



GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF FOUR FRACTIONS FROM COMFREY ROOTS AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT:

The current study aimed to evaluate the metabolite profile of four subsequent extracts from comfrey roots (hexane, chloroform, ethyl acetate, and 95% ethanol) and its antimicrobial potential. The extracts were obtained by subsequent maceration in each solvent for a duration of each extraction 24 hours. The resulting extracts were evaporated to dryness and analyzed by gas chromatography coupled with mass spectrometry (GC-MS). The agar diffusion method was used to evaluate the antimicrobial potential of comfrey root extracts against Gram-positive and Gram-negative bacteria, yeasts, and fungi. The highest extractive yield was found in 95 % ethanol fraction of 2.12%. It was detected high extractable contents of fatty acids, sterols, phenolic acids, amino acids, organic acids, sugars, and polyols in the obtained fractions; ethyl acetate and ethanol fractions showed the highest number of compounds—32 and 29, respectively. In the hexane and chloroform fractions dominated fatty acids and sterols. The results from antimicrobial activity showed that hexane and chloroform fractions showed moderate antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Bacillus amyloliquefaciens* 4BCL-YT, *Micrococcus luteus* 2YC-YT, and *Pseudomonas aeruginosa* ATCC 9027. In general, hexane and ethyl acetate fractions showed low activity against fungi, against which other fractions were inactive. Only ethyl acetate and ethanol fraction showed antimicrobial against *Candida albicans* NBIMCC 74. In general, hexane and ethyl acetate fractions showed the highest range of antimicrobial activity against tested microorganisms. This research enriched the knowledge for metabolite composition of comfrey root extracts and demonstrated their potential antimicrobial effect for future application in cosmetics and pharmacy.

Keywords: antimicrobial activity, comfrey root extract, GC-MS analysis,

INTRODUCTION

Comfrey (*Symphytum officinale* L.) is a medicinal plant from Boraginaceae family. It was widely spread across Europe, but it can also be found in some parts of Asia and South America [1, 2]. Most of the researches were focused on polyphenolic compounds [1, 3, 4]. The profile of comfrey root phenolic compounds was reported by many researchers [1, 2, 3, 4, 5, 6], as dominating compounds were phenolic acids (rosmarinic, caffeic, and salvianolic acids, flavonoids, and allantoin). However, comfrey roots were also a rich source of flavonoids, inulin and sugars [3], fatty acids [1] and allantoin [1]. The compounds in comfrey root that are active in the treatment of sprains, arthritis, fractures, and hematoma comprises allantoin, rosmarinic acid, derivatives of hydroxycinnamic acid, muco-polysaccharides, vitamins A, B and C, saponins, triterpenoid saponins, tannins, Ca, K, and Se [1, 3, 6]. Due to the rich phytochemical compounds, comfrey roots were used for bone breakages, sprains and rheumatism, skin problems, hematomas, problems with joints wounds, gout, and thrombophlebitis [6]. Despite the numerous studies about the phytochemical profile of comfrey roots, many gaps still exist. Therefore, the investigation of polar and unpolar extracts from comfrey roots continues. Most of the extracts possessed antioxidant, antiinflammatory and antimicrobial portential due to the bioactive compounds containing in them [3, 4, 5, 7].

The antimicrobial potential of comfrey root water and ethanol extracts were tested against several bacterial strains, especially against *Staphylococcus aureus* ATCC 25923 [5, 6], as well as antifungal activity against

Bipolaris oryzae [6]. It was reported that the antimicrobial potential of the investigated extracts could be due mainly to the phenolic compounds (caffeic and chlorogenic acids, luteolin glycoside) and allantoin, also to the synergy between different compounds [6]. However, the leaves and water root extracts were mainly analyzed for the antimicrobial potential of the comfrey plant [6]. Therefore, the evaluation of fractional extracts of comfrey roots still remains chalanges.

The aim of the current study was to elucidate the metabolite profile of four subsequent extracts from dry comfrey roots (hexane, chloroform, ethyl acetate, and 95% ethanol) and to evaluate their antimicrobial potential.

MATERIALS AND METHODS:

Plant material

The plant material (*Symphytum officinale* L. roots) was produced from Bilki Ltd, Sofia, Krasno selo (LOT L22), and it was purchased for their on-line shop www.bilki.bg. The dry roots were finely ground on a laboratory homogenizer and then sieved through a 0.5 mm.

