Original Article

Determination of Some Flavonoids and Antimicrobial Behaviour of Some Plants' Extracts

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ABSTRACT

C. sativa, *C. intybus*, *L. stoechas*, *V. officinalis* and *G. glabra* plants were extracted by using 65% ethanol to isolate their active constituents. The antimicrobial activities of extracts were investigated against 15 microorganisms by using the disk diffusion method, MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration) and MFC (Minimal Fungicidal Concentration) tests. Furthermore, the presence of eight flavonoids were analysed by using HPLC. It was found that *C. sativa* is active against *C. albicans*, *E. faecalis*, *S. enteritidis* and *S. typhimurium* with MIC values of 26.02 µg/mL, 13.01 µg/mL, 416.25 µg/mL and 832.50 µg/mL respectively, where *C. intybus* is active against *C. albicans* and *E. faecalis*, with MIC values of 13.01 µg/mL and 6.50 µg/mL, respectively. On the other hand, *L. stoechas* and *V. officinalis* were observed to be active against only *S. enteritidis* with MIC values of 52.03 µg/mL and 26.02 µg/L respectively, where *G. glabra* was active against only *E. faecalis*, with a MIC value of 52.03 µg/mL. The extracts of plant samples showed antibacterial activity against tested microorganisms at different levels. But the activities against *C. albicans* and *E. faecalis* is noteworthy. The flavonoids were determined at different amounts in extracts.

Keywords: Soxhelet Extraction, Antimicrobial Activity, Flavonoid, MIC, Disc Diffusion.

INTRODUCTION

The evolution and spread of antibiotic resistance, as well as the evolution of new strains causing diseases, attract the attention of many scientist. Our ability to treat diseases dependent on the development of new agents, and one potential source of novel drugs is traditional medicine.[1]

Some compounds of plants are the basis of many of the modern pharmaceuticals. Bioactive compounds are normally accumulated as secondary metabolites in plant cells but their concentration varies according to several factors such as the part of plant, season, climate and etc. Leaves are one of the highest accumulated plant part of such compounds and people are generally preferred them for therapeutic purposes against microorganisms causing diseases.[2]

Flavonoids are a broad class of low molecular weight, plant phenolic compounds characterized by the flavan nucleus. In the literature, about 4000 flavonoids were identified.[3] The subclasses such as flavonol, flavone, flavanone, is a flavone and anthocyanidin occur by substitutions of some groups to the core structure.[4] Flavonoids have beneficial health effects due to their antioxidant and chelating capacities observed as metal-chelating, antioxidant enzyme activation, reduction of α-tocopherol radicals and inhibition of oxidase enzymes in organisms.[4,5] In this paper the antimicrobial activity of ethanol (65%) extracts of *C. sativa*, *C. intybus*, *L. stoechas*, *V. officinalis* and *G. glabra* were tested against 15 microorganisms by using the disk diffusion, minimum inhibitory concentration

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and minimum bactericidal/fungicidal concentration tests. In addition, composition of catechin, epicatechin, rutin, naringin, myricetin, luteolin, naringenin and apigenin were found with normal-phase HPLC in the obtained extracts.

MATERIALS AND METHODS

Plant Samples

Cannabis sativa (seed), *Cichorium intybus* (whole plant), *Lavandula stoechas* (inflorescens), *Valeriana officinalis* (root) *and Glycyrrhiza glabra* (root) were obtained from Özşen Lokman Hekim Company located in Ankara/Turkey, Gimat at 2016.

Extraction method

The plants were washed with water and air dried under shade. The dried plant sample were ground in a mixer. 10-30 g ground samples were extracted with 250 mL of ethanol (%65) in a Soxhlet apparatus by continuous heat extraction for 24 hours. Extracts were prepared according to previous studies and stored at 4°C for further studies.[6]

Determination of Antimicrobial Activities

Preparation of Extract Stock

Extract stocks to test the antimicrobial activity were prepared by dissolving 1 mg of extract in each 3 mL of ethanol for disk diffusion test, where the solvent was distilled water for MIC tests. The extract stock prepared for MIC test was sterilised through 0.45 µm filter (Millipore).

Strains

Bacillus subtilis DSMZ 1971, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium, Escherichia coli* ATCC 25922, *Klebsiella pneumoniae, Pseudomonas aeruginosa* DSMZ 50071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis, Salmonella kentucky, Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* DSMZ 20044 were used in the study.

