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Determination of antioxidant, antimicrobial and antitumor activity of bryophytes from Mount Ida (Canakkale, Turkey)

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In this study, active components of bryophytes were extracted from the samples collected from Mount Ida (Canakkale-Turkey). Three different extraction methods (Soxhlet, supercritical fluid extraction-SFE, Soxhlet after SFE) were employed for 2016 samples while direct Soxhlet extraction was carried out for 2017 samples using methanol as extracting solvent. Initially, antioxidant activity tests of the crude extracts were performed for all the samples. Antioxidant properties were determined to be fairly weak or moderate in the majority of the species studied. *Marchantia polymorpha* (T1), a liverwort, showed high extraction yield and antioxidant activity. Similarly, high activity was detected in *Hypnum cupressiforme* (T9) and *Neckera complanata* (T10) species. The antioxidant capacity of the tested species often sheds light on other activities. Antimicrobial properties were also tested for all extracts obtained in 2017. *Thuidium tamariscinum* (T8) was positive against *Acinetobacter haemolyticus* ATCC 19002 bactericin; T1 and *Isothceum myurum* (T11) were positive against *Bacillus subtilis TCC6633* bacteria and all other species were negative. Following these results, we have concentrated on the most active species and carried out antitumoral tests. Again, fractioned polar isolate of T1 was the only antiproliferative species against HeLa and A549 lung cancer cells.

Keywords: Antimicrobial, Antioxidant, Antitumor activity, Bryophyte.
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Bryophytes have various activities due to the wide variety of substances they contain. Recent studies have found that mosses have antioxidant activity. The richness of species in terms of active metabolite makes it possible to use it as an antioxidant as well as antimicrobial. Traditionally, bryophytes are used in the treatment of skin diseases, inflammation and antiviral treatment.[1,2] Research into the antimicrobial activity of mosses has increased in the last decade.[3-6] Some recent

One of the flavonoids, luteolin, is a commonly found glycoside in the plant kingdom. Luteolin is found in many plant families and has been identified in Bryophyta, Pteridophyta, Pinophyta, and Magnoliophyta. Preclinical studies have shown that this flavone has a variety of pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and anticancer activities. The ability of luteolin to inhibit angiogenesis, induce apoptosis, inhibit carcinogenesis in animal models, reduce *in vivo* tumor growth and sensitize cytotoxic effects of some anticancer drugs administered to tumor cells demonstrate that this flavonoid has a reduced cancer effect and chemotherapeutic potential. Modulation of ROS levels, inhibition of topoisomerase 1 and 2, reduction of NF kappa B and AP1 activity, P53 stabilization and PI3K, STAT3, IGF1R, HER2 inhibition are possible mechanisms involved in the biological activities of luteolin. Recent investigations have shown that [illegible]g and therapeutic potential.[7] Other studies confirmed presence of Communin A and B from *Polytrichum commune* as well as benzonaphthoxanthenone, ohioenesin H, and 11 new compounds. These new compounds can be used for human lung cancer (A549), human liver cancer (Hep62), human intestinal cancer and showed cytotoxic activity against 5 human cell lines such as human breast adenocarcinoma (MDA-MB-435) and human T-cell leukemia (6T-CEM).[9]

The aim of this study is to purificate and characterize chemicals from natural plant resources of Mount Ida (Canakkale, Turkey) and determination of

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their antioxidant, antimicrobial, and antitumoral properties.

Materials and Methods

Bryophyte samples

Bryophyte samples were collected from Mount Ida in November 2016 and June 2017. Voucher specimens were identified by Dr Ozlem Tonguc Yayintas and deposited in Canakkale Onsekiz Mart University, School of Applied Sciences, Turkey. Following species were investigated: *Marchantia polymorpha* L. (T1), *Polytrichum juniperinum* Hedw. (T2), *Grimmia pulvinata* (Hedw.) Sm. (T3), *Hedwigia ciliata* (Hedw.) P. Beauv. (T4), *Thamnobryum alopecurum* (Hedw.) Nieuwl. Ex Gangulee (T5), *Plagiomnium undulatum* (Hedw.) T.J. Kop (T6), *Antitrichia curtipendula* (Timmex Hedw.) Brid. (T7), *Thuidium tamariscinum* (Hedw.) Schimp. (T8), *Hypnum cupressiforme* Hedw. (T9), *Neckera complanata* (Hedw.) Huebener (T10) and *Isothecium myurum* Brid. (T11). The roots and damaged parts were removed. Above ground parts of the plant materials were used after careful cleaning, washing and drying in an oven at 40°C.