Extraction procedure

The finely ground comfrey roots (100 g) were successively extracted by maceration with the solvents in the following order: n-hexane, CHCl₃, ethyl acetate and 95% ethanol in a solid-to-solvent ratio of 1:10 (w/v). Each extraction was conducted at room temperature on a magnetic stirrer for 24 h. The solvents were removed after each extraction step through vacuum evaporation. The yield of dry extracts was calculated, and they were used for further analysis. The extracts was stored in closely thight flasks under nitrogen atmosphere at -18°C.

Gas Chromatography-Mass Spectrometry

The dry n-hexane and chlorophorm extracts obtained from comfrey roots were saponified with an ethanolic solution of 2 M KOH under reflux for 1.5 h, as previously described [8]. These extracts, together with ethyl acetate and 95% ethanol extracts, were analyzed on a gas chromatograph Agilent Technology Hewlett Packard 7890 A, connected with a mass detector Agilent Technology 5975 C inert XL EI/CI MSD at 70 eV, under conditions mentioned earlier [9].

Antimicrobial activity

For performing this analysis the dry extracts from comfrey roots was dissolved in methanol to the final concentration 10 mg/ml. These extracts were tested for their antimicrobial activity against gram-positive and gram-negative bacteria, yeasts, and fungi. The agar well diffusion method was used for the antimicrobial activity [10]. The selected microorganisms from the collection of the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria, were used. The anysis was performed against twenty microorganisms: six Gram positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus amyloliquefaciens* 4BCL-YT, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* NBIMCC 8632,

Micrococcus luteus 2YC-YT and *Enterococcus faecalis* ATCC 19433), six Gram negative bacteria (*Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* NBIMCC 1672, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380 and *Pseudomonas aeruginosa* ATCC 9027), two yeasts (*Candida albicans* NBIMCC 74 and *Saccharomyces cerevisiae* ATCC 9763) and six fungi – *Aspergillus niger* ATCC 1015, *Fusarium moniliforme* ATCC 38932, *Aspergillus flavus*, *Penicillium chrysogenum*, *Rhizopus* sp. and *Mucor* sp. (plant isolates). After the incubation (24/48 h), the inhibition effect was determined by measuring the diameter around the wells.

Statistical analysis

All determinations were performed in triplicate (n = 3) and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using MS Excel 2015. ANOVA, with Tukey's test of difference, was considered statistically significant when p < 0.05.

RESULTS:

Yield of comfrey root extracts

Table 1 showed the extraction yields from maceration. The extraction yield was expressed as the mass of obtained dry extract (g) per 100 g of dry plant material. The highest extractive yield was obtained with 95% ethanol (after pretreatment of comfrey roots with n-hexane, chloroform, ethyl acetate) – 2.12 g/100 g, while the lowest yield was detected for ethyl acetate fraction – 0.17 g/100 g.

Table 1. Extractive yield from subsequent solvent extraction from comfrey roots

No.	Fraction	Yield, g/100g dry roots
1.	Hexane	0,37±0,05
2.	Chloform	0,24±0,02
3.	Ethyl acetate	0,17±0,05
4.	95% ethanol	2,12±0,11
5.	Hexane after saponification	0,27±0,02
6.	Chloroform after hydrolysis	0,07±0,01

Gas Chromatography-Mass Spectrometry analysis of comfrey root fractions

The results for detected phytochemical compounds in n-hexane and chlorophorm fractions were presented (Table 2). In both fractions were detected fatty acids and sterols. In n-hexane fraction were found 5 sterols and 14 fatty acids, while in chloroform fraction were found 4 sterols and 12 fatty acids. Lanosterol (31,93% of TIC) was the dominating compound in n-hexane fraction, folowed by linoleic and palmitic acid (19,93 and 18,80% of TIC, respectively). Cycloartenyl acetate, n-heptadecanoic acid, cis-15-Tetracosenoic acid, n-tetradecanoic acid (myristic

acid) and n-Pentanoic acid (margaric acid) were detected only in hexane fraction, while hexanedioic acid (adipic acid) and nonanedioic acid (azelaic acid) were found only in chloroform fraction. From sterols campesterol, α -sitosterol, lupenone and lanosterol were found in both extracts, as lanosterol was the most abandoned compound

in hexane fraction. Cycloartenyl acetate was found only in hexane fraction. In chloroform extract dominated fatty acids: palmitic acid – 32,74% of TIC and linoleic acid - 23,84 % of TIC. In both fraction, unsaturated α -Linolenic acid was presented in amount around 7% of TIC.

