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# Phytochemical screening, antiproliferative and cytotoxic activities of the mosses *Rhytidiadelphus triquetrus* (Hedw.) Warnst. and *Tortella tortuosa* (Hedw.) Limpr.

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## Abstract

The paper presents information about the phytochemical analysis, antiproliferative and cytotoxic activities of *Rhytidiadelphus triquetrus* and *Tortella tortuosa* extracts. The cytotoxic activities of some extracts shows highest antiproliferative activities were detected with Lactate Dehydrogenase Leakage Assay. Sixteen components obtained from hexane extracts were determined by GC/MS. Palmitic acid was identified as the main component. The phenolic components of the other extracts were determined by HPLC-TOF/MS. 4-hydroxy benzoic acid, salicylic acid, gallic acid, caffeic acid, and gensitic acid were detected as the main components in all extracts. The hexane, chloroform, ethyl acetate extracts of studied mosses and the EtOAc and hexane extracts of RT showed statistically significant antiproliferative activities.

**Keywords:** Anticancer; cytotoxic; C6 cancer cell; HeLa cancer cell; mosses

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## 1. Introduction

Bryophytes are placed taxonomically between algae and pteridophytes and are represented by 24,000 taxa in the world (Asakawa et al., 2013). They are generally small plants and grow on different substrata such as soils, rocks, trees etc. (Schofield, 2001). Mosses exhibit different morphological variation with respect to liverworts and hornworts. Moss branching pattern is referred to as acrocarpous and pleurocarpous (Vanderpoorten and Goffinet, 2009). The acrocarpous moss *Tortella tortuosa* (Hedw.) Limpr. (TT) belonging to the family Pottiaceae is mainly found in Europe as far north as Svalbard and in Turkey, Cyprus, Caucasus, North and East Asia, Madeira, the Canary Islands, Algeria, Morocco, North America, Greenland, Peru, and Tierra del Fuego. Its length is between 1-8 cm and grows on rocks, in rock crevices, soil in turf, in flushes (Smith, 2004). TT was investigated earlier for its antifungal and antibacterial effects (Elibol et al., 2013) and antimicrobial activity (Savaroglu et al., 2011). The pleurocarpous moss *Rhytidiadelphus triquetrus* (Hedw.) Warnst. (RT) belonging to the family Hylocomiaceae is mainly distributed in Europe north to northern Fennoscandia, Faeroes, Iceland, Caucasus, Turkey, Asia, Central Africa, Madeira, and North America. RT is up to 20 cm long, and grows in basic to acidic habitats (Smith, 2004). The seasonal variation in flavonoid concentration (Brinkmeier, et al., 1999) and characterization of chemical composition (Kļaviņa et al., 2012) of RT was performed previously.

Detailed chemical studies on many bryophyte taxa have increasingly appeared since the 1960s (Schofield, 2001). In recent years, the presence of a large number of compounds showing biological activity in some bryophytes has been introduced to science world (Asakawa et al., 2013). Different steroids, fatty acids and some organic compounds (terpenoids, flavonoids, lignins, antibiotics, lipids, sterols, etc.) were obtained as the potentially significant chemical compounds from bryophytes (Sabovljević et al., 2001). The various biologically active substances of bryophytes exhibit antimicrobial, antifungal, antitumor, anticancer, and insecticidal activities (Asakawa, 2007; Üçüncü et al., 2010).

To our knowledge, this is the first report on the detailed chemical characterization, antiproliferative and cytotoxic activities of RT and TT extracts from Turkish material. In this study, we determined the phytochemical analysis, antiproliferative and cytotoxic effects of the extracts obtained from the aforementioned mosses. As a part of ongoing natural product researches, our results showed that the hexane and ethyl acetate extracts of RT and TT have high antiproliferative activities against C6 glioma cells and human cervical carcinoma (HeLa) cells at higher concentrations. Also, the results have potential use of these natural products for cancer and tumor treatments.

## 2 Materials and methods

### 2.1 Chemicals

The solvents for extraction were obtained from Merck. The chemicals used for the antiproliferative and cytotoxic activities were provided from Roche. Hexane, chloroform, ethyl acetate, methanol, water, water/ethyl acetate and water/n-butanol extracts of the materials were prepared from non-polar to polar solvent systems.

Fatty acids and other phenolic compounds were analysed by GC-MS. Antiproliferative activities of all extracts were determined using the BrdU ELISA method against HeLa and C6 cells.

### 2.2 Plant Materials

The mosses RT and TT were collected from soil in year 2013. RT was gathered at an altitude of 1689 m, latitude 40° 49′ 734″ N, longitude 33° 46′ 634″ E. The woodland habitats were dominated by *Pinus sylvestris* L. and *Juniperus communis* L. var. *saxatilis* Pall. stand. TT was also collected from soil in the similar locality, at an altitude of 1679 m, latitude 40° 49′ 695″ N, longitude 33° 46′ 779″ E. After the species identification, the materials were dried at room temperature (25 °C) in the shade and crushed with liquid nitrogen.

### 2.3 Extraction Procedure

The whole plants of RT (74.54 g) and TT (297.34 g) were extracted with increasing polarity of hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) and using maceration method at 25 °C for a week. The extracts were filtered and concentrated in a rotary evaporator and refluxed with water for

the residues. The water extracts were filtered, extracted with EtOAc (5x 300 mL; W-EtOAc) and n-butanol (BuOH; 5x 300 mL; W-BuOH) and evaporated (Demirtas et al., 2013).