Preparation of the Bryophytes extracts

The specimens were subjected to extraction, testing and further isolation. In the extraction process, conventional automatic Soxhlet extraction, supercritical fluid extraction (SFE) and Soxhlet extraction after SFE were applied. The crude extract obtained in the solvent extraction was used in the relevant tests, and when the activity was determined, the isolation process started. For this purpose, 10-100 g mass of moss extract was fractionated with increasing polar solvent from hexane to methanol. The lower fractions (depending on the number of constituents) thus obtained were subjected again to the relevant tests. Pure organic solvents were used in the extraction process and all were supplied by Sigma. Shimadzu brand UV-GB (Japanese) spectrometer and Agilent (USA) HPLC system were used for chemical analysis. Gradient system with acetonitrile-water mobile phase for phenolic standards was used for HPLC.

Procedure of extraction

Three different extraction methods were applied for Bryophyte species. These are i) Super critical fluid extraction (SFE) modified to 5% ethanol in samples (to extract apolar and partially polar compounds), ii) automatic soxhlet extraction (with methanol to extract more polar compounds) from the remaining material after SFE and iii) direct Soxhlet extraction with methanol. These methods were applied only for 2016 samples. Direct Soxhlet extraction with methanol was applied for 2017 samples since it was the most appropriate method to obtain active principles.

In vitro antioxidant activity tests-DPPH method

Bryophyte samples tested by this method were dissolved in methanol at specific concentrations to prepare stock solutions. 50 µL of micropipette was prepared from these stock solutions and 5 mL of 0.004% (w/v) 2,2-diphenylpicrylhydrazyl (DPPH) solution was added to them and left for inhibition. Color transformations and antioxidant activities were tested by comparison with control (solvent). After incubation in the dark for 30 min the absorbance values of the samples were measured at 517 nm and the inhibition % were calculated using the absorbance values of each sample and blank control test. The inhibition % obtained are plotted versus the bryophyte extract mass, and the concentrations providing 50% color expression are calculated as IC_{50} values[10].

Antimicrobial activity tests

Agar well diffusion method was used to investigate the antimicrobial effect of bryophyte extracts. The test microorganisms (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus fecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 90028) adjusted to the McFarland 0.5 concentration were cultivated by the Muller Hinton Agar spreading method (of $5x10^5 CFU.mL^{-1}$). Subsequently, wells with a diameter of 6 mm were opened on the medium and extracts were placed in 50 µL volumes in each of the wells. 50 µL volume of sterile distilled water was used as a negative control, ampicillin (10 µg) and chloramphenicol (30 µg) were added to bacterial plates in 50 µL volumes, and amphotericin B (10 µg) was added to yeast cultures as positive controls. Plates were incubated at 37°C for 24 and 48 h, and at the end of the incubation period, inhibition zones were observed around the wells.

Antitumoral (Cytotoxicity) tests

The cytotoxic effect of the test substances was studied using cancer cell lines of A549 and HeLa cells. Briefly, cell lines were first replicated in T75 cell culture dishes at 37^0C, 5% CO_2 using appropriate media (RPMI 1640, DMEM or EMEM containing 10% FBS, gentamicin/fungicide). The cells prepared in the form of $1x10^6$ mL^{-1} suspension when they are to

be used in the test were dispensed into 96-well cell culture plates as 100 µL portions and left to incubate for 24 hours at 37°C, 5% CO_2 and humidified medium. Test items and negative controls after adding half-log (3.16 fold) dilutions of triplicate (100 µL for each well) starting from 200 µg/mL in culture medium containing 10% FBS and gentamicin/fungizone, cells were incubated in the presence of test substance for 48 h at 37 °C, 5% CO_2 and humidified environment.

Culture plates, including control wells without test substance, were subjected to MTT (3- [4,5-dimethylthiazole-2,5-diphenyl tetrazolium bromide) test to determine the cell growth intensity at the end of the incubation period. The different doses of the extract (25, 50, 100 and 200 µg/mL) were tested for 24 h on A549 and HeLa cells ($1x10^6$ per mL) cultured on 96 well microplates. After 24 h, the cells were washed with PBS and incubated with 1 mg/mL MTT (DMSO) to 100 µL per well, and the resulting color density was read on plates at 570 nm on an EZ read 400 microplate reader (Biochrom, Cambridge, UK). The experiments were carried out in four replicates and absorbance values for extract concentration were plotted.

