Anadolu Üniversitesi Bilim ve Teknoloji Dergisi A- Uygulamalı Bilimler ve Mühendislik
Anadolu University Journal of Science and Technology A- Applied Sciences and Engineering

ANADOLU UNIVERSITY

2017 - Volume: 18 Number: 2
Page: 439- 455
DOI: 10.18038/aubtda.284496
Received: 06 January 2017 Revised: 25 April 2017 Accepted: 09 May 2017

IN VITRO BIOLOGICAL ACTIVITY STUDIES ON *TANACETUM ABROTANIFOLIUM* (L.) DRUCE (ASTERACEAE)

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ABSTRACT

Tanacetum L. (Asteraceae) species have been used by local people in folk medicine for centuries in Turkey to treat antipyretic, headache, tinnitus, dizziness and against difficulties in childbirth. We performed detailed biological activity screening tests of *Tanacetum abrotanifolium*, collected from its natural habitats in the eastern region of Turkey, for the first time. To obtain active compound from aerial parts of *T. abrotanifolium* by using chromatographic techniques, anticancer, antimicrobial and antioxidant assays were applied. Total phenol, flavonoid, β-carotene and lycopene contents were determined quantitatively. The isolation procedure was carried out successfully through thin layer and column chromatographies. Successively fractionation and chromatographic process led to the isolation of luteolin-7-*O*-glucoside, an agent of anticancer, antimicrobial and antioxidant. This active compound was isolated from aerial parts of *T. abrotanifolium* for the first time.

Keywords: *Tanacetum abrotanifolium*, Luteolin-7-*O*-glucoside, Anticancer activity, Antimicrobial potency, Antioxidant capacity

1. INTRODUCTION

The unique natural products isolated from plants are major sources for anticancer, antimicrobial and antioxidant compounds. Including the most ancient civilizations, people always benefited from plants to prevent or treat diseases and from their therapeutic properties originating from their biologically active natural compounds. There is a growing interest on plants for the discovery of new drugs [1,2]. The natural compounds isolated from plants and plant extracts provide unlimited opportunities for novel and suitable additives and drug treatments because of their unmatched range of chemical diversity [3,4]. Plant extracts containing physiologically active phytochemicals have immense potential for producing new drugs with great benefit to mankind. In this context, a systematic screening of secondary metabolites of folk herbs and medicinal plants may result in the discovery of novel and effective anticancer, antioxidant and antimicrobial compounds [5].

The studies showed that some famous species of *Tanacetum* L. like Feverfew (*Tanacetum parthenium* L.) and tansy (*Tanacetum vulgare* L.) exhibit significant anti-inflammatory, anticancer, antioxidant and antimicrobial effects. Parthenolides (sesquiterpene lactone) and flavonoids were held responsible for these powerful biological activities [6-10].

According to our detailed literature search, there is a limited number of the study regarding the type of *T. abrotanifolium* [11,12]. For this purpose we have tried to determine the bioavailability of this *Tanacetum* species. Firstly, the aerial parts of the plant were extracted with different solvents using fast and reliable methods to determine antioxidant activities such as ABTS cation radical scavenging activity, DPPH free radical-scavenging activity, antioxidant capacity by phosphomolybdenum method, ferrous chelating capacity and reducing power. Likewise quantitative amounts of bioactive compounds in these extracts such as the total phenol, total flavonoids, β-carotene and lycopene were determined. Secondly, antimicrobial activities were determined against two yeast and six bacterial species which contain bacterial species of gram positive and gram negative. Thirdly, anticancer activities were tested

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against cancer cells HeLa (Human Endometrial Cancer Cells) and C6 (Rat Brain Tumor Cells). Finally, isolation of active compound was performed in the solvent system from the active extract.

2. MATERIALS AND METHODS

2.1. Plant Material

T. abrotanifolium was collected from the vicinity of Ilicalar Çır waterfall and around in the province of Bingol in Turkey in July 2011. Mentioned place is situated in height 2400 m and coordinates N:40°39′1449″-E:39°09′3661″. The species identification of plant material was determined by Prof. Dr. Lütfi BEHÇET, he is a botanist at Biology Department in Bingöl University and it was stored in the Herbarium of the University of Bingöl (Voucher No: BIN 780).

2.2. Test Microorganisms and Cells

To determine the antimicrobial activities, a total of eight microorganisms including six bacteria species (*Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* EMCS, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* NCTC 5384, *Salmonella enterica* ATCC 13311) two yeast species (*Candida albicans* ATCC 96268, *Saccharomyces cerevisiae* RSKK 04017) have been used. For antiproliferative activities C6 and HeLa cells were used.

2.3. Extraction and Isolation Procedures

Aerial part of the plant was pulverized with laboratory type mill, dried in the shade in a chamber. For biological activity tests; powdered plant samples (2 g) were macerated in different solvent systems including *n*-hexane, dichloromethane, acetone, ethyl acetate, methanol: dichloromethane (1:1), methanol and cold water separately. After maceration process solvents in extract solutions were evaporated at 40°C under low pressure. After evaporation process, the solvent free extracts were obtained including *n*-hexane (HE), dichloromethane (DE), acetone (AE), ethyl acetate (EE), methanol: dichloromethane (1:1, MDE), methanol (ME) and cold water (CWE) extracts. All operations were performed in triplicate. For isolation procedure; 980 g of powdered plant sample was macerated with solvent system of methanol: dichloromethane (5x5L; 1:1;v/v). Concentration of the extract under vacuum gave 178 g of dry crude extract. 170 g portion of the extract was applied to coarse separation using vacuum-liquid chromatography (10x100 cm; silica gel Merck). *n*-Hexane, dichloromethane, ethyl acetate and methanol were used as mobile phases to obtain Fr 1, Fr 2, Fr 3 and Fr 4 coded fractions, respectively. As sub-fraction 3 (Fr 3) exhibited high biological activities, isolation process was continued with this fraction. For this, a glass column (4x140 cm) and Silica gel 60 (500 g, Merck) were used. Isolation process was initiated with methanol: dichloromethane (1:22; v/v) solvent system, polarity was increased gradually and finished with 100% methanol, and fractions were collected in 200 mL volumes (120 fractions in total). Similar fractions were combined according to their thin layer chromatography (TLC) profiles. Fractions 60-100 gave a pure compound (PC) in the amount of 78 mg as a yellowish solid (Figure 1).

