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RESEARCH ARTICLE

Fagonia cretica: Identification of compounds in bioactive gradient high performance liquid chromatography fractions against multidrug resistant human gut pathogens

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ABSTRACT

Plants are alternative source of natural medicines due to secondary active metabolites. *Fagonia cretica* extracts and Gradient High-Pressure Liquid Chromatography fractionations were checked against multidrug-resistant gastrointestinal pathogens including, *Salmonella typhi*, *Escherichia coli* and *Shigella flexneri*. ESI-MS/MS analysis of bioactive HPLC fractions was performed to elucidate antibacterial compounds. *F. cretica* extracts exhibited potential antibacterial activity. Twenty-four (24) HPLC fractions were obtained from methanol, ethanol and aqueous extracts of *F. cretica*. Eighteen (18) fractions showed antibacterial activity, while no activity was observed by the remaining six (6) fractions. HPLC fractions, F1 (25^{g} ± 0.20 mm) and F2 (15^{f} ± 0.12 mm) of aqueous extract exhibited activity against multidrug resistant GI pathogens. Gallic acid, quinic acid, cyclo-l-leu-l-pro, vidalenolone, liquirtigenin, rosmarinic acid and cerebronic acid were identified in F1 fraction of aqueous extract, while succinic acid, cyclo (l-Leu-l-Pro) and liquirtigenin were identified in F2 fraction of aqueous extract through ESI-MS/MS analysis. *F. cretica* extracts and HPLC fractions showed potential activity against MDR GI pathogens. Vidalenolone, Cyclo-1-leu-1-pro and Cerebronic acid are first time reported in *F. cretica.* Further characterization of bioactive compounds from *F. cretica* may be helpful to elucidate antibacterial therapeutic molecules.

Keywords: *F. cretica*; HPLC; MDR; MS-analysis.

INTRODUCTION

The historical perspective of herbs used as drugs is thousand years of age (Butler, 2004). The use of herbal extracts and secondary metabolite in medicinal treatment are of more importance with antibacterial properties. Because of the presence of phytochemicals including terpenoids, tannins, alkaloids, steroids and flavonoids, plants have antibacterial potential (Nascimento *et al*., 2000).

Recent emergence of antibiotic resistance in human pathogenic bacteria diverge efforts to quest compounds in alternative sources like plants. Among these plants *F. cretica*, a member of the Zygophyllaceae is distributed in tropical and dried areas of the world and are used for therapeutic purpose ranging from topical infections to lethal diseases (Qureshi *et al*., 2016). *F. cretica,* powdered in water, is used for ulcers and skin injuries and even traditionally in-home medication to treat dehydration, fatigue, dysentery, allergies, liver and gastrointestinal problems (Baquar, 1989; Hussain *et al*., 2007).

The role of *F. cretica* extract in cell cycle arrest and cell death in breast cancer cells via tumor suppression genes expression (Lam *et al*., 2012). Several studies elaborately the antibacterial and antifungal potential, chemical constituents, pharmacological activities of *F. cretica* (Qureshi *et al*., 2016; Naz *et al*., 2021; Rehman *et al*., 2021).

In the undertaken study, the antibacterial activity of *F. cretica* HPLC fractions were checked against Multi drug resistant pathogens causing gastrointestinal infections in human. The bioactive fractions were processed for compounds identification using ESI-MS/MS analysis. Findings of the work will be helpful to further characterize the identified compounds for therapeutic use against GI infections.

MATERIALS AND METHODS

Collection and processing of F. cretica

F. cretica plant was collected from Takht Bhai, Mardan, Khyber Pakhtunkhwa, Pakistan and deposited with a voucher number (F.C 258), at the herbarium of Department of Botany, Abdul Wali Khan University Mardan. The *F. cretica*, plant grinding procedure was performed as reported previously (Wendakoon *et al*., 2012). Briefly

F. cretica was cleaned and dried in the shade before being sliced into small pieces and pulverized.

Extracts preparation of F. cretica

The powdered plant was extracted as previously described by (Abachi *et al*., 2013). Following crushing, the powdered were dissolved in various solvents with a proportion (1:10). About 100 grams powdered *F. cretica* was absorbed in one liter each of methanol, ethanol and refined water. To improve and speed up the soaking process the solutions were incubated for 72 hours at 32 °C on shaking. With the help of disinfected filter paper, the slurry was filtered and evaporated through a rotational evaporator at 44 °C.

