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Chemoprofiling and antimicrobial activity of medicinal herbs used in the treatment of inflammatory bowel disease

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ABSTRACT

Inflammatory bowel disease (IBD) is a term utilized to illustrate two different chronic disorders of the gastrointestinal tract i.e., Crohn's disease and ulcerative colitis. The symptoms of IBD are mainly characterized by inflammation, including abdominal pain, chronic diarrhoea, weight loss, shortening of the colon and rectal bleeding. The objective of this study was to evaluate the antimicrobial activity and Gas Chromatography-Mass Spectrometry (GC-MS) analysis of herbs used in the treatment of IBD in Saudi Arabia. Ethanolic extracts of five different herbs from Saudi Arabia namely *Pimpinella anisum* (Anise), *Foeniculum vulgare* (Fennel), *Matricaria chamomilla* (Chamomile), *Linum usitatissimum* (Linseed), and *Punica granatum* (Pomegranate) were prepared by Soxhlet extraction. The systemic chemical composition of the extracts was identified by GC-MS with their relative concentrations. The ethanolic extract of *P. anisum*, *F. vulgare*, *M. chamomilla*, *L. usitatissimum*, and *P. granatum* showed the presence of 35, 42, 34, 37, and 47 chemical components in these extracts, respectively. The five extracts and an equal mixture of them were examined for their antimicrobial activity by broth dilution method against different organisms. These included Gram-positive (*Staphylococcus aureus*), Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) bacteria and one yeast (*Candida albicans*). *P. anisum, F. vulgare*, *M. chamomilla, L. usitatissimum*, *P. granatum* and the mixture of all five extracts had good activity against *E. coli* (MIC=3.125, 0.050, 6.25, 0.050 and 0.100 mg/ml, respectively). *P. granatum* also had a MIC of 3.125 mg/ml against *S. aureus*. In conclusion. the plants' extracts and an equal mixture of them showed a narrow spectrum of antimicrobial activity against *S. aureus*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa* and *C. albicans*.

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Introduction

Inflammatory bowel disease (IBD) is a complex inflammatory disorder of gastro-intestinal tract (GIT) including Crohn's disease (CD) and ulcerative colitis (UC) (1). It may occur in teenagers and adults affecting both men and women in equal proportions (2). The symptoms of IBD include weight loss, chronic diarrhoea, abdominal pain, rectal bleeding and colon shortening.

Increased antibiotic use, irregular immune response, gut microbial flora, dietary changes, decreased exposure to parasites and other infections, genetic and environmental factors, are all known to contribute to the pathogenesis of IBD, even though the exact cause of the disease is still unknown (3-5). IBD is growing widespread worldwide, with the biggest increases occurring in developing nations and in young children (6). IBD in children and adolescents can manifest itself in a variety of ways. IBD patients make their first appearance in about 25% of cases before they turn 20. With the peak onset occurring in adolescence, IBD affects 4% of children under the age of five and 18% of children under the age of 10 (7).

The microbial flora differs between sick and healthy individuals. In a thorough investigation, it was discovered that individuals with IBD had larger concentrations of Bacteroidetes and *Escherichia coli* (*E. coli*) in their normal gut flora. However, other microbes were introduced as helpful bacteria that prevent IBD, such as Lactobacillus and Bifidobacterium (8). Corticosteroids, aminosalicylates and immunosuppressants are all now used in treatment plans. They have adverse effects and therapeutic limitations. Additionally, recent failures of IBD therapeutic targets, such as interleukin (IL)-13, IL-17, chemokine receptor-9 and interferon-γ have shown that IBD single-target therapy is challenging due to pathologic heterogeneity (9-11). As a result, it is necessary to find alternate treatment approaches with different therapeutic objectives.

It is expected that natural medicines, such as herbs or extracts may offer effective alternative remedies for IBD as currently existing medication regimens show a wide range of adverse effects. However, based on natural products no successful remedy against IBD has been developed to date. Fruits of *Pimpinella anisum* (*P. anisum*) Linn. (Umbelliferae) and *Foeniculum vulgare* (*F. vulgare*) Mill. (Umbelliferae), flowers of *Matricaria chamomilla* (*M. chamomilla*) L. (Asteraceae), seeds of *Linum usitatissimum* (*L. usitatissimum*) Linn. (Linaceae), and fruit peel of *Punica granatum* (*P. granatum*) L. (Punicaceae) alone or in specific combinations have been given by local herbal practitioners in Saudi Arabia against IBD (12). Thus,

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based on ethnobotanical knowledge and continuation of our previous work (12), we aimed to explore the antimicrobial activity against six different microorganisms and Gas Chromatography-Mass Spectrometry (GC-MS) analysis of these five herbs.

Materials and Methods

Materials

All five herbs were purchased from the local market of Taif, Saudi Arabia. The selected herbs are culinary/spices and are very commonly used in Saudi Arabia. They were authenticated by their morphological and microscopical characters as described (13).

Extraction

The air-dried coarse powder (10 gm) of each herb was extracted with ethanol (100 ml) individually in a Soxhlet apparatus at 60 °C for two hours. The extracts were filtered using a layer of cotton and Whatman filter paper (12). Filtered extracts were stored at -20 °C for further use (12).

GC-MS analysis

On a Gas Chromatography/Mass Spectrometry (Shimadzu QP-2010) using an HP-20M column (50 m×0.32 mm× 0.30 µm), the ethanolic extracts were analyzed. With a flow rate of 1.21 mL/min, helium was employed as the carrier gas in this experiment. The oven temperature was 80°C for 1 minute and then was controlled isothermally for 2 minutes. The detector was used at 280 ºC, the Injector port at 270 ºC, and split ratio was 1:50. The injected volume of the sample was 1 µL, and the recording was performed at 70 eV with a 1.5-second scan duration. The Chemstation program was used to manage mass spectra and chromatographs (14, 15).

Identification of components

By comparing mass spectra and their fragmentation patterns acquired by GC/MS analysis with those kept in the spectrometer database of NIST14, WILEY8 libraries, and published literature, distinct components were identified (16-18).