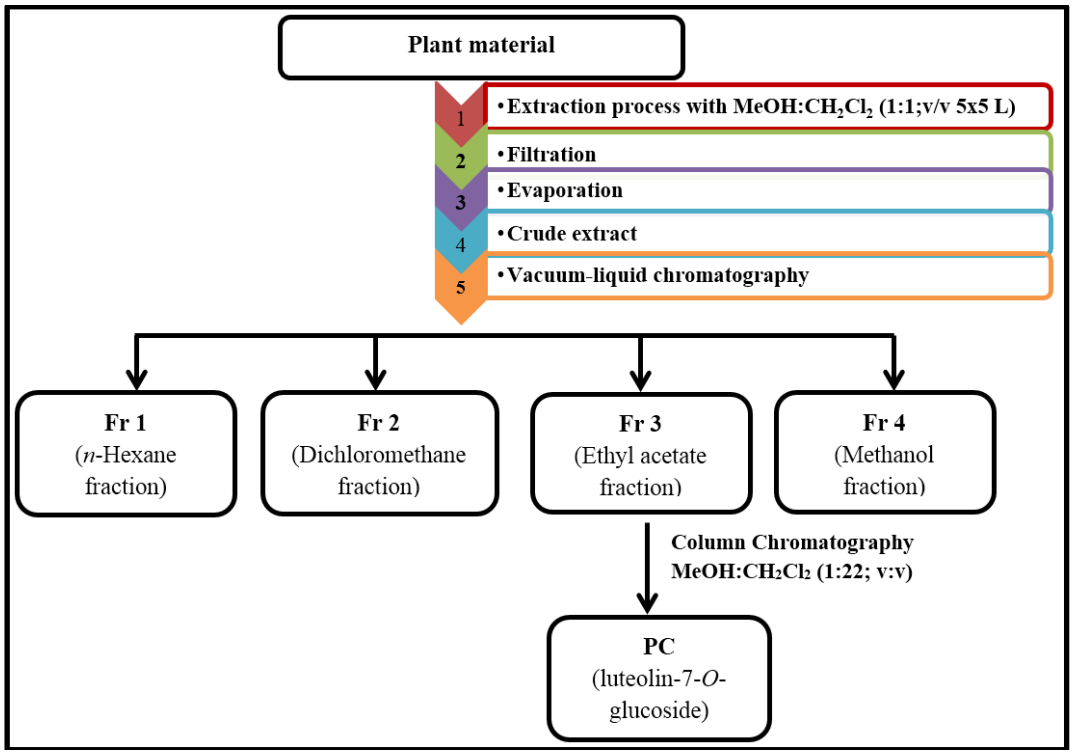

Figure 1. Flowchart for isolation process of PC from aerial parts of *T. abrotanifolium*

2.4. Quantitative Determination of Bioactive Secondary Metabolites

2.4.1. Determination of total phenolic content

Total phenolic content (TPC) of samples was determined using the Folin-Ciocalteu reagent according to the method by Gecibesler et al. [13]. 100 µL of each sample at a concentration of 1 mg/mL pipetted into a test tube, along with 4.5 mL of distilled water, 100 µL Folin–Ciocalteu reagents then fully mixed. After incubation for 10 min at laboratory conditions, 3 mL of 20% (w/v) Na_2CO_3 was added to each test tube, stirred and placed in a water bath at 40°C for 20 min. Samples were cooled to laboratory temperature and absorbance were recorded at 760 nm by UV-VIS spectrophotometer. Experiments were repeated three times for each sample. The total amount of phenolic compounds was determined as the gallic acid equivalent (GAE) using an equation obtained from a standard gallic acid (y = 0.0015 x total phenols (µg GAE/g dried weight (DW)) + 0.0178, r^2:0.9984).

2.4.2. Determination of total flavonoid content

For the total flavonoid content (TFC), the method based on Dewanto et al. [14] with a minor modification was used. At a concentration of 1 mg/mL sample solutions were mixed with 150 µL $NaNO_2$(aq) (15% in water) and left at laboratory conditions for 5 min before adding 75 µL of $AlCl_3$ (10% in water). After 5 min, 1 mL of NaOH(aq) (4% in water) was added. Total volume was adjusted to 5 mL with distilled water and mixed. The total flavonoid content in the samples was measured spectrophotometrically (UV-VIS) at 510 nm. Different concentrations of quercetin (15–480 µg/mL) were used for calibration. The quantification was carried out using a calibration curve (y = 0.0018 x total flavonoid (µg QEE/g dried weight (DW)) + 0.0253, r^2:0.9973). The results were expressed in µg quercetin equivalents (QEE)/g dried weight as mean of tree replicates.

2.4.3. Determination of β-carotene and lycopene content

β-carotene and lycopene amounts in the extracts were determined according to the method of Nagata and Yamashita [15]. The dried extract (100 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm. Content of β-carotene and lycopene was calculated according to the following equations:

$$\text{Lycopene (mg/100mL)} = -0.0458\text{x}A_{663} + 0.372\text{x}A_{505} - 0.0806A_{453} \quad (1)$$

$$\beta - \text{carotene (mg/100mL)} = -0.216\text{x}A_{663} - 0.304\text{x}A_{505} + 0.452A_{453} \quad (2)$$

The assays were carried out in triplicate; the results were mean values ± standard deviations and expressed as mg β-carotene or lycopene/100 g dried weight.

2.5. Real-Time Cell Proliferation Assays

Antiproliferative tests were performed against HeLa and C6 cells by using xCELLigence Real Time Cell Analyzer (RTCA) in an incubator (5% CO_2 and humidity, 37°C). Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 2% penicillin-streptomycin was used as cell culture medium during assessments. Firstly, 50 µL of medium was added to each well of E-Plate 96 and the plate was left in the hood for 15 min and in the incubator for 15 min to let both the E-Plate's golden electrode well bottoms and medium reach a thermal equilibrium. Then, the E-Plate was inserted into the RTCA station in the incubator and a background measurement was performed. After ejection of the E-Plate from the station, 100 µL HeLa cell suspensions were added to the wells to obtain a $1.0\text{x}10^4$ cell/well and $2.0\text{x}10^4$ cell/well concentration in each well for MDE and PC respectively, except three wells. These wells were left without cell to check if there would be an increase in cell index (CI) originating from the medium. 100 µL medium was added to these wells instead of cell suspension. After leaving the E-Plate in the hood for 30 min, the E-Plate was inserted into the RTCA station and the second step measurement was initiated for 60 min for MDE tests and 80 min for PC tests. In this period, the cells adapted to the bottom of the wells and entered into a growth and division phase. After this step, the E-Plate was ejected from the station. The solutions of MDE and PC in dimethyl sulfoxide (DMSO) (final concentration of DMSO was less than 1% in each wells) and medium were added to the wells to obtain final concentrations of 250 and 500 µg/mL of MDE and 100, 50 and 10 of PC µg/mL in each well. The final volume of the wells was completed to 200 µL with medium. After this addition step, the E-Plate was inserted into the station and the main measurement period was initiated for 48 hours. All the measurements were done in 10 min intervals and triplicated.

