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Isolation and characterization of bioactive principles from leaves of *Foeniculum vulgare* and effect of anti tubercular perspectives

Pharmacology

KEYWORDS: Foeniculum vulgare; Mycobacterium tuberculosis $H_{37}RV$; Alamar blue assay method

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ABSTRACT

Foeniculum vulgare is a perennial herb native to southern Europe and meditteranean sea. Folklore claims states that it has a wide range of biological potentials which are unsung in nature. Preliminary phytochemical screening reveals the chloroform extract of the leaves of ***Foeniculum vulgare*** possess antitubercular perspectives against $H_{37}RV$ (ATCC No: 27294) using MABA method. Chromatographic fractionation of chloroform extract of ***F.vulgare*** resulted in the isolation of bioactive compounds such as monoterpene, 1,8 –cineole and estragole. Characterization was performed by interoperating the IR, C^{18} NMR, H^1 NMR & Mass data. MIC of the fractions (hexane: ethylacetate 95:5;85:15;75:25) exhibited at 25µg/mL. Phytochemical qualitative investigations revealed the presence of secondary metabolites such as alkaloids, phenols, glycosides, tannins, saponins and flavanoids.

INTRODUCTION

India has the highest burden of tuberculosis in the world with over 2 million incident cases amounting to more than 5 th global burden. According to WHO reports which states that approximately 8 million cases, out of which 7.6 million cases were newly reported with MDR & XDR-TB. Due to the prevailing extremity, it is a challenging task for the synthetic medicinal chemists to explore the new drug molecules to combat [1].Hence forth, chemists switch over to explore the unsung biological potentials of the phytodrugs as an alternative rescue force to combat against these dreadful diseases. Fennel is a perennial herb commonly found in Asian continent such as India, Pakistan, China and Egyptian countries. Fennel has listed as one of the nine anglo –saxon sacred herbs [2].Folklore studies revealed that fennel **(*Foeniculum vulgare*)** has been possessing antispasmodic, carminative, diuretic, expectorant, laxative, stimulant and anti ulcer properties.[3]. Herbal medicine explores the pharmacological aspects of fennel in the treatment of gastro enteritis, hernia repair, indigestion and abdominal pain [4]. These unsung potentials made our intrigue to investigate and explore the antimycobacterial properties of the leaf extracts of ***F.vulgare.***

EXPERIMENTAL

The leaves of the fennel ***(F.vulgare)*** for the proposed study was collected from Nacharam vaillage, Medak district, Telangana. The plant was authenticated by Prof. P.Jayaraman, PARC, Chennai and authenticated as PARC/2013/2053. The leaf samples were washed with water, shade dried and pulverized by passing through the sieve No:40 mesh. Thus obtained powder (100g) was subjected to successive cold extraction process with the aid of solvents of increasing polarity 9hexane, chloroform and methanol) for 72 hrs. The corresponding extracts were concentrated on a rota vap under reduced pressure to get the viscous mass. The corresponding percentage of yield were found to be 0.7 % w/w,1.7%w/w and 0.8% w/w respectively. Preliminary phytochemical investigation were done to explore the various phytoconstituents such as steroids, phenols, alkaloids, flavanoids, tannins and glycosides. The extracts were subjected to preliminary antimycobacterial screening $H_{37}RV$ (ATCC Bo: 27294) using microplate Alamar blue assay method (MABA).Bioactive chloroform extract was subjected to column chromatography to carry out isolation of the compounds from leaves of *F.vulgare* by gradient elution technique. The eleutropic series comprising of hexane, ethyl acetate, methanol and their mixtures was used. Each eluate obtained from the column was monitored by thin layered chromatography (TLC) on a precoated silica gel G plates. Similar TLC eluates were combined, pooled and subjected to evaporation under reduced pressure. The offered compounds from eluates were labeled as 1 to 3. The isolated compounds were structurally characterized on the basis of interpretation of spectral data. The various chemicals used were AR grade. H1 NMR and 13C NMR spectra were recorded on Bruker Advance II 400 NMR Spectrometer in $CDCl_3$ and DMSO –d using TMS as internal standard. The chemical shift values are expressed in δ units while J value in Hz.The IR spectra were recorded on FT –IR perkin elmer IR spectrophotometer using KBR pellet. EI – Mass spectra were recorded on VG -70S11250 J EI mass spectrometer. All spectroscopic studies were done at BioNeemtech, India Pvt.Limited, Chennai, Tamil nadu, India.

