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The Phenolic Compounds from *Hypericum Perforatum* and Their Antimicrobial Activities

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Abstract

Hypericum perforatum (St. John's wort) *(H. perforatum)* is a plant which has been used as a medicinal herb since ancient times and grow in Europe, West Asia and North Africa. In this work, the crude extracts of *Hypericum Perforatum* grown in Muğla were obtained by the extraction with acetone:water and water. The extracts were chemically investigated for their total phenol contents by the Folin–Ciocalteu method. The phenolic compounds in the extract with acetone:water (7:3, v/v, 150.44 mg GA/g material) was determined as almost five fold higher than water extraction process (33.01 mg GA/g material). The thin layer chromatographic (TLC) analysis revealed the presence of polyphenolic compounds such as tannins.

Antimicrobial activity of the samples was assayed separately using an agar diffusion method against *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Candida krusei* ATCC 4243. *Staphylococcus aureus* and *Bacillus subtilis* type organisms have showed less resistance to phenolic extract while the inhibition zone was not formed when *Candida albicans* used in the case of acetone:water extract. The phenolic compounds obtained by water extraction had inhibitory effect against *Escherichia coli* and *Bacillus subtilis* organisms.

Key Words: *Hypericum perforatum*, Tannins, Total phenol content, TLC, Antimicrobial activity, Muğla.

INTRODUCTION

Hypericum perforatum (St. John's wort) has been used for centuries in the treatment of burns, bruises, swelling, inflammation, and anxiety, as well as bacterial and viral infections. In addition, *H. perforatum* has become popular herbal medicine quickly in the world for the treatment of mood disorders, since its effectiveness in the therapy for mild to moderate depression with a smaller side effects than that of traditional antidepressant medications has been claimed in the United States. Numerous studies have proven the clinical efficacy of *H. perforatum* in both human and animal behavioral models of depression [1]. It is also a popular folk medicine for treating kidney and gallbladder stones, liver related diseases such as jaundice and liver cancer, viral infections such as hepatitis and tuberculosis, malaria, diabetes and fever [2]. Plant extracts are red fluid including a great many of biological asset matters. This plant is

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rich in antioxidants such as vitamin C, tannin, carophyliene, piene, limonene, myrecene, flavonoids, hyperic, carotene. It is a common plant in European and Anatolian.

Plants accumulate a wide variety of 'secondary' compounds, including alkaloids, terpenes and phenolics. They do have diverse biological activities ranging from toxicity to hormonal mimicry and may play a role in protecting plants from herbivory and disease. The group of phenolic compounds known as tannins is clearly distinguished from other plant secondary phenolics in their chemical reactivities and biologicial activities. Two identified tannin groups are hydrolysable tannins (derivatives of gallic acid; 3,4,5-trihydroxylbenzoic acid) and condensed tannins (flavanoids). Hydrolysis of ellagitannins yields ellagic acid and condensed tannins such as flavan-3-ol and flavonol [3]. Tannins which have high molecule weight polyphenols can form multiple hydrogen binding and inhibate enzymes [4].

TLC is a relatively cheap but powerful technique to screen plant extracts for the presence of different types of phenolic compounds. Extracts are spotted onto layers of cellulose or silica gel that are attached to glass plates or plastic sheets. Plates can then be sprayed with various reagents to detect the tannin compounds. The vanilin / HCl spray gives red / pink spots with flavan-3-ols and CTs. Under UV-light, Galloyl esters and gallotannins appear as violet fluorescent spots that are enhanced on fumigation with ammonia vapour. Ellagic acid produces a violet spot that darkens on exposure to ammonia vapour [5].

The plants are analyzed for antimicrobial activity against several microorganisms (bacteria and fungi). The phloroglucinol derivatives found frequently in the lipophilic fractions of several *Hypericum* species have demonstrated antifungal and antibacterial activities against microorganisms such as *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis* and *Nocardia garde*n [6-10]. Their presence could justify the popular use of some *Hypericum* species as wound healing agents and in the treatment of some microbiological diseases [6-11]. Other substances present in some species of *Hypericum* have also shown antimicrobial activity against various bacteria and fungi including the benzopyrans [12,13], xanthones [14], flavonoids [15] and tannins, recognized antimicrobial compounds [16].