### 2.4 Esterification Procedure of the Hexane Extracts

The each RT and TT hexane extracts were taken as 11 mg and 1 molar of KOH/methanol solution (5 mL) were added to the samples. The resulting mixtures were vigorously mixed by vortex. The mixtures were added into 3 mL of hexane and vortexed. 1 mL hexane phases were taken and diluted with 7 mL hexane and analyzed by GC-MS (Demirtas and Sahin, 2013).

### 2.5 Gas Chromatography (GC)

Fatty acids analyzed by GC-MS (Agilent Technologies 7890A model GC system, 5975C inert MSD with Triple-Axis Detector) using BPX-20 capillary column (30 mx 0.25 mm, 0.25 µm film thickness; 5% phenyl polysilphenyl IN-siloxane), 70 eV ionization voltage, and FID detector. Oven temperature was between 50 and 120 ºC at 5 ºC/min and 120 -240 ºC at 10 ºC/min and hold for 5 minutes. 1.0 µL of diluted extracts 300:1 were injected in the split mode. The injector and detector temperatures were adjusted to 220 ºC and 290 ºC, respectively. Helium was used as a carrier gas and the flow rate 1 mL/min and the samples were determined with 1/1000 dilutions (Demirtas and Sahin, 2013).

### 2.6 Gas Chromatography / Mass Spectrometry (GC/MS)

GC/MS analysis was performed by gas chromatography mass spectrometer using BPX-20 column with autosampler and column (30m x 0.25mm x 0.25µm film). GC/MS detection system was used for electron ionization (ionization energy 70 eV). Helium was used as a carrier gas with the flow rate of 1.3 mL/min and diluted to 1/1000 (Demirtas and Sahin, 2013).

### 2.7 The Analyzed Phenolic Standards

The phenolic content of chloroform, ethyl acetate, methanol, water, water/ethyl acetate and water/n-butanol extracts were performed by HPLC-TOF/MS. The qualitative and quantitative analysis of some phenolics were investigated and found as gallic acid, gentisic acid, catechin, 4-hydroxybenzoic acid, protocatechuic acid, caffeic acid, 4-hydroxy benzaldehyde, rutin, *p*-coumaric acid, ferulic acid, apigenin-7-glycoside, naringenin, kaempferol, rosmarinic acid, salicylic acid, quercetin, ellagic acid, resveratrol, and chlorogenic acid (Eser et al., 2016).

### 2.8 HPLC-TOF/MS Analysis

The phenolic contents were determined with HPLC System Agilent Technologies 1260 Infinity 6210 Time of Flight (TOF) LC/MS detector and Agilent Poroshell 120 EC-18 (2.7 mm, 4.0 x 50 mm) column individually. Mobile phase A and B were used with ultrapure water 0.1% formic acid and acetonitrile, respectively. The flow rate was 0.4 mL/min, the column temperature was 35 °C, and the injection volume also 5µL. Solvent program was as follows: 0 min 10% B; 0-1 min 10% B; 1-10 min 80% B; 10-19 min 80% B; 19-19.10 minutes 10% B; and 19.10-29.1 min 10% B. The retention times of standard compounds and m/z values were used to determine the components of the extracts. MS-TOF device was in negative ionization mode; the gas temperature was 325 °C, gas flow 11.0 L/min, and the nebulizer was also 45 psi (Eser et al., 2016).

### 2.9 Preparation of Stock Solutions of Extraction

Sample solutions were prepared in dimethyl sulfoxide (20 mg/mL) and diluted with Dulbecco's modified eagle's medium. The volume of dimethyl sulfoxide is below 0.1% in all experiments. The samples were filtered using 0.22 micron sterile filters and stored at -20 °C (Demirtas et al., 2017).

### 2.10 Cell Culture and Antiproliferative Activities

Human cervix carcinoma (HeLa) and rat brain tumor (C6) cells were used for antiproliferative activity tests. The cells were treated in Dulbecco's modified eagle's medium (DMEM, Sigma), supplied with 10% (v/v) fetal bovine serum (Sigma, Germany) and PenStrep solution (Sigma, Germany) in a 5% $CO_2$ humidified atmosphere at 37 °C. The proliferation assays were carried out in 96-well microplate (COSTAR, Corning, USA) at a density of $3\times10^4$ cells in per well and incubated in 5% $CO_2$, at 37 °C for 24 hours. 5-Fluorouracil (5-FU) was used as positive control. The extracts were determined on 100, 75, 50, 40, 30, 20, 10,

and 5 mg/mL concentrations and all operations performed in a laminar flow. Cell proliferation assay was determined according to the manufacturer's protocol by BrdU Cell Proliferation ELISA (Roche, Germany) (Demirtas et al., 2009; Demirtas and Sahin, 2013; Karakus et al., 2013; Sahin Yaglioglu et al., 2013). All tests were repeated three times with three replications. Measurements were measured in ELISA reader (Chromate, Microplate Reader P4300 Series, USA) at 450 nm. The inhibition of cell proliferation was calculated as follows: (1- Abs treatments /Abs control) x100.

