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Demonstration of biological activities of extracts from *Isodon rugosus* Wall. Ex Benth: Separation and identification of bioactive phytoconstituents by GC-MS analysis in the ethyl acetate extract

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Abstract

Background: Since long, natural sources have been explored for possible managements of various diseases. In this context, the study is designed to evaluate *Isodon rugosus* Wall. ex Benth for biological potentials including antibacterial, anthelmintic, insecticidal, anti-termites and anti-Pharaoh activities followed by GC-MS analysis of active fraction to identify various bioactive compounds.

Methods: *I. rugosus* was investigated against eight bacterial strains using well diffusion method and microdilution method with ceftriaxone as positive control. Similarly, the insecticidal activity was carried out against *Tribolium castaneum*, *Rhyzopertha dominica*, *Monomorium pharaonis* and *Heterotermis indicola* following contact toxicity method. Likewise, anthelmintic activity was performed against *Ascaridia galli* and *Pherethima posthuma* using albendazole as positive control, in which the paralysis and death times of the worms were observed. The GC-MS analysis of the most active solvent fraction was performed for identifications of various bioactive compounds.

Results: Among the tested samples of *I. rugosus*, flavonoids and ethyl acetate fraction exhibited high antibacterial activities. The crude saponins showed highest anthelmintic activity against *Pherethima posthuma* and *Ascaridia galli* with death times of 27.67 and 29.22 min respectively at concentrations of 40 mg/ml. In insecticidal activity, chloroform fraction and saponins exhibited notable results against *R. dominica* (60 and 70%) and *T. castaneum* (70 and 76%) at concentration of 200 mg/ml. In anti-termite assay, all the plant samples showed overwhelming results, i.e. all the 25 termites were killed on the 3rd day. Similarly, in anti-Pharaoh activity, the chloroform, ethyl acetate and saponins fractions were most potent, each exhibiting LD₅₀ of <0.1 mg/ml. In GC-MS analysis, total of 57 compounds were identified. Some of the bioactive compounds identified in GC-MS analysis are palmitic acid, hinokiol, α -amyrin, phytol, ethyl linolate, cyclohexanone, hinokione, methyl palmitate, ethyl palmitate and stigmasterol acetate.

Conclusions: Based on our current results, it can be concluded that *I. rugosus* possess strong antibacterial, insecticidal and anthelmintic potentials having crude saponins and ethyl acetate as the most active fractions. The GC-MS analysis and biological assays reveal that ethyl acetate fraction is a suitable target for the isolation of diverse array of bioactive compounds.

Keywords: Antibacterial, MICs, Anthelmintic, *Ascaridia galli*, Anti-termites, Anti-Pharaoh, *Isodon rugosus*, GC-ms

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Background

Infectious diseases are among the leading health problems, accounting for 41% of global disease burden [1]. Diseases caused by bacteria and parasites are still challenging to public health. Their impact is particularly large in developing countries due to unavailability of relevant medicines and the emergence of widespread drug resistance [2–4]. The clinical efficacy of many existing antibiotics has been endangered by the emergence of MDR pathogens [5, 6]. To triumph over MDR pathogens, it is important to develop novel and more effective antimicrobial agents, especially with a different mechanism of action [7]. It is obvious that despite synthetic compounds, natural raw materials are the key sources for novel therapeutic agents for the management of various ailments like infectious diseases [8, 9].

Helminthiasis is among the most widespread parasitic infections in humans, distressing an enormous population of the world. Approximately two billions people are suffering from parasitic worms infections [10]. Majority of helminths mediated infections are limited to tropical regions and cause huge hazard to health leading to undernourishment, anaemia, eosinophilia, physical, mental sluggishness and pneumonia [11]. Parasitic infections also cause lymphatic filariasis leading to elephantiasis, onchocerciasis called river blindness and schistosomiasis. These infections are more common in populations living in endemic areas with poor hygiene and socio-economic problems [12]. Livestock and crops are also affected by parasitic worms thus leading to reduction in crops and milk production [13]. There is a substantial improvement in helminths control but due to emergence of anthelmintic resistance to currently available drugs. So, it is important to search alternative strategies against gastrointestinal nematodes [14]. Similarly, most of the available insecticidal from chemical sources are hazardous to human health and are associated with environmental risks [15]. Therefore, alternative resources must be explored to substitute the chemical pesticides. Pesticides from natural sources are advantageous as they are selective in targeting specific species and also have unique modes of action [16].

Isodon rugosus Wall. ex Benth. belongs to the family Lamiaceae. *Isodon*, an important genus of this family is a rich source containing a large number of bioactive compounds. The *Isodon* species have been used as anti-tumor, anticancer, antimicrobial, insecticide and anthelmintic [17, 18]. *Isodon rugosus* has been used ethnomedicinally as an antimicrobial and anthelmintic [19, 20]. This plant has also been used traditionally against skin infections and for the treatment of scabies [21, 22]. This specie has also been verified scientifically to possess variety of pharmacological activities [23]. Based on the published literature, the

current study is designed to evaluate various fractions of *I. rugosus* for the antimicrobial, anthelmintic and insecticidal potentials along with identifications and sorting out of bioactive compounds by GC-MS analysis.

Methods

Plant collection and extraction

The plant was collected from Dir (KPK), Pakistan in the month of June and was identified by plant taxonomist at Department of Botany, Shaheed Benazir Bhutto University Dir (KPK), Pakistan. The plant sample was kept at the herbarium of the same university with voucher number 1016AZ. Fresh aerial parts (11 kg) were separated from the rest of the plant and rinsed with clean water to remove any dust particles followed by shade drying for 23 days. The dried plant parts were cut into small pieces and grinded into coarse powder with the help of a grinder. The powdered material (6 kg) was macerated in 80% methanol (25 L) for 18 days and the process was repeated three times. The filtrates were combined and concentrated under reduced pressure using rotary evaporator [24, 25]. A brown semisolid mass (380 g) of the crude methanolic extracts was obtained.

Fractionation

The crude methanolic extract was transferred into a separating funnel and diluted with 500 ml of distilled water. *n*-Hexane (500 ml) was added to it with vigorous shaking and kept for a while to form two layers. The *n*-hexane layer was separated and repeated the same procedure three times by adding 500 ml of *n*-hexane each time. All the *n*-hexane layers were combined and concentrated at reduced pressure using rotary evaporator. The final concentrated weight of *n*-hexane fraction was 19 g. In a similar way like *n*-hexane fraction, the chloroform and ethyl acetate fractions were extracted sequentially weighing 27 and 80 g respectively. The dry water residue (aqueous fraction) was collected at the end weighing 125 g [26, 27].

Extraction of crude saponins

The crude saponins from *I. rugosus* were obtained by adding 20 g of the plant powder in a conical flask having 100 ml of 20% ethanol. The sample was heated in water bath for 4 h at 55 °C with continuous stirring. The mixture was filtered and extracted again with 200 ml of 20% ethanol. The volume was heated up to 60 °C in water bath and got 40 ml of greenish color residue. The residue was added into the separating funnel followed by the addition of diethyl ether (20 ml) with vigorous shaking. After vigorous shaking, the separating funnel was put in a stand to get two distinct layers. The upper diethyl ether layer was discarded while the lower aqueous layer obtained was diluted with *n*-butanol (60 ml). This extract was washed with 10 ml of 5% sodium

chloride solution. The solution was evaporated by keeping in water bath to obtain the dried saponins [26].

Extraction of Flavonoids

Plant sample having weight of 20 g was heated at 50 °C in 200 ml of HCl (2 M) under reflux for 30 min. The plant sample was filtered using Whatman No. 42 filter paper after getting cold. The filtrate obtained was poured into a separating funnel and added equal volume of ethyl acetate. The total amount of flavonoids present in the plant sample was precipitated, which were collected from the sample using a filter paper. The weight of flavonoids was 1.4 g (7%) [28].

Gas chromatography (GC) analysis

Ethyl acetate fraction was subjected to GC analysis and the GCMS was done by means of an Agilent USB-393752 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HHP-5MS phenylmethylsiloxane 5% with capillary column (30 m × 0.25 mm × 0.25 μm; Restek, Bellefonte, PA) equipped with an FID detector. Initially, the temperature of oven was maintained at 70 °C for 1 min, and then increased the required temperature slowly and step wise to 180 °C at the rate of 6 °C/min for 5 min, and at the final stage increased the temperature to 280 °C at the rate of 5 °C/min for 20 min. The temperatures of the Injector and detector were set at 220 °C and 290 °C, respectively. Helium was used as a carrier gas with a flow rate of 1 ml/min [29].