Antimicrobial activity testing

Microbroth dilution method was used to determine the minimum inhibitory concentration (MIC) of the tested extracts on Muller Hinton broth (Oxoid, UK). The investigated herbal extracts were principally tested for antimicrobial susceptibility (AST) with concentrations ranging from 50µg/ml to 100 mg/ml at two-fold dilutions. The following organisms were tested: *Staphylococcus aureus* (ATCC 29213, a Gram-positive organism), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Proteus mirabilis* (ATCC 14153), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 10231, a yeast). 100 mg/ml stock solutions of the tested extracts were made in DMSO (Sigma-Aldrich, US). Using various concentrations of the examined substances, the broth microdilution method was performed in 96 well microtiter plates. Positive (Ciprofloxacin) and negative (no organism) controls were employed. Following successive dilutions of the tested extracts, 100 ml of double-strength Muller Hinton broth containing the tested isolates at the required density was pipetted into all wells of the plate and mixed thoroughly by pipetting 3–6 times to complete the volume to 200µl/well. The plates were incubated for 18 hours at 37 °C as described (19). The test organisms' inoculum preparation (direct colony suspension method) was performed as follows: A few colonies of the investigated isolates at their log phase were suspended in sterile normal saline using the direct colony suspension method. The turbidity of the prepared suspensions was matched with a 0.5 McFarland standard, which is equivalent to 1-2x10^8 Colony-forming unit (CFU)/ml. The final inoculum concentration in each well of the microtiter plates was 5x10^5 CFU. The diluted broth cultures were used within 15 minutes of preparation (19).

Results

GC-MS analysis

GC-MS analysis of the ethanolic extract of the fruits of *P. anisum* showed the presence of 35 chemical compounds (Figure 1). Components with their retention times (RT), relative concentrations (RC, %), molecular formulae (MF) and molecular weights (MW) are shown in Table 1. The predominant compounds identified were anethole (48.06%), longifolene (5.72%), p-anisaldehyde (4.95%), 3-tert-butyl-4-hydroxyanisole (4.49%), p-acetonylanisole (2.69%), estragole (2.38%), n-hexadecanoic acid (1.87%) and beta-bisabolene (1.08%). However, limonene (0.04%), vitamin E (0.30%), gamma-sitosterol (0.34%), beta-famesene (0.35%), ethyl oleate (0.35%), linalool (0.36%), squalene (0.40%), beta-amyrin (0.45%), and stigmasterol (0.49%) were detected in trace amounts. GC-MS analysis of the ethanolic extract of *F. vulgare* fruits displayed the presence of 42 components (Figure 2). Compounds with their RT, RC, MF and MW are presented in Table 2. The major components detected were anethole (37.25%), p-allylanisole (16.78%), fenchone (10.05%), D-limonene (9.83%), 3-tert-butyl-p-hydroxyanisole (2.28%), (1.95%) p-anisaldehyde (1.89%), and alpha-pinene (1.75%). On the other hand, beta-myrcene (0.61%), camphor (0.32%),

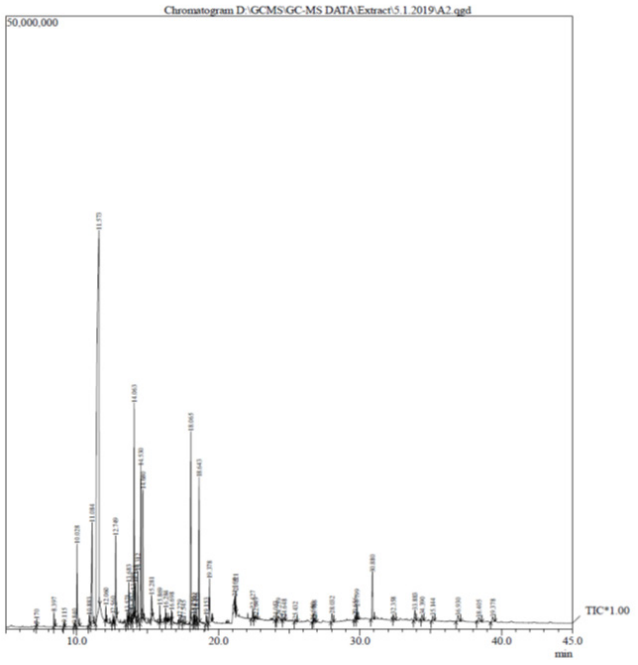

Figure 1. GC-MS spectrum of the ethanolic extract of the fruits of *P. anisum*.

Table. 1 Phytoconstituents present in the ethanolic extract of *P. anisum* fruits using GC-MS analysis.

Peak	RT	RC	Chemical compound	MW	MF
1	7.17	0.04	Limonene	136	C10H16
2	8.39	0.36	Linalool	154	C10H18O
3	9.11	0.08	Geijerene	162	C12H18
4	9.84	0.07	1-Dodecene	168	C12H24
5	10.02	2.38	Estragole	148	C10H12O
6	10.88	0.38	Trans-anethole	148	C10H12O
7	11.08	4.95	p-Anisaldehyde	136	C8H8O2
8	11.57	48.06	Anethole	148	C10H12O
9	12.06	0.26	delt-EIemene	204	C15H24
10	12.56	0.24	Alpha-Copaene	204	C15H24
11	12.74	2.69	p-Acetonylanisole	164	C10H12O2
12	13.57	0.35	beta-Famesene	204	C15H24
13	13.68	0.77	alpha-Himachalene	204	C15H24
14	13.85	0.27	4,5-Dehydro-isolongifolene	202	C15H22
15	13.98	0.51	alpha-Curcumene	202	C15H22
16	14.06	5.72	Longifolene	204	C15H24
17	14.14	0.57	7-epi-Sesquithujene	204	C15H24
18	14.31	1.08	beta-Bisabolene	204	C15H24
19	14.53	4.49	3-tert-Butyl-4-hydroxyanisole	180	C11H16O2
20	15.86	0.33	Isospathulenol	220	C15H24O
21	16.28	0.33	1-Hydroxy-1-(4-methoxyphenyl)propan-2-one	180	C10H12O3
22	16.69	0.26	1-(4-Methoxyphenyl)propane-1,2-diol	182	C10H14O3
23	17.27	0.20	Tetradecanoic acid	228	C14H28O2
24	17.54	0.18	17-Pentatriacontene	490	C35H70
25	18.25	0.15	Neophytadiene	278	C20H38
26	19.37	1.87	n-Hexadecanoic acid	256	C16H32O2
27	21.22	0.35	Ethyl oleate	310	C20H38O2
28	22.64	0.22	cis-9-Hexadecenal	238	C16H30O
29	26.68	0.05	p-Anisoin	272	C16H16O4
30	28.03	0.41	Tetracontane	562	C40H82
31	29.79	0.40	Squalene	410	C30H50
32	34.39	0.30	Vitamin E	430	C29H50O2
33	36.93	0.49	Stigmasterol	412	C29H48O
34	38.40	0.34	gamma-Sitosterol	414	C29H50O
35	39.37	0.45	beta-Amyrin	426	C30H50O