This study represents the first investigation of the extraction, total phenol content and TLC analysis results of *Hypericum perforatum* plant grow in Muğla. The antimicrobial activity of the extracts against various test organisms were also reported.

MATERIALS AND METHODS

Materials

Muğla region's *Hypericum Perforatum* plant, Folin-Ciocalteu reagent, Na_2CO_3, butane-2-ol, acetic acid, toluene, acetone, formic acid, silica gel plate were purchased from Merck. The standart, gallic acid was from Sigma. Gentamisin (10 µg/disc) and Flukonazol (25 µg/disc) discs were purchased from Sigma Chemical Company. Brain heart infusion was purchased from Oxoid (Wesel, Germany). All other chemicals were of reagent grade and were used as received. Distilled water was used through out the research.

Preparation of Plant Extract

The aerial part of the *Hypericum perforatum* was collected from Yerkesik region of Muğla. The dried and powdered plant material were extracted with two different extraction soluution. The plant material (0.625 gr) was extracted with 25 ml distilled water at 45°C (12 h), 75°C (5 h), 100°C (1 h) and the rest of the time the solution was kept at room

temperature. The procedure was performed three times with each plant extract [17]. Then the three extracts were joined. The same procedure was performed for acetone:water (70:30, v/v) solution [18].

Analysis of Total Phenols In Extracts

Distillate water of 6 ml was added to 100 µL plant extract. And then 500 µL of Folin-Ciocalteu reagent was added, shaked for 30 sec. The final volume was completed with distilled water after addition of the saturated sodium carbonate (Na_2CO_3, 75 g/l, 1,5 ml) solution and again shaked 30 sec. The sample was incubated for 60 min at room temperature. The absorbance was measured at 725 nm and 760 nm wavelenghts in UV-Visible spectrophotometer (Labomed Inc.). Gallic acid (GA) was used as standart. The results were expressed as gram of gallic acid per kilogram of extract (g GA / kg extract) [19].

Thin Layer Chromatography (TLC) of Phenolic Compounds

One and two dimensional TLC was applied to plant extracts. The mobile phase was butane-2-ol:acetic acid:water (14:1:5, v/v/v) solvent system for two dimensions [20]. The other mobile phase was toluene:acetone:formic acid (60:60:10, v/v/v) solvent system which is used for hydrolyzable tannins [18]. TLC was performed to each 2 extracts by loading 15 µL of the extracts on a silica TLC plate. The plate is then placed into a solvent. As the solvent migrates up the plate, the various phenolic compounds are separated. When the solvent reached to the top, the plate is removed and the solvent is dried off. In order to apply the second dimension, the plate was then turned by 90° and placed into a second solvent in order to achieve separation of HTs and CTs. After the plates were dried they were sprayed with vanilin-sulphuric acid spray reagent or ammonia vapour for visualization of the spots [21, 22]. Then the plates were heated in an oven for 5 minutes at 120 C° in order to complete colour formation and observe the spots easily.

Antimicrobial Activity Test Method

Antimicrobial activity of plant extracts was tested by agar diffusion test method [23, 24]. Twenty-five mL of sterile agar (brain heart infusion) was poured into Petri dishes. The agar was left to set and 0.1 mL of an appropriate bacterial suspension was distributed in it. A 9 mm core of agar was removed from the seeded agar and the hole was closed against the dish bottom with pure agar. Holes were filled up with 0.1 mL of each plant extract. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 and *Candida krusei* ATCC 14243 were used as test organisms. The control experiments were done by incubation of cultures with solutions that did not contain phenolic compounds and also with antimicrobial pharmaceutics, gentamisin sulphate (10 µg/disc) and flukonazol (25 µg/disc).

All extraction studies and inhibition zone diameter values were repeated three times and the results were reported as average values.