6- Isopropyl -3 –methyl cyclohexane -1,3,4 –triol **(1)** was obtained on elution with hexane: ethyl acetate (9.5 :0.5).It is a colorless liquid, B.P: 176-177°C, R_f value = 0.2. It is a cyclic ether and a monoterpenoid. IR (Kbr, $V_{maxcm-1}$) : 3424.5,2024.8,1627.3,727. ^{1}HNMR (δ,$CDCl_3$) : 1.110 (s, 3H,1XCH_3), 1.160 (s, 3H, 1XCH_3), 2.0 -2.5 (m, 5H, 3X CH, 2X CH_2), 3.4-3.6 (t, 3H, 2XCH, 1XOH), 4.795 (d, 2H, 2XOH). ^{13} C NMR (δ, $CDCl_3$) : 14.152 (1C, 1XCH_3), 22.706 (1C, C- CH_3), 29.393,29.715 (2C, Cyclic carbon), 70.0-73.0 (3C, CH-OH). MS (m/z relative intensity) : 188 (M^+), 142.85, 129.046,106.94.

1,8 Cineole **(2)** was obtained on elution with hexane : ethylacetate (8.5 :1.5). It is a colorless liquid, B.P : 282° C, R_f Value = 0.3. It is a 1,4,4 – trimethyl -2- oxa bicycle (2.2.2) octane. IR (Kbr, $V_{max\ cm-1}$): 3656.3,2728.32. ^{1}H NMR (δ, $CDCl_3$) : 1.169-1.715 (t, 9H, 3XCH_3), 2.013-2.089 (m, 8H, 4XCH_2), 2.116-2.533 (s, 1H, 1XCH). ^{13} CNMR (δ, $CDCl_3$) : 29.649 (1C, C-CH_3), 30.700 (1C , CH_3), 35.0-41.0 (4C – Cyclic CH). MS (m/z relative intensity) : 155 (M^+), 170.58, 201.54.

Estragole **(3)** was obtained on elution with hexane: ethylacetate (7.5:2.5).It is a colorless liquid, B.P : 216° C, R_f Value = 0.5, It is a P-allyl anisole. IR (KBr, $V_{max\ cm-1}$) 2467.2,1396,2180. ^{1}H NMR (δ, $CDCl_3$): 1.419-2.391 (m, 4H, 4XCH), 3.514 (s, overlapped, 3H, 1XOCH_3),7.294 (s, 2H, 1XCH_2). ^{13} C NMR (δ, $CDCl_3$) : 27.337, 29.705 (4C, C-CH), 50.924 (1C, C-CH_3), 130-140 (2C, aryl carbon). MS (m/z relative intensity) : 149 (M^+), 167.71,206.17.

BIOEVALUATION/ANTIMYCOBACTERIAL ACTIVITY:

The extract/fractions from the leaves of ***F.vulgare*** were evaluated against *Mycobacterium tuberculosis* ($H_{37}RV$ strain, ATCC No: 27294) using MABA technique. 200µL of sterile deionized water was added to all outer perimeter wells. The wells were maintained in sterile conditions to minimize the evaporation of medium during incubation. MABA plate consist of 96 wells. In each plate, 100µL of middle brook 7H9 broth has been added admixed with the drugs in a serial dilutions.The final drug concentrations tested were 0.2-100µg/mL.Plates were convered and sealed with parafilm to avoid contamination.The plates were incubated at 37° C+/-1°C for 5 days, 25µL of freshly prepared 1:1 mixture of alamar blue reagent associated with 10% tween 80 was added. 50µL of *M.tb* starin (ATCC No: 27294, $H_{37}RV$) was added and the plates were incubated for 48- 64

hrs at 37ºC+/-1ºC. Pyrazinamide, ciprofloxacin and streptomycin was used as a standard with respective concentrations such as 3.125,3.125 &4.25µg/mL respectively.The blue color retained in the well indicating the no growth of *Mycobacterium* sp. Pink color was scoring the indictor of growth of *M.tb* strain. MIC was determined as lowest drug concentration which prevent color change from blue to pink.