RT: Retention Time; RC: Relative concentration; MW: Molecular weight; MF: Molecular formula.

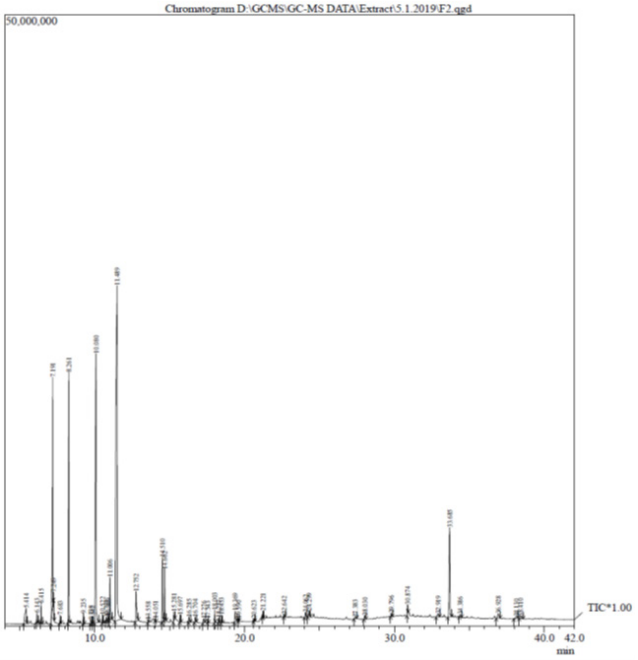

Figure 2. GC-MS spectrum of the ethanolic extract of the fruits of *F. vulgare*.

beta-phellandrene (0.31%), gamma-sitosterol (0.29%), gamma-terpinene (0.27%), fenchol (0.22%), vitamin E (0.18%), carvone (0.17%), ethyl oleate (0.17%), squalene (0.15%), beta-famesene (0.08%), and germacrene D (0.08%) were identified in lower percentages. Ethanolic extract of flowers of *M. chamomilla* led to the characterization of 34 chemical compounds by using the GC-MS technique (Figure 3). Compounds with their RT, RC, MF and MW are presented in Table 3. The chief identified components were bisabolol oxide A (21.04%), pentacosane (9.70%), beta-famesene (8.49%), tetracontane (6.21%), n-hexadecanoic acid (4.76%), 7-methoxy-coumarin (3.84%), hexatriacontane (3.70%), alpha-bisabolol oxide B (2.61%), heneicosane (2.30%), vitamin E (1.99%), bisabolone oxide (1.99%), and spathulenol (1.15%). Neophytadiene (0.86%), gamma-sitosterol (0.72%), germacrene D (0.62%), anethole (0.35%), germacrene B (0.20%), phytol (0.14%), artemisia ketone (0.12%), and limonene (0.09%) were detected in lower quantities (Table 3).

GC-MS analysis of the ethanolic extract of *L. usitatissimum* seeds showed the presence of 37 chemical

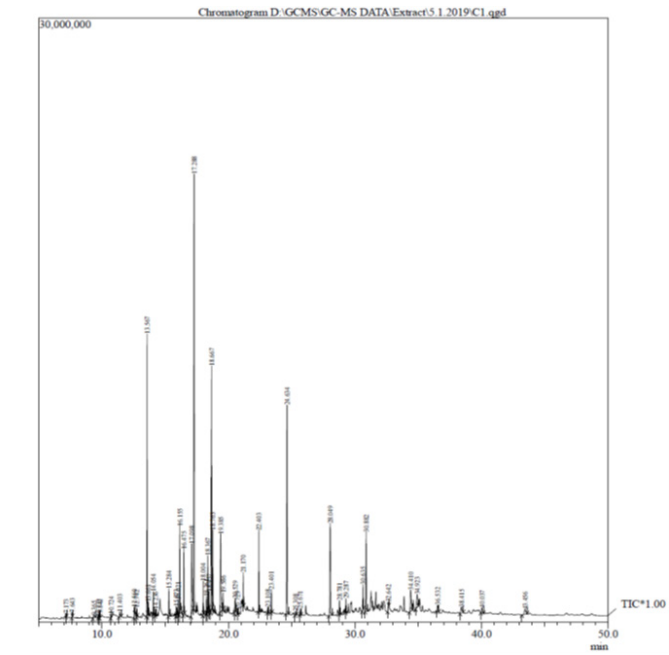

Figure 3. GC-MS spectrum of the ethanolic extract of the flowers of *M. chamomilla*.