2.6. Determination of Antimicrobial Activity by Disc Diffusion

The antimicrobial activity of the plant extract or compound was determined by disc diffusion method with slight modifications [16,17]. The dried plant extracts or compounds were dissolved in DMSO at a concentration of 10 mg/mL. Prior to use the plant extracts were filtered using nylon membrane filter (0.22 µm). From suspension cultures of microorganisms used in the test 200 µL aliquots, that contains approximately 106 colonies according to the equation Mc Farland 0.5, was transferred to petri dishes containing Mueller Hinton Agar allowed to spread to the surface homogenously. Then, samples of 10 µL were injected into 6 mm diameter discs and placed with the help of a sterile forceps to petri dishes containing medium and microorganisms. Petri dishes incubated for 1 hour at 4°C then for 24 hours at 37°C. Negative control was used by injecting DMSO of 10 µL on the same size discs. Gentamicin was used as reference antimicrobial agent. Anti-microbial activity was determined by measuring the diameter of the inhibition zone in millimeters, including disc diameter (6 mm) with digital calipers.

2.7. Determination of *In vitro* Antioxidant Activities

2.7.1. DPPH free radical-scavenging activity

The samples were tested with DPPH free radical according to the method previously defined by Zovko Koncic et al. [18] with some modifications. Shortly, 0.5 mL of samples prepared at different concentrations (12.5–400 µg/mL) were taken into test tubes and stirred with 2.5 mL of 2 mM DPPH solution. The mixture was stirred thoroughly and incubated for 30 min in dark laboratory conditions. The absorbance at a wavelength of 517 nm was measured by UV spectrophotometry. DPPH free radical scavenging activities (FRSA) were calculated using the following equation.

$$\mathrm{FRSA}(\%) = [\mathrm{A}_0 - \mathrm{A}_1/\mathrm{A}_0]\mathrm{x}100 \qquad (3)$$

A_0 is the absorbance value without specimen and A_1 is the absorbance in the presence of specimen. As opposed to increasing concentration of specimens, decline of absorbance is an indication that destroyed DPPH radical. Antioxidant activity results are expressed as IC_{50} value (µg extract/mL) that reduces by half the effective concentration of DPPH radicals and was calculated by interpolation from linear regression analysis.

2.7.2. Ferrous chelating capacity

The ferrous chelating capacity of samples was conducted following the method used by Decker and Welch [19] with slight modifications. 2 mL of samples of concentration labeling between 25 and 250 µg/mL were added to 50 µL of 2 mM $FeCl_2$(aq). The reaction mixture was well mixed and incubated in laboratory conditions. The reaction was initiated by the addition of 100 µL ferrozine (5 mM). After 10 min of incubation period, the absorbance of the solution was measured at 562 nm using a UV–visible spectrophotometer. For the ferrous-chelating activity, IC_{50} values were calculated using the equation as described above was used for DPPH free radical scavenging activity.

2.7.3. Assay of reducing power

The reducing power was determined according to the method of Oyaizu [20] with slight modifications. Various concentrations of samples (2.5 mL) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 mL of 10% trichloroacetic acid (w/v) addition, the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. BHA, BHT and α-tocopherol were used as standards. It was indicated that high absorbance of the sample was good reducing power in the reaction conditions.

2.7.4. Evaluation of total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of samples was evaluated by the method of Prieto, Pineda, and Aguilar [21] with slight modifications. The antioxidant capacity of the samples was measured spectrophotometrically using a phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of specific green phosphate/Mo(V) compounds. An aliquot of 0.1 mL of sample solution (100 µg/mL) was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent

used for the sample and it was incubated under same conditions as rest of the sample. Stock solutions of α-tocopherol were prepared in methanol. The total antioxidant activity was expressed as equivalents of α-tocopherol (mmol α-tocopherol/g of DW).

2.7.5. ABTS cation radical scavenging activity

To determine the antioxidant activity of samples, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) cation radical-scavenging activity was employed in this study with slight modifications [22]. The cationic ABTS radical was generated by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was kept at ambient temperature for 24 h until the reaction was complete and the absorbance was stable. Briefly, 1.9 mL of an $ABTS^{\bullet+}$ was added to 0.1 mL of different concentrations of standard (trolox) or samples. After 10 min, the absorbance was read at 734 nm, and distilled water was used as a blank (each measured in triplicate). A standard curve was obtained from different concentrations of $ABTS^{\bullet+}$ (10-400 µg/mL). To determine the $ABTS^{\bullet+}$ remaining in the reaction medium was used an equation ($y = 1.691 \times [ABTS^{\bullet+}] - 0.0016$, r^2:0.9976). The IC_{50} is the concentration of an antioxidant that is required to quench 50% of the initial $ABTS^{\bullet+}$ under the experimental conditions. Trolox, a well-known antioxidant, was used as a positive control.

2.7.6. Statistical analysis

All the experimental data results were carried out in triplicate. The results were expressed as mean value and standard deviation (mean ± SD). Differences between the samples were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test using SPSS software version 23 and $p < 0.05$ probability level was regarded as significant.

3. RESULTS AND DISCUSSIONS

3.1. Bioactive Secondary Metabolites

In this study, aerial parts of *T. abrotanifolium* were extracted using seven different solvents that are *n*-hexane, dichloromethane, acetone, ethyl acetate, methanol, methanol: dichloromethane (1:1), and distilled cold water. The effects of these solvent systems in extracting phenolics, flavonoids and carotenoids from the plant were quantitatively measured and compared. Among all the extracts, MDE has the highest value of TPC, hence its extraction solvent is considered as the most efficient solvent system for extracting phenolic compounds from *T. abrotanifolium*. The percentage of extractable compounds ranged from 1.65 % to 16.09 % based on dry plant material (Table 1). The total phenolic content of the extracts of *T. abrotanifolium* varied between 22.36±0.83 µg and 352.33±1.85 GAE µg/g DW. The results given in Table 1.