**2.11 Lactate Dehydrogenase (LDH) Leakage Assay**

Cytotoxic activities of the samples were tested using the manufacturer's procedures Cell LDH Cytotoxicity Assay (Roche 04744926001, Germany). C6 cells were used for cytotoxic activity tests. The cells were amplified in DMEM, supplied with 10% (v/v) fetal bovine serum and PenStrep solution in a 5% $CO_2$ humidified atmosphere at 37 °C. The cytotoxicity assay was carried out in 96-well microplate cells at a density of $5\times10^3$ cells in per well and incubated in 5% $CO_2$, at 37 °C for 24 hours. 5-FU was used as a positive control. The tests were repeated three times with three replications. The absorbance of the samples was measured in ELISA reader at 492 nm and the cytotoxicity values calculated using the following formula:

Cytotoxicity (%) = (experimental value - low control) / (high control-low control) x 100

**2.12 Statistical Analysis**

*In vitro* assay results are the mean of nine values (± SD). Differences between the groups were assessed using by one-way analysis of variance (ANOVA) ($p < 0.01$) and the differences between the administration groups were analyzed by multiple comparison test (Duncan).

**2.13 Calculation of $IC_{50}$ and $IC_{75}$ Values**

$IC_{50}$ and $IC_{75}$ values of the extracts and 5-FU were calculated by ED50 Plus v1.0 software (Vargas, 2000).

**3. Results and discussion**

**3.1 Extractions**

The amounts and yields of RT and TT extracts are given in Table 1. The extracts were obtained using the hexane, chloroform, ethyl acetate, methanol, water, water/ethyl acetate, and water/n-butanol. The highest yield was obtained with methanol and the lowest yield of water/ethyl acetate for RT, and water and water/ethyl acetate for TT, respectively. The highest amount was obtained from water/n-butanol and the lowest from ethyl acetate for RT, and water/n-butanol and hexane for TT, respectively. The highest yield (4.05%) was obtained from methanol extract for RT in comparison with other solvents and plant.

Table 1. The amounts and yields of RT and TT extracts.

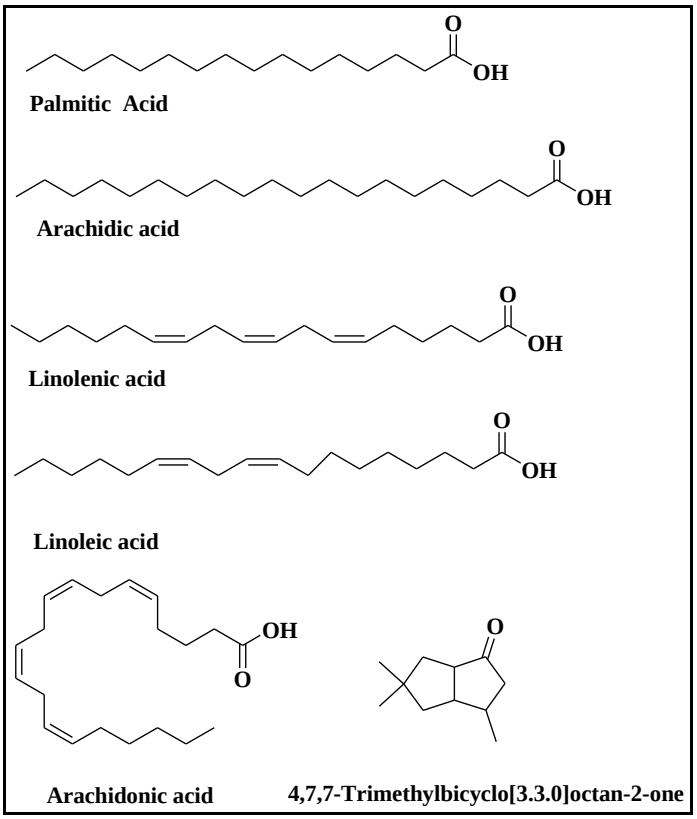
| Extracts | RT | | TT | |
|---|---|---|---|---|
| | Amount (g) | Yield (%) | Amount (g) | Yield (%) |
| Hexane | 0.70 | 0.92 | 0.45 | 0.15 |
| $CHCl_3$ | 2.26 | 3.03 | 5.88 | 1.97 |
| EtOAc | 0.24 | 0.32 | 0.53 | 0.18 |
| MeOH | 3.02 | 4.05 | 3.66 | 1.23 |
| Water | 2.38 | 3.56 | 10.99 | 3.84 |
| W/EtOAc | 98.90 | 0.13 | 160.00 | 0.053 |
| W/BuOH | 581.00 | 0.77 | 618.30 | 0.207 |

**3.2 GC-MS Analysis**

RT and TT hexane extracts were identified as eight components for RT and fifteen for TT by GC-MS (Table 2). Arachidic acid (25.39%) was obtained as main component in RT with linolenic acid (20.70%), arachidonic acid (17.60%), 4,7,7-trimethylbicyclo [3.3.0] octan-2-one (14.67%) and palmitic acid (13.15%). However, the main component for TT was linolenic acid (52.97%) and other components obtained as linoleic acid (8.53%), palmitic acid (5.52%) (Table 2, Scheme 1).

Table 2. GC-MS analysis results of RT and TT mosses.