Table 2. Chemical compounds identified by GC-MS analysis in hexane and chloroform, subsequent fractions from comfrey roots

Peak	Retention time	Retention index	Compound	% of TIC sample	
				Hexane	Chloroform
1	8,05	1522	Hexanedioic acid (Adipic acid)	-	1,91
2	11,71	1787	Nonanedioic acid (Azelaic acid)	-	1,48
3	12,52	1842	n-Tetradecanoic acid (Myristic acid)	0,62	-
4	13,79	1939	n-Pentanoic acid (Margaric acid)	0,33	-
5	15,32	2016	cis-9-Hexadecenoic acid (Palmitoleic acid)	0,71	0,80
6	15,77	2041	n-Hexadecanoic acid (Palmitic acid)	18,80	32,74
7	17,28	2140	n-Heptadecanoic acid	0,25	-
8	18,09	2177	γ -Linolenic acid	7,84	7,34
9	18,49	2205	Linoleic acid	19,93	23,84
10	18,55	2218	Oleic acids	4,38	5,58
11	18,62	2222	α -Linolenic acid	1,76	1,75
12	18,92	2239	Octadecanoic acid (Stearic acid)	1,95	2,83
13	21,68	2436	Eicosanoic acid	0,80	2,21
14	25,01	2637	Docosanoic acid	0,47	1,72
15	27,49	2808	cis-15-Tetracosenoic acid	0,68	-
16	27,82	2841	Tetracosanoic acid	0,26	1,07
17	33,09	3194	Campesterol	1,87	1,39
18	34,40	3265	β -Sitosterol	4,93	3,91
19	34,94	3280	Lupenone	0,72	1,49
20	35,45	3327	Lanosterol	31,93	5,80
21	36,37	3390	Cycloartenyl acetate	0,89	-

- Not detected, TIC - total ion current

The results for detected phytochemical compounds in ethylacetate and 95 % ethanol extracts from comfrey roots after pretreatment with n-hexane and chlorophorm fractions were summerized in Table 3.

Table 3. Chemical compounds identified by GC-MS analysis in ethyl acetate and 95% ethanol, subsequent fractions from comfrey roots

Peak	Retention time	Retention index	Compound	% of TIC	
				Ethyl acetate	95% Ethanol
1	4,70	1268	Leucine 2TMS	0,32	4,70
2	4,87	1284	Isoleucine 2TMS	0,19	4,87
3	5,09	1293	Proline 2TMS	0,46	5,09
4	5,79	1262	Glycerol 3TMS	4,50	3,40
5	6,21	1305	Succinic acid 2TMS	3,64	2,16

6	6,69	1346	Fumaric acid 2TMS	0,95	0,70
7	7,06	1397	Glutaric acid 2TMS	0,31	-
8	7,12	1403	n-Tetradecane	1,52	-
9	8,05	1478	Malic acid 3TMS	1,99	2,60
10	8,11	1485	Threitol 4TMS	1,40	-
11	8,32	1506	Salicylic acid 2TMS	2,48	1,49
12	9,18	1555	Cinnamic acid 1TMS	2,87	0,76
13	9,36	1602	n-Hexadecane	0,24	-
14	9,47	1620	p-hydroxy-Benzoic acid 2TMS	3,35	1,02
15	10,54	1761	Azelaic acid 2TMS	0,98	-
16	11,75	1838	Quinic acid 5TMS	3,48	-
17	11,75	1800	Citric acid 4TMS	-	0,85
18	11,87	1855	Fructose oxime 5TMS isomer	8,23	10,29
19	11,96	1864	Fructose oxime 5TMS isomer	6,66	8,33
20	12,04	1870	Galactose oxime 6TMS isomer	0,43	0,54
21	12,38	1881	Glucose oxime 6TMS isomer	0,78	0,98
22	12,94	1897	Galactose oxime 6TMS isomer	1,09	1,36
23	13,11	1902	Glucose oxime 6TMS isomer	1,41	1,76
24	13,35	1914	Mannitol 6TMS	0,42	0,52
25	13,41	1921	Sorbitol 6TMS	0,25	0,31
26	13,60	1938	Coumaric acid 2TMS	-	0,92
27	13,50	1934	Ascorbic acid 5TMS	1,84	-
28	15,68	2039	Palmitic acid 1TMS	21,90	29,81
29	16,97	2087	Ferulic acid 2TMS	1,92	1,53
30	17,12	2144	Caffeic acid 3TMS	2,68	2,15
31	18,35	2205	Linoleic acid 1TMS	6,21	7,46
32	18,45	2209	Oleic acid 1TMS	2,58	2,84
33	18,56	2218	Linolenic acid 1TMS	0,53	0,50
34	18,90	2240	Stearic acid 1TMS	8,95	11,14
35	22,02	2424	Eicosanoic acid 1TMS	0,83	-
36	24,24	2618	Sucrose 8TMS isomer	2,48	2,72
37	24,90	2821	Sucrose 8TMS isomer	1,54	1,61