Gradient HPLC fractionations of F. cretica

Plant extracts were processed by dissolving in 60% methanol for HPLC fractionation as described (Cock, 2008). Briefly, 20 mg/ml of crude extract was dissolved in 60% methanol and centrifuged at 4000 rpm for five minutes. The debris was discarded after centrifugation and the supernatant was filtered (0.45 µm). To stop column blockage, the HPLC column was washed with 60% methanol until the sample was subjected to HPLC fractionation. The HPLC fractionation was carried out at 250 nm under pressures ranging from 1820 to 1950 psi. The HPLC fractions were obtained in Eppendorf tubes.

Collection of multidrug-resistant (MDR) gastrointestinal bacterial pathogens

Three different MDR bacterial pathogens (*Escherichia coli*, *Salmonella typhi* and *Shigella flexneri*) identified biochemically, were collected from the Department of Microbiology, Kohat University of Science and Technology. The MDR profile of *Escherichia coli*, *Salmonella typhi* and *Shigella flexneri* is already published by our group (Khan *et al*., 2015; Nisa *et al*., 2022; Yaseen *et al*., 2022).

Antibacterial activity of crude extracts and HPLC fractions of F. cretica

F. cretica crude extract and HPLC fractions antibacterial activity were checked using the well diffusion method. The bacterial strains (*Escherichia coli*, *Salmonella typhi* and *Shigella flexneri*) were inoculated on Muller Hinton agar. 10 µl, 20 µland 30 µl of *F. cretica* crude extract were poured into separate wells of each plate. While checking the antibacterial activity of HPLC fractions of *F. cretica*, 50 µl volume was used. For the antibacterial activity of crude extract and HPLC fractions ethanol, methanol and water were used as solvent control and antibiotics discs (AZM, SAM, CIP, CRO) were used as antibiotic control. Bacterial plates were incubated for 24 hours at 37°C. The results were interpreted as described earlier (Valgas *et al*., 2007).

ESI-MS/MS analysis for bioactive compounds identification

The fractions that exhibited antibacterial activity were subjected to ESI-MS/MS (LTQ XL, Linear Ion Trap Mass spectrometer, Thermo Scientific, USA). An electron spray ionization (ESI) mode was used to identify bioactive compounds through the direct insertion method, both at positive and negative modes. The sample flow rate was set to 8 µl/min. The capillary temperature was maintained at 292°C and voltage 4.7 at kV. The mass range was set at 50 to 2000 m/z. During MS/MS collision mediated dissociation energy (CID) was maintained at 10 and 25, depending on the structure of the parent molecular ion. The proportion of methanol and acetonitrile was 80:20 (v/v) as a portable stage for the HPLC parts of *F. cretica*. The MS parameters for each compound were optimized to ensure the most positive ionization, particle move conditions, and accomplished the ideal sign of both the forerunner and section particles by imbuing the analytes and physically turning the boundaries. For the entire collection of analytes, the source boundaries were indistinguishable (Steinmann & Ganzera, 2011).

Data analysis

Experimental results were reported as standard error of the mean. ANOVA and Duncan test was incorporated for Group comparisons using *p*-value less than 0.05 was considered significant. The online database software (www.chemspider.com) was used for structure parameters of bioactive compounds.

RESULTS

Antibacterial activity of F. cretica extracts against MDR GI pathogens

The crude ethanolic, methanolic and aqueous extracts obtained from the whole *F. cretica* plant were tested against MDR pathogens (*E. coli*, *S. flexneri* and *S. typhi*) in different volumes (10, 20, 30 µl). All the crude extracts showed some inhibitory activity as listed in Table 1.