Preparation of Inocula

All strains were incubated according to their requirements as it was previously mentioned.[6] The inocula was prepared according to previous studies and adjusted.[7,8] Thus standard inocula contained adjusted number of microorganisms.[9,10]

Disk Diffusion Test

The disk diffusion test was applied as it was mentioned in the previous studies.[11,12] 20, 50 and 100 µL of extracts were loaded on sterile disks as mentioned before.[13] The inoculation process was as described in the previous studies.[13] Inhibition zones were defined in mm.[13]

Minimum Inhibitory Concentration (MIC) Test

A broth microdilution MIC test was applied as mentioned before.[14] Two-fold dilutions of the extracts were prepared ranging from 33 mg/mL to 6.50 µg/mL.

Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) Test

The wells, where no visual growth were observed in MIC test were used for further analysis called MBC and MFC tests, which were conducted according to previous studies.[14]

Controls

Empty disks and sterilized broth medium were used as negative controls, where broth medium inoculated with each microorganism was used as positive control of microorganisms.[15]

Statistical Analysis

All tests were done in triplicates. Statistical analysis was conducted as mentioned in previous studies.[16] $p < 0.05$ was considered as statistically significant.

HPLC Analysis

HPLC analysis were carried out by using an Agilent Eclipse XDB C18 5 µm with 4.6 x 250 mm column and studied at the column temperature of 30°C. Flavonoids measurements were determined at 280 nm after 20 µL injection volume. Standard solvents were prepared in the ethanol-distilled water (65-35) mixture. For HPLC analysis, mobile phase A (containing water-10% formic acid (95-5)), mobile phase B (containing acetonitrile-10% formic acid (5-95)) were used. Mobil phases have been flowed for during 39 min.

RESULT AND DISCUSSION

The HPLC results of the extracts are presented in **Table 1**, and the antimicrobial activity test results are presented in **Table 2.**

While myricetin, naringenin and apigenin were detected in all extracts, catechin was only determined in extract of *C. intybus*, epicatechin was only found in extract of *G. glabra*. Rutin in other extracts apart from *G. glabra*, naringin in other extracts apart from *C. sativa*, luteolin in other extracts apart from *V. officinalis*, were detected. The most abundant flavonoids were observed as myricetin (in extracts of *C. sativa* and *L. stoechas*), luteolin (in extract of *C. intybus*) and apigenin (in extract of *C. sativa*) **Table 1**.

Table 1: HPLC analyses results of flavonoids (µg g^{-1} plant)

	Catechin	Epicatechin	Rutin	Naringin	Myricetin	Luteolin	Naringenin	Apigenin
C. sativa	-	-	8.55	-	426.47	12.37	5.76	167.96
C. intybus	18.26	-	3.12	29.07	5.91	71.60	1.49	3.59
L. stoechas	-	-	5.21	0.58	805.81	0.68	2.20	11.94
V. officinalis	-	-	0.56	0.54	71.34	-	0.35	1.14
G. glabra	-	10.59	-	0.52	25.78	5.51	7.17	15.89

Table 2: Disk Diffusion Test and MIC (µg/mL) and MBC/MFC (mg/mL) results of extracts (mm)

	C. sativa			***C. intybus***			***L. stoechas***			***V. officinalis***			***G. glabra***		
	20 µL	50 µL	100 µL	20 µL	50 µL	100 µL	20 µL	50 µL	100 µL	20 µL	50 µL	100 µL	20 µL	50 µL	100 µL
B. subtilis	-	-	-	-	-	-	-	-	-	-	8	7	-	-	-
C. albicans	7	8	9	-	7	7	-	-	-	-	-	-	-	-	-
E. faecalis	9	11	13	-	-	7	-	-	-	-	-	-	-	-	7
S. enteritidis	-	8	9	-	-	-	-	8	7	-	8	10	-	-	-
S. typhimurium	-	8	14	-	-	-	-	-	-	-	-	-	-	-	-
No activity:	*E. aerogenes*, *E. faecium*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, *P. fluorescence*, *S. infantis*, *S. kentucky*, *S. aureus*, *S. epidermidis*														

	C. sativa		***C. intybus***		***L. stoechas***		***V. officinalis***		***G. glabra***	
	MIC	MBC MFC	MIC	MBC MFC	MIC	MBC MFC	MIC	MBC MFC	MIC	MBC MFC
B. subtilis	-	-	-	-	-	-	>3.33	-	-	-
C. albicans	26.02	>3.33	13.01	>3.33	-	-	-	-	-	-
E. faecalis	13.01	1.665	6.50	0.8325	-	-	-	-	52.03	3.33
S. enteritidis	416.25	>3.33	-	-	52.03	>3.33	104.06	3.33	-	-
S. typhimurium	832.50	>3.33	-	-	-	-	-	-	-	-