Table. 2 Phytoconstituents present in the ethanolic extract of *F. vulgare* fruits using GC-MS analysis

Peak	RT	RC	Chemical compound	MW	MF
1	5.41	1.75	alpha-Pinene	136	C10H16
2	6.14	0.31	beta-Phellandrene	136	C10H16
3	6.41	0.61	beta-Myrcene	136	C10H16
4	7.19	9.83	D-Limonene	136	C10H16
5	7.24	0.41	trans-beta-Ocimene	136	C10H16
6	7.68	0.27	gamma-Terpinene	136	C10H16
7	8.26	10.05	Fenchone	152	C10H16O
8	9.23	0.32	Camphor	152	C10H16O
9	9.73	0.15	Menthol	156	C10H20O
10	9.84	0.09	1-Dodecene	168	C12H24
11	10.08	16.78	p-Allylanisole	148	C10H12O
12	10.52	0.22	Fenchol	154	C10H18O
13	10.77	0.17	(-)-Carvone	150	C10H14O
14	10.88	0.26	cis-Anethole	148	C10H12O
15	11.00	1.89	p-Anisaldehyde	136	C8H8O2
16	11.48	37.25	Anethole	148	C10H12O
17	12.75	1.95	Anisic ketone	164	C10H12O2
18	13.55	0.08	(E)-beta-Famesene	204	C15H24
19	14.05	0.08	Germacrene D	204	C15H24
20	14.51	2.28	3-tert-Butyl-p-hydroxyanisole	180	C11H16O2
21	14.66	1.75	3-(4-Hydroxy-3-methoxyphenyl)-2-oxopropanoic acid	210	C10H10O5
22	15.28	0.43	1-Hxadecene	224	C16H32
23	15.69	0.25	Apiole	222	C12H14O4
24	17.27	0.27	Tetradecanoic acid	228	C14H28O2
25	17.54	0.11	1-Heptadecene	238	C17H34
26	18.00	0.35	Neophytadiene	278	C20H38
27	19.36	0.64	n-Hexadecanoic acid	256	C16H32O2
28	19.59	0.09	Heptadecanoic acid, ethyl ester	298	C19H38O2
29	20.62	0.11	6-Octadecenoic acid, methyl ester	296	C19H36O2
30	21.22	0.17	Ethyl oleate	310	C20H38O2
31	22.64	0.10	Palmitoyl chloride	274	C16H31ClO
32	24.06	0.34	3-Cyclopentylpropionic acid, 2-dimethylamino ethyl ester	213	C12H23NO2
33	27.38	0.13	3,13-Octadecadien-1-ol	266	C18H34O
34	28.03	0.26	Tetratetracontane	618	C44H90
35	29.79	0.15	Squalene	410	C30H50
36	30.87	0.38	2,6,10,14-Tetramethyl hexadecane	282	C20H42
37	32.91	0.33	Octadecanal	268	C18H36O
38	33.68	6.77	Nonacosan-10-one	422	C29H58O
39	34.38	0.18	Vitamin E	430	C29H50O2
40	36.92	0.49	Stigmasta-5,22-dien-3-ol	412	C29H48O
41	38.13	0.22	11-Oxo-eicosanoic acid, methyl ester	340	C21H40O3
42	38.41	0.29	gamma-Sitosterol	414	C29H50O

RT: Retention Time; RC: Relative concentration; MW: Molecular weight; MF: Molecular formula.

Table. 3 Phytoconstituents present in the ethanolic extract of *M. chamomilla* flower using GC-MS analysis.

Peak	RT	RC	Chemical compound	MW	MF
1	7.17	0.09	Limonene	136	C10H16
2	7.64	0.12	Artemisia ketone	152	C10H16O
3	9.84	0.11	1-Undecene	154	C11H22
4	10.72	0.09	Linalyl acetate	196	C12H20O2
5	11.40	0.35	Anethole	148	C10H12O
6	12.56	0.40	Decanoic acid	172	C10H20O2
7	12.74	0.24	1-Tetradecene	196	C14H28
8	13.56	8.49	beta-Famesene	204	C15H24
9	13.66	0.42	7-Methyl-1-naphthol	158	C11H10O
10	14.05	0.62	Germacrene D	204	C15H24
11	14.23	0.20	Germacrene B	204	C15H24
12	15.28	1.15	(-)-Spathulenol	220	C15H24O
13	15.87	0.25	4,5,9,10-Dehydro-isolongifolene	200	C15H20
14	16.02	0.50	2,3,4,5,8,8a-Hexahydro-6, 3a(1h)-azulenol	222	C15H26O
15	16.15	2.61	alpha-Bisabolol oxide B	238	C15H26O2
16	16.47	1.99	Bisabolone oxide	236	C15H24O2
17	17.09	3.84	7-methoxy-Coumarin	176	C10H8O3
18	17.28	21.04	Bisabolol oxide A	238	C15H26O2
19	18.00	0.86	Neophytadiene	278	C20H38
20	18.26	0.53	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	C20H40O
21	19.38	4.76	n-Hexadecanoic acid	256	C16H32O2
22	19.58	0.72	Hexadecanoic acid, ethyl ester	284	C18H36O2
23	20.72	0.14	Phytol	296	C20H40O
24	21.17	0.88	9,12-Octadecadienoic acid	280	C18H32O2
25	22.40	2.30	Heneicosane	296	C21H44
26	23.10	0.19	3-Methyl-octadecane	268	C19H40
27	24.63	9.70	Pentacosane	352	C25H52
28	25.67	0.28	3-Methylpentacosane	366	C26H54
29	28.04	6.21	Tetracontane	562	C40H82
30	29.28	0.61	1-iodo-Hexacosane	492	C26H53I
31	30.88	3.70	Hexatriacontane	506	C36H74
32	34.41	1.99	Vitamin E	430	C29H50O2
33	38.41	0.72	gamma-Sitosterol	414	C29H50O
34	43.45	0.75	Lup-20(29)-ene-3,28-diol	442	C30H50O2

RT: Retention Time; RC: Relative concentration; MW: Molecular weight; MF: Molecular formula.