F. cretica methanol, aqueous and ethanol extracts, HPLC fractions, against MDR GI pathogens

Twenty-four (24) HPLC fractions were collected from *F. cretica* methanol, aqueous and ethanol extracts and were checked against the *E. coli, S. typhi, S. flexneri.* Most of the fractions showed activity against MDR GI bacterial pathogens. In methanolic extracts, fraction F2 was more active against *E. coli* (16^{i} ± 0.26 mm) followed by fraction F1 (11^{g} ± 0.14 mm). The aqueous fractions, F1 and F2 exhibited potential activity against all MDR pathogens. F1 exhibited maximum zone of inhibition against *E. coli* (16^{i} ± 0.08 mm), *S. typhi* (25^{g} ± 0.20 mm), *S. flexneri* (15^{g} ± 0.08 mm) and F2 against *E. coli* (12^{h}± 0.02 mm), *S. typhi* (15^{f} ± 0.12 mm) and *S. flexneri* (3^{c} ± 0.04 mm). In ethanolic fractions, F1 and F4 have minimum inhibitory zone (Figure 1 and Table 2).

F. cretica bioactive compounds identified by ESI-MS/MS analysis

F1 and F2 fractions of aqueous extract were processed for ESI-MS/MS analysis. Seven compounds including Gallic acid, Quinic acid, Cyclo-l-leu-l-pro, Vidalenolone, Liquirtigenin, Rosmarinic acid and Cerebronic acid were identified in F1 fraction of aqueous extract while Succinic acid, Cyclo (l-Leu-l-Pro) and Liquirtigenin were identified in F2 fraction of aqueous extract (Table 3).

Table 1. Antibacterial activity of *Fagonia cretica* extracts against MDR GI bacteria

Plant used	Solvents extracts	Volume used	MDR bacteria inhibitory zone (mm)		
			E. coli	*S. typhi*	*S. flexneri*
Fagonia cretica	Methanolic	10 µl	$10^{c} \pm 0.23$	$14^{e} \pm 0.37$	$20^{h} \pm 0.44$
		20 µl	$10^{c} \pm 0.35$	$16^{f} \pm 0.34$	$16^{f} \pm 0.57$
		30 µl	$12^{d} \pm 0.26$	$29^{k} \pm 0.46$	$25^{j} \pm 0.18$
	Aqueous	10 µl	$7^{b} \pm 0.17$	$18^{g} \pm 0.47$	$12^{d} \pm 0.88$
		20 µl	$6^{a} \pm 0.26$	$20^{h} \pm 0.43$	$12^{d} \pm 0.57$
		30 µl	$11^{d} \pm 0.26$	$22^{i} \pm 0.57$	$18^{g} \pm 0.39$
	Ethanolic	10 µl	$12^{d} \pm 0.20$	$10^{c} \pm 0.52$	$15^{f} \pm 0.38$
		20 µl	$14^{e} \pm 0.46$	$12^{d} \pm 0.52$	$7^{b} \pm 0.37$
		30 µl	$16^{f} \pm 0.14$	$8^{b} \pm 0.40$	$21^{hi} \pm 0.39$
	Antibiotic control		AZM 0	SAM 0	SAM 0
	Solvent control, methanol, aqueous, ethanol		0	0	0

Note: Mean ± standard error of given value and mean with different letters within a column represent a statistically significant difference. Azithromycin (AZM) and Ampicillin (SAM) antibiotics were used as control, methanol, aqueous, ethanol as a solvent control. Superscript letters (a, b, c, d, e, f, g, h, i, j, k) indicate significantly different means between different volumes of crude extract of plants according to one-way ANOVA and Duncan test.