The total flavonoid content was assayed by aluminum colorimetric assay, calculated from regression equation of calibration curve and expressed in quercetin equivalents. The data presented in Table 1 indicates that the highest flavonoid content of 206.11±1.91 µg quercetin/g of dried samples was found in the MDE extract of *T. abrotanifolium*. Table 1 shows a list of the total phenolic (TPC), total flavonoid (TFC), lycopene and *β*-carotene content of the seven extracts of *T. abrotanifolium*. The differences in polarities of the solvents might influence the chemical composition and yields of the extracts or fractions. Therefore, the selection of an appropriate solvent system is one of the most important steps in optimizing the recovery of TPC, TFC, lycopene, *β*-carotene and other bioactive compounds from a sample.

Table 1. The percentage of yield and phytochemical compositions of crude extracts in different solvents extracted from *T. abrotanifolium*

Extract	Yield (%)	Total phenol µg GAE/g DW	Total Flavonoids, µg QEE/g DW	*β*-carotene (mg/100 g DW)	Lycopene (mg/100 g DW)
HE	1.65	nd	nd	28.72±2.82^{a}	41.6±0.22^{a}
DE	3.1	81.17±0.15^{c}	nd	113.41±1.69^{d}	73.9±2.93^{c}
AE	14.95	162.12±3.22^{d}	112.97±4.84^{c}	97.59±0.23^{c}	127.1±1.12^{d}
EE	12.05	63.26±0.14^{b}	85.34±6.86^{b}	34.85±1.72^{a}	49.6±2.22^{b}
MDE	16.09	352.33±1.85^{e}	206.11±1.91^{e}	59.72±2.59^{b}	65.3±1.95^{c}
ME	9.1	246.16±1.71^{e}	142.53±4.28^{c}	41.65±1.72^{a}	24.6±0.42^{a}
CWE	12.3	22.36±0.83^{a}	34.33±0.19^{a}	nd	nd

Abbreviations: GAE: gallic acid equivalent; QEE: Quercetin equivalent; DW: dry weight. nd: not determined; HE: Hexane Extract DE dichloromethane Extract, AE: Acetone Extract, EE: Ethyl acetate Extract, MDE: Methanol: Dichloromethane (1:1) Extract, ME: Methanol Extract, and CWE Cold Water Extract, Values correspond to the mean ± standard error. Values with different letters are significantly different ($p < 0.05$).

As comparisons were made between the seven solvent extractions of *T. abrotanifolium*, MDE gave the highest score of TFC whereas CWE gave the lowest score. Thus, solvent system of MDE may also be considered as the most efficient solvent system for extraction of flavonoids from the plants. This solvent system was often used as an extraction solvent system for flavonoids in many studies [23, 24]. Flavonoids were well known to occur in plant extracts and to possess many different biological activities [25].

The *β*-carotene and lycopene contents were determined by measuring the absorption of crude extracts at different wavelengths. The rich *β*-carotene and lycopene contents were evidenced for DE extract with a value of 113.41±1.69 mg/100 g DW and AE extract with a value of 127.1±1.12 mg/100 g DW, respectively, among different polarity solvent extracts in contrast to CWE extract. Earlier, significant amounts of β-carotene and lycopene were analyzed in other solvent-dependent extracts. In a previous study, β-carotene contents in medicinal aromatic plants were analyzed at range of 34.22 ± 0.22 and 183.05±0.75 mg/100 g DW [26]. Karaboduk et al. determined the lycopene content as 0.0946±0.0004 and 0.3126±0.0037 mg/g in methanol and ethanol extracts respectively, while it was not found in water extract [27]. All these studies are very compatible with our current work. The considerable amounts of carotenoids such as lycopene and β-carotene found in the medicinal plants. In terms of these components, the abundant medicinal aromatic plant species will be the appropriate resource to prevent diseases associated with heart diseases and cancer oxidative stress [28].

3.2. The Chemical Structure Elucidation of PC

The PC was isolated as a yellowish appearance. Molecular formula of compound was found to be $C_{21}H_{20}O_{11}$ using spectroscopic instruments. Corresponding to the formula molecular ion peak was identified by HPLC-TOF/MS as 447.0948 [M-H]-(m/z) (Figure 2). FT-IR spectral data of the compound showed the carbonyl group and OH absorption peaks at 1654 and 3303 cm^{-1} respectively. The melting point of the compound was found to be 205°C. Full ^{13}C and ^{1}H NMR data was tabulated as Table 2. As a result of all spectral data the chemical structure of PC was elucidated as a luteolin-7-*O*-glucoside. The obtained spectral data are consistent with the data existing in the literature [29].

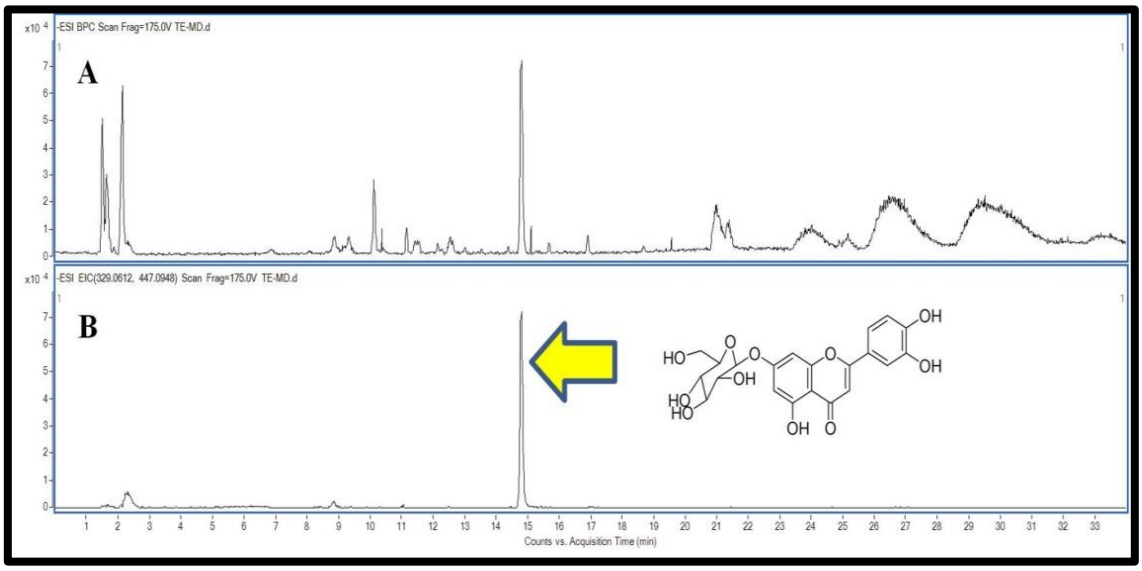

Figure 2. HPLC-TOF/MS chromatogram of MDE t **(A)** and PC isolated from *T. abrotanifolium* **(B)** (MDE: Methanol: Dichloromethane (1:1) Extract, PC: Purified Compound)