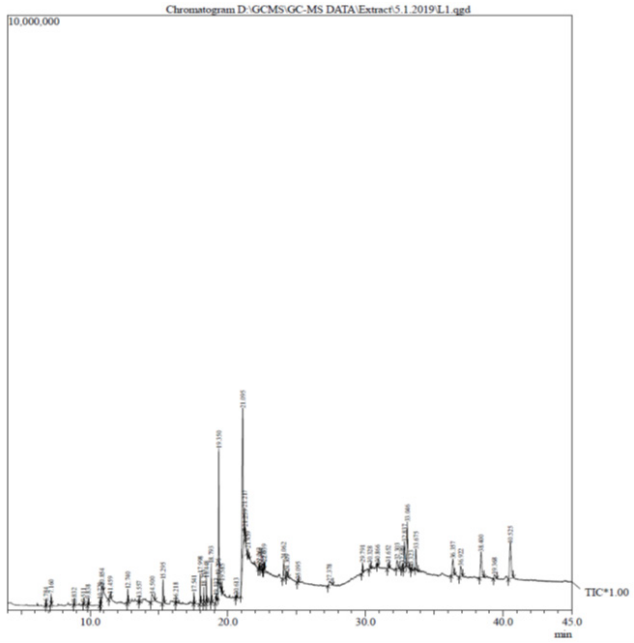

Figure 4. GC-MS spectrum of the ethanolic extract of the fruits of *F. vulgare*.

components (Figure 4). Compounds with their RT, RC, MF and MW are presented in Table 4. The predominant identified components were 7-tetradecenal (26.87%), n-hexadecanoic acid (10.36%), 9,19-cyclolanost-24-en-3-ol (8.32%), gamma-sitosterol (5.70%), vitamin E (5.32%), oleoyl chloride (4.45%), ergost-5-en-3-ol (3.08%), 5-hydroxymethylfurfural (2.67%), beta-sitosterol (2.46%), fumaric acid, 2-dimethylaminoethyl nonyl ester (2.47%), 1,6-anhydro-beta-D-glucopyranose (2.09%), stigmasterol (2.04%), geranyllinalool (1.97%), campesterol methyl ether (1.72%), neophytadiene (1.47%), and 9-octadecenal (1.09%). On the other hand, ethyl oleate (0.85%), squalene (0.74%), stigmasterol acetate (0.65%), alpha-springene (0.47%), hexadecanoic acid ethyl ester (0.44%), 1-hexadecanol (0.43%), linalyl acetate (0.28%), beta-famesene (0.19%), and hexadecadienoic acid methyl ester (0.17%) were identified in minute concentrations (Table 4). Ethanolic extract of *P. granatum* fruit peel led to the detection of 47 chemical compounds by GC-MS analysis (Figure 5). Compounds with their RT, RC, MF and MW are presented in Table 5. The major components identified were 6-octadecenoic acid (17.32%), D-allose (17.11%), n-hexadecanoic acid (13.97%), 5-hydroxymethylfurfural (8.88%), 1,6-anhydro-beta-D-glucofuranose (6.68%), oleic anhydride (5.30%), stigmast-5-en-3-ol, oleate (3.52%), 3-methyl-2,5-furandione (2.79%), 1,2,4-benzenetriol (1.67%), cis-9-hexadecenal (1.66%), and gamma-sitosterol (3.88%). Isosorbide (0.62%), beta-sitosterol (0.54%), vitamin E (0.52%), stigmasterol (0.52%), cis-isomyristicin (0.40%), squalene (0.39%), palmitic acid beta-monoglyceride (0.35%), palmitoleic acid (0.29%), hexadecanoic acid ethyl ester (0.27%), ethyl oleate (0.22%), limonene (0.19%), diosphenol (0.15%), 9-octadecenoic acid methyl ester (0.13%), 1,3,4-eugenol methyl ether (0.05%), and D-glucitol (0.03%) were identified in low concentrations (Table 5).

Antimicrobial activity testing

The five ethanolic extracts and an equal mixture of them were examined for their antimicrobial activity by broth dilution method against different Gram-positive and

Table. 4 Phytoconstituents present in the ethanolic extract of *L. usitatissimum* seeds using GC-MS analysis.

Peak	RT	RC	Chemical compound	MW	MF
1	9.83	0.39	1-Undecanol	172	C11H24O
2	10.72	0.28	Linalyl acetate	196	C12H20O2
3	10.85	2.67	5-Hydroxymethylfurfural	126	C6H6O3
4	12.74	0.70	3-Hexadecene	224	C16H32
5	13.55	0.19	beta-Famesene	204	C15H24
6	14.50	2.09	1,6-Anhydro-beta-D-glucopyranose	162	C6H10O5
7	17.54	0.55	1-Heptadecene	238	C17H34
8	17.99	1.47	Neophytadiene	278	C20H38
9	18.44	1.19	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	C20H40O
10	18.79	1.97	Geranyllinalool	290	C20H34O
11	19.29	0.47	alpha-Springene	272	C20H32
12	19.35	10.36	n-Hexadecanoic acid	256	C16H32O2
13	19.58	0.44	Hexadecanoic acid, ethyl ester	284	C18H36O2
14	20.61	0.17	Hexadecadienoic acid, methyl ester	266	C17H30O2
15	21.09	26.87	7-Tetradecenal	210	C14H26O
16	21.21	0.85	Ethyl oleate	310	C20H38O2
17	21.25	0.33	9-octadecenoic acid	282	C18H34O2
18	21.45	0.43	1-Hexadecanol	242	C16H34O
19	22.38	0.24	Glycidyl palmitate	312	C19H36O3
20	22.63	0.45	cis-11-Hexadecenal	238	C16H30O
21	24.06	2.47	Fumaric acid, 2-dimethylaminoethyl nonyl ester	313	C17H31NO4
22	24.30	1.09	9-Octadecenal	266	C18H34O
23	25.09	0.32	Tetradecanal	212	C14H28O
24	27.37	1.19	1,3,12-Nonadecatriene-5,14-diol	294	C19H34O2
25	29.79	0.74	Squalene	410	C30H50
26	30.32	0.53	1-Stearoyl-1H-imidazole	334	C21H38N2O
27	30.86	0.22	2-Methylhexacosane	380	C27H56
28	32.30	1.72	Campesterol methyl ether	414	C29H50O
29	32.68	0.65	Stigmasterol acetate	454	C31H50O2
30	32.83	4.45	Oleoyl chloride	300	C18H33ClO
31	33.04	5.32	Vitamin E	416	C28H48O2
32	33.32	0.27	1-Bromo-tetracosane	416	C24H49Br
33	33.67	2.46	beta-Sitosterol	414	C29H50O
34	36.35	3.08	(3beta,24r)-Ergost-5-en-3-ol	400	C28H48O
35	36.92	2.04	Stigmasterol	412	C29H48O
36	38.40	5.70	gamma-Sitosterol	414	C29H50O
37	40.52	8.32	(3beta)-9,19-Cyclolanost-24-en-3-ol	426	C30H50O

RT: Retention Time; RC: Relative concentration; MW: Molecular weight; MF: Molecular formula.