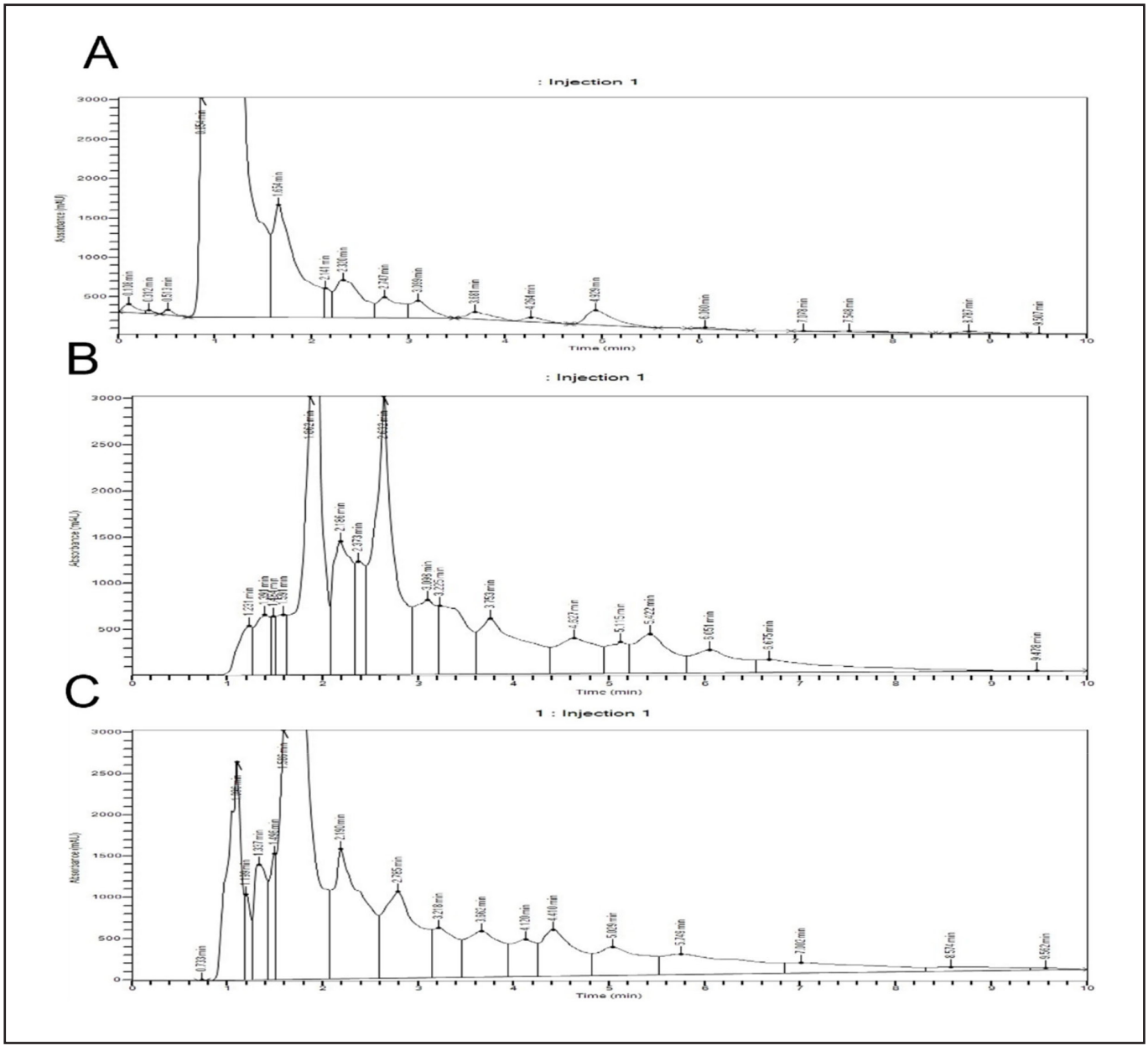


Figure 1. (A) Chromatogram HPLC fractions of *F. cretica* methanol extract, (B) Aqueous extract, (C) Ethanol extract.

Table 2. Antibacterial activity of *Fagonia cretica* methanol, aqueous and ethanol extracts HPLC fractions