RESULTS AND DISCUSSION

Total phenol content

Hypericum perforatum plant sample was extracted and studied by performing total phenols content analysis, TLC of phenolic compounds and antimicrobial activities through six various organisms. The results of analysis of total phenolics based on the absorbance values reacted with Folin-Ciocalteu's reagent, expressed as gallic acid equivalents. The results were given Table 1.

The lowest value was determined for water extract with an average value of 33.01 mg GA/g material

Table 1. The results of total phenolics of *Hypericum perforatum* in Muğla, Turkey.

	725 nm *mg GA/g material	760 nm*mg GA/g material
Extraction with aceton:water (7:3, v/v)	150.44	155.64
Extraction with water	33.01	34.38

*average of three experiments

measured in 725 nm wavelenght. The highest phenolics contents was found in % 70 aceton extract with an average value of 150.44 mg GA/g material. The presence of organic solvent, acetone caused high extraction performance of phenolics. The methanolic extraction of *H. perforatum* in ultrasonic bath for 2 h resulted with a total phenolic content of 191 mg GA/g material [25].

TLC analysis

Extracts are spotted onto layers of silica gel that are attached to plastic sheets. The bottom of the plate is then placed into the solvent and let the various phenolic compounds are separated. After the plates were dried, they were subjected to the second separation process with the same solvent system when two dimensional TLC was applied. The plates were sprayed with vanilin-sulphuric acid spray reagent for visualization of the spots with flavane-3-ols and condensed tannins. The other spray reagent was ammonia vapour which darkens violet ellagic acid spot on exposure [21].

Two dimensional TLC of different plant extracts were shown in Figure 1. The separation of phenolics were presented as dark points on the figure after exposure to ammonia vapor and vanilin-sulphuric acid spray reagent. The first separation results were given as I, II, III, IV, and V while integers represent the spots obtained from separation of the second dimension.

Under UV light, two dimensional thin layer chromatographic separation shows four and five phenolic compounds for water and acetone:water extract, respectively under the same conditions. The spot number 4 in both extract darkened after fumigation to ammonia vapour which is the indicator of ellagic acid. On the other hand, the hydrolizable tannins, galloyl esters and gallotannins, appeared as violet fluorescent spots for both extracted solutions. The spots with numbers 1, 2, and 3 in water extract and numbers 1, 2, 3, and 5 in acetone:water extract indicate the separated phenolic compounds which are greatly hydrolyzable tannins.