Table. 5 Phytoconstituents present in the ethanolic extract of *P. granatum* fruit peel using GC-MS analysis.

Peak	RT	RC	Chemical compound	MW	MF
1	5.66	2.79	3-methyl-2,5-Furandione	112	C5H4O3
2	7.16	0.19	Limonene	136	C10H16
3	7.57	0.42	cis-2-Nonene	126	C9H18
4	8.97	0.03	D-Glucitol	182	C6H14O6
5	9.30	0.93	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	144	C6H8O4
6	9.63	0.11	2,6-dimethyl-7-Octen-3-ol	156	C10H20O
7	9.83	0.15	1-Dodecanol	186	C12H26O
8	10.43	0.62	Isosorbide	146	C6H10O4
9	10.70	8.88	5-Hydroxymethylfurfural	126	C6H6O3
10	11.97	0.95	2-Heptanol, acetate	158	C9H18O2
11	12.74	0.40	1-Tridecene	182	C13H26
12	12.90	0.05	1,3,4-Eugenol methyl ether	178	C11H14O2
13	13.09	0.53	1,5-Pentandiol, diacetate	188	C9H16O4
14	13.31	1.67	1,2,4-Benzenetriol	126	C6H6O3
15	13.96	0.15	Diosphenol	168	C10H16O2
16	14.52	0.40	cis-Isomyristicin	192	C11H12O3
17	15.01	17.11	D-Allose	180	C6H12O6
18	15.27	0.21	1-Hexadecene	224	C16H32
19	16.33	6.68	1,6-Anhydro-beta-D-glucofuranose	162	C6H10O5
20	16.72	0.17	Undecanal	170	C11H22O
21	17.27	0.72	Tetradecanoic acid	228	C14H28O2
22	17.54	0.19	1-Heptadecene	238	C17H34
23	19.16	0.29	Palmitoleic acid	254	C16H30O2
24	19.40	13.97	n-Hexadecanoic acid	256	C16H32O2
25	19.58	0.27	Hexadecanoic acid, ethyl ester	284	C18H36O2
26	20.61	0.13	9-Octadecenoic acid, methyl ester	296	C19H36O2
27	21.11	17.32	6-Octadecenoic acid	282	C18H34O2
28	21.21	0.22	Ethyl oleate	310	C20H38O2
29	21.27	0.59	Octadecanoic acid	284	C18H36O2
30	21.45	0.21	Undec-10-ynoic acid, undecyl ester	336	C22H40O2
31	22.26	0.67	9,12,15-Octadecatrien-1-ol	264	C18H32O
32	24.06	0.50	Fumaric acid, 2-dimethylaminoethyl nonyl ester	313	C17H31N
33	24.29	0.60	9-Octadecenal	266	C18H34O
34	24.52	0.35	Palmitic acid beta-monoglyceride	330	C19H38O4
35	27.38	1.66	cis-9-Hexadecenal	238	C16H30O
36	29.79	0.39	Squalene	410	C30H50
37	30.33	1.31	3-Methyl-3-(palmitoylperoxy)butyl palmitate	596	C37H72O5
38	30.87	0.84	Tetratetracontane	618	C44H90
39	31.66	0.54	beta-Sitosterol	414	C29H50O
40	32.33	0.61	Glyceryl monooloeate	358	C21H42O4
41	32.69	0.48	3beta-Stigmasta-5,22-dien-3-ol, acetate	454	C31H50O2
42	32.84	5.30	Oleic anhydride	546	C36H66O3
43	33.08	0.45	beta-Stigmast-5-en-3-ol	414	C29H50O
44	33.68	3.52	Stigmast-5-en-3-ol, oleate	678	C47H82O2
45	34.38	0.52	Vitamin E	430	C29H50O2
46	36.92	0.52	Stigmasterol	412	C29H48O
47	38.42	3.88	gamma-Sitosterol	414	C29H50O

RT: Retention Time; RC: Relative concentration; MW: Molecular weight; MF: Molecular formula.

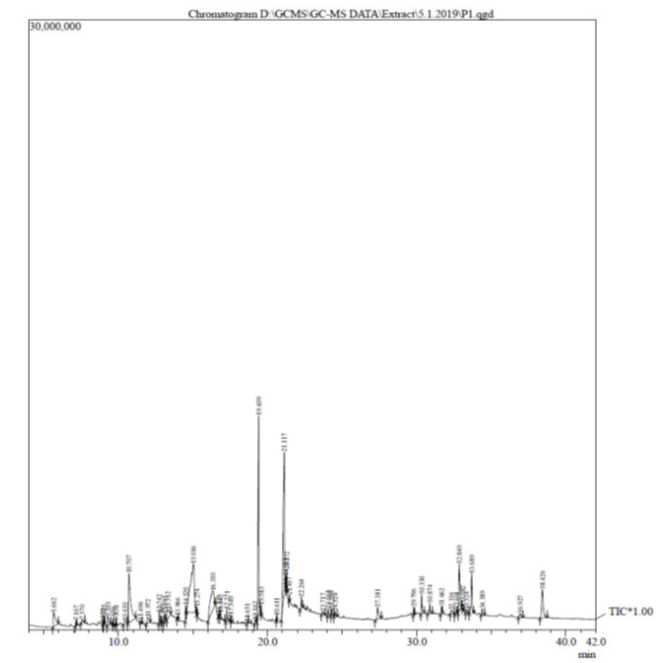

Figure 5. GC-MS spectrum of the ethanolic extract of the *P. granatum* fruit peel.