Ethyl acetate and ethanol fractions showed the highest number of compounds—32 and 29, respectively. In both fractions were detected amino acids, polyols, fatty acids, organic and phenolic acids, as well as sugars. However, only in ethyl acetate fraction were alkanes found (n-tetradecane and n-hexadecane). Four amino acids were found in ethyl acetate fraction, while in 95 % ethanol, only three were found – leucine, isoleucine and proline, as essential amino acid leucine and isoleucine obtained 4.7 and 4.8 % of TIC. From organic acids - succinic, fumaric and malic acid were found in both fractions, while citric acid was detected only in 95 % ethanol fraction. Ascorbic acid was found only in ethyl acetate fraction. From phenolic acids, five representatives (salicylic, cin-

amic, p-hydroxy-Benzoic, ferulic and caffeic acids) were found in both fractions, while quinic acid was detected in ethyl acetate extract and coumaric acid was presented in 95 % ethanol extract. Glycerol, mannitol and sorbitol and sugars (fructose, glucose, galactose and sucrose) were found in both fractions, however, their content was higher in 95% ethanol extract. Palmitic, linoleic, oleic, stearic acids were detected in four fractions (Table 2 and Table 3), as palmitic acid was the dominating compound in chloroform, ethyl acetate and ethanol fractions. Saturated stearic acid was the second most abandoned compound in 95% ethanol and ethyl acetate fractions, while fructose oxime 5TMS isomer was the third wide-spread compound.

Antimicrobial activity of subsequent fractions from comfrey roots

Four subsequent extracts from comfrey roots were tested for their antimicrobial activity against 20 microor-

ganisms: gram-positive and gram-negative bacteria, yeasts, and fungi. However, comfrey root extracts demonstrate potential only against 13 microorganisms and this antimicrobial activity presented in Table 4.

Table 4. Antimicrobial potential of the extracts from comfrey root.

No.	Microorganisms	Inhibition zones, mm*			
		Hexane fraction	Chloroform fraction	Ethyl acetate fraction	95% Ethanol fraction
1	<i>Bacillus subtilis</i> ATCC 6633	11	11	8	8
2	<i>Bacillus amyloliquefaciens</i> 4BCL-YT	11	-	10	8
3	<i>Staphylococcus aureus</i> ATCC 25923	8	8	8	-
4	<i>Listeria monocytogenes</i> NBIMCC 8632	9	8	9	8
5	<i>Micrococcus luteus</i> 2YC-YT	12	13	11	8
6	<i>Escherichia coli</i> ATCC 25922	10	10	10	10
7	<i>Proteus vulgaris</i> ATCC 6380	8	8	8	8
8	<i>Pseudomonas aeruginosa</i> ATCC 9027	12	12	12	9
9	<i>Candida albicans</i> NBIMCC 74	-	-	8	8
10	<i>Aspergillus niger</i> ATCC 1015	10	-	9	-
11	<i>Penicillium chrysogenum</i>	-	11	-	-
12	<i>Rhizopus</i> sp.	8	-	8	-
13	<i>Fusarium moniliforme</i> ATCC 38932	-	-	10	-

The results from antimicrobial activity showed that hexane and chloroform fractions showed moderate antimicrobial activity against *Micrococcus luteus* 2YC-YT and *Pseudomonas aeruginosa* ATCC 9027. In general, hexane and ethyl acetate fractions showed low activity against fungi, against which other fractions were inactive. Only ethyl acetate and ethanol fraction showed antimicrobial activity against *Candida albicans* NBIMCC 74. None of the extracts demonstrated antifungal activity against four bacteria *Enterococcus faecalis* ATCC 29212, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* NBIMCC 1672, *Klebsiella pneumonia* ATCC 13883, yeasts *Saccharomyces cerevisiae* ATCC 9763 and two fungi *Aspergillus flavus* and *Mucor* sp. The most active were hexane and chloroform fraction, that could be explained with the presence of fatty acids and sterols, that could disrupt and effect permeability of microbial cell membrane.