In such studies, the selective toxic effect of test substances on cancer cells (selective toxicity index = SI) was found to be normal in non-linear dose response curve (cell cytotoxicity 50, CC50) (SI = Normal cell toxic effect / Cancer cell toxic effect) compared to the toxic effect (CC50) of human cell (human fibroblast cell=HFC). However, 50% cytotoxicity concentrations were not calculated as the working concentrations were for screening purposes and were kept at a wide range of concentrations. CC50 and SI values can be determined after the approximate concentration range of the active species. These studies can be continued with the species that are specifically active in the progeny.

Result and Discussion

Extraction of Bryophyte species and determination of antioxidant activity

Bryophyte species have been subjected to extracorporeal extractions given in the material and method section. Antioxidant activity measurements was evaluated as the concentration giving the 50% inhibition (IC_{50}), from the Concentration-% Inhibition graph (Fig. 1).

The IC_{50} value of each sample was determined from the corresponding graph and given in Table 1.

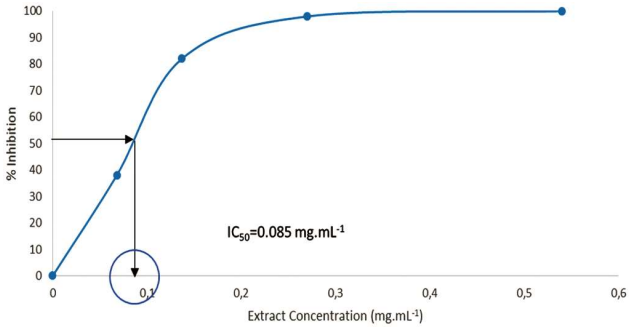

Fig. 1 — Typical graph of concentration-% inhibition for *Marchantia polymorpha*

Table 1 — Antioxidantactivities (IC_{50}:mg/mL) of bryophytesbyradicalscavengingcapacitymethod (DPPH)afterdifferentextractionmethods

Sample	*November 2016* Super Critical Extraction (SFE) Modified with 5% Ethanol		*November 2016* Soxhletextraction with methanol after SFE		*November 2016* Direct Soxhlet extraction with methanol		*June 2017*,*** Direct Soxhlet extraction with methanol			
	Average	Activity	Average	Activity	Average	Activity	Average	Activity	Average	Activity
T1	0.230	Partly weak			0.250	Partly weak			0.085	Very high
T2	-	Not observed	-	Not observed						
T3	-	Not observed	-	Not observed						
T4					0.220	Partly weak				
T5	-	Not observed	-	Not observed						
T6					-	Very weak			0.750	Weak
T7									0.260	Partly weak
T8									0.240	Weak
T9									0.085	Very high
T10									0.075	Very high
T11									0.590	Weak

*The samples collected during the period of June 2017 were analyzed after direct soxhlet extraction with methanol due to high extraction yield and high antioxidant activity.

**In these studies, samples of June 2017 were only subjected to direct soxhlet extraction with methanol because the activity was observed intensively in.

As can be seen in Table 1, T1 and T4 methanol extracts obtained by directly Soxhlet extraction showed weak activity in November 2016. T1, T9 and T10 species showed high antioxidant activity in June 2017 period. These results show that these species potential natural antioxidant source especially in the field of health and food industry.

Determination of antimicrobial activity of Bryophyte species

In current study, Results obtained with the agar well diffusion method in order to investigate the antimicrobial effect of bryophyte species extracts collected in June 2017 are given in Table 2.

As shown in Table 2, T8 was positive against *A.haemolyticus* ATCC 19002 bactericide. T1 and T11 were positive against *B. subtilis* TCC6633 bacteria, but all other species were negative.

Determination of antitumoral activity of Bryophyte species

Due to its high antioxidant and antimicrobial activity, the antitumoral effect of T1, the liverwort species, and moss species (T9and T10) were investigated. The antiproliferative effect obtained from the indicator absorbance values for different concentrations (µg/mL) of the extracts, are plotted for T1 (Fig. 2).

