract of *A. tetracantha* leaves (AEAT) against the causative agents in diabetic foot infections and to understand the major phyto-constituents.
Methods: Susceptibility analysis was carried out by disc diffusion method at two different concentrations of AEAT '*viz*' 500 &1000 µg/ml. UHPLC-ESI MS/MS was carried over with C_{18} RP column using gradient mobile system and the spectrum obtained was interpreted.
Results: All the strains tested were more susceptible to AEAT with maximum of 15mm inhibition observed for *Aspergillus niger* and *Klebseilla pneumoniae*. UHPLC-ESI MS/MS study confirmed the presence of Isorhamnetin-3-O-rutinoside, Myricetin and Friedelin in AEAT.
Conclusion: To conclude AEAT is found to be a good source of lead compounds in diabetic foot infections.

Keywords: *Azima tetracantha*, UHPLC ESI-MS/MS, antimicrobial

INTRODUCTION

Azima tetracantha (Salvadoraceae) is a well known medicinal herb, termed '*Mulsangu*' in Tamil and '*Kundali*' in Sanskrit. Root, root bark and leaves of *A. tetracantha* are used with food as a remedy for rheumatism, diuretic and as stimulant [1,2]. Traditionally Indian medical practitioners use *A. tetracantha* in inflammatory conditions, cough, asthma, small pox and diarrhoea [3,4]. The major phyto-constituents reported in *A. tetracantha* are azimine, azecarpin, carpine, isorhamnitine-3-O-rutinoside, friedelin, lupeol, glutinol and β-sitosterol [5,6]. *A. tetracantha* is reported to have antifungal [7] antitumour [8], antidiabetic [9], antidiarrhoeal [10] and hepatoprotective [11] activities.

Diabetes mellitus has an increase susceptibility to infections due to the complications developed in various physiological systems and thereby weaken the immune system [12]. Infections are the important contributing factor to the morbidity of diabetic patients with foot problems [13]. Prevention and treatment of such wound is of great importance, as they can lead to foot amputation. The most important characteristics of the diabetic foot infection are often poly-microbial in nature and frequently harbours anaerobic organisms synergistically present along with the aerobes. The most common aerobic organisms encountered are the Gram positive *cocci*, including *Staphylococcus aureus*, coagulase-negative *Staphylococcus* and *Streptococcus* species. The Gram negative pathogens frequently cultured include *Proteus* species, *Escherichia coli* and various other species of *Enterobacteriaceae* family. Fungal infections of the toe nails are the most common skin condition affecting the diabetic patients [14]. Since *A. tetracantha* leaf extract is documented as antidiabetic, it is worthwhile to evaluate the plant against the causative microbial agents for diabetic associated foot infections.

MATERIALS AND METHODS

Preparation of Plant Extract

The aerial part (leaves) of *A. tetracantha* was collected from the Panayur area of Madurai, Tamilnadu as raw material, during the second week of February and a voucher specimen is stored in Ultra College of Pharmacy (015/UCP) and the plant material was authenticated by a renowned scientist. About 100 g of coarse powdered leaf in1.5 L water is boiled, cooled and filtered. The filtrate is evaporated to dryness in desiccator and stored in refrigerator (Yield- 26.5% w/w). The aqueous extract of *A. tetracantha* (AEAT) was subjected to preliminary phytochemical analysis [15].

UHPLC - ESI MS / MS analysis

Polar and semi-polar molecules in AEAT were separated and identified using UHPLC-ESI MS/MS. AEAT was chromatographed over C_{18} RP column (Acclaim 120 Å, 2.1 x 150mm, 3.0 µm, Dionex USA). Liquid Chromatography conditions are fixed as UV at 330nm and 0.2 ml/min flow rate. UHPLC was conditioned at-Ml/min flow rate, with gradient mobile system start at solution A (ACN in 1% acetic acid) for 0.2 min and 99% of solution B (water in 1% acetic acid). This was then brought to 75% solution A at 16th min and then reaching at 100% solution A at 19th min to 5% Solution A at 21st min and was maintained at same condition till run ends at 30th min. Nebulizer was set at 30.5 psi with 6.0 L/min N_2 flow rate. Masses were analyzed in 50-1000 *m/z* range, keeping capillary voltage of 4500 V with dry heater temperature at 280^{o} C. Absorbance was read arbitrary at 330nm. Exact mass of each eluted compound and their fragmented pattern (MS/MS) were identified using ESI-Q-II TOF (Bruker, Germany) at negative mode.