Gas chromatography–mass spectrometry (GC/MS) analysis

Ethyl acetate fraction was subjected to GC/MS analysis and the processed was done by means of an Agilent USB-393752 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HHP-5MS phenylmethylsiloxane 5% with a capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Restek, Bellefonte, PA) prepared with an Agilent HP-5973 mass selective detector in the electron impact mode (ionization energy: 70 eV) working under similar experimental background as illustrated for GC.

Identification of components

Identification of the compounds present in the ethyl acetate fraction of *I. rugosus* was carried out on the basis of comparison of their relative retention indices of each component with those of the authentic compounds present in the literature. Further identifications of the compounds were carried out from the spectral data obtained from the Wiley and NIST libraries and additional identifications were carried out by completed comparisons of the fragmentation pattern of the mass spectra with the reported in literature [30].

Antibacterial investigations

Bacterial strains

Eight bacterial strains including *Escherichia coli* (739), *Klebsiella pneumoniae* (700603), *Pseudomonas aeruginosa* (27853), *Enterococcus faecalis* (29212), *Proteus mirabilis* (13315), *Staphylococcus aureus* (29213), *Bacillus cereus* and *Salmonella typhi* were used to investigate antibacterial potential of plant's samples. The samples were provided by the Microbiology Laboratory of Department of Microbiology, Quaid-e-Azam University, Islamabad. Bacteria were preserved in freeze-dried condition at 4 °C in stab slant agar until later use.

Preparation and standardization of inoculum

Bacterial cultures were grown for 24 h at 37 °C and suspensions with cell density of 1×10^8 CFU/ml, were prepared using McFarland standard and was further diluted to a cell density of 1×10^6 CFU/ml using a UV visible spectrophotometer (Thermo electron corporation USA) at 625 nm and the standardization was maintained for the period of the study [31].

Antibacterial assay

Preliminary antibacterial activity of plant extracts were investigated using well assay technique [32, 33]. Briefly, Nutrient agar plates were prepared and inoculated with the test organisms aseptically under laminar flow hood and were properly labeled. Wells of 5 mm diameter were made in the plates using sterile cork borer. Samples were prepared having concentrations of 10 mg/ml. From each sample, 100 μl of extract was added into each well using micropipette. The standard drug ceftriaxone was used as positive control, which was added into the central well contained in nutrient agar media in each Petri plate. After inoculation and addition of the samples, the Petri plates were kept in incubator at 37 °C for 24 h. Diameter of inhibitory zones were determined around each bore after 24 h and were compared with positive control. Experiments were performed in triplicate and the data obtained was represented as mean ± SEM.

Determination of MICs

MICs were determined using microdilution method as described by National Committee for Clinical Laboratory Standards (NCCLS) [34]. Briefly, the plant's samples were prepared at concentrations of 50 mg/ml (stock solution) in sterile distilled water and serially diluted with distilled water to 20, 17.5, 15, 12.5, 10, 7.5, 5, 2.5, 1 and 0.5 μg/ml using nutrient broth followed by inoculation with 0.2 ml suspension of the test organisms. After 24 h of incubation at 37 °C, the test tubes were visually observed for growth. The lowest concentration at which no growth of the microorganism was observed was

Table 1 List of all compounds identified in the GC-MS analysis of ethyl acetate fraction of *Isodon rugosus*

S. No	Compound Label	RT	Name	Formula	Hits (DB)
1.	Cyclohexanone (CAS) \$\$ Anon \$\$ Anone \$\$ Nadone \$\$ Hexanon \$\$ Sextone	3.858	Cyclohexanone	C6H10O	10
2.	Ethanol, 2-ethoxy-, acetate (CAS) \$\$ 2-Ethoxyethyl acetate \$\$ Oxitol acetate	3.936	Oxitol acetate	C6H12O3	10
3.	Cyclopentane, 1,1,3,4-tetramethyl-, trans- (CAS)	4.726	Nf	C9H18	10
4.	1-(4-Tosyloxybutyl)-3-(1-methoxy-1-methylethoxy)clopentene	4.842	Nf	C20H30O5S	1
5.	(1.alpha.,5.alpha.,6.alpha.,7.alpha.)-6,7-(Z,E)-Dipropenyl-3-oxabicyclo[3.2....	5.428	Nf	C12H18O	6
6.	Butanedioic acid, ethyl methyl ester (CAS) \$\$ 1-Ethyl 4-methyl succinate	7.031	Nf	C7H12O4	10
7.	N-Butylpropargylamine	7.156	Butylpropargylamine	C7H13N	10
8.	2-Cyclohexen-1-one, 3,5,5-trimethyl- (CAS) \$\$ 3,5,5-Trimethyl-2-cyclohexenone	7.366	Nf	C9H14O	10
9.	Succinic acid, butyl ethyl ester	8.151	Nf	C10H18O4	10
10.	Butanedioic acid (CAS) \$\$ Succinic acid (CAS) \$\$ Asuccin \$\$ Amber acid	8.234	Amber acid	C4H6O4	10
11.	Undecane, 4-methyl- (CAS) \$\$ 4-Methylundecane \$\$ 4 -methyl - undecane	8.456	Nf	C12H26	10
12.	1-(1,1-dimethyl-2-propenyl)-1-cyclohexene	10.256	Nf	C11H18	10
13.	Dodecane, 2-methyl- (CAS) \$\$ 2-Methyl-dodecane \$\$ 11-Methyl-dodecane	11.258	Nf	C13H28	10
14.	(+)-(E)-N,N-Di-tert-butoxycarbonyl-1,3-diphenylprop-2-enamine	12.008	Nf	C25H31NO4	1
15.	Benzene, 1-(bromomethyl)-3-nitro- \$\$ m-(Bromomethyl) nitrobenzene	12.284	Nf	C7H6BrNO2	10
16.	rac-gamma.(2)-muurolene	12.418	rac-gamma(2)-muurolene	C16H24	10
17.	Decane, 3-bromo- \$\$ 3-Bromodecane #	12.54	Nf	C10H21Br	1
18.	Acetic Acid 2-(4-Hydroxyphenyl)ethyl Ester	13.523	Nf	C10H12O3	10
19.	Tetradecane, 1-chloro- \$\$ Myristyl chloride \$\$ Tetradecyl chloride	13.738	Myristyl chloride	C14H29Cl	10
20.	alpha-Amorphene \$\$ ALPHA. AMORPHENE \$\$ 6.alpha.-Cadin-4,9-diene, (-)-	14.153	alpha-Amorphene	C15H24	10
21.	alpha-Copaene \$\$ Copaene (CAS) \$\$ Copaen \$\$ (-)-alpha-Copaene	14.516	Copaen	C15H24	10
22.	alpha-Cadinol \$\$ Cadin-4-en-10-ol \$\$ 1.alpha.-Cadinol \$\$ 10-ALPHA-CADINOL	14.64	alpha-Cadinol	C15H26O	10
23.	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	15.859	Nf	C10H12O3	10
24.	(-)-Loliolide \$\$ Loliolide \$\$ Loliolid \$\$ Digiprolactone \$\$ Calendin	16.428	Loliolide	C11H16O3	2
25.	Neophytadiene \$\$ 7,11,15-TRIMETHYL,3-METHYLENE-1-HEXADECENE	17.314	Neophytadiene	C20H38	10
26.	R-7-endo-p-phenylbenzoyloxy-6-anti-(beta-phenylethenyl)-2-oxa-bicyclo[3.3....	17.447	Nf	C27H24O3	1
27.	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)	18.25	Nf	C20H40O	10
28.	Hexadecanoic acid, methyl ester (CAS) \$\$ Methyl palmitate \$\$ Uniphat A60	19.316	Methyl palmitate	C17H34O2	10
29.	n-Hexadecanoic acid \$\$ Hexadecanoic acid \$\$ n-Hexadecoic acid \$\$ Palmitic acid	20.498	Palmitic acid	C16H32O2	10
30.	Hexadecanoic acid, ethyl ester (CAS) \$\$ Ethyl palmitate \$\$ Ethyl hexadecanoate	21.386	Ethyl palmitate	C18H36O2	10
31.	CIS-11,14,17-EICOSATRIENOIC ACID ME	25.548	Nf	C21H36O2	10
32.	Phytol \$\$ 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	26.041	Phytol	C20H40O	10

Table 1 List of all compounds identified in the GC-MS analysis of ethyl acetate fraction of *Isodon rugosus* (Continued)