As can be seen in Fig. 2, the viability of Hela cells decreased and the proliferation was inhibited by increased concentration of T1-butanol fraction. Proliferation of cells was inhibited by 13.04% at 25 µg/mL dose, 29.01% at 50 µg/mL dose, 40.83% at 100 µg/mL dose and 81.57% at 200 µg/mL dose. Similarly, the proliferation of A549 cells was inhibited by 8.75% at 25 µg/mL dose, 10.26% at 50 µg/ml dose, 18.26% at 100µg/mL dose and 46.60% at 200 µg/mL dose. T1extract showed cytotoxic and antiproliferative effects on both HeLa and A549 cell lines, but seems to be more effective on HeLa cells. The crude methanol (unisolated) extract of T9 increased its antiproliferative effects on HeLa cancer cells. Proliferation was inhibited in HeLa cells by 11.92% at the dose of 25 µg/mL, 18.73% at the dose of 50 µg/mL, 38.01% at the dose of 100 µg/mL and 54.47% at the dose of 200 µg/mL in the Hela cells after the extract application. T9 extract has a strong antiproliferative effect on HeLa cancer cells and moderate antiproliferative effects in lung A549 cells.

However, the antiproliferative effect of T10 crude extract on HeLa cancer cells are weak. All doses of the crude methanol extract T10-isolation decreased cell viability at certain doses. However, it is thought that this decrease does not differ between doses, and that the extract is not effective on A549 lung cancer cells.

Isolation of active components

Because of its high antioxidant and antimicrobial activity, isolation studies of active ingredients from T1 were performed. For this purpose, a total of 16 g samples were used. Firstly, for example, a methanol extract was prepared by the Soxhlet system and the antioxidant activity was found to be 0.0846 mg/mL by DPPH method. A series of increasing polarity extractions were performed to separate the active components. These processes are schematically

Table 2 — Results of antimicrobialactivitytests of Bryophytes species extracts by agar diffusion method (June 2017)

Bacteria Tested	*M. polymorpha* (T1)	*P.undulatum* (T6)	*A. curtipendula* (T7)	*T. tamariscinum* (T8)	*H. cupressiforme* (T9)	*N. complanata* (T10)	*I. myurum* (T11)
S. aureus ATCC 25923	-	-	-	-	-	-	-
E.faecalis ATCC 29212	-	-	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-	-	-
K. pneumoniae ATCC 13883	-	-	-	-	-	-	-
P. aeruginosa ATCC 27853	-	-	-	-	-	-	-
A. haemolyticus ATCC 19002	-	-	-	+ (7-8 mm)	-	-	-
B. subtilis ATCC 6633	+ (11 mm)	-	-	-	-	-	+ (9 mm)
C. albicans ATCC 10231	-	-	-	-	-	-	-