Table 2. ^{1}H and ^{13}C NMR data for PC (in DMSO-d_6; ^{13}C: 100 MHz; ^{1}H: 400 MHz)

C/H	**δ_C**	**δ_H (Hz)**
C_2	164.9	
C_3	103.6	6.76 s
C_4	182.3	
C_5	161.5	
C_6	99.9	6.45 s (*J=1.9*)
C_7	163.4	
C_8	95.1	6.79 s (*J=1.9*)
C_9	157.4	
C_{10}	105.7	
$C_{1'}$	121.8	
$C_{2'}$	113.9	7.42 d (*J=2.0*)
$C_{3'}$	146.2	
$C_{4'}$	150.4	
$C_{5'}$	116.4	6.91 d (*J=8.4*)
$C_{6'}$	119.6	7.46 dd (J=8.4 J=2.0)
4'-OH		10.41 s
3'-OH		12.98 s
5-OH		9.47 s
Glucose		
1"	100.2	5.07 d (*J*=7.6)
2"	76.1	3.55
3"	69.4	3.27
4"	71.1	3.91
5"	76.2	3.46
6"	61.0	3.73 d (*J*=8.1)
	61.0	3.45 d (*J*=8.1)

3.3. Antioxidant Activities

In this study, the antioxidant activities were evaluated by using *in vitro* antioxidant models, including total antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and iron-chelating capacity. The relationship between the antioxidant activity and the total content of phenol and flavonoids were also studied.

The total antioxidant capacity of extracts, sub-fractions, purified compound and the positive standard of trolox was assessed by the formation of phosphomolybdenum complexes. This method is based on the reduction of Mo (VI) to Mo (V) by the sample analyte, and this reaction is accompanied by the appearance of a green colour. The total antioxidant capacity was measured by monitoring the absorbance of the reaction mixture at 695 nm. The total antioxidant capacity expressed as α-tocopherol equivalents (mmol α-tocopherol/g DW) as shown in Figure 3. All the samples showed a potent total antioxidant activity that was surprisingly higher in MDE with value of 45.45±1.54 mmol α-tocopherol/g DW and significantly lower levels in CWE with value of 6.51±0.32 mmol α-tocopherol/g DW. The activity values of other samples were found to range from 8.72±0.21 to 36.50±1.27 mmol α-tocopherol/g DW.

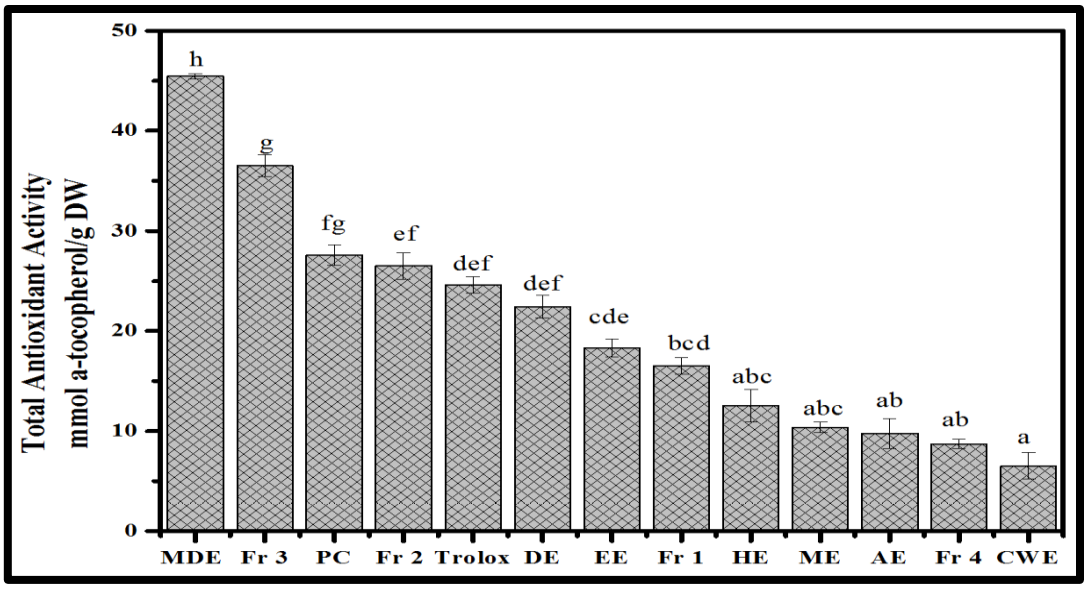

Figure 3. Total antioxidant activity of different solvent extracts, sub-fractions, purified compound and trolox based on the reduction of Mo(VI) to Mo(V). Values with different letters are significantly different ($p < 0.05$)

The free radical-scavenging activities of different solvent extracts of *T. abrotanifolium* were determined using the DPPH assay and the results were given in Table 3. The results, expressed as IC_{50} values, were calculated by regression analysis. The DPPH radical has been widely used to test the free radical-scavenging ability of various natural products [30-32]. The DPPH radical scavenging assay of purified compound from MDE and its sub-fractions of *T. abrotanifolium* were measured in comparison with BHT as standard antioxidant. As shown in Table 3, purified compound exhibited strong DPPH radical activities with IC_{50} value of 7.26±0.89 µg/mL. The MDE, Fr 3 and purified compound showed potent free radical-scavenging activity on the DPPH radical compared to other samples and, overall, the activity decreased in the following order: PC>Fr 3>BHT>Fr 2>Fr 4>Fr 1>MDE>ME>EE>DE>HE>AE>CWE. The BHT was used as reference compound. The high IC_{50} value of CWE indicates that it is a poor free radical scavenger. There was a statistically significant difference (p<0.05) in IC_{50} values in antioxidant activity between extracts and PC. The antagonistic effect could be responsible for the high antioxidant activity.

Our investigation revealed that all tested different polarity solvent extracts, fractions and purified compound showed significant scavenging activities against $ABTS^{\bullet+}$. The non-polar DE extract showed lower activity in comparison with the polar extracts and fractions. In this investigation, the polar extract/fraction (AE, EE, MDE, ME, Fr 3 and Fr 4) and the non-polar extract/fraction (HE, DE, Fr1 and Fr2) showed significant difference (p<0.05) except for CWE with value of IC_{50}. (Table 3) These data suggested that polar solvent and its proportional systems might be a better choice to extract bioactive component from *T. abrotanifolium*.