33.	17-Octadecynoic acid	27.132	Nf	C18H32O2	10
34.	Linoleic acid ethyl ester \$\$ Ethyl linoleate \$\$ Ethyl linolate \$\$ Mandenol	27.616	Mandenol	C20H36O2	10
35.	Acetic acid, chloro-, hexadecyl ester \$\$ Chloro-acetic acid hexadecyl ester	27.817	Nf	C18H35ClO2	10
36.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- \$\$ Methyl linolenate	27.831	Methyl linolenate	C19H32O2	10
37.	Octadecanoic acid, ethyl ester (CAS) \$\$ Ethyl stearate \$\$ Ethyl octadecanoate	28.617	Ethyl stearate	C20H40O2	10
38.	Neophytadiene \$\$ 7,11,15-TRIMETHYL,3-METHYLENE-1-HEXADECENE	29.321	Neophytadiene	C20H38	10
39.	Hexadecanoic acid, ethyl ester (CAS) \$\$ Ethyl palmitate \$\$ Ethyl hexadecanoate	33.473	Ethyl palmitate	C18H36O2	10
40.	Naphthalene, 1,2,3,4-tetrahydro-1-isopropyl-1,2,4,4,7-pentamethyl-	35.4	Nf	C18H28	1
41.	3,4,8-trimethyl-9-oxy-1-trimethylsilyloxybicyclo[4.3.1]non-1-ene	35.883	Nf	C15H26O2Si	10
42.	Di-(2-ethylhexyl)phthalate \$\$ 1,2-BENZENEDICARBOXYLIC ACID	36.417	Nf	C24H38O4	10
43.	Hinokione \$\$ (+)-Hinokione	36.604	Hinokione	C20H28O2	10
44.	[1, 3, 4]thiadiazolo[3,2-a]pyrimidin-7-one, 6-(2,5-dimethoxybenzylidene)-5-imi...	37.056	Nf	C15H14N4O3S	1
45.	Hinokiol \$\$ (+)-Hinokiol	37.242	Hinokiol	C20H30O2	4
46.	7,8,9-Trimethoxy-4,5-dihydro-1H-benzo[g]indazole	39.446	Nf	C14H16N2O3	1
47.	13b-methyl-3-propyl-1,3,4,6,7,8,13,13b-octahydro-2 h-pyrido [1',2':1,2]azepino...	40.14	Nf	C20H28N2	10
48.	7,7-Dimethyl-6,7-dihydro-5H-benzo[f]pyrano[2,3-h]quinoxaline	40.607	Nf	C17H16N2O	2
49.	3-[(3,4-dimethoxy-benzylamino)-methyl]-8a-methyl-5-methylene-decahydro-napht...	40.934	Nf	C24H33NO4	1
50.	1-Hexadecanol (CAS) \$\$ Cetal \$\$ Ethal \$\$ Ethol \$\$ Cetanol \$\$ Cetylol \$\$ Adol 52	43.494	Cetylol	C16H34O	10
51.	dl.alpha.-Tocopherol \$\$ (+/-)-.alpha.-Tocopherol \$\$ Vitamin E	45.326	Vitamin E	C29H50O2	7
52.	1,3-Diethyladamantane	49.926	Nf	C14H24	2
53.	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)- \$\$ Stigmasterol acetate	50.163	Stigmasterol acetate	C31H50O2	6
54.	Viminalol \$\$ Urs-12-en-3-ol, (3.beta.)- (CAS) \$\$.alpha.-Amyrin \$\$ ALPHA-AMYRIN	50.299	Viminalol	C30H50O	10
55.	4,6-Dimethoxy-2,3-diphenyl-7-(1-pyrrolin-2-yl)indole	51.53	Nf	C26H24N2O2	3
56.	Stigmast-4-en-3-one \$\$ 4-Stigmasten-3-one \$\$ Sitostenone	51.622	Sitostenone	C29H48O	1
57.	4-Hydroxy-1,2-dimethoxyanthraquinone	62.475	Nf	C16H12O5	3

considered as MIC of the plant extract against the relevant strains of the microorganisms.

Anthelmintic investigations

For the anthelmintic activity of *I. rugosus*, adult earthworms (*Pherethima posthuma*) and roundworms (*Ascaridia galli*) were used. Earthworms having average length of 7–8 cm and width 0.1–0.2 cm were collected from the muddy soil near Department of Pharmacy, University of Malakand, KPK, Pakistan. The selection of earthworms (*P. posthuma*)

is based on close physiologic and anatomic resemblance with the human intestinal round worm parasites *Ascaris lumbricoides* [35]. Roundworms were collected from the intestines of chicken. The chickens were slaughtered, their intestines were dissected after the removal of fecal material with the help of normal saline and roundworms were collected from their intestines. These roundworms (*A. galli*) have close resemblance with that of the roundworm parasite (*Ascaris lumbricoides*) found in the human intestine. Both types of worms were divided into different groups,

each group containing six (6) worms. All the extracts, their fractions, crude saponins and albendazole solutions were prepared in 10, 20 and 40 mg/ml. From each solution, 25 ml of the sample was transferred to sterilized Petri dishes (150 × 15 mm) followed by addition of earthworms using forceps. Observations were made for the time taken when the worms lost their motility as paralysis time and death was concluded when no movement was observed even with vigorous shaking in hot water at 50 °C. Both paralysis and death times were recorded for different fractions and were compared with the positive control [36].

Insecticidal investigations

Anti-beetle

The anti-beetle activity was carried out against *R. dominica* (grain borer) and *T. castaneum* (flour beetle) following the procedure previously described [37]. Various dilutions of extracts were prepared in methanol, i.e., 200, 100 and 50 mg/ml and filter paper was wetted with the 5 ml of each test sample solution in Petri dishes and kept overnight to evaporate the solvent. After evaporation of the solvents, 20 healthy insects were shifted to each Petri dish and kept for 24 h at room temperature with 50% of relative humidity. After 24 h, the results were checked by counting the number of dead and alive insects. Permethrin was used as positive control while the volatile solvents were used as negative control.

Anti-termite investigations

The anti-termite activity of crude saponins, methanolic extracts and resultant fractions of *I. rugosus* was conducted following standard procedure [38]. In this procedure the plant's samples were assayed against *Heterotermes indicola* (termites). Sterilized filter papers were kept in Petri dishes equal to the size of the Petri dishes. Samples were prepared by dissolving 2 mg/ml of each extract of the plant in respective solvents. The filter papers were kept for 24 h so that the solvents become fully evaporated from it. Each filter paper was kept in the labeled Petri dishes. In each Petri

dish, 25 termites were placed and were kept for 24 h at room temperature. After 24 h, the number of dead and alive termites was counted. Similarly, the results were also recorded on the second and third day. This activity was performed in triplicate and the average termites killed each day were recorded. Permethrin was used as positive control. The LD₅₀ of each sample was calculated by graph prism pad software and Microsoft Excel.

Anti-Pharaoh (ants) investigations

The anti-Pharaoh activity was performed by the direct contact toxicity method described by Ahn et al. [37]. From each fraction, solutions of 0.5, 1, 2, 4, 6 and 8 mg/ml concentrations were prepared. Each concentration was added into Petri dish containing sterilized filter paper and kept for 12–15 h for evaporation of the solvent. After evaporation, 20 Pharaoh ants were added into each Petri dish. The Petri dishes containing distilled water treated filter paper served as a control. The Petri dishes were placed at room temperature for 24 h and numbers of dead and alive ants were observed in each Petri dish. Permethrin was used as positive control. The percent mortality of each sample was calculated and was compared with the positive control.

Statistical analysis

Each experiment was performed in three replicates and values were expressed as mean ± SEM. two way ANOVA followed by multiple comparison Bonferroni's test. The *P* values less than 0.05 were considered as statistically significant. LD₅₀ values were calculated by a linear regression analysis among the percent inhibition against the extract concentrations via Excel program and Graph-Pad Prism software.