DISCUSSION:

The highest yield of comfrey roots were obtained with the fourth used solvent – 95% ethanol. It was previously reported that in maceration, the yield was highly dependent on the solvent (especially mixtures of methanol, ethanol, or acetone/water), not from the time of extraction [1]. In our study, the yields obtained with non-

polar solvent n-hexane and chloroform were lower than this in 95% ethanol, that showed the domination of the polar compounds in the comfrey roots. The yield with 95% ethanol was comparable with some data obtained with 100% ethanol and methanol for 12h maceration [1].

In four subsequent extracts from comfrey roots were found 52 compounds (alkanes, fatty acids, organic acids, phenolic acids, sugars, polyols, amino acids and sterols. A total of 44 compounds were identified earlier in comfrey root (included anthraquinones, organic, phenolic and fatty acids, and their derivatives), as 39 phytochemicals that have never been reported in this sample matrix [1]. The n-hexane and chloroform fraction contained mainly lipids – saturated and unsaturated fatty acids and sterols. Linoleic acid was the second widespread fatty acid in hexane fraction. α -Linolenic acid was detected only in hexane and chloroform fractions as the third most abundant fatty acid. In our case, this unsaturated fatty acid occupied 13% and 9%, respectively, from the total fatty acid content in hexane and chloroform fractions. Our data were in good agreement with Hansen CE, et al. [11] who reported α -linolenic acid in comfrey root and stems (12-16%) from total fatty acids. The current confirmed the finding that fatty acids were the most abundant in *S. officinale* root extracts [1]. Palmitic and stearic acids were both wide spread com-

pounds in all fractions (tables 2 and 3). Cinnamic and quinic acids dominated in ethyl acetate fraction, however, many phenolic acids were remained in the subsequent 95 % ethanol extraction. However, other researches also reported the presence of phenolic acids but diluted ethanol solutions as in 60% ethanol, 65% ethanol and 50 % ethanol extracts obtained by maceration or ultrasonic extraction [2,12,13,14]. Moreover, it is well known that comfrey roots are a good source of phenolic acids [2, 3, 6, 14]. Caffeic, p-coumaric and m-hydroxybenzoic acids were also detected in ethyl acetate and 95% ethanol fractions that coincided with previous report for their presence in comfrey roots [2, 6]. Most of detected in our study phenolic acids coincided with reports of Luca et al. [14] found in 60 % ethanol comfrey extracts. However, rosmarinic acid and chlorogenic acids were not detected in this study, contrary to previous report [14, 15]. The third most abandoned metabolites in comfrey root ethyl acetate and 95 % ethanol fractions were fructose oxime. The sugars were detected in these both fractions. This was in good agreement with our previous report for fructose, glucose and sucrose presence in 95% ethanol comfrey root extract [3].

The antimicrobial potential of comfrey fractional extracts

It was reported earlier that comfrey root extracts have antimicrobial activity, effective against several bacterial strains including *Staphylococcus aureus* and *Escherichia coli*, and also show antifungal properties. This is explained with the presence of compounds like phenolic acids (such as rosmarinic and salvianolic acid) and potentially other bioactive molecules [15]. In the current research comfrey root extracts showed antimicrobial activity against 13 microorganisms. It was the first detailed study about the evaluation of antimicrobial activity of comfrey root fractional extracts. n-hexane, chloroform, and ethyl acetate fractions demonstrated moderate antimicrobial activity against Gram negative bacteria *Pseu-*

domonas aeruginosa ATCC 9027, while only n-hexane and chloroform showed moderate activity against Gram positive bacterial *Micrococcus luteus* 2YC-YT. This could be explained with presence of fatty acids and sterols in these hydrophobic fractions. In general all comfrey extracts possessed low activity against *Escherichia coli* ATCC 25922 with an inhibition zone 10 mm, which was in good agreement with [4, 6]. This study enrich the information about antimicrobial activity of comfrey root extracts.

CONCLUSION:

A detailed characterization of phytochemical compounds in four subsequent comfrey root extracts was carried using GC-MS. Moreover, the antimicrobial potential of comfrey root extracts were evaluated. The ethyl acetate and 95 % ethanol were evaluated as the richest fractions of phytocomponents with the promising antibacterial activity, while only ethyl acetate demonstrated low antifungal activity. The most active comfrey root extracts were n-hexane and chlorophorm fractions against Gram-negative, Gram-positive bacteria and fungi. This could be explained with the rich lipid component in these unpolar fractions that could effect the microbial cells. The current study provided useful information with respect to further utilization of extracts from comfrey (*S. officinale*) root rich of active compounds for future application in cosmetics and pharmacy.

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