| No | RT | Isomer | Compound names | % Area | |
|---|---|---|---|---|---|
| | | | | RT | TT |
| Saturated fatty acids | | | | | |
| 1 | 30.357 | C16:0 | Palmitic acid | 13.15 | 5.52 |
| 2 | 31.026 | C16:0 | Palmitic acid, isopropyl ester | 2.34 | 1.02 |
| 3 | 35.724 | C18:0 | Stearic acid | 1.74 | 0.71 |
| 4 | 40.199 | C20:0 | Arachidic acid | 25.39 | 0.40 |
| | | | Total | 42.62 | 7.65 |
| Mono unsaturated fatty acids | | | | | |
| 5 | 36.228 | C18:1 | Oleic acid | | 2.77 |
| 6 | 36.250 | C18:1 | 7-Octadecenoic acid | | 2.70 |
| 7 | 36.388 | C18:1 | 11- Octadecenoic acid | 4.41 | 0.56 |
| | | | Total | 4.41 | 6.03 |
| Polyunsaturated fatty acids | | | | | |
| 8 | 37.286 | C18:2 | Linoleic acid | | 8.53 |
| 9 | 38.883 | C18:3 | Linolenic acid | 20.70 | 52.97 |
| 10 | 41.995 | C20:2 | 11,13-Eicosadienoic acid | | 0.94 |
| 11 | 43.443 | C20:4 | Arachidonic acid, ethyl ester | | 4.48 |
| 12 | 43.466 | C20:4 | Arachidonic acid | 17.60 | 3.97 |
| 13 | 45.423 | C20:5 | 5,8,11,14,17-Eicosapentaenoic acid (EPA) (omega 3) | | 4.19 |
| | | | Total | 38.30 | 75.08 |
| Other compounds | | | | | |
| 14 | 25.459 | | 1-Octadecene | | 0.77 |
| 15 | 31.278 | | 1-Heptadecene | | 1.18 |
| 16 | 39.970 | | 4,7,7-Trimethylbicyclo[3.3.0]octan-2-one | 14.67 | |
| | | | Total | 14.67 | 1.95 |
| | | | General Total | 100.00 | 98.80 |




Scheme 1. The main components of RT and TT hexane extract.

Arachidic acid provides for producing of detergents, photographic materials and lubricants (Patil and Chavan, 2012). Alpha-linolenic and linoleic acids which cannot be synthesized by the human body (Burr et al., 1930) are known to be essential fatty acids for humans (Burr et al., 1930; Whitney and Rolfes, 2008). Arachidonic acid is abundant in the brain, muscle, and liver (Smith et al., 2011). The fatty acid has an important role in the regulation of signaling enzymes, and can also act as a vasodilator (Baynes and Dominiczak, 2005). Palmitic acid is mainly used to produce soaps with high saponification value, cosmetics, and release agents (Bwai, et al., 2013).

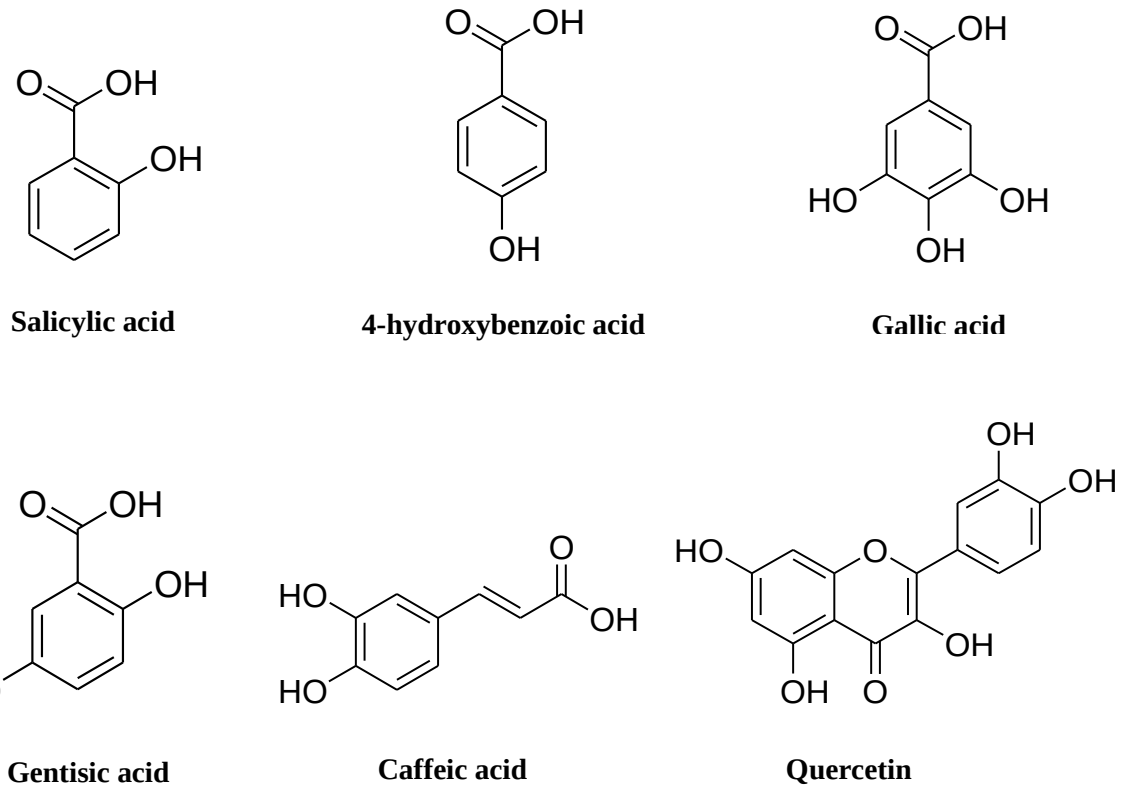
TT hexane extract exhibited linolenic acid as the main component and showed significantly antiproliferative activity at 64.06% inhibition against C6 cells. (Narisawa et al. (1991) found that the linolenic acid has an anti-tumor promoting effect. Their study results showed that a diet with 12% perilla oil rich in α-linolenic acid inhibited colon tumor development in rats.