Results

Gas chromatography mass spectroscopy

The GC-MS analysis of ethyl acetate fraction revealed the identifications of 57 compounds which have been

Table 2 Various parameters major compounds in GC-MS of ethyl acetate fraction of *Isodon rugosus*

S.No	RT	Height	Height %	Area	Area %	Area Sum %	Base Peak m/z	Width
1.	3.857	54,336	6.81	66,940	1.91	0.65	55.1	0.05
2.	3.938	57,317	7.19	70,955	2.03	0.69	43.1	0.054
3.	7.029	40,590	5.09	80,194	2.29	0.78	115	0.07
4.	7.367	581,648	72.95	1E + 06	29.17	9.92	82	0.107
5.	8.15	74,641	9.36	122,166	3.49	1.19	101	0.054
6.	9.698	44,503	5.58	70,074	2	0.68	70.1	0.06
7.	11.73	62,937	7.89	90,543	2.59	0.88	93	0.05
8.	21.386	542,089	67.99	2E + 06	66.56	22.64	88	0.171
9.	27.829	797,352	100	3E + 06	100	34.02	79	0.178
10.	29.319	754,398	94.61	3E + 06	78.36	26.66	68.1	0.137

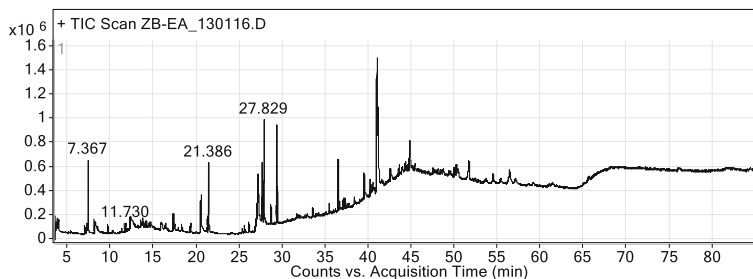


Fig. 1 Chromatogram of Ethyl acetate fraction of *Isodon rugosus*

enlisted in Table 1. Various parameters of major compounds in this fractions i.e., retention time, percent area, base peak, width etc. has also been summarized in Table 2. The GC-MS chromatogram of ethyl acetate fraction (Fig. 1) showed the peak areas for various compounds including the bioactive compounds. The structures of the bioactive compounds are shown in Fig. 2. The integration pattern of dominant compounds determined by GC-MS analysis is provided in Fig. 3.

Antibacterial studies

Sensitivity pattern

The bacterial strains were exposed to different antibiotic groups to determine their susceptibility pattern as shown in Table 3. All strains were found resistant to more than three antibiotics and therefore were termed as multi drug resistant (MDR) strains.

Zone of inhibitions

Results of antibacterial activity (ZOI) are shown in Table 4. Among all the tested samples, flavonoids were most active against all the strains and its antibacterial potential was comparable to the standard drug ceftriaxone. Among other fractions, ethyl acetate was most effective against *S. aureus* and *S. typhi* displaying inhibitory zones of 29.3 ± 0.28 and 28.2 ± 0.35 mm respectively. Crude saponins were also effective against *S. aureus*, *E. coli*, *B. cereus* and *K. pneumoniae* causing inhibitory zones of 27.2 ± 0.23 , 24.1 ± 0.52 , 26.3 ± 0.34 and 21.3 ± 0.39 mm respectively. The crude methanolic extract and chloroform fraction were inactive against *B. cereus*, whereas the aqueous fraction was inactive against *E. coli* and *E. faecalis*. *n*-Hexane fraction was also inactive against *E. coli*. All the test samples were approximately least active against *E. coli*.

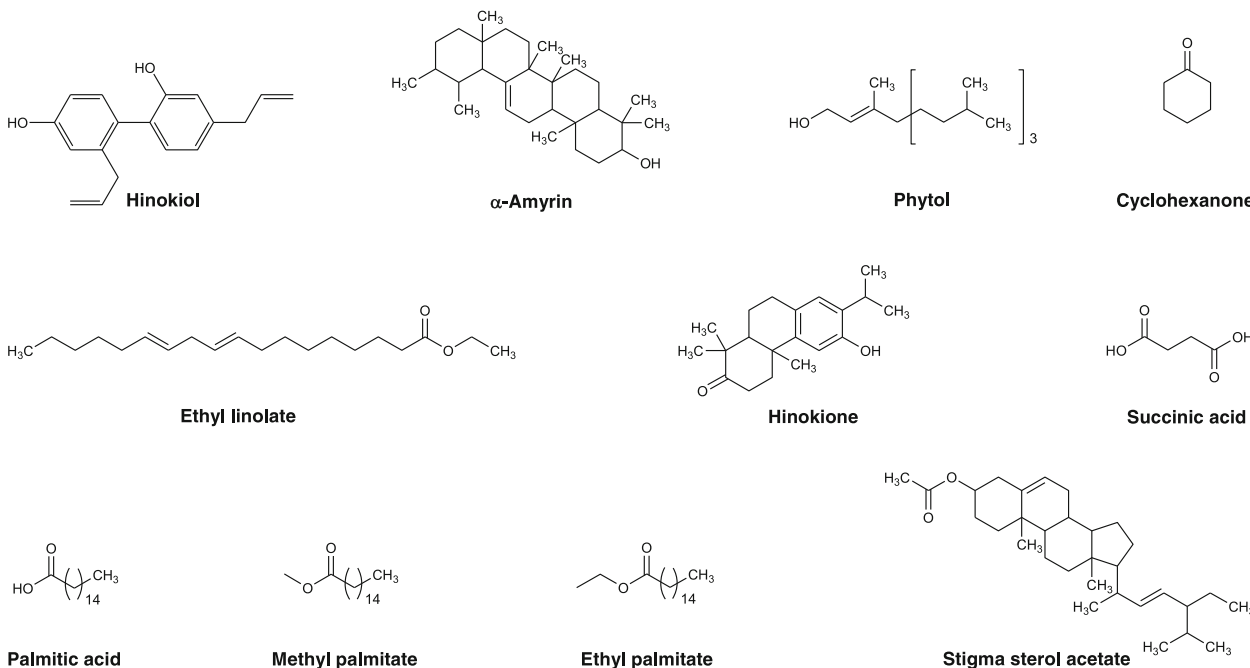


Fig. 2 Bioactive compounds identified in the ethyl acetate fraction of *Isodon rugosus*