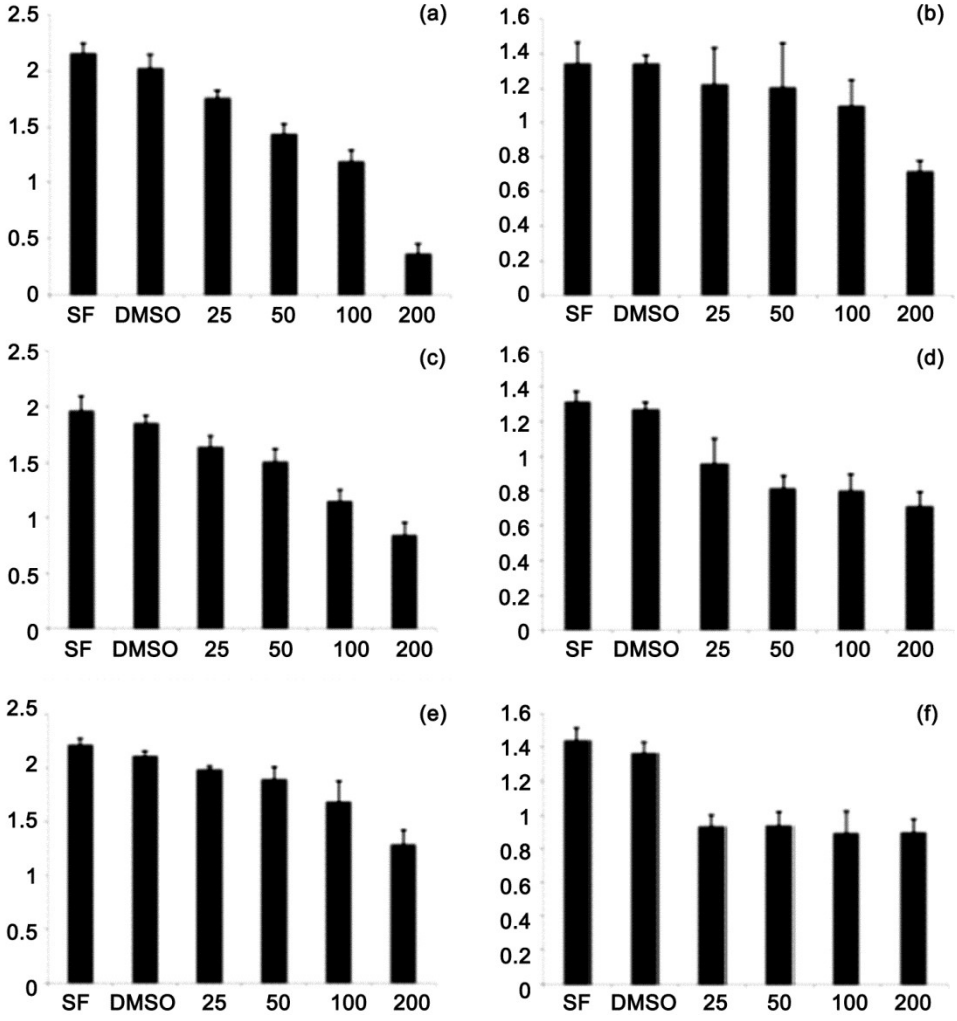

Fig. 2 — *Marchatia polymorpha*-post-isolation butanol extract a) Antiproliferative effect on HeLa Cancer Cells, *b)* A549 antiproliferative effect on lung cancer cells. *Hypnum cupressiforme* crude extract *c)* Antiproliferative effect on HeLa Cancer Cells, *d)* A549 antiproliferative effect on lung cancer cells. *Neckera complanata* crude extract *d)* Antiproliferative effect on HeLa Cancer Cells, *e)* A549 antiproliferative effect on lung cancer cells.

shown in Fig. 3. After each isolation step the solvent was removed in the evaporator and extract was subjected to the DPPH test again. DPPH test was carried out all samples and findings were given in Table 3.

Aqueous phase seems to be very active and contains the active principles. Therefore,the aqueous phase was subjected to further HPLC analysis to determine its phenolic acid contents. The phenolic acid content of this sample was determined by an optimized HPLC method and quantifications were carried out by phenolic acid standards. The contents of phenolic acids are given in Table 4.

The major phenolic acid was rosmarinic acid (73.14%) which exhibits high antioxidant properties. Thus confirming the high antioxidant activity.

Table 3 — DPPH test results of theextracts

Extract	DPPH Test (IC_{50}) (µg/mL)
Hexane Extract	297
Chloroform Extract	No activity
Ethyl Acetate Extract	No activity
Aqueous Phase	75
n-Butanol Extract from Aqueous Phase	70

However, previous studies and publications do not provide information on the determination of rosmarinic acid in T1. It is generally known to contain gallic acid,vanillic acid and caffeic acid but not rosmarinic acid. Its presence should be confirmed by LC-MS methods.On the other hand, other phenolic compounds might be present in aqueous phase. Buthanol extraction