Antimicrobial study

Antimicrobial study was performed by disc diffusion method [16]. MTCC strains like *Escherichia coli* MTCC 118, *Proteus vulgaris* MTCC 426, *Bacillus subtilis* MTCC 619 and *Aspergillus niger* MTCC 872 were procured from IMTECH Chandigarh. Clinical isolates *Candida albicans,* Staphylococcus *aureus, Klebseilla pneumonia* and *Pseudomonas fluorescence* were obtained from Vijay Lab, Madurai, characterized and stored. A weighed quantity of appropriate media was dissolved in sterile water and autoclaved at 121^{o}C for 15 min. In lukewarm condition, media was poured in Petri Plates and allowed for solidification. 24 hr old cultures were spread on to the surface of the solidified agar aseptically and carefully using a sterile L bend rod. Discs were immersed in different test concentrations (500 and 1000 µg/ml) of the extract and allowed to evaporate the solvent dimethyl sulfoxide. All the discs were placed on to the surface of agar, maintaining proper distance. Plates were incubated at

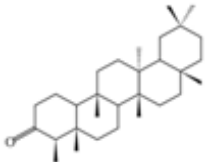
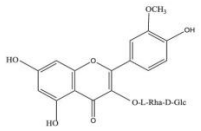
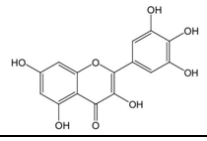
appropriate temperature and time in an inverted position. After incubation the zone of inhibition was measured using a metric ruler.

RESULTS

Phytochemical analysis

Preliminary phytochemical screening of AEAT showed the presence of terpenoids, alkaloids, saponins, tannins, phenolic compounds, flavonoids and steroids. The characterisations of the compounds using UHPLC ESI MS/MS analysis in negative ion mode are furnished in Table 1.

Table : 1 UHPLC-ESI MS/MS Analysis of AEAT

[M-H] *m/z* (g mol^{-1})	Retention Time (min)	Fragmentation in MS HPLC-ESI-MS^n	Identity	Molecular Formula	Structural Formula
425.5	22.2-22.6	125.2, 205.3, 273.4	Friedelin	$C_{30}H_{50}O$	
622.6	14.4-14.5	315	Isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	
317.3	24.5-24.7	151.2, 179.2, 317.3	Myricetin	$C_{15}H_{10}O_8$	

The mass fragmentation pattern shows a molecular ion peak at *m/z* 425.5 (Fig 1). The loss of a methyl group was indicated by the presence of a peak at *m/z* 411. The peak recorded in between Retention time 22.2 to 22.6 min showed other significant ions at *m/z* 125.2, 205.3 and 273.4 were attributed to the fragmentation of A, B, C and D rings, respectively. To the molecular weight 425.5g mol^{-1}, the corresponding molecular formula extracted from mass data bank is found to be $C_{30}H_{50}O$. These results were confirmed with small molecular data base and from previous studies [17] the identified compound is Friedelin.

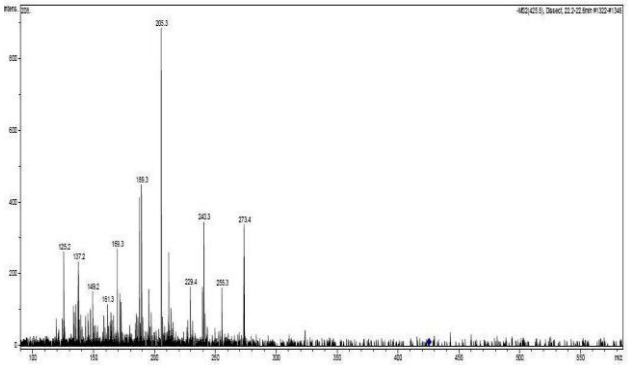


Fig: 1 Mass spectra of Friedelin from AEAT

The peak recorded in between Retention time 14.4 to 14.5 min showed one major signal in the mass spectra (Fig 2). This correlates with Isorhamnetin which exhibits specific fragmentation with the loss of methyl radical, thus giving *m/z* 315. To the molecular weight 622.6 g mol^{-1}, the corresponding molecular formula is found to be $C_{28}H_{32}O_{16}$ from mass data bank. These results were confirmed with small molecular data base and from previous studies [18] and the identified compound is Isorhamnetin-3-O-rutinoside.

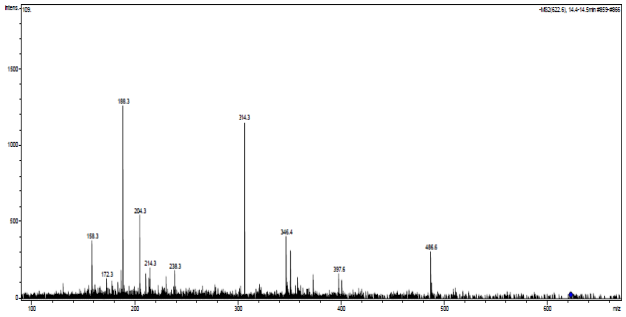


Fig: 2 Mass spectra of Isorhamnetin-3-O-rutinoside from AEAT

The peak recorded in between Retention time 24.5 to 24.7 min showed three major signals in the mass spectra (Fig 3). The peak corresponding to the electron spray ionization at *m/z* 151.2, *m/z* 179.2 and the fragment ion at *m/z* 317.3 and having molecular weight of 317.3 g mol^{-1} corresponds to the molecular formula $C_{15}H_{10}O_8$. These results were confirmed with small molecular data base and from previous studies [19] and the identified compound is Myricetin.