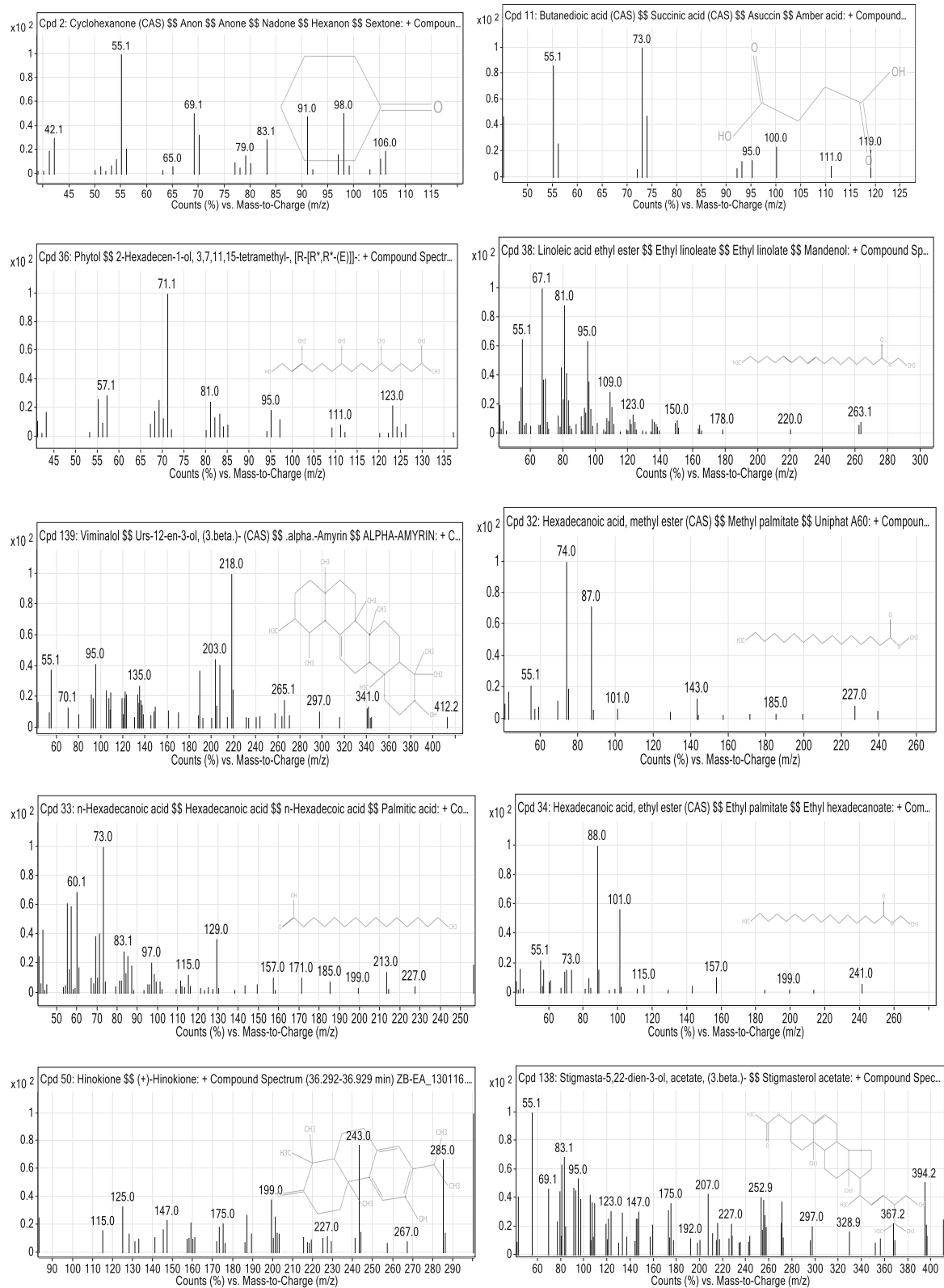


Fig. 3 Mass spectra of bioactive compounds in ethyl acetate fraction of *Isodon rugosus*

Table 3 Antibiotic Susceptibility pattern of selected bacterial strains to different antibiotic groups

Bacterial strains	Diameter of the inhibitory zone (mm) Mean \pm SEM (n = 5)					
	Ciprofloxacin	Cefixime	Amoxicillin	Gentamycin	Cephadrine	Cloxacillin
<i>Escherichia coli</i> (739)	24.67 \pm 1.20	18.33 \pm 0.88	07.67 \pm 0.67	26.67 \pm 0.67	06.67 \pm 0.88	09.33 \pm 0.33
<i>Klebsiella pneumoniae</i> (700603)	19.00 \pm 0.57	06.67 \pm 0.67	08.00 \pm 0.57	22.00 \pm 0.57	08.67 \pm 0.88	06.67 \pm 1.20
<i>Pseudomonas aeruginosa</i> (27853)	22.33 \pm 0.88	16.67 \pm 1.20	07.67 \pm 1.20	20.67 \pm 1.20	10.33 \pm 1.45	08.33 \pm 0.33
<i>Enterococcus faecalis</i> (29212)	16.33 \pm 0.33	12.33 \pm 0.88	09.33 \pm 0.88	26.67 \pm 0.67	14.00 \pm 1.15	06.67 \pm 0.67
<i>Proteus mirabilis</i> (13315)	18.67 \pm 1.20	06.67 \pm 0.33	10.67 \pm 0.33	27.33 \pm 0.88	10.00 \pm 0.57	12.33 \pm 0.88
<i>Staphylococcus aureus</i> (29213)	26.67 \pm 0.67	08.33 \pm 0.33	14.00 \pm 0.57	29.00 \pm 0.57	12.33 \pm 0.88	08.00 \pm 0.57
<i>Bacillus cereus</i>	23.00 \pm 0.57	11.67 \pm 0.67	11.33 \pm 0.88	26.00 \pm 1.15	12.67 \pm 0.67	09.67 \pm 1.20
<i>Salmonella typhi</i>	23.33 \pm 0.33	26.67 \pm 1.20	7.67 \pm 1.20	24.33 \pm 0.33	10.00 \pm 1.15	07.00 \pm 1.15

Minimum inhibitory concentration (MICs)

Among all the tested samples, crude methanolic extract was found most effective against *S. aureus* and *B. cereus* with minimum inhibitory concentrations (MICs) of 2.50 ± 0.00 and 2.66 ± 1.30 $\mu\text{g/ml}$. MIC of *n*-hexane fraction against *B. cereus* was 1.33 ± 0.60 $\mu\text{g/ml}$ which was comparable to positive control ceftriaxone displaying MIC of 0.83 ± 0.16 $\mu\text{g/ml}$. The chloroform fraction was most effective against *K. pneumonia* and *E. coli*, whose visible growth was inhibited at 1.16 ± 0.66 and 1.00 ± 0.00 $\mu\text{g/ml}$ respectively. Saponins fraction was most potent against *P. mirabilis*, *S. aureus* and *K. pneumonia* with MICs of 1.00 ± 0.00 , 0.83 ± 0.16 and 1.00 ± 0.00 $\mu\text{g/ml}$ respectively. Flavonoids were also found equally effective against the tested microbes. All the results of minimum inhibitory concentrations (MICs) are summarized in Table 5.

Anthelmintic activity

All the tested fractions were effective in concentration dependent manner as shown in Table 6. The saponins fraction was observed to be most effective against *Pheretima posthuma* with an average death time of 44.00 ± 2.00 , 33.67 ± 3.05 and 27.67 ± 1.53 min at

concentrations of 10, 20, 40 mg/ml respectively. The anthelmintic activity observed for saponins and chloroform fractions were comparable to the positive control albendazole. The order of anthelmintic activity for various fractions of the plant against *Pheretima posthuma* was in an ascending order of saponins > chloroform > ethyl acetate > *n*-hexane > methanolic extract > aqueous fraction. Moreover, crude saponins exhibited a significant result against *Ascaridia galli* with an average death time of 29.22 ± 0.61 min at 40 mg/ml concentration. Whereas, average death time for the standard drug albendazole was 33.6 ± 1.76 at the same tested concentration.

Insecticidal activity

In finding out the insecticidal potentials of different fractions, saponins caused mortality to a significant level, i.e. 70.67 ± 1.20 and $76.65 \pm 1.65\%$ mortality against *R. dominica* and *T. castaneum* respectively at a concentration of 200 mg/ml, while at a concentration of 50 mg/ml saponins exhibited 61.66 ± 1.20 and $66.67 \pm 0.67\%$ mortality against *R. dominica* and *T. castaneum* respectively. Among other fractions, chloroform was more effective against *R. dominica* and *T. castaneum* showing

Table 4 Antibacterial activity of *Isodon rugosus* against various bacterial strains expressed in mm as ZOI

Samples	<i>P. m</i>	<i>S. a</i>	<i>E. c</i>	<i>B. c</i>	<i>S. t</i>	<i>K. p</i>	<i>P. a</i>	<i>E. f</i>
Ir.Cr	17.2 \pm 0.37	9.1 \pm 0.34	7.2 \pm 0.55	—	28.2 \pm 0.40	18.2 \pm 0.35	9.3 \pm 0.31	21.3 \pm 0.36
Ir.Hex	07.3 \pm 0.34	9.1 \pm 0.52	—	7.1 \pm 0.49	18.2 \pm 0.37	17.2 \pm 0.37	8.3 \pm 0.25	23.3 \pm 0.36
Ir.Cf	11.2 \pm 0.32	19.2 \pm 0.47	7.1 \pm 0.26	—	6.1 \pm 0.46	15.3 \pm 0.35	9.2 \pm 0.31	19.3 \pm 0.39
Ir.EtAc	9.2 \pm 0.40	29.3 \pm 0.28	21.2 \pm 0.40	17.1 \pm 0.46	28.2 \pm 0.35	18.2 \pm 0.40	7.1 \pm 0.46	8.2 \pm 0.37
Ir.Aq	21.2 \pm 0.31	9.2 \pm 0.31	—	13.2 \pm 0.20	9.2 \pm 0.40	9.2 \pm 0.40	17.2 \pm 0.37	—
Ir.Sp	18.2 \pm 0.43	27.2 \pm 0.23	24.1 \pm 0.52	26.3 \pm 0.34	8.2 \pm 0.37	21.3 \pm 0.39	9.2 \pm 0.40	19.3 \pm 0.38
Ir.FlV	33.1 \pm 0.49	16.2 \pm 0.54	12.2 \pm 0.54	31.3 \pm 0.31	16.2 \pm 0.23	27.3 \pm 0.35	19.2 \pm 0.38	13.2 \pm 0.45
Ceft	35.2 \pm 0.37	31.1 \pm 0.34	9.2 \pm 0.44	29.3 \pm 0.42	19.2 \pm 0.37	25.4 \pm 0.40	36.1 \pm 0.34	21.3 \pm 0.41

Results are expressed as zone of inhibition (ZOI). Data is represented as mean \pm SEM, (n = 3).