RESULTS AND DICUSSION:

Preliminary phytochemical studies explore the presence of alkaloids, glycosides, tannins, flavanoids, phenols and terpenoids. Compound **1** was obtained on elution with hexane : ethyl acetate (9.5 :0.5).The appearance of the absorption band at 3424.5 cm^{-1} confirms the presence of hydroxyl group. Other absorptions appeared at 2024.8,1627.3 cm^{-1} confirms the presence of aromatic CH and carbonyl group. The presence of the absorption at 727 cm^{-1} confirms the presence of aromatic CH bending. In ^{1} H NMR spectra of a compound **1** in $CDCl_3$, a singlet at 1.10 indicating the presence of terminal methyl groups. Three signals of 3.4-3.6 & 4.795 δ integrating the three protons each were assigned to three hydroxyl groups. A ^{13} C triplet assigned at 70.0 -73.0 integrating three carbon atoms at CH –OH group. The MS fragmentation pattern lends further support to the proposed structure. Based upon above data and literature survey, this compound could be characterized as 6- isopropyl -3- methyl cyclohexane -1,3,4 –triol (**1**).

Compound **2** was obtained on elution with hexane : ethyl acetate (8.5 ;1.5). The IR of the compound showed a peak at 3464.2 cm^{-1} confirms the presence of hydroxyl group. Other absorptions at 2728.32 appeared in their IR spectra. ^{1} HNMR spectra of compound **2** in $CDCl_3$ a singlet2.116-2.533 indicates the presence of oxo linkage. ^{13} C NMR spectra, peaks mentioned at 29.649 and 30.700 assigned to methyl carbon atoms. The MS fragmentation pattern lends further support to proposed structure. Based upon above data, the identity of compound **2** was characterized as 1,8 cineole.

Compound 3 was obtained as colorless liquid on elution with hexane : ethyl acetate (7.5 :2.5). The IR absorption at 2467.2, 1.396 and 2.180 cm^{-1} indicated the presence of aromatic CH stretching and alkyl CH stretching. The ^{1} H NMR spectra of this compound, peak mentioned at 50.924 assigned for methoxy carbon. The MS fragmentation pattern leands further support to propsed structure. Based upon above data and comparison with literature, the compound was characterized as 1- allyl -4-methoxy benzene (**3**).

ANTIMYCOBACTERIAL ACTIVITY:

Antimycobacterial activity of chloroform extract fractions of leaves of ***F.vulgare*** was tested against *Mycobacterium tuberculosis* (H_{37} RV – ATCC 27294) at two fold serial dilution technique ranging from 0.8µg/mL - 100µg/mL concentrations. A perusal of activity data presented in Table -1 revealed that FC1- FC3 exhibited activity with 80 % inhibition at 25µg/mL against the standard drugs at the concentration of 3.125 & 4.25µg/mL by MABA technique (Fig.1).

CONCLUSION:

The present study explore the chloroform extract of ***F.vulgare*** offers a bioactive molecules against *Mycobacterium tuberculosis*.

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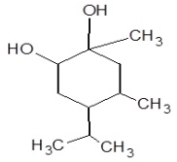
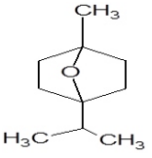
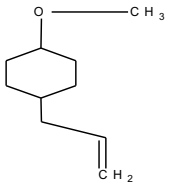




Compound 1 **Compound 2**



Compound 3

Structures of compound 1-3

Table no: 1 Antimycobacterial activity of isolated compounds of leaf extracts o*f Foeniculum vulgarae*

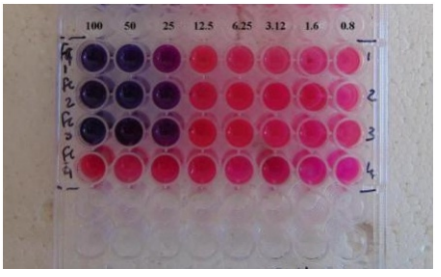
S.No	Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	Fc1	S	S	S	R	R	R	R	R
2	FC2	S	S	S	R	R	R	R	R
3	FC3	S	S	S	R	R	R	R	R

F=Fennel C= Compound, S=Sensitive, R=Resistant

Figure no: 1 Screening of isolated compounds by Alamar blue Anti Tb method.

(a) Standard drug

b) Screening of isolated compounds against Mycobacterium tuberculosis (compounds 1-4)



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