Table 3. Antioxidant activities of crude extracts, sub-fractions and purified compound isolated from *T. abrotanifolium*

	DPPH$^{\bullet}$	**ABTS$^{\bullet+}$**	**Chelating Activity**
Extract/sub-fraction/compound		**IC_{50}(µg/mL)**	
HE	142.91±2.43^{i}	211.56±2.09^{h}	252.12±0.13^{g}
DE	115.43±1.59^{h}	304.43±2.79^{i}	355.43±1.72^{i}
AE	197.26±2.15^{j}	85.26±0.88^{cd}	87.26±2.33^{d}
EE	97.01±0.42^{g}	42.01±1.12^{b}	101.01±2.22^{de}
MDE	61.13±3.21^{e}	71.59±0.16^{c}	37.13±0.85^{ab}
ME	73.87±1.12^{f}	87.87±0.19^{d}	129.87±0.36^{f}
CWE	283.26±2.25^{k}	192.53±1.52^{g}	315.26±3.17^{h}
Fr 1	47.68±2.39^{d}	128.72±2.74^{f}	47.68±2.12^{ab}
Fr 2	26.84±0.93^{bc}	105.84±1.72^{e}	66.21±0.27^{c}
Fr 3	14.79±0.01^{a}	27.79±0.59^{ab}	32.79±0.65^{a}
Fr 4	31.16±1.25^{c}	95.16±2.84^{de}	108.16±0.91^{e}
PC	7.26±0.89^{a}	18.13±1.18^{a}	39.26±0.52^{ab}
BHT	21.7±0.25^{b}	nd	nd
Trolox	nd	16.3±1.52^{a}	nd
Vit. E	nd	nd	48.7±1.31^{b}

	Reducing Power (700nm)			
Extract/sub-fraction/compound	**12.5 (µg/mL)**	**25(µg/mL)**	**50(µg/mL)**	**100(µg/mL)**
HE	0.051±0.00^{a}	0.055±0.00^{a}	0.067±0.00^{a}	0.069±0.01^{a}
DE	0.091±0.00b^{c}	0.091±0.01^{ab}	0.141±0.01^{bc}	0.173±0.03^{b}
AE	0.104±0.02^{c}	0.153±0.01^{cd}	0.199±0.02^{e}	0.221±0.02^{d}
EE	0.098±0.01^{c}	0.129±0.00^{bc}	0.157±0.01b^{cd}	0.174±0.03^{b}
MDE	0.173±0.01^{e}	0.271±0.01^{fg}	0.388±0.03^{i}	0.469±0.05^{g}
ME	0.175±0.00^{e}	0.287±0.02^{fg}	0.395±0.04^{i}	0.485±0.01^{g}
CWE	0.107±0.01^{cd}	0.126±0.01^{bc}	0.131±0.00^{b}	0.143±0.00^{b}
Fr 1	0.075±0.00^{b}	0.186±0.00^{d}	0.324±0.03^{h}	0.387±0.00^{f}
Fr 2	0.278±0.02^{g}	0.296±0.01^{g}	0.309±0.00^{fg}	0.361±0.02^{e}
Fr 3	0.305±0.03^{h}	0.495±0.02^{i}	0.513±0.05^{j}	0.569±0.01^{k}
Fr 4	0.145±0.01^{e}	0.162±0.00^{cd}	0.178±0.01^{de}	0.193±0.01^{b}
PC	0.107±0.00^{cd}	0.196±0.01^{de}	0.301±0.04^{f}	0.479±0.01^{g}
BHT	0.138±0.01^{e}	0.276±0.02^{fg}	0.395±0.01^{i}	0.515±0.03^{h}
Trolox	nd	nd	nd	nd
Vit. E	0.127±0.00^{de}	0.242±0.01^{ef}	0.317±0.01^{fg}	0.532±0.01^{ij}
BHA	0.205±0.01l^{f}	0.393±0.02^{h}	0.405±0.03^{i}	0.551±0.04^{jk}

Abbreviations: DPPH$^{\bullet}$: scavenging of diphenyl-picrylhydrazyl radical; $ABTS^{\bullet+}$: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; HE: Hexane Extract DE: Dichloromethane Extract, AE: Acetone Extract, EE: Ethyl acetate Extract, MDE: Methanol: Dichloromethane (1:1) Extract, ME: Methanol Extract, and CWE Cold Water Extract. IC_{50}: expressed in µg/mL. nd: not determined BHT Butylated hydroxy toluene, Vit.E: Vitamin E, Trolox (as reference), and PC: Purified Compound. Values with different letters are significantly different (p < 0.05).

The chelating capacity of different polarity extracts/fractions and pure compound isolated from *T. abrotanifolium* were assessed using solutions of ferrous ion. As shown in table 3, HE, DE and CWE extracts slightly inhibited with IC_{50} values as 252.12±0.13, 355.43±1.72 and 315.26±3.17 μg/mL the formation of the red-colored complex, respectively.

Fr 3 appeared to be better chelators of iron (II) ions compared to the positive control Vit E in this assay. MDE and PC demonstrated excellent chelating ability with IC_{50} values of 37.13±0.85 and 39.26±0.52 μg/mL respectively. These results suggested that MDE extract from *T. abrotanifolium*, Fr 3 obtained from its fractionations and PC are too strong Fe^{2+} chelators.

The reducing power is summarized in Table 3 and results obtained at different concentration of the polar and non-polar extract, column fractions and purified compound showed significant levels among the samples. As shown in Table 3, the Fr 3 from MDE of *T. abrotanifolium* exhibited the most strong reducing power with absorbance of 0.569±0.01 at concentration 100 μg/mL and had no significant difference when compared with standard antioxidant compounds BHT, BHA and Vit-E of absorbance 0.515±0.03, 0.551±0.04 and 0.532±0.01 respectively, while HF yielded the lowest activity at all concentrations. The reducing power of all samples arranged in the following order: Fr 3>Vit-E>BHA>BHT>ME>PC>MDE>Fr 1>Fr 2>AE>Fr 4>EE> DE>CWE>HE.