Keys:

P. m *Proteus mirabilis*, *S. a* *Streptococcus aureus*, *E. c* *Escherichia coli*, *B. c* *Bacillus cereus*, *S. t* *Salmonella typhi*, *K. p* *Klebsiella pneumonia*, *P. a* *Pseudomonas aeruginosa*, *E. f* *Enterococcus faecalis*, *Ir.Cr* Crude methanolic extract, *Ir.Hex* *n*-hexane fraction, *Ir.Cf* Chloroform fraction, *Ir.EtAc* Ethyl acetate fraction, *Ir.Aq* Aqueous fraction, *Ir.Sp* Saponins, *Ir.FlV* Flavonoids, *Ceft* Ceftriaxone

Table 5 Minimum inhibitory concentrations for different fractions of *Isodon rugosus* against the tested bacterial strains expressed as $\mu\text{g/ml}$

Samples	<i>P. m</i>	<i>S. a</i>	<i>E. c</i>	<i>B. c</i>	<i>S. t</i>	<i>K. p</i>	<i>P. a</i>
Ir.Cr	6.66 \pm 0.83	2.50 \pm 0.00	8.33 \pm 0.83	2.66 \pm 1.30	12.50 \pm 2.88	8.33 \pm 0.83	4.16 \pm 1.66
Ir.Hex	18.33 \pm 0.83	16.66 \pm 0.83	2.83 \pm 1.16	1.33 \pm 0.60	8.33 \pm 2.20	10.83 \pm 2.20	12.50 \pm 1.44
Ir.Cf	2.50 \pm 0.00	3.66 \pm 1.96	1.00 \pm 0.00	5.33 \pm 2.60	2.01 \pm 0.50	1.16 \pm 0.66	2.50 \pm 0.00
Ir.EtAc	7.50 \pm 1.44	7.50 \pm 1.44	4.16 \pm 0.83	2.83 \pm 1.16	3.33 \pm 0.83	1.33 \pm 0.60	2.83 \pm 1.16
Ir.Aq	9.16 \pm 0.83	11.66 \pm 0.83	10.00 \pm 1.44	10.83 \pm 2.20	7.50 \pm 1.44	5.00 \pm 1.44	5.00 \pm 1.44
Ir.Sp	1.00 \pm 0.00	0.83 \pm 0.16	5.00 \pm 1.44	2.50 \pm 0.00	4.50 \pm 1.89	1.00 \pm 0.00	3.33 \pm 0.83
Ir.FlV	2.83 \pm 1.16	1.00 \pm 0.00	1.50 \pm 0.50	6.66 \pm 2.20	2.00 \pm 0.50	2.66 \pm 1.30	5.00 \pm 1.44
Ceftriaxone	0.50 \pm 0.00	0.66 \pm 0.16	2.50 \pm 0.00	0.83 \pm 0.16	0.66 \pm 0.16	0.50 \pm 0.00	1.33 \pm 0.60

Data is represented as mean \pm SEM, (n = 3)

Keys:

P. m *Protius mirabilis*, *S. a* *Streptococcus aureus*, *E. c* *Escherichia coli*, *B. c* *Bacillus cereus*, *S. t* *Salmonella typhi*, *K. p* *Klebsiella pneumonia*, *P. a* *Pseudomonas aeruginosa*, *E. f* *Enterococcus faecalis*, *Ir.Cr* Crude methanolic extract, *Ir.Hex* n-hexane fraction, *Ir.Cf* Chloroform fraction, *Ir.EtAc* Ethyl acetate fraction, *Ir.Aq* Aqueous fraction, *Ir.Sp* Saponins, *Ir.Fl* Flavonoids

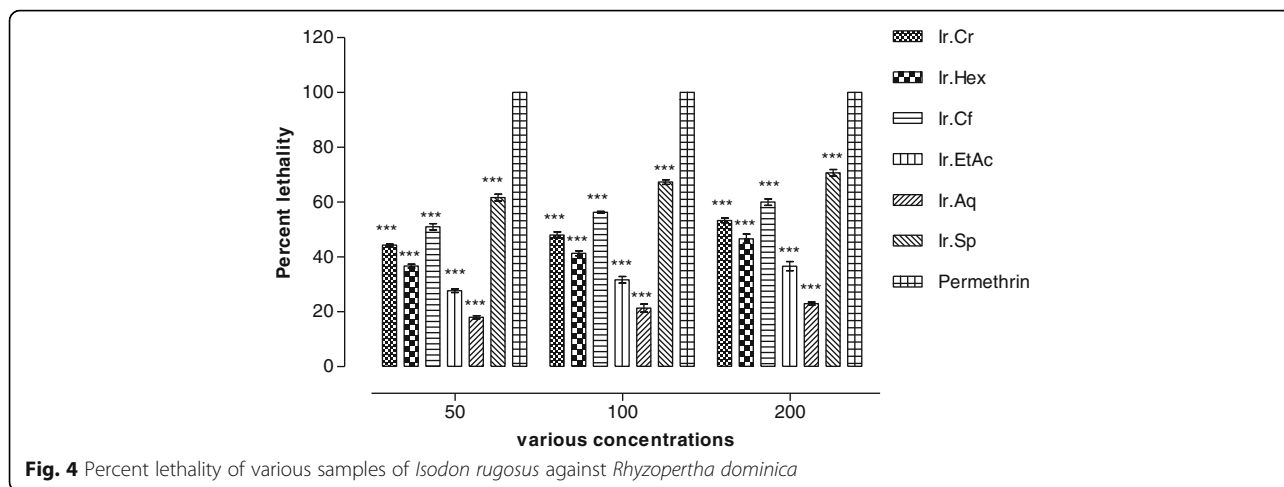
Table 6 Anthelmintic activity of aerial part of *Isodon rugosus* against *Pheretima posthuma* and *Ascaridia galli*

Samples	Concentration (mg/ml)	<i>Pheretima posthuma</i>		<i>Ascaridia galli</i>	
		Paralysis time in Minutes (mean \pm SD)	Death time in Minutes (mean \pm SD)	Paralysis time in Minutes (mean \pm SD)	Death time in Minutes (mean \pm SD)
Ir.Cr	10	24.33 \pm 1.15***	67.00 \pm 2.00***	20.25 \pm 0.57***	69.39 \pm 0.60***
	20	18.67 \pm 1.53**	60.00 \pm 2.00***	17.01 \pm 0.46***	61.18 \pm 0.67***
	40	11.33 \pm 1.56 ^{ns}	40.67 \pm 1.53***	13.48 \pm 0.52***	53.19 \pm 0.36***
Ir.Hex	10	23.33 \pm 2.08***	67.33 \pm 2.08***	28.89 \pm 0.52***	87.34 \pm 0.78***
	20	17.00 \pm 1.00*	62.33 \pm 1.53***	17.32 \pm 0.96***	71.36 \pm 0.56***
	40	11.67 \pm 1.53 ^{ns}	49.00 \pm 1.00***	3.43 \pm 0.67**	56.63 \pm 0.38***
Ir.Cf	10	14.00 \pm 1.00 ^{ns}	50.00 \pm 1.00 ^{ns}	14.53 \pm 0.67 ^{ns}	53.01 \pm 0.44***
	20	10.00 \pm 2.00 ^{ns}	37.67 \pm 1.53 ^{ns}	11.95 \pm 0.34 ^{ns}	41.77 \pm 0.40 ^{ns}
	40	7.33 \pm 1.53 ^{ns}	30.67 \pm 2.52 ^{ns}	9.85 \pm 0.50 ^{ns}	34.39 \pm 0.55 ^{ns}
Ir.EtAc	10	18.00 \pm 2.00 ^{ns}	52.00 \pm 2.00 ^{ns}	18.13 \pm 0.49***	58.37 \pm 1.16***
	20	13.00 \pm 2.00 ^{ns}	45.00 \pm 2.00*	13.63 \pm 0.40**	49.31 \pm 0.82***
	40	10.00 \pm 2.00 ^{ns}	38.67 \pm 1.53**	10.29 \pm 0.56*	42.25 \pm 0.89***
Ir.Aq	10	21.00 \pm 2.00**	61.00 \pm 2.00***	19.66 \pm 0.88***	68.54 \pm 0.81***
	20	19.00 \pm 2.00**	56.00 \pm 2.00***	15.4 \pm 0.61***	57.15 \pm 0.85***
	40	15.00 \pm 2.00**	47.67 \pm 1.53***	13.40 \pm 0.69***	52.85 \pm 0.50***
Ir.Sp	10	13.33 \pm 1.53 ^{ns}	44.00 \pm 2.00 ^{ns}	8.40 \pm 0.9***	43.00 \pm 1.00**
	20	9.00 \pm 2.00 ^{ns}	33.67 \pm 3.05 ^{ns}	8.33 \pm 0.58 ^{ns}	32.79 \pm 0.54***
	40	5.67 \pm 1.53 ^{ns}	27.67 \pm 1.53 ^{ns}	4.33 \pm 0.58 ^{ns}	29.22 \pm 0.61**
Albendazole	10	12.33 \pm 2.08	48.67 \pm 1.53	13.10 \pm 1.85	47.40 \pm 1.50
	20	10.33 \pm 2.57	37.33 \pm 1.53	9.80 \pm 1.60	40.20 \pm 2.25
	40	7.00 \pm 2.00	28.67 \pm 1.53	7.10 \pm 1.01	33.60 \pm 1.76
Negative control	-----	-----	-----	-----	-----