### 3.3 HPLC-TOF/MS Analysis

Phenolic acids and their analogs have broad biological activities and some phenolic acids play an important role in preventing cancer (Clifford, 2000). The salicylic acid from the choloroform, 4-hydroxybenzoic acid from the ethyl acetate and methanol, gallic acid from water, 4-hydroxybenzoic acid from the water-ethyl acetate, gentisic acid and 4-hydroxybenzoic acid from the water/n-butanol extracts were determined as the main components of RT (Table 3, Scheme 2). The EtOAc extracts of RT was found the higher antiproliferative activity against C6 cells than even 5-FU ($IC_{75}$: 27.60).

Table 3. The phenolic components and amount of RT and TT extracts (mg phenolic compound/100 g dried plant).

| Phenolic components | MeOH | | EtOAc | | $CHCl_3$ | | Water | | W/EtOAc | | Water/BuOH | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RT | TT | RT | TT | RT | TT | RT | TT | RT | TT | RT | TT |
| Gallic Acid | - | - | 0.07 | - | - | - | 1.09 | 0.80 | 0.12 | 0.02 | 0.02 | 0.04 |
| Gentisic Acid | 1.06 | 0.29 | 0.08 | 0.05 | - | - | - | - | 1.17 | 0.64 | 0.07 | 0.09 |
| Catechin | - | - | - | - | - | - | - | - | - | - | - | - |
| 4-Hydroxybenzoic Acid | 2.36 | 0.20 | 0.72 | 0.10 | - | - | - | - | 4.96 | 1.35 | 0.07 | - |
| Protocatechuic Acid | - | - | 0.04 | - | - | - | - | - | 0.08 | 0.02 | - | 0.01 |
| Caffeic Acid | 0.59 | 0.08 | 0.04 | 0.01 | - | 0.13 | - | - | 0.12 | 0.01 | 0.01 | 0.02 |
| 4-Hydroxy Benzaldehyde | - | - | 0.01 | 0.03 | - | 0.08 | - | - | 0.13 | 0.03 | - | - |
| Rutin | 0.09 | 0.02 | 0.01 | - | - | - | - | - | - | - | - | 0.003 |
| *p*-Coumaric Acid | - | - | 0.02 | - | - | - | - | - | - | 0.02 | - | - |
| Ferulic Acid | 0.15 | - | 0.03 | 0.004 | - | - | - | - | 0.04 | 0.02 | - | - |
| Apigenin-7-Glucoside | - | - | - | - | - | - | - | - | - | - | - | - |
| Naringenin | 0.13 | - | 0.02 | 0.006 | - | 0.02 | - | - | 0.01 | 0.001 | - | - |
| Kaempferol | - | - | - | - | - | - | - | - | - | - | - | - |
| Rosmarinic Acid | 0.17 | - | - | - | - | - | - | - | 0.02 | - | - | - |
| Salicylic Acid | 0.10 | 0.03 | 0.01 | 0.004 | 0.16 | 0.05 | - | - | - | 0.10 | - | 0.01 |
| Quercetin | - | - | - | - | - | - | - | - | 1.12 | - | - | - |
| Chlorogenic Acid | - | - | - | - | - | - | - | - | 0.02 | - | - | 0.03 |
| Ellagic Acid | - | - | - | 0.02 | - | - | - | - | 0.01 | - | - | - |
| Resveratrol | - | - | - | - | - | - | - | - | 0.01 | 0.003 | - | - |




Scheme 2. The main phenolic components of RT and TT extract.

The caffeic acid from the chloroform, 4-hydroxybenzoic acid from the ethyl acetate and water/ethyl acetate, gentisic acid from methanol and water/n-butanol, and gallic acid from water extracts were determined as the main components of TT (Table 3, Scheme 2). The gallic acid was determined as the main component of TT and RT water extracts. According to literature, gallic acid was determined to significantly inhibit to human cancer cells of esophageal cancer cells (Faried et al., 2007).

### 3.4 Antiproliferative and Cytotoxicity Activities

The antiproliferative activities of TT and RT solvent extracts were determined against HeLa cell lines and compared with 5-fluorouracil (5-FU) as used standard (Figures. 1A-B and 2A-B). The antiproliferative activities were exhibited as very low activity at all doses with dose-dependent increases exception of hexane, chloroform and ethyl acetate extracts of RT as seen in Figure 2A. The EtOAc and hexane extracts of RT exhibited the highest activities at 100, 75, 50 and 40 µg/mL concentrations and only the EtOAc extract at 30 µg/mL. The methanol extract exhibited the lowest activity at all concentrations (Figure 2A).