3.4. Real-Time Cell Proliferation Assays

The MDE and PC were tested for their ability to inhibit the proliferation of HeLa and C6 cancer cells by the xCELLigence system, Roche. To monitor real-time kinetic responses and dynamic growth of cancer cells against the added samples, they were allowed to form into log growth phase by continuously monitoring them. For this process HeLa and C6 cells were precipitated in E-plate 96 and monitored as real-time until composed stage of the log growth phase at that the moment added different concentrations of samples. After interaction of the cells with samples, changes in cell number related Cell Index values (CI) were recorded instantly as Cell Index (CI) Real-time cell analyzers (xCELLigence system, Roche) with microelectronic impedance sensors which were developed to monitor a variety of live cell characteristics such as cell growth [33], adhesion [34], differentiation, migration [35], cytotoxicity [36], receptor tyrosine kinase activation, G protein-coupled receptor–ligand interactions, host–pathogen interactions [37] and β-cell injury [38].

Proliferation assays conducted with HeLa and C6 cancer cell line at different concentrations (250 and 500 μg/mL) of the crude extract of *T. abrotanifolium* has demonstrated significant antiproliferative activity compared with control and 5-fluorouracil (5FU) that is anticancer agent (Figure 4A).

In order to figure out selective inhibition of PC, dose-dependent antiproliferative effects were investigated by RTCA and examined at three different concentrations (10, 50 and 100 μg/mL) (Figure 4B). PC clearly demonstrated a selective cytotoxicity against both HeLa cancer cell line at the concentration of 50 μg/mL and C6 cancer cells in all concentrations at 15-27 and 36-45 hours.

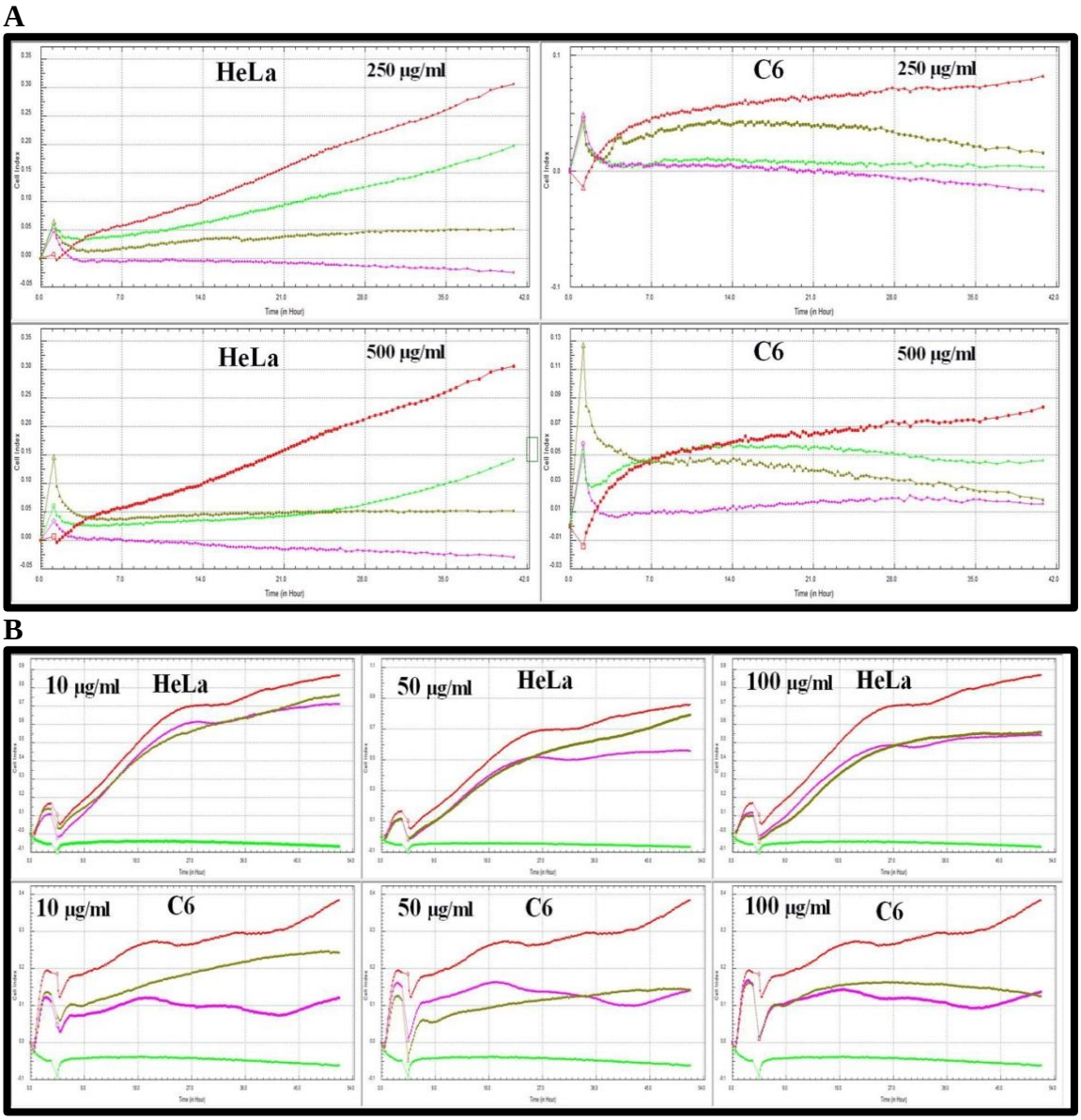

Figure 4. (A) The proliferation assays of the crude extract (MDE) of *T. abrotanifolium* [■MDE ■DMSO ■5-FU ■Ctrl]. **(B)** Anti-proliferative effects of purified compound (PC) on HeLa and C6 cells. [■PC ■5-FU ■Ctrl ■Medium]. Proliferation of the cells monitored by Roche

3.5. Antimicrobial Activity

Table 4 shows the results of antimicrobial activity of crude extracts in different solvent, sub-fractions and pure compound on selected microorganisms and showed good antimicrobial activity against 6 bacteria and 2 yeasts. While there was not an observable difference in the susceptibility of gram (+) and gram (-) bacteria to the antimicrobial effect of crude extract, significant effects were observed between sub-fractions and purified compound. EE was the most effective among crude extracts in different polarity solvent on studied microorganisms. *S. aureus* and *S. cerevisiae* were found to be the most sensitive bacteria and yeast respectively against the EE of *T. abrotanifolium*. DE was found to be generally effective against gram (-) bacteria. Surprisingly CWE revealed weak antimicrobial activity against the only *Salmonella enterica*, but did not show any antifungal effect against fungi species. Among polar (Fr 3, Fr 4) and non-polar (Fr 1, Fr 2) sub-fractions from column chromatography, Fr 3

inhibited the growth of both gram (+)/gram (-) bacteria and fungi species. Also PC exhibited the most powerful antibacterial effect against *K. pneumonia*, while against *S. enterica* did not show any effect. The HE showed weak antimicrobial and anticandidal activity against gram (-)/ gram (+) bacteria and yeasts tested, even showed no activity against species of *L. monocytogenes* and *E. coli*.