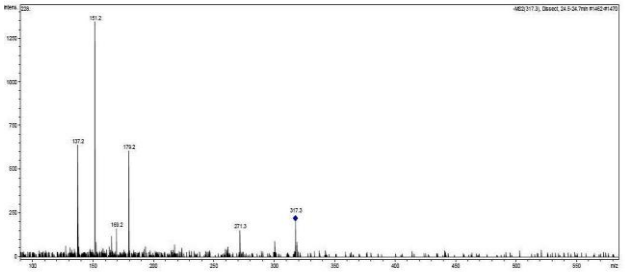


Fig: 3 Mass spectra of Myricetin from AEAT

Antimicrobial Study

Obtained crude extracts after pooled together was subjected to antimicrobial screening using disc diffusion method. AEAT exhibit potent antimicrobial activity against all the tested strains. Its inhibitory effect is proportional to the concentration gradient which is evident from the results. The susceptibility analysis of *A.tetracantha* is briefly expressed in Table 2.

Table: 2 Antimicrobial activity of AEAT

S. No	Strains Tested	Zone of Inhibition (mm)		
		AEAT 500 µg/ml	AEAT 1000 µg/ml	Std
1	*Candida albicans* *	11	13	15
2	*Aspergillus niger* MTCC 872	11	15	17
3	*Bacillus subtilus* MTCC 619	12	13	16
4	*Klebsiella pneumonia* *	11	14	17
5	*Pseudomonas aeruginosa* *	8	13	17
6	*Proteus vulgaris* MTCC 426	7	12	19
8	*Staphylococcus aureus* *	7	13	14
9	*Pseudomonas fluorescence* *	9	12	10
10	*Escherichia coli* MTCC 118	7	15	19

Bacterial Standard - Kanamycin, Fungal Standard – Clotrimazole (*) - Clinical Strains,

DISCUSSIONS

Traditionally *A. tetracantha* has been used to treat many diseases. Diabetes mellitus (DM) is a chronic disorder caused by partial or complete insulin deficiency which produces inadequate glucose control and leads to acute and chronic complications while *A.tetracantha* extract lowers blood glucose by a pancreatotrophic action and thereby exhibit potent antidiabetic activity [9]. The skin is colonized with an indigenous microbial flora. The normal flora may act as a competitive inhibitor of pathogenic microbes. Breaks in the skin, such as leg ulcers, burns and surgical or traumatic wounds and infections like Diabetic foot infections allow colonization with a broader range of bacteria [20]. As the Diabetic foot infections are often polymicrobial, a combination of antimicrobial agents would be more effective, than a single agent alone [14].

Current study demonstrates the antimicrobial effect of *A.tetracantha* leaf aqueous extract on selected pathogens which are the causative agents for diabetic foot infections. The results showed promsing effects regarding the inhibition of selected pathogens. Also an antimicrobial activity of Hexane, ethyl acetate, and methanol leaf extracts of *A.tetracantha* were reported to be potent against clinical pathogens and fungi [21].

AEAT led to the confirmation of three compounds *'viz'* Friedelin, Isorhamnetin-3-O-rutinoside and Myricetin using LC-MS technique. Myricetin is a novel compound that has an excellent antidiabetic effect at cellular level and being under process in management of prevention and treatment of Diabetes Mellitus [22]. Myricetin also proved to be a potent antibacterial agent against six different strains of both Gram positive and negative [23].

Friedelin from *A.tetracantha* is a strong antifungal agent [24] and also able to stimulate glucose uptake up to 1.8 fold compared with insulin-treated cells by mimic insulin action and that would be useful in the treatment of diabetes type-2 [25]. Isorhamnetin-3-O-rutinoside, may acts as insulin regulator to promote regeneration of beta-cells of the Islets of Langerhans [9]. 3-O-rutinosides of isorhamnetin, quercetin and Kaempferol etc, from *Calotropis procera* showed antimicrobial activity against the test microorganisms including the Gram Positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) were more susceptible than the Gram negative (*Pseudomonas aeruginosa and Salmonella enteritidis*) and the yeast species were more susceptible than the filamentous fungi [26].

Myricetin has been reported very well for its antibacterial activity. The triterpenoid, friedelin and flavonoid glycoside Isorhamnetin-3-O-rutinoside reported to possess excellent antifungal activity. These three compounds possess very good antidiabetic activity also. So the activity reported here may be attributed to the presence and combined efficiency in AEAT.

COCLUSION

From the outcome of this study, it may be concluded AEAT possesses antimicrobial and hypoglycaemic property which are very well effective in the prophylaxis and treatment of the Diabetic foot infections.

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