Values significantly different (*: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$) as compared to positive control drug. ns: Non significantly different in comparison to control group

Keys:

Ir.Cr Crude methanolic extract, *Ir.Hex* n-hexane fraction, *Ir.Cf* Chloroform fraction, *Ir.EtAc* Ethyl acetate fraction, *Ir.Aq* Aqueous fraction, *Ir.Sp* Saponins.



60.00 ± 1.15 and 70.33 ± 0.88% mortality against *R. dominica* and *T. castaneum* respectively at concentration of 200 mg/ml, while showed 51.00 ± 1.15 and 59.00 ± 0.57% mortality against *R. dominica* and *T. castaneum* respectively at a concentration of 50 mg/ml. The methanolic extract and *n*-hexane fraction also showed moderate insecticidal effects against both the insects as shown in Figs. 4 and 5.

Anti-termite activity

The anti-termite activity of various samples of *I. rugosus* showed prominent lethality against termites. The highest activity was attributed to saponins, killing almost all the termites on the first day as shown in Table 7. The second highest activity was shown by ethyl acetate fraction which demonstrated 93.00 ± 0.57% lethality on the first day and 100% lethality on the second day. Other fractions including chloroform, *n*-hexane and aqueous fractions showed moderate activity causing 77.32 ± 1.32,

50.33 ± 0.88 and 48.33 ± 0.88% lethality respectively on the first day. On the third day almost all the insects were killed by all the plant’s samples.

Anti-Pharaoh (ants) activity

The determination of anti-Pharaoh ants potentials of *I. rugosus* reveal that among all the plant’s samples, saponins showed 100% activity against Pharaoh at all the tested concentrations except at 0.5 mg/ml which was 96.33 ± 0.88%. The second highest activity can be attributed to the ethyl acetate fractions of *I. rugosus* which displayed 72.00 ± 1.15, 77.67 ± 0.67, 80.33 ± 0.33, 86.33 ± 0.88, 90.67 ± 0.67 and 100.0 ± 0.57% lethality at concentrations of 0.5, 1, 2, 4, 6 and 8 mg/ml respectively. The LD₅₀ shown by saponins, ethyl acetate and chloroform fractions were smaller than 0.1 mg/ml, whereas LD₅₀ for methanolic extract, *n*-hexane and aqueous fractions were 0.85, 1.13 and 3.37 mg/ml respectively as shown in Table 8.

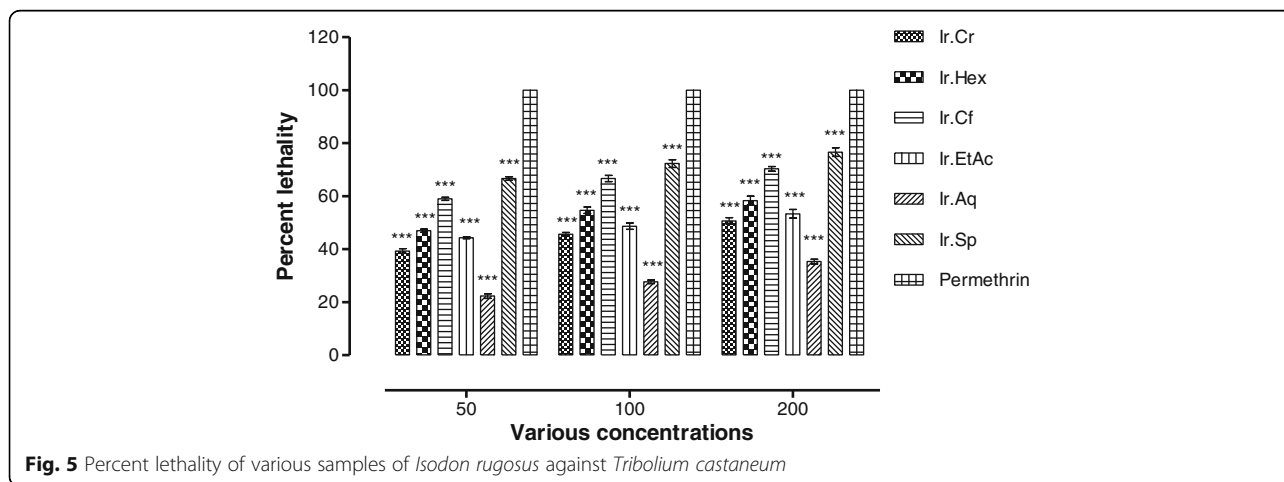


Table 7 Anti-termite effect of various samples of *Isodon rugosus*

Samples	Number of Termites treated	Days	Percent lethality (mean \pm SEM)
Ir.Cr	25	1	45.00 \pm 1.15 ^{***}
		2	77.32 \pm 1.32 ^{***}
		3	100.0 \pm 0.00 ^{ns}
Ir.Hex	25	1	50.33 \pm 0.88 ^{***}
		2	69.32 \pm 1.32 ^{***}
		3	100.0 \pm 0.00 ^{ns}
Ir.Cf	25	1	77.32 \pm 1.32 ^{***}
		2	96.67 \pm 1.20 ^{ns}
		3	100.0 \pm 0.00 ^{ns}
Ir.EtAc	25	1	93.00 \pm 0.57 ^{ns}
		2	100.0 \pm 0.00 ^{ns}
		3	100.0 \pm 0.00 ^{ns}
Ir.Aq	25	1	48.33 \pm 0.88 ^{***}
		2	74.00 \pm 0.57 ^{***}
		3	100.0 \pm 0.00 ^{ns}
Ir.Sp	25	1	100.0 \pm 0.00 ^{ns}
		2	100.0 \pm 0.00 ^{ns}
		3	100.0 \pm 0.00 ^{ns}
Permethrin	25	1 2 3	

100.0 \pm 0.00100.0 \pm 0.00100.0 \pm 0.00Data is represented as mean \pm SEM; (n = 3). Values significantly different

(***) P < 0.001) as compared to the control drug

Keys:

Ir.Cr Crude methanolic extract, Ir.Hex n-hexane fraction, Ir.Cf Chloroform fraction, Ir.EtAc Ethyl acetate fraction, Ir.Aq Aqueous fraction, Ir.Sp Saponins

Discussion

The origin and nature of drug used against specific diseases make a sound difference in the therapy and prognosis. The efficacy and safety of synthetic and natural drugs have been investigated and compared by multiple reporters [39, 40]. Similarly, the use of drugs derived from natural sources have been found comparatively safe and have been preferred against synthetic drugs [41]. Several groups of natural compounds have been reported to possess variety of potentials, for examples, flavonoids have been shown to possess antimicrobial activity that is also obvious in our current study [42]. Similarly, parasitic worms causing infections of human beings and animals are well-known to have negative health consequences and a significant reduction in body ability to resist other diseases. To find compounds with anthelmintic activity, a variety of substances have been investigated using different species of worms like earthworms, ascaris, heterakis and nippostrongylus [42, 43]. Among all these species, earthworms have been used generally for the preliminary evaluation of anthelmintic compounds in-vitro due to physiological similarity with intestinal worms, their reaction to anthelmintics and are

easily accessible [44]. It has been established that all those anthelmintics which are toxic to earthworms are regarded as anthelmintic agents. To minimize losses caused by helminths infections, commercial anthelmintics have been used for decades. However, the threats of anthelmintic resistance, poor availability, risk of residue and high cost especially to low income people in developing countries have led to the need of other alternative control methods [45]. Screening and proper evaluation of the claimed medicinal plants could offer the possible alternatives that may both be sustainable and environmentally acceptable instead of those having toxic effect [46].