S. No	Plant used	Solvent extract	HPLC fractions	MDR bacteria inhibitory zone (mm)		
				E. coli	*S. typhi*	*S. flexneri*
1	*Fagonia cretica*	Methanol extract	F1	$11^{g} \pm 0.14$	$3^{e} \pm 0.10$	0.0^{a}
			F2	$16^{i} \pm 0.26$	0.0^{a}	$4^{d} \pm 0.08$
			F3	$7^{e} \pm 0.26$	$3^{b} \pm 0.30$	$5^{d} \pm 0.11$
			F4	0.0^{a}	$3^{b} \pm 0.24$	0.0^{a}
			F5	$4^{d} \pm 0.21$	$3^{b} \pm 0.21$	$6^{e} \pm 0.37$
			F6	$2^{b} \pm 0.57$	$3^{b} \pm 0.32$	$5^{d} \pm 0.18$
			F7	$2^{bc} \pm 0.18$	0.0^{a}	$2^{c} \pm 0.10$
			F8	$2^{b} \pm 0.46$	0.0^{a}	$2^{c} \pm 0.88$
2		Aqueous extract	F1	$16^{j} \pm 0.08$	$25^{g} \pm 0.20$	$15^{g} \pm 0.08$
			F2	$12^{h} \pm 0.20$	$15^{f} \pm 0.12$	$3^{c} \pm 0.44$
			F3	$2^{b} \pm 0.46$	$9^{d} \pm 0.14$	$2^{b} \pm 0.20$
			F4	$2^{b} \pm 1.00$	$3^{b} \pm 0.10$	0.0^{a}
			F5	$3^{c} \pm 0.23$	$3^{b} \pm 0.15$	0.0^{a}
			F6	0.0^{a}	$3^{b} \pm 0.41$	0.0^{a}
			F7	0.0^{a}	0.0^{a}	0.0^{a}
			F8	0.0^{a}	0.0^{a}	0.0^{a}
3		Ethanol	F1	$1^{b} \pm 0.08$	$9^{d} \pm 0.20$	0.0^{a}
			F2	0.0^{a}	$29^{h} \pm 0.37$	0.0^{a}
			F3	$7^{e} \pm 0.18$	0.0^{a}	0.0^{a}
			F4	$2^{b} \pm 0.08$	0.0^{a}	0.0^{a}
			F5	$4^{d} \pm 0.29$	0.0^{a}	0.0^{a}
			F6	$8^{f} \pm 0.88$	0.0^{a}	0.0^{a}
			F7	0.0^{a}	0.0^{a}	0.0^{a}
			F8	$9^{f} \pm 0.26$	0.0^{a}	0.0^{a}
4	Antibiotic control			0.0^{a} CIP	$8^{c} \pm 0.20$ CRO	$14^{d} \pm 0.29$ CRO
5	Solvent control, Methanol, aqueous, ethanol			0	0	0

Note: ±= Standard error of specified value, means with different letters within a column represent a statistically significant difference. Antibiotic controls Ciprofloxacin (CIP), Ceftriaxone (CRO) and solvent controls methanol, aqueous and ethanol. The cited values are calculated after subtracting well diameter in mm. Superscript letters (a, b, c, d, e, f, g, h, i) indicate significantly different means between different volumes of crude extract of plants according to one-way ANOVA and Duncan test.

DISCUSSION

Single plant processing in various formulations may exhibit effective antibacterial potential. Due to the vast number of bioactive compounds found in medicinal plants, they must be isolated and then processed for antibacterial activity (García-Sosa *et al*., 2006). From literature mining, HPLC fractionation of *F. cretica* extracts against *S. typhi*, *E. coli* and *S. flexneri* is not yet reported. Antibacterial activity of *F. cretica* crude extract against MDR *S. flexneri, E. coli,* and *S. typhi* were checked in different volumes (10, 20, and 30 µl). The maximum zone of inhibition was exhibited by *F. cretica* 30 µl methanolic extract against *S. typhi* (29^{k} ±0.46 mm) and minimum zone of inhibition was showed by 20 µl aqueous extract against MDR *E. coli* ($6^{a} \pm 0.26$ mm).

A previous study, the antibacterial potential of *F. cretica* ethanolic, aqueous and methanolic extracts against different bacteria was checked in various concentration. In a study the aqueous and methanolic extracts had higher antibacterial activity than ethanolic extract (Sajid *et al*., 2011). Khushnood *et al*. (2021) used agar well diffusion method and reported that methanol fraction showed significant antibacterial and antifungal potential as compared to aqueous extract. In our study, using the same approach, methanol extract exhibited higher inhibitory activity against MDR GI pathogens. Dastagir (2012) reported the promising activity of *F. cretica* methanolic and n-hexane extracts against some Gram positive and Gram negative bacteria. Saleem *et al*. reported *F. cretica* antidiabetic activity in a concentration (38.1 µg/ml) (Saleem *et al*., 2014). Recently in one study by Saima *et al*. (2021) used HPLC and reported phenolic compounds including ferulic acid, gallic acid cinnamic acid and several other in ethyl acetate, ethanol and methanol fractions.