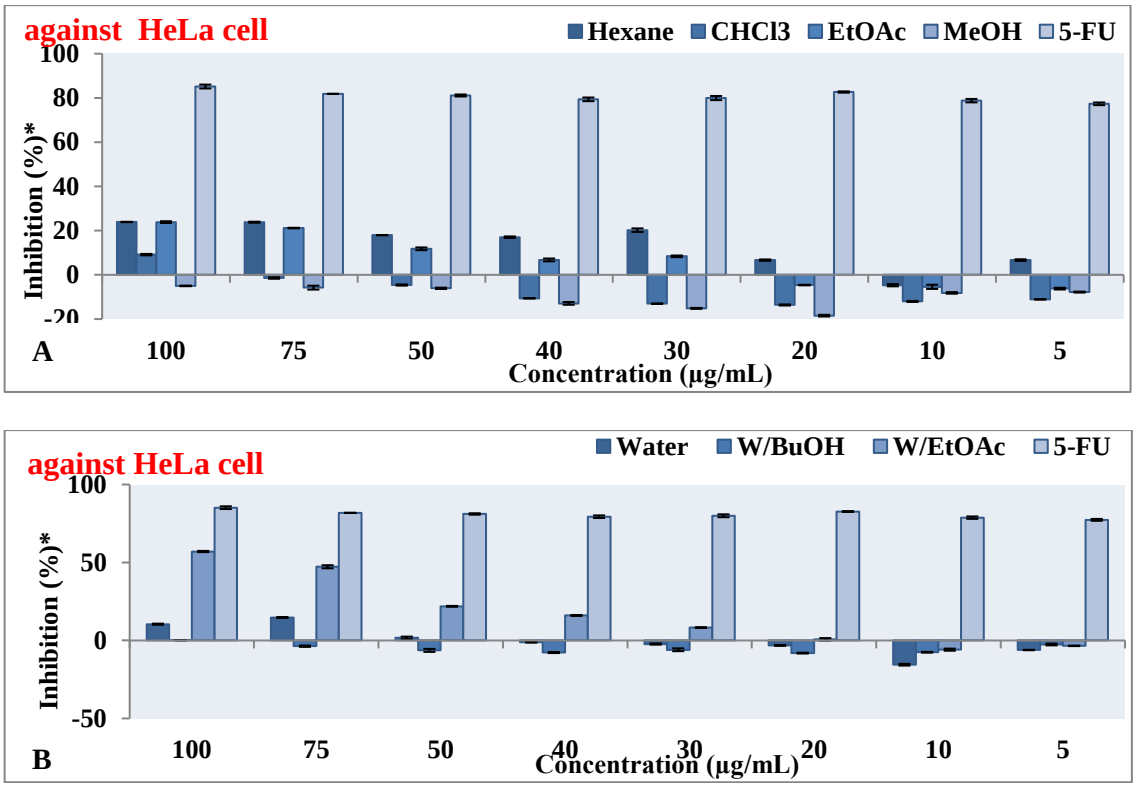



Figure 1. The antiproliferative activity of *Tortella tortuosa* organic solvent **(A)** and water **(B)** extracts against HeLa cells (* p <0.01.).

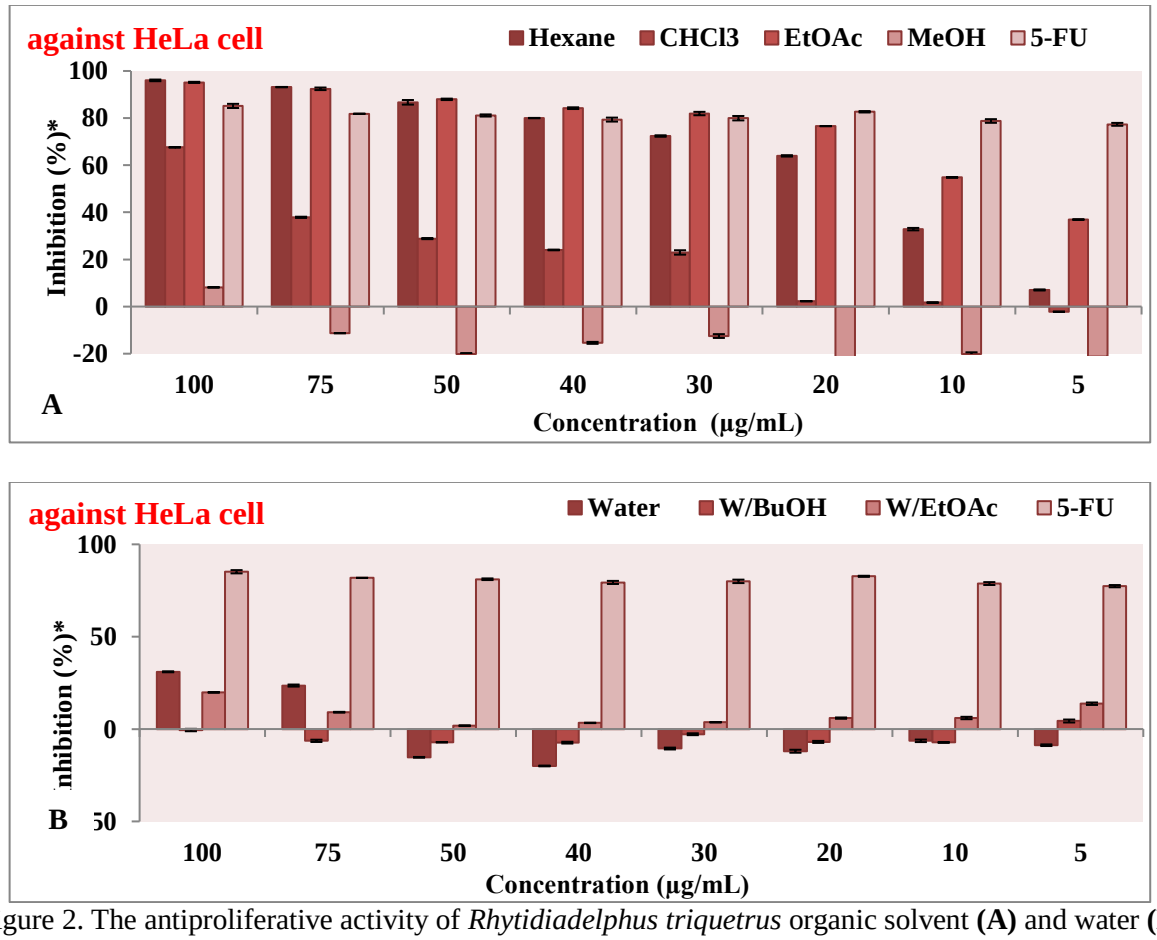



Figure 2. The antiproliferative activity of *Rhytidiadelphus triquetrus* organic solvent **(A)** and water **(B)** extracts against HeLa cells (* p <0.01.).