Disk diffusion test results given in **Table 2** show that *C. sativa* is active against *C. albicans*, *E. faecalis*, *S. enteritidis* and *S. typhimurium* with inhibition zones between 7-14 mm, where *C. intybus* is active against *C. albicans* and *E. faecalis* with an inhibition zone of 7 mm. On the other hand, *L. stoechas* was observed to be active against only *S. enteritidis* with inhibition zones between 7 - 8 mm depending on the amount of extract loaded on disks, where *V. officinalis* was observed to be active against *B. subtilis* and *S. enteritidis* with inhibition zones between 7 - 10 mm and *G. glabra* was active against only *E. faecalis* with an inhibition zone of 7 mm. To the extracts and microorganism combination which presented antimicrobial activity were chosen for MIC value determination. **Table 2** show that *C. sativa* is active against *C. albicans*, *E. faecalis*, *S. enteritidis* and *S. typhimurium* with MIC values of 26.02 µg/mL, 13.01 µg/mL, 416.25 µg/mL and 832.50 µg/mL respectively, where *C. intybus* is active against *C. albicans* and *E. faecalis*, with MIC values of 13.01 µg/mL and 6.50 µg/mL respectively. On the other hand *L. stoechas* and *V. officinalis* were observed to be active against only *S. enteritidis* with MIC values of 52.03 µg/mL and 26.02 µg/mL respectively, where *G. glabra* was active against only *E. faecalis*, with a MIC value of 52.03 µg/mL. For *V. officinalis* although an activity was observed against *B. subtilis* MIC test showed that the concentration range tested was inactive against *B. subtilis*, which means that the MIC value is higher than 3.33 mg/mL. Furthermore, MBC/MFC test showed that all the MIC values observed were bacteriostatic/fungistatic concentrations, which means they only inhibit the reproduction of microorganisms. The "cidal" concentrations, which kills microorganisms were observed to be 1.665 mg/mL for *C. sativa* against *E. faecalis*, 0.8325 mg/mL for *C. intybus* against *E. faecalis* and 3.33 mg/mL for both *V. officinalis* and *G. glabra* against *S. enteritidis* and *E. faecalis* respectively. It was not possible to identify the "cidal" concentrations for other plant extract and microorganism combinations, which presented an activity in MIC test, and were given as MBC/MFC>3.33 mg/mL.

CONCLUSION

Catechin and epicatechin are the least common flavonoids in the studied plants, whereas myricetin, naringenin and apigenin were found as more common flavonoids. However, the plant extracts seem to be rich in the studied flavonoids. According to the results of antimicrobial activities, it can be suggested that some studied plant extracts may be used as antimicrobial agents. For example, *C. Sativa* extract against *C. albicans*, *S. enteritidis*, *S. typhimurium*: *C. intybus* extract against *C. albicans*: *L. stoechas* extract against *S. enteritidis* and *V. officinalis* extract against *B. subtilis*, can be used. Furthermore, their active compounds and activities especially for *C. sativa* need to be analysed in further studies.

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CONFLICT OF INTEREST

None

ABBREVIATION USED

HPLC: High Performance Liquid Chromatography.

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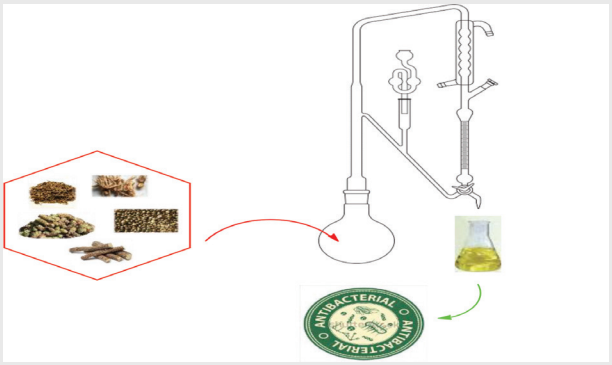
PICTORIAL ABSTRACT



SUMMARY

- *C. sativa, C. intybus, L. stoechas, V. officinalis* and *G. glabra* plants were extracted by using 65% ethanol solution.
- The antimicrobial activities of extracts were investigated against 15 microorganisms by using the disk diffusion method, MIC, MBC and MFC tests.
- Furthermore, the presence of eight flavonoids were analysed by using HPLC. The flavonoids were determined at different amounts in extracts.

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Mahmut GÜR has been working as Assistant Professor in Kastamonu University at Forest Industrial Engineering department, Kastamonu. He is interesting in the development of synthetic molecules. He has published a number of publications in different Journals in National and International Repute.

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