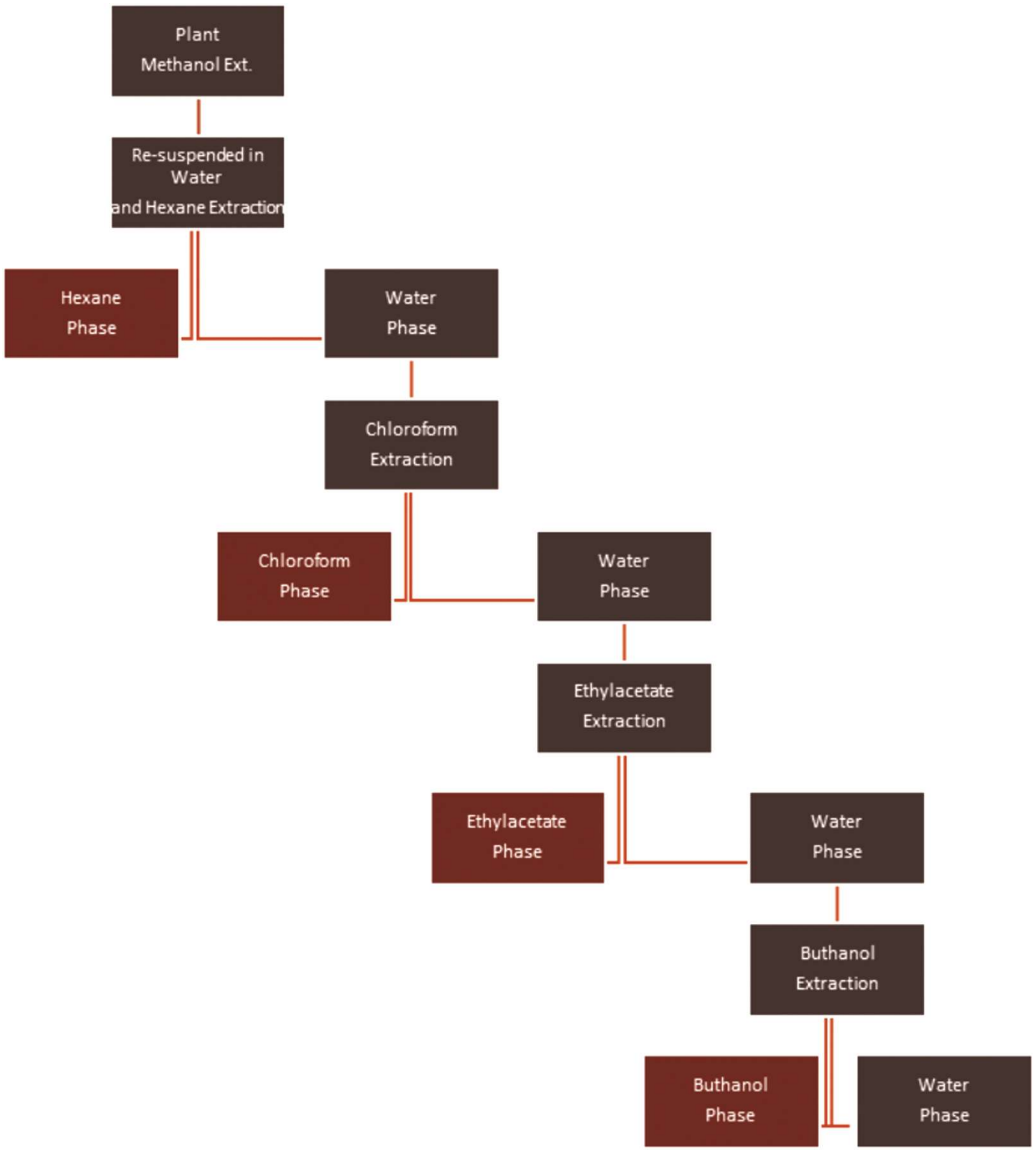

Fig. 3 — Separationsteps of activecomponents

Table 4 — Phenolicacidcontent of *Marchantia polymorpha*

Compound Number	Compound Name	Retention Time	Area	Area%	Height	Calculated Quantity
1	Gallic Asit	4.941	37.579	12.24	3449	0.090
2	4-Hydroxy benzoic acid	13.758	9608	3.13	1153	0.047
3	Chlorogenic Acid	14.112	6985	2.28	551	0.031
4	Vanillic Acid	16.127	8522	2.78	856	0.036
5	Caffeic Acid	16.644	5427	1.77	505	0.012
6	Syrinic Acid	17.304	3191	1.04	354	0.008
7	Kumaric Acid	23.839	3920	1.28	336	0.006
8	Routine	28.024	1333	0.43	122	0.012
9	Benzoic Acid	31.129	871	0.28	124	0.015
10	Cinnamic Acid	34.058	1349	0.44	100	0.002
11	Rosmarinic Acid	37.031	224521	73.14	20076	1.199
12	Quarcetin	42.742	3674	1.20	609	0.190

was carried out to separate possible phenolic compounds from aquous phase. As seen from Table 3 buthanol phase was quite active. To determine its content a rough analysis of these components was carried out by thin layer chromatography (TLC) taking the literature data into account. The sample taken from the butanol extract was first dissolved in butanol and the silica gel was applied to the TLC layer. A mixture of n-butanol-water-acetic acid (12: 2: 1) was prepared to separate the components and placed in a TLC tank. The layer was placed in the mobile phase to separate the components. R_f values were found to be 0.90 for apigenin and 0.81 for luteolin. Literature data indicate that apigenin first follows luteolin and $R_f$values are 0.90 and 0.81, respectively. The appearance of the TLC layer under the 254 nm UV lamp is compatible with this data. Probably these two components are responsible for the activity.

Conclusion

As a result, In this study, active compounds obtained from T1*,* T9*,* and T10 obtained from purification and isolation of some chemicals from our plant sources are natural and novel and are an important antioxidant that can be applied to the industry and especially to the health sector for use in medicine, food, cosmetics and other industrial sectors in general as potential antimicrobial, antiviral and antitumoral sources.

Acknowledgement

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