The solvent extract (antiproliferative) activities of TT and RT were determined against C6 cell lines compared with 5-FU (Figures 3A-B and 4A-B). The highest activities were obtained from hexane and EtOAc extracts at higher concentrations of 100, 75, 50 mg/mL and lowest activities obtained from methanol extract for all doses of RT as seen in Figure 4A. The highest HeLa cell activity was similarly obtained for the C6 cell lines at the same solvent extracts, hexane and EtOAc. Also the lowest cell activities were obtained from the same solvent extract of methanol for both C6 and HeLa. Similar lower activities were also observed for all water and mixture of water extracts as seen in Figures 1B, 2B, 3B and 4B.

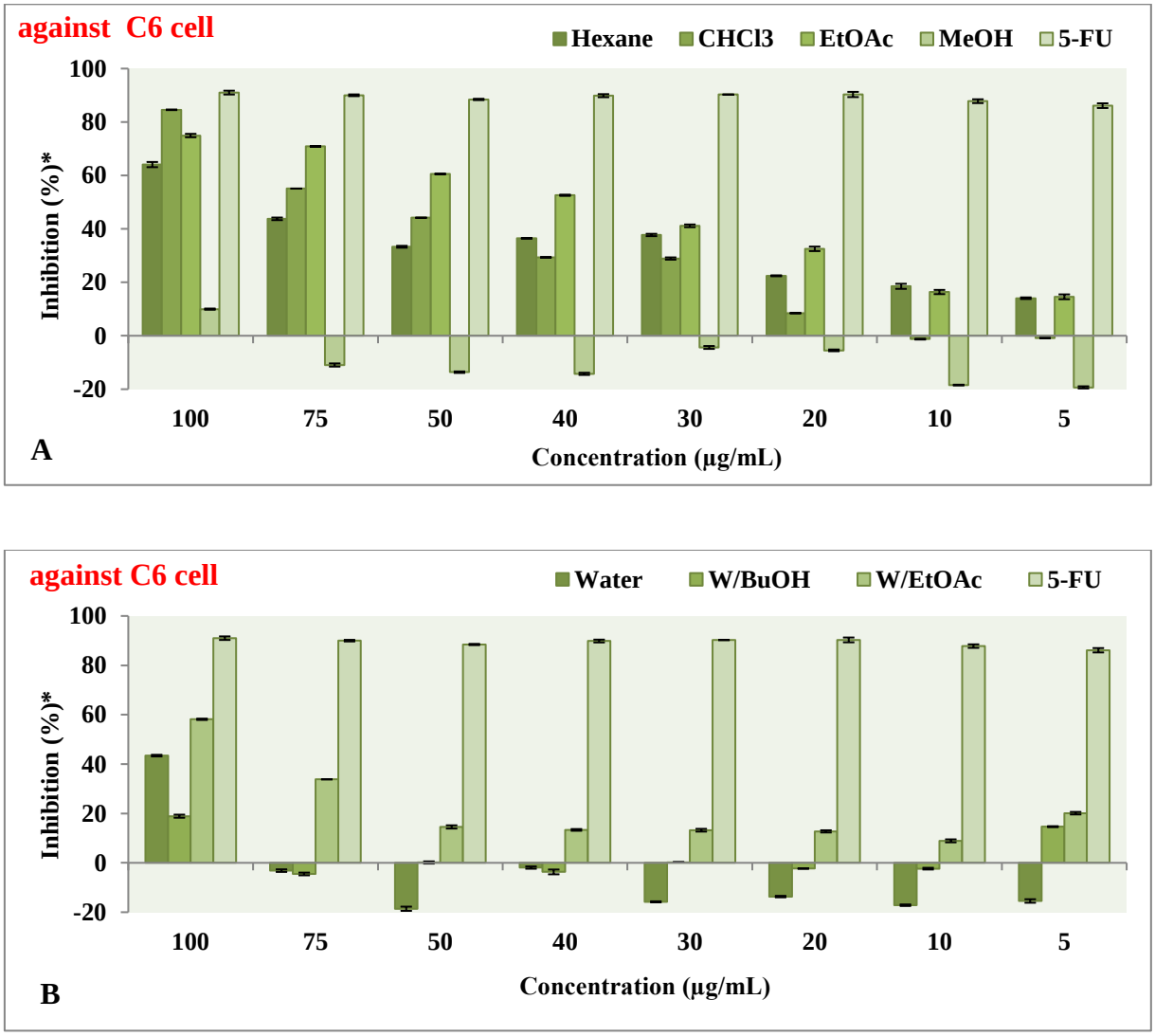



Figure 3. The antiproliferative activity of *Tortella tortuosa* organic solvent **(A)** and water **(B)** extracts against C6 cells (* p <0.01.).