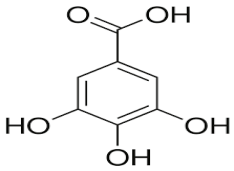
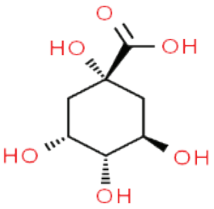
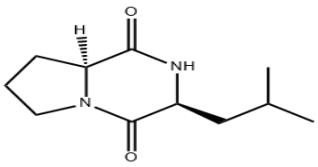
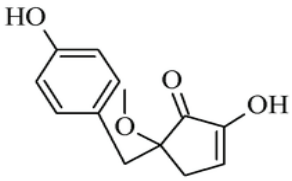
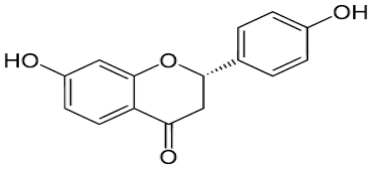
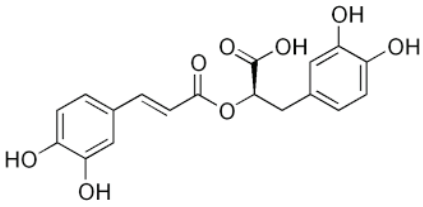
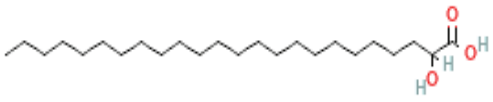
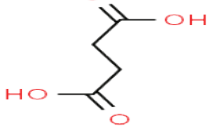
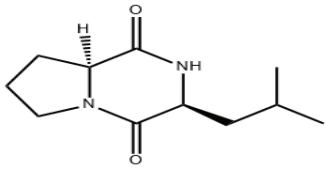
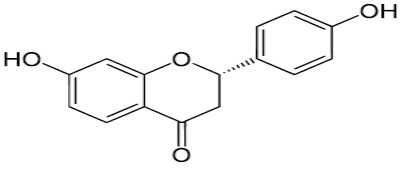
HPLC fractions from *F. cretica* aqueous, methanolic and ethanolic extracts were screened against MDR pathogens. HPLC bioactive fractions were further subjected to mass spectrometric identification. In F1 aqueous extract, gallic acid, quinic acid, cyclo-l-leu-l-pro, vidalenolone, liquirtigenin, rosmarinic acid, cerebronic acid while in F2, succinic acid, cyclo (l-Leu-l-Pro), liquirtigenin were found and these fractions were more effective against MDR GI pathogens.

Shekarchi *et al*. reported that rosmarinic acid (RA) is reported in many genera of Labiatae and exhibits biological activities such as antiviral, antibacterial and antioxidant (Shekarchi *et al*., 2012).

Liquirtigenin as bioactive compound is already reported in *Glycyrrhiza glabra* HPLC fractions. The study used similar approach to identify bioactive compounds in *Glycyrrhiza glabra* fractions against some selected MDR pathogens (Rahman *et al*., 2018).

It is to worth mention that that some of the identified bioactive compounds listed in the study are reported in *F. cretica*; however, Vidalenolone, Cyclo-1-leu-1-pro and Cerebronic acid are first time reported in *F. cretica.* Vidalenolone extracted from red algae exhibited anticancer activity (Yoo *et al*., 2002). Further in-depth functional characterization of the identified compounds might be helpful to uses these compounds as therapeutic molecules against MDR GI pathogens.

Table 3. *Fagonia cretica* bioactive compounds identified by ESI-MS/MS analysis

S. No.	Fraction source	Fraction ID	Compound	Molecular formula	Structure
1	Aqueous	F1	Gallic acid	$C_7H_6O_5$	
			Quinic acid	$C_7H_{12}O_6$	
			Cyclo-1-leu-1-pro	$C_{11}H_{18}N_2O_2$	
			Vidalenolone	$C_{13}H_{14}O_4$	
			Liquirtigenin	$C_{15}H_{12}O_4$	
			Rosmarinic acid	$C_{18}H_{16}O_8$	
			Cerebronic acid	$C_{24}H_{48}O_3$	
		F2	Succinic acid	$C_4H_6O_4$	
			Cyclo (l-Leu-l-Pro)	$C_{11}H_{18}N_2O_2$	
			Liquirtigenin	$C_{15}H_{12}O_4$	

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Conflicts of interest

The author declares that they have no conflicts of interests.

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