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
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.

**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**


**Preparation of Inocula**

All strains were incubated according to their requirements as it was previously mentioned. The inocula was prepared according to previous studies and adjusted. Thus standard inocula contained adjusted number of microorganisms.

**Disk Diffusion Test**

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

**Minimum Inhibitory Concentration (MIC) Test**

A broth microdilution MIC test was applied as mentioned before. 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.

**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.

**Statistical Analysis**

All tests were done in triplicates. Statistical analysis was conducted as mentioned in previous studies.  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: HPLC analyses results of flavonoids (µg g⁻¹ plant)

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

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

<table>
<thead>
<tr>
<th></th>
<th>C. sativa</th>
<th>C. intybus</th>
<th>L. stoechas</th>
<th>V. officinalis</th>
<th>G. glabra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 µL</td>
<td>50 µL</td>
<td>100 µL</td>
<td>20 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. albicans</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>-</td>
<td>8</td>
<td>9</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>-</td>
<td>8</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

No activity: E. aerogenes, E. faecium, E. coli, K. pneumonia, P. aeruginosa, P. fluorescence, S. infantis, S. kentucky, S. aureus, S. epidermidis

<table>
<thead>
<tr>
<th></th>
<th>C. sativa</th>
<th>C. intybus</th>
<th>L. stoechas</th>
<th>V. officinalis</th>
<th>G. glabra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC/MFC</td>
<td>MIC</td>
<td>MBC/MFC</td>
<td>MIC</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. albicans</td>
<td>26.02</td>
<td>&gt;3.33</td>
<td>13.01</td>
<td>&gt;3.33</td>
<td>-</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>13.01</td>
<td>1.665</td>
<td>6.50</td>
<td>0.8325</td>
<td>-</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>416.25</td>
<td>&gt;3.33</td>
<td>-</td>
<td>52.03</td>
<td>&gt;3.33</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>832.50</td>
<td>&gt;3.33</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

REFERENCES
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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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Ergin Murat Altuner is working as an Associate Professor in Kastamonu University, Faculty of Science and Arts, Department of Biology, Kastamonu, TURKEY. He completed his B.Sc. degree in Ankara University, M.Sc. degree in Middle East Technical University and Ph.D. degree in Ankara University.