negative organisms as well as one yeast. These included Gram-positive (*S. aureus*), Gram-negative (*E. coli*, *K. pneumoniae*, *P. mirabilis* and *P. aeruginosa*) as bacterial strains and one yeast (*C. albicans*). The MIC data of the five extracts and an equal mixture of them are shown in Table 6. *P. anisum, F. vulgare*, *L. usitatissimum*, *P. granatum* and the mixture of all five extracts had good activity against *E. coli* (MIC=3.125, 0.050, 6.25, 0.050 and 0.100 mg/ml, respectively). *P. granatum,* also, showed a MIC of 3.125 mg/ml against *S. aureus*. On the other hand, the remaining extracts showed very high MIC (≥6.25mg/ml) against *K. pneumoniae*, *P. mirabilis, P. aeruginosa* and *C. albicans* (Table 6). These data show that the extracts and an equal mixture of them had a narrow spectrum of antimicrobial activity against some Gram-positive and Gram-negative organisms with no antifungal activity.

Discussion

Native healers in Saudi Arabia favored using a single herbs or a combination of herbs to treat IBD. Using specific combinations of *P. anisum* (Anise), *F. vulgare* (Fennel), *M. chamomilla* (Chamomile), *L. usitatissimum* (Linseed), and *P. granatum* L. (Pomegranate), indigenous natural healers have recommended them for the treatment of IBD and its symptoms based on ethnobotanical knowledge (12). The systemic chemical composition of these extracts was studied by GC-MS with their RT, RC, MF and MW.

Table 6. Antimicrobial activity and MIC values (mg/ml) of tested plant extracts against Gram-positive, Gram-negative organisms and *C. albicans.*

Extract	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	*C. albicans*
P. anisum	100	3.125	>100	>100	100	>100
F. vulgare	50	≤0.050	>100	100	100	100
M. chamomilla	100	>100	>100	>100	>100	>100
L. usitatissimum	>100	6.25	>100	100	100	100
P. granatum	3.125	0.050	>100	12.5	50	>100
Mixture	12.5	0.100	>100	12.5	50	25
Ciprofloxacin	≤0.5 µg	≤ 0.5 µg	≤ 0.5 µg	≤ 0.5 µg	≤ 0.5 µg	>1024 µg

By comparing mass spectra, fragmentation patterns, spectrometer database libraries and published literature, components present in extract were identified (16-18).

GC-MS analysis of the ethanolic extract of the fruits of *P. anisum* showed the presence of 35 chemical compounds. The predominant compounds identified were anethole (48.06%) and longifolene (5.72%). However, limonene (0.04%), vitamin E (0.30%), gamma-sitosterol (0.34%) and stigmasterol (0.49%) were detected in trace amounts. Earlier findings reported the identification of 43 compounds in the ethanolic extract of *P. anisum* by GC/MS. The most important components reported were butanoic acid, 2-methyl-, 2-methoxy-4-(2-propenyl) phenyl ester (23.21%) and anethole (12.52%) (20). However, in the present study, the percentage of bioactive anethole (48.06%) was much higher. In other studies, tran-anethole was detected at concentrations of 71.52% (21) and 89.24% (22) as the main component in the volatile oil of *P. anisum* fruits.

GC-MS analysis of the ethanolic extract of *F. vulgare* fruits displayed the presence of 42 components. The major components detected were anethole (37.25%), p-allylanisole (16.78%), and fenchone (10.05%). However, gamma-sitosterol (0.29%), vitamin E (0.18%), and squalene (0.15%) were identified in lower percentages. In earlier findings, 57 compounds were identified in GC-MS analysis of a methanolic extract of *F. vulgare*. The major components detected were trans-anethole (31.49%), 2-pentanone (25.01%) and fenchone (11.68%) (23). In the current study, the percentage of anethole and fenchone was very close to the later report. However, 2-pentanone was not detected in the extract. Ahmed et al. 2019, reported the essential oil analysis of Egyptian and Chinese fennel (24). Egyptian fennel showed 27 constituents with estragole (51.04%), limonene (11.45%), l-fenchone (8.19%) and trans-anethole (3.62%) as major components. On the other hand, the Chinese fennel showed 30 constituents with trans-anethole (54.26%), estragole (20.25%), l-fenchone (7.36%) and limonene (2.41%) as major components (24).

Ethanolic extract of flowers of *M. chamomilla* led to the characterization of 34 chemical compounds by using the GC-MS technique. The chief identified components were bisabolol oxide A (21.04%), beta-famesene (8.49%) and vitamin E (1.99%), while gamma-sitosterol (0.72%), anethole (0.35%), phytol (0.14%), and limonene (0.09%) were detected in lower quantities. Previously, Sayyar et al. 2018, reported only 8 components in GC-MS analysis of *M. chamomilla* flower ethanolic (70%) extract, where heptacosane (33.53%), tetracosahexaene (16.71%), and 1,2,2-trimethylcyclopropylamine (13.96%) were detected as the major compounds (25). In another study, *M. chamomilla* essential oil was reported with 13 compounds, in which α-bisabolol oxide A (48.22%), α-bisabolol oxide B (23.31%), α-bisabolol (12.1%) and β-farnesene (5.21%) were the major components identified (26). However, the results of the current study displayed bisabolol oxide A (21.04%) and α-bisabolol oxide B (2.61%) in lesser percentages than an earlier report (26).