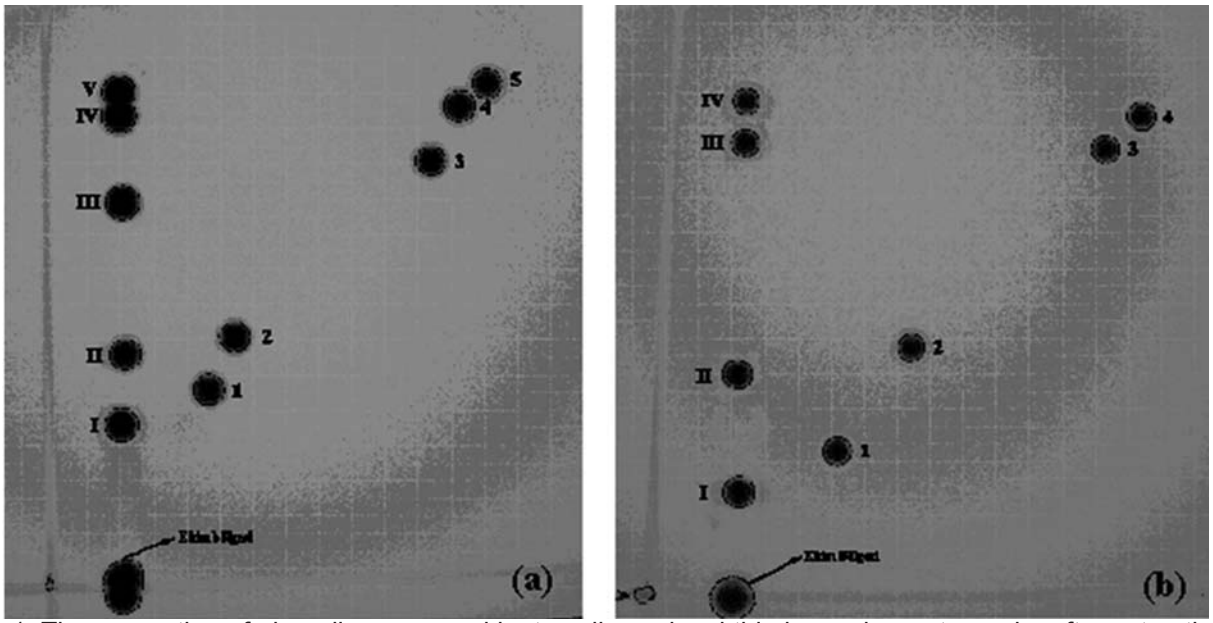

Figure 1. The separation of phenolic compound by two dimensional thin layer chromatography after extraction with (a) aceton:water (7:3, v/v) and (b) water.

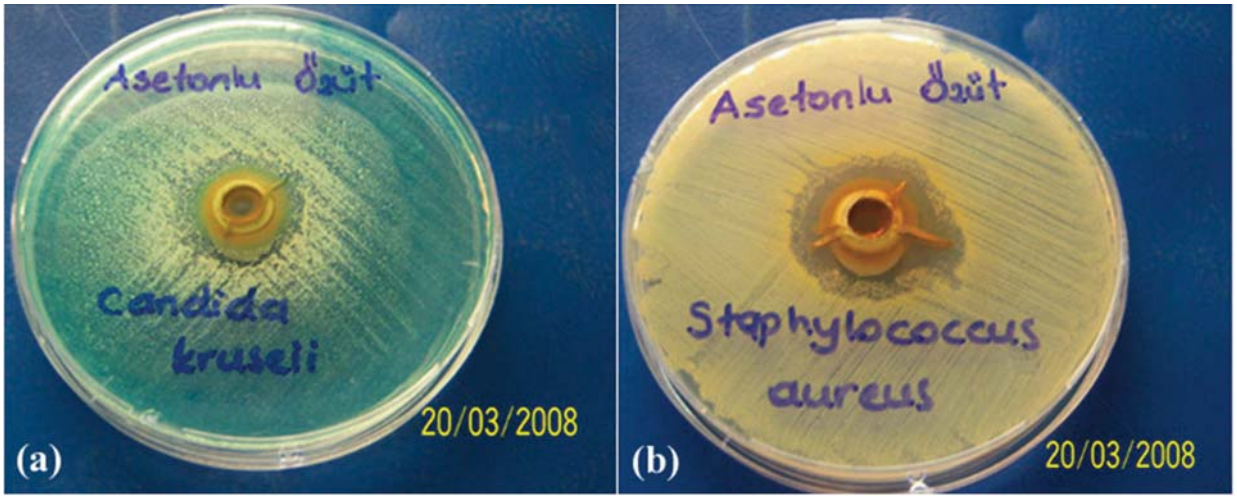

Figure 2. The formation of inhibition zones after injection of plant extract into the agar holes.

The vanillin/sulphuric acid spray gave red/pink spots with flavane-3-ols and condensed tannins which was the spots number 2 and 4 in acetone:water extract indicates the presence of high molecular weight phenolic compounds of condensed tannins. The spot number 1 in water extract gave pink colour with this spray so, again it comes from the presence of flavane-3-ols.

Toluene:acetone:formic acid (60:60:10, v/v/v) solvent system was also used to separate the extract of plant material with 70 % acetone and water systems in silica plates. It was observed that a high accumulation in the spot point is present besides the separated phenolic compounds (figure not given). The accumulation of phenols indicates that the extract contain high molecule weight of phenolic compound which are greatly tannins [23]. The similar result was not observed in the case of extraction with water. The chemical analysis revealed the presence of polyphenolic compounds such as tannins and flavonoids. [26]

Recently, the methanolic extracts from *H. myrianthum* and *H. ternum* species were evaluated chemically and tannin, flavonoid and phenolic acids were the prominent compounds [17]. The aerial parts of *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *angustifolium* were investigated for their chemical composition and antimicrobial activity. Spectrophotometric analysis indicated that *H. perforatum* subsp. *perforatum* is richer in flavonoids and tannins than the other subspecies [27].