Results of the current study reveal that saponins were most effective against *P. posthuma* exhibiting an average death time of 27.67 \pm 1.53 min which was comparable with the standard drug albendazole with average death time of 28.67 \pm 1.53 at the same concentration (40 mg/ml). Furthermore, crude saponins were most effective against *Ascaridia galli* with average death time of 29.22 \pm 0.61 min at 40 mg/ml. The average death time for standard drug albendazole was 33.6 \pm 1.76 at the same concentration. Researchers have proven that saponins possess excellent anthelmintic potential which has also been depicted in our investigations [36].

In the current study, we observed that saponins were also active against insects in a similar way reported earlier [47]. Moreover, the saponins have also been reported to possess anthelmintic, antitussive, anticancer, fungicidal and antiviral properties [48–51]. Actually the saponins help the plants to protect against pests and parasites [49, 52–55]. Saponins may cause inhibition of food uptake in the insects' gut and interrupt in their digestion which lead to stop their growth [47]. They are considered as first line choice for the proper eradication of insects due to their protease inhibitory properties and interaction with cholesterol by disturbing the synthesis of moulting hormones [56]. The saponins isolated from various plants have been reported to possess toxicity profile and have been demonstrated with prominent haemolytic potential. The haemolytic potential and toxicity profile of the saponins make it inevitable that the saponins of each and every plant should be subjected for the evaluation of safety profile in regards to the human body.

The GC-MS analysis showed 57 compounds in the ethyl acetate fraction of *I. rugosus*. The bioactive compounds identified were palmitic acid, hinokiol, α -amyrin, phytol, ethyl linolate, cyclohexanone, hinokione, methyl palmitate, ethyl palmitate and stigma sterol acetate. The literature survey of the bioactive compounds reveals the importance of *I. rugosus* regarding various biological activities. The compounds identified in this plant have been reported previously with significant biological activities. Cyclohexanone possess strong antibacterial

Table 8 Anti-Pharaoh ant effect of various samples of *Isodon rugosus*

Samples	Dose (mg/ml)	Total treated	No. repeated	Mortality(%) mean \pm SEM	LD ₅₀ (mg/ml)
Ir.Cr	0.5	20	3	41.33 \pm 0.88 ^{***}	0.85
	1	20		52.00 \pm 0.57 ^{***}	
	2	20		57.67 \pm 0.67 ^{***}	
	4	20		58.00 \pm 0.57 ^{***}	
	6	20		70.00 \pm 0.00 ^{***}	
	8	20		81.33 \pm 0.88 ^{***}	
Ir.Hex	0.5	20	3	39.67 \pm 1.20 ^{***}	1.13
	1	20		49.00 \pm 1.15 ^{***}	
	2	20		53.00 \pm 0.57 ^{***}	
	4	20		56.67 \pm 0.67 ^{***}	
	6	20		58.00 \pm 0.57 ^{***}	
	8	20		80.67 \pm 1.20 ^{***}	
Ir.Cf	0.5	20	3	67.33 \pm 1.45 ^{***}	<0.1
	1	20		68.67 \pm 0.67 ^{***}	
	2	20		73.33 \pm 0.88 ^{***}	
	4	20		76.00 \pm 1.15 ^{***}	
	6	20		85.00 \pm 0.57 ^{**}	
	8	20		95.33 \pm 0.88 ^{ns}	
Ir.EtAc	0.5	20	3	72.00 \pm 1.15 ^{***}	<0.1
	1	20		77.67 \pm 0.67 ^{***}	
	2	20		80.33 \pm 0.33 ^{**}	
	4	20		86.33 \pm 0.88 [*]	
	6	20		90.67 \pm 0.67 ^{ns}	
	8	20		100.0 \pm 0.57 ^{ns}	
Ir.Aq	0.5	20	3	29.00 \pm 1.15 ^{***}	3.37
	1	20		41.33 \pm 0.88 ^{***}	
	2	20		44.67 \pm 1.20 ^{***}	
	4	20		53.33 \pm 0.33 ^{***}	
	6	20		66.00 \pm 0.57 ^{***}	
	8	20		80.67 \pm 1.76 ^{***}	
Ir.Sp	0.5	20	3	96.33 \pm 0.88 ^{ns}	<0.1
	1	20		100.0 \pm 0.00 ^{ns}	
	2	20		100.0 \pm 0.00 ^{ns}	
	4	20		100.0 \pm 0.00 ^{ns}	
	6	20		100.0 \pm 0.00 ^{ns}	
	8	20		100.0 \pm 0.00 ^{ns}	
Permethrin	0.5	20	3	93.33 \pm 4.40	<0.1
	1	20		98.33 \pm 1.66	
	2	20		100.0 \pm 0.00	
	4	20		100.0 \pm 0.00	
	6	20		100.0 \pm 0.00	
	8	20		100.0 \pm 0.00	
Distilled Water	Negative control	20	3	0.00	0.00

Data is represented as mean \pm SEM; (n = 3). Values significantly different (*: $P < 0.05$, **: $P < 0.01$ and *** $P < 0.001$) as compared to the control drug

Keys:

Ir.Cr Crude methanolic extract, *Ir.Hex* n-hexane fraction, *Ir.Cf* Chloroform fraction, *Ir.EtAc* Ethyl acetate fraction, *Ir.Aq* Aqueous fraction, *Ir.Sp* Saponins.

activity [57]. Red and white wine containing succinic acid have been reported with strong antibacterial activity [58]. The succinic acid derivatives have been reported to treat the infants urinary tract infection [59]. Similarly, the succinic acid have also been reported for antibacterial potential produced by yeast during fermentation [60]. Palmitic acid have been reported to possess antimicrobial potential [61, 62]. Phytol have been reported for its antibacterial [63], antimicrobial [64] and insecticidal potentials [62]. Hinokiol isolated from *Magnolia officinalis* have been used for antibacterial activity [65]. Ethyl linolate have been used for the treatment of acne vulgaris [66] and topically as antibacterial agent [67]. In the same way, stigmaterol acetate has also been used as antibacterial agent [68]. Likewise, alpha-amyrin possess strong antibacterial activity [69]. Cyclohexanone have been reported to used as a solvent and as a building block in the production of various organic compounds such as insecticidal [70]. Methyl palmitate have been reported to possess insecticidal activity [71]. Palmitic acid have been reported to possess strong anti mosquitoes activities isolated from *Acanthus montanus* [72]. Ethyl palmitate is larvicidal and insecticidal agent and has been used in insecticidal sprays [73]. Similarly, hinokione isolated from *Sabina vulgaris* have been reported to possess insecticidal potential [74]. Stigmaterol acetate has mosquito repellent property [75]. Alpha-Amyrin have been reported for insecticidal activity [69].

Conclusions

It can be concluded that *Isodon rugosus* possess strong antibacterial, insecticidal and anthelmintic potentials. The gas chromatography-mass spectroscopy analysis also elucidated a collection of biologically important compounds putatively known for their antibacterial, insecticidal and anthelmintic potentials. Based on the experimental analysis, it may also be deduced that *Isodon rugosus* is a good source of antibacterial, insecticidal and anthelmintic compounds. The overwhelming role of ethyl acetate fraction in various biological assays and the GC-MS analysis confirm that this fraction might be an appropriate target for the isolation of bioactive compounds.

Abbreviations

B. c: *Bacillus cereus*; E. c: *Escherichia coli*; E. f: *Enterococcus faecalis*; GC-MS: gas chromatography-mass spectrometry; Ir.Aq: aqueous fraction; Ir.Cf: chloroform fraction; Ir.Cr: Crude methanolic extract; Ir.EtAc: ethyl acetate fraction; Ir.Fl: flavonoids; Ir.Hex: n-hexane fraction; Ir.Sp: saponins; K. p: *Klebsiella pneumoniae*; LD₅₀: median lethal dose; MDR: multi drug resistant; MIC: minimum inhibitory concentration; NCCLS: National Committee for Clinical Laboratory Standards; P. a: *Pseudomonas aeruginosa*; P. m: *Protius mirabilis*; S. a: *Streptococcus aureus*; S. t: *Salmonella typhi*; SEM: standard error mean; ZOI: zone of inhibition

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Availability of data and materials

The data supporting our research results is present within the manuscript.

Authors' contributions

AZ and SA carried out experimental work, data collection and evaluation, literature search and manuscript preparation under the supervision of AS. MA, AS and FU refined the manuscript for publication. All authors read and approved the final manuscript for publication.

Competing interests

The authors declare that they have no competing interest.

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