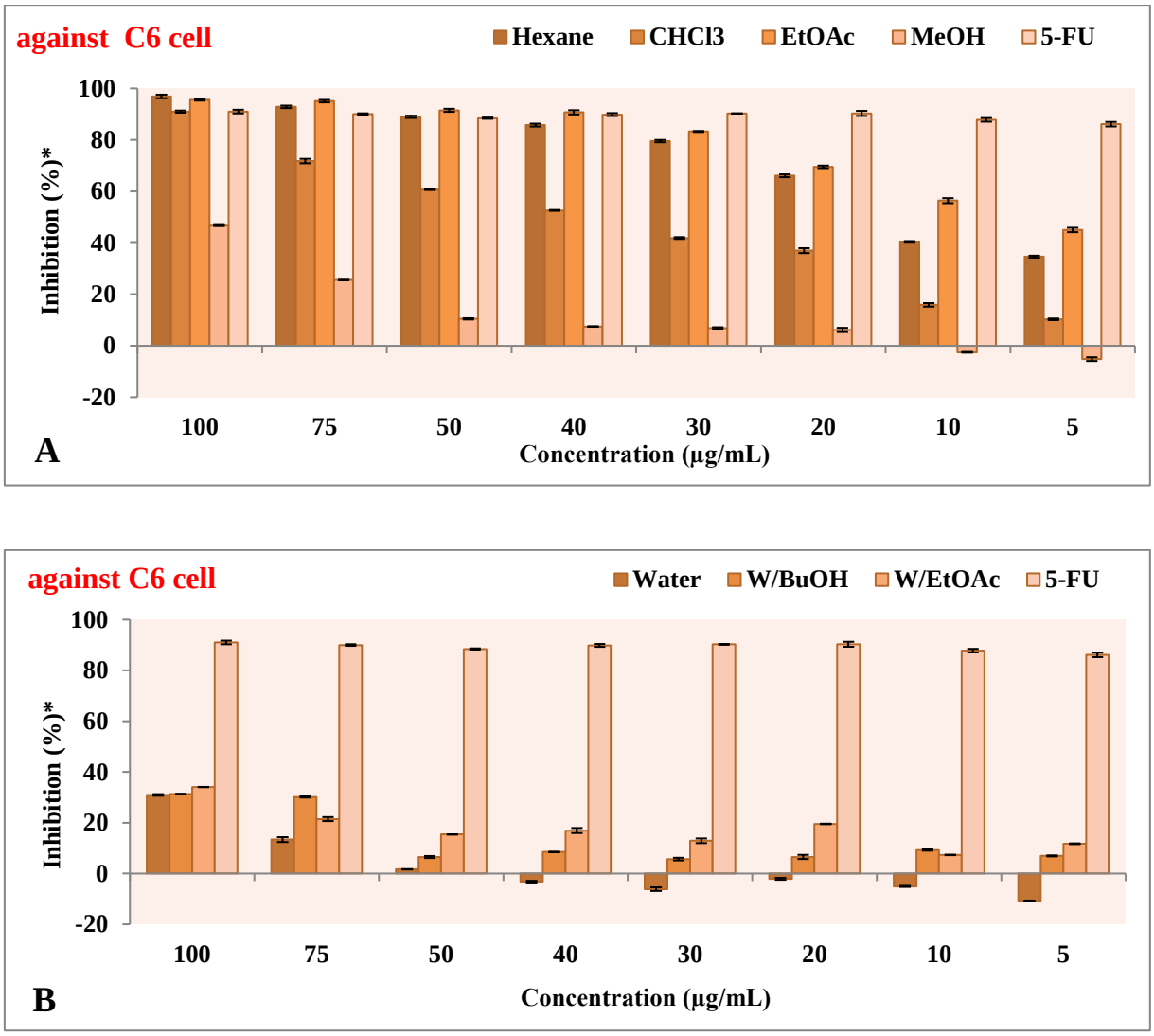



Figure 4. The antiproliferative activity of *Rhytidiadelphus triquetrus* organic solvent **(A)** and water **(B)** extracts against C6 cells (* p <0.01.).

The $IC_{50}$ and $IC_{75}$ values of all the extracts are given in Table 4. The results showed the lower toxicities of the extracts compared with 5-fluorouracil exception of W/EtOAc extract obtained from TT as seen in Table 5.

Table 4. The $IC_{50}$ and $IC_{75}$ values of all the extracts against HeLa cell.

| Sample | TT | | RT | |
|---|---|---|---|---|
| | $IC_{50}$ (µg/mL) | $IC_{75}$ (µg/mL) | $IC_{50}$ (µg/mL) | $IC_{75}$ (µg/mL) |
| Hexane | 33.26 | 57.26 | 17.94 | 48.16 |
| Cloroform | 94.62 | 104.31 | 57.08 | 81.61 |
| EtOAC | 55.60 | 72.82 | * | 31.49 |
| MeOH | 138.60 | 122.20 | 112.33 | 120.19 |
| Water | 70.76 | 85.17 | 80.75 | 97.94 |
| W/EtOAc | 59.53 | 76.74 | 65.01 | 124.05 |
| W/BuOH | 139.22 | 139.93 | * | * |

*values could not be determined.

Table 5. Cytotoxicity percentages of the effective extracts.

| Sample | Cytotoxicity (%) * |
|---|---|
| TT Hexane | 7 |
| RT Hexane | 7 |
| TT Chloroform | 20 |
| RT Chloroform | 16 |
| TT EtOAc | 23 |
| RT EtOAc | 16 |
| TT W/EtOAc | 37 |

* All tests were done three times and triplicate ($p<0.01$).

## 4. Conclusion

The phytochemical contents and their antiproliferative activities of TT and RT were examined and responsible compounds found as unsaturated fatty acids like linoleic, linolenic and arachidonic. The hexane and ethyl acetate extracts of RT showed high antiproliferative activities against C6 and HeLa cell lines at higher concentrations. The extracts except for W/EtOAc were also found to be less toxic than the 5-FU used as a standard. The highest activities and lowest cytotoxicities were obtained from hexane extracts of RT and TT. These results may be used for continuing studies of anticancer drugs in future.

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