GC-MS analysis of the ethanolic extract of *L. usitatissimum* seeds showed the presence of 37 chemical components. The predominant identified components were 7-tetradecenal (26.87%), n-hexadecanoic acid (10.36%), gamma-sitosterol (5.70%) and vitamin E (5.32%). On the other hand, ethyl oleate (0.85%), squalene (0.74%) and stigmasterol acetate (0.65%) were identified in minute concentrations. In a previous study, only 17 compounds were detected in *L. usitatissimum* seeds ethanolic extract of which squalene (45.27%), 9,12,15-octadecatrienoic acid (24.67%) and oleic acid (10.16%) were present with highest percentages (27). However, in the present study squalene (0.74%) was identified in a trace amount, and 7-tetradecenal (26.87%) and n-hexadecanoic acid (10.36%) were detected as major components. In another study, Kaur et al., 2017 reported 10 components in the petroleum ether extract of *L. usitatissimum* seeds, of which naphthalene decahydro-4a-methyl-1-methylene-7-(1-methylethenyl) (57.99%) and epiglobulol (14.38%) were the major constituents (28).

Ethanolic extract of *P. granatum* fruit peel led to the detection of 47 chemical compounds by GC-MS analysis. The major components identified were 6-octadecenoic acid (17.32%), D-allose (17.11%), n-hexadecanoic acid (13.97%), and gamma-sitosterol (3.88%). However, beta-sitosterol (0.54%), vitamin E (0.52%), stigmasterol (0.52%), squalene (0.39%), limonene (0.19%) and diosphenol (0.15%) were identified in low concentrations. Al-Tai & Al-Mayyahi, 2021, reported 2H-pyran-2-one (25.27%), hexadecanoic acid, ethyl ester (16.32%), furfural (12.12%), ethyl oleate (10.89%) and 3-fluoro-benzenemethanol (6.58%) as major compounds and few fatty acid esters in trace amounts in the ethanolic extract of *P. granatum* fruit peel by GC-MS analysis (29). The results of the previous study are not consistent with the current study in which 6-octadecenoic acid (17.32%), D-allose (17.11%), n-hexadecanoic acid (13.97%) and 5-hydroxymethylfurfural (8.88%) were identified as major components. In another report, glycerin (27.03%), hydroxymethylfurfurole (21.18%), guanosine (13.34%) and D-allose (4.64%) were detected as major constituents with a comparatively less percentage of fatty acids (30).

GC-MS analysis showed the presence of anethole as the main active constituent in the ethanolic extract of both

P. anisum and *F. vulgare,* and as trace in *M. chamomilla*. Anethole has anti-inflammatory and antioxidant activities as described in previous studies. It shows potential against acetic acid-induced colitis and reduced *E. coli*-induced intestinal barrier disruption and intestinal inflammation (31, 32). On the other hand, vitamin E was detected by GC-MS in all the plant extract. In earlier reports, vitamin E exhibited anti-inflammatory and antioxidant potential against colitis by protecting intestinal barrier function and by modulating the gut microbiota in tested animals (33, 34). As per the above-mentioned points, anethole and vitamin E could be responsible for the effectiveness of these plants against IBD.

The five ethanolic extracts and an equal mixture of them were also examined for their antimicrobial activity by broth dilution method against different Gram-positive and Gram-negative organisms as well as one yeast. Microbial infections usually contribute to the pathogenesis of IBD. Therefore we tried to examine the antimicrobial activity of the extracts against a wide range of infectious microorganisms. These included Gram-positive (*Staphylococcus aureus*), Gram-negative bacteria (*E. coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*). All of them live in the GIT and some are implicated in IBD." *P. anisum, F. vulgare*, *L. usitatissimum*, *P. granatum* and the mixture of all five plant extracts had a good activity against *E. coli*. *P. granatum,* also, showed a considerable MIC against *S. aureus*. On the other hand, the remaining extracts showed very high MIC against the remaining tested organisms. *P. anisum* (10%v/v) was recently shown to have antimicrobial activity against *E. coli, S. aureus* and *C. albicans* (35). Contrasting this study's findings, the antifungal activity of *P. anisum* (anise) and *F. vulgare* (fennel) essential oils were recently proven *in vitro* against ten *Candida* isolates (36). This can be attributed to using different strains of this yeast. Also, the antimicrobial activity of *M. chamomilla* was recently reviewed (37), although we did not find any antimicrobial or antifungal activity in this extract. In addition, *L. usitatissimum* was shown to have antimicrobial activity against *E. coli, S. aureus, K. pneumoniae* and *C. albicans* at 100mg/ml by disc diffusion method (38), which we consider as a very weak antimicrobial and antifungal activity as determined by MIC microbroth dilution method. Furthermore, a methanolic extract (50µl) of yellow *P. granatum* showed 26, and 9mm zones of inhibition against *S. aureus*, and *E. coli*, while that of red *P. granatum* L. (100µl) were 27, and 15 mm against the same organisms, respectively (39). In summary, the above data show that these extracts used in IBD in Saudi Arabia had a narrow spectrum of antimicrobial activity against some Gram-positive and Gram-negative organisms with no antifungal activity.

GC-MS analysis showed the presence of various bioactive compounds in the ethanolic extract of these herbs. Anethole was the chief active constituent detected in *P. anisum* and *F. vulgare* ethanolic extract. However, bisabolol oxide A, 7-tetradecenal and 6-octadecenoic acid were the major components identified in *M. chamomilla*, *L. usitatissimum* and *P. granatum* ethanolic extracts, respectively. Anethole and vitamin E both exhibited anti-inflammatory and antioxidant potential against colitis. However, the potential of other detected bioactive compounds is to be evaluated against IBD. *P. anisum, F. vulgare*, *M. chamomilla, L. usitatissimum*, *P. granatum* and a mixture of all five extracts showed good activity against *E. coli*. However, these plant extracts and an equal mixture of them had a narrow spectrum of antimicrobial activity against Gram-positive (*S. aureus*), Gram-negative (*K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*) bacteria and one yeast (*C. albicans*).

Conflict of interest

The Authors declare that there is no conflict of interest.

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Data availability

All the data pertinent to this manuscript are included herein.

Ethical consideration

Our study did not require ethical board approval because it is a lab-based experimental study with no human or animal samples.

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