The crude extracts isolated in the different polarity solvent from aerial parts of *T. abrotanifolium* generally showed moderate antimicrobial activity with zones of inhibition between 6.5 and 11 mm, but purified compound and sub-fractions obtained from column chromatography process showed satisfactory antimicrobial activity with zones of inhibition between 7 and 20.5 mm, although generally having a high zone diameter.

Table 4. Antimicrobial activity of crude extracts, sub-fractions and purified compound isolated from *T. abrotanifolium*

Microorganisms	**Crude extracts in different solvent**						
	HE	**DE**	**EE**	**AE**	**MDE**	**ME**	**CWE**
Bacteria (Gram-negative)							
Klebsiella pneumoniae	7.0±0.5	10.0±0.6	10.0±0.7	10.5±0.6	8.5±0.6	9.0±0.4	-
Salmonella enterica	7.0±0.7	10.0±0.4	8.0±0.4	6.5±0.6	-	6.5±0.8	6.5±0.6
Escherichia coli	-	10.0±0.2	8.0±0.5	8.0±0.7	6.5±0.4	9.0±2.4	-
Bacteria (Gram-positive)							
Staphylococcus aureus	6.5±0.0	9.0±0.9	11.0±0.2	7.0±0.7	6.5±0.7	7.0±0.3	-
Bacillus subtilis	7.0±1.5	9.0±1.7	10.0±0.0	8.0±0.8	9.0±0.3	7.0±0.2	-
Listeria monocytogenes	-	7.0±0.3	7.5±0.6	10.0±0.4	7.0±0.5	8.0±0.4	-
Yeast							
Candida albicans	6.5±0.0	10.0±0.6	10.0±0.7	9.0±0.0	7.0±0.3	9.0±0.2	-
Saccharomyces cerevisiae	7.0±0.9	9.5±0.9	11.0±3.4	9.0±0.3	7.0±0.2	9.0±0.4	-
Microorganisms	**Sub-fractions and pure compound**						
	Fr 1	**Fr 2**	**Fr 3**	**Fr 4**	**PC**	**NC**	**RC**
Bacteria (Gram-negative)							
Klebsiella pneumoniae	13.0±1.5	16.0±1.1	10.0±0.2	12.5±0.3	20.5±0.6	-	30.0±0.8
Salmonella enterica	11.0±0.1	-	18.0±0.7	-	-	-	24.0±0.9
Escherichia coli	11.0±0.6	10.0±1.4	8.0±0.9	7.0±0.4	16.5±0.7	-	25.0±0.1
Bacteria (Gram-positive)							
Staphylococcus aureus	18.5±0.8	14.7±0.8	11.0±0.9	19.5±0.2	16.5±0.7	-	25.0±0.0
Bacillus subtilis	-	13.5±0.3	10.0±0.7	18.0±0.8	11.3±2.7	-	28.0±0.4
Listeria monocytogenes	13.0±0.6	17.4±0.2	7.5±0.3	10.8±0.5	7.0±1.0	-	22.0±0.5
Yeast							
Candida albicans	12.5±0.8	10.0±0.3	10.0±0.4	19.0±1.4	19.8±0.1	-	22.0±0.0
Saccharomyces cerevisiae	9.5±0.9	17.5±1.8	11.0±0.6	12.5±0.6	7.0±0.3	-	27.0±0.3

Abbreviations: NC: negative control (used DMSO); RC: reference compound (used Gentamicin) PC: Purified Compound HE: Hexane Extract DE: Dichloromethane Extract, AE: Acetone Extract, EE: Ethyl acetate Extract, MDE: Methanol: Dichloromethane (1:1) Extract, ME: Methanol Extract, and CWE: Cold Water Extract. Results were expressed as mean ± SD and include disc diameter (6 mm)

4. CONCLUSION

T. abrotanifolium is used by the people for healing purposes, even sold by herbalists, in Turkey. Phytochemical investigation and detailed biological activity studies of this species have not been reported so far. The present study revealed that the rich bioactive components of *T. abrotanifolium* have pharmacological utility with the three main biological activities, i.e. anti-cancer, anti-microbial and anti-oxidant, extracted and fractioned with solvents of different polarities instead of conventional methods. According to the analysis results the biological activity of the extract or its sub-fractions depends on the polarity of the organic and inorganic solvent used. For example, hexane and dichloromethane solvents extracted the lipophilic ingredients-rich extracts with high abundance of fatty acids, saturated hydrocarbons, terpenoids and steroids (sugar group free), while the solvents such as acetone, ethyl acetate, methanol and water extracted the hydrophilic extracts rich in phenolics, polyphenols, phenolic

acids and their glycosidic forms. Also when methanol: dichloromethane (1:1; v: v) solvent system is used, all polar and apolar-specific secondary metabolites pass through the extract**.** The present study indicated that the bioactive compounds content of the extracts was solvent-dependent. As a result of chromatographic procedures and biological activity studies, luteolin-7-O-glucoside was isolated as the considerable bioactive component from the aerial parts of *T. abrotanifolium.* We believe that this study will provide useful information for further investigations on this species and be supportive to researchers uncovering different pharmacological effects of *T. abrotanifolium*. Previous studies to determine different biological activity potential of some medicinal plants specific to Turkey were a good prospect to continue with those studies [39-42]. Therefore the available literature reports are insufficient for *T. abrotanifolium* and to establish the exact medicinal value hence, further detailed phytochemical and pharmacological studies are warranted. Considering all the studied pharmacological activities, we conclude that MDE is better as a crude extract than the other extracts and column sub-fractions for total antioxidant activity. The studies explored the biological activities of compounds present in *T. abrotanifolium* extracts and subfractions. As a result *T. abrotanifolium* species prepared the ground for future drug development, clinical and scientific studies such as chemistry, phytochemistry, molecular biology and genetic pharmacology, toxicology, pharmacognosy and polypharmacological studies etc.

ACKNOWLEDGMENT

This study was supported by the grant from the Scientific and Technological Research Council of Turkey (TUBITAK; 111T560) and the Scientific Research Project Department of Bingol University (Project No: BAP-21-217-2014 and BAP-21-322-2015). İHG would like to express gratitude to Prof. Dr. İbrahim Demirtaş for his supervisor of this study. İHG is very grateful to Serkan Koldaş and Fatma Caf for antiproliferative and antimicrobial assays.

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