Antimicrobial activity

Antimicrobial activity of the above mentioned samples were assayed separately using agar diffusion method as described previously. The organisms was *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, and *Candida krusei* ATCC 4243. The inhibition zone

Table 2. Antimicrobial activity of *Hypericum perforatum* extracts.

	Inhibition zone diameters (mm)			
	Extraction with water	Extraction with acetone:water	Gentamisin 10 µg/disc	Flukonazol 25 µg/disc
Pseudomonas aeruginosa	-	14	20	-
Escherichia coli	14	16	22	-
Staphylococcus aureus	-	27	21	-
Bacillus subtilis	17	22	18	-
Candida albicans	-	-	-	20
Candida krusei	-	21	-	-

diameters were reported after 24 h incubation time. Table 2 shows the inhibition zone diameters obtained for each microorganism.

The results indicate that phenolic compounds extracted with acetone:water mixture has high antimicrobial activity against all the tested microorganisms. *Staphylococcus aureus* type organism has less stability toward the phenolics of *Hypericum perforatum* and therefore the biggest inhibition zone diameter was measured. The small diameter size indicate high resistance to chemicals of the extract. The control experiments were done with each extraction solution and no inhibition zone formation was observed. The phenolics from water extraction inhibited *Escherichia coli* and *Bacillus subtilis* type organisms but acetone:water extraction leads to bigger inhibition which can be seen from the results. Figure 2 shows the inhibition of phenolics when *Candida krusei* and *Staphylococcus aureus* type organisms were used.

Three plant species, *Hypericum connatum*, *Hypericum caprifoliatum*, *Hypericum polyanthemum* (Guttiferae), growing in Southern of Brazil were chemically investigated and tested for their antiviral activity against feline immunodeficiency virus (FIV) by Schmith and coworkers. The cytotoxic properties of the aqueous extract (AE), the aqueous extract with low tannin concentration (LTCAE), and the methanolic extract (ME) were tested for their cytotoxic properties in concentrations of 50–150 µg/mlLTCAE and ME (10–50 µg/ml) were analyzed for antiviral activity by inhibition of CPE and measuring FIV genome from cell culture supernatant. LTCAE of all species in this work did not cause any inhibition of FIV while AE was toxic to Crandell feline kidney cells for the three species in all concentrations. The results suggest that some plants of the genus *Hypericum* from Southern Brazil contain compounds with potential antiviral activity against lentiviruses [26]. Six species of *Hypericum* growing in southern Brazil were extracted with methanol and crude extracts were analyzed for antimicrobial activity against several microorganisms (bacteria and fungi). None of the crude methanolic extracts showed activity against *S. epidermidis, Escherichia coli* or *Saccharomyces cerevisiae. H. caprifoliatum* showed activity against *Staphylococcus aureus* and *H. polyanthemum. H. ternum* extracts were active against *Bacillus subtilis* [17]. The chemical composition and antimicrobial activity of *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *angustifolium* were investigated. The MeOH extracts, dichloromethane and petroleum ether fractions were effective against *Staphylococcus aureus*, *S. epidermidis, Enterococcus faecalis* and *Bacillus subtilis* [27].

CONCLUSION

The chemical investigations indicated that the acetone:water extract of Hypericum perforatum contain high amount of phenolic compounds and some of these compounds showed tannic structure. The antimicrobial activity was observed against all tested organisms except *Candida albicans*. However, as indicated by TLC chromatograms, full chemical identification of both acetone:water and water extracts of the species is needed. Again high molecular weight phenolic compounds, tannins, concentrations and identification needs to be done besides their antimicrobial activity.

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