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## **Abstract:**

Natural populations of *Ruta graveolens* L. and *Artemisia abrotanum* L. in northern Saudi Arabia may be a rich source of natural polyphenols with potential biological activities. Therefore, tentative high-performance liquid chromatography-diode array detection was used to analyze the polyphenol contents of leaf extracts. *R. graveolens* mainly contained the phenolic acids chlorogenic acid and p-coumaric acid and the flavonoids rutoside and quercetin, whereas those of *A. abrotanum* mainly contained the phenolic acids isochlorogenic acid and rosmarinic acid and the flavonoid quercetin. Leaf extracts of both species showed antioxidant activities due to the presence of quercetin, chlorogenic acid, and p-coumaric acid as well as antiproliferative and cytotoxic activities against cancer cells, which may be attributed to necrotic cell accumulation during the early and late apoptotic periods. Both species also exhibited antibacterial activity, although the activity was higher in *R. graveolens* due to the high contents of quercetin and other polyphenols. Finally, both species exhibited antifungal activities, which were associated with specific polyphenols. This is the first study to confirm the richness of polyphenols and wide spectrum of biological activities in natural populations of *R. graveolens* and *A. abrotanum* in northern Saudi Arabia.

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Article

# Polyphenol Content and Biological Activities of *Ruta graveolens* L. and *Artemisia abrotanum* L. in Northern Saudi Arabia

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**Abstract:** Natural populations of *Ruta graveolens* L. and *Artemisia abrotanum* L. in northern Saudi Arabia may be a rich source of natural polyphenols with potential biological activities. Therefore, tentative high-performance liquid chromatography–diode array detection was used to analyze the polyphenol contents of leaf extracts. *R. graveolens* mainly contained the phenolic acids chlorogenic acid and p-coumaric acid and the flavonoids rutoside and quercetin, whereas those of *A. abrotanum* mainly contained the phenolic acids isochlorogenic acid and rosmarinic acid and the flavonoid quercetin. Leaf extracts of both species showed antioxidant activities due to the presence of quercetin, chlorogenic acid, and p-coumaric acid as well as antiproliferative and cytotoxic activities against cancer cells, which may be attributed to necrotic cell accumulation during the early and late apoptotic periods. Both species also exhibited antibacterial activity, although the activity was higher in *R. graveolens* due to the high contents of quercetin and other polyphenols. Finally, both species exhibited antifungal activities, which were associated with specific polyphenols. This is the first study to confirm the richness of polyphenols and wide spectrum of biological activities in natural populations of *R. graveolens* and *A. abrotanum* in northern Saudi Arabia.

**Keywords:** *Ruta graveolens*; *Artemisia abrotanum*; leaf extract; phenolic acids; flavonoids; antiproliferative; cytotoxicity; antioxidant; antibacterial; antifungal

## 1. Introduction

Medicinal plants are rich sources of polyphenols, which have important biological activities. For instance, polyphenols may slow aging through their antioxidant activities [1] or control microorganisms growth through their antibacterial and antifungal activities [2,3]. Furthermore, polyphenols have been shown to act as anticancer agents [4–7] and food preservatives [8].

The antioxidant activity of polyphenols is related to their ability to scavenge free radicals, modulate metal chelation, and activate detoxification enzymes, stimulating their antioxidant mechanism [4,9–14]. The antiproliferative and apoptotic activities of polyphenols against cancer cells are attributed to the control of cell cycle arrest as well as the molecular regulation of cancer-related genes [4,9–13].

*Ruta graveolens* L. (*Rutaceae*) is a shrubby evergreen plant that is native to Southern Europe and used in traditional and alternative medicine. The fresh and dried leaves and stems of this plant can be used for cooking or decoction and tea infusion, whereas essential oils extracted from the leaves and shoots are used for several purposes in the pharmaceutical industry [15–18], such as in the treatment of inflammation and infections and as antidotes for scorpion and snake venoms [19]. Its leaves and stems have been found to contain many compounds, such as rutoside, coquisagenine, skimmianine, graveoline, furocoumarins (psoralens), and xantotoxine (8-methoxy psoralen) [20,21], as well as 2-undecanone, 2-heptanol acetate, 1-dodecanol, geyrene, and 2-nonanone [18]. However, the phytochemical profiles of *R. graveolens* may be affected by environmental factors (temperature, light, soil fertility, and humidity) and genetic diversity both among and within populations [22,23]; therefore, some natural populations are richer sources of secondary metabolites than others.

*Artemisia abrotanum* L. (*Astraceae*) is a deciduous shrub that is native to Asia, Europe, and Africa. The leaves and stems of this plant are used in traditional and alternative medicine to improve digestion and liver function, induce menstrual flow, reduce fever, treat malaria, and control intestinal worms [24]. The major components of the essential oils of this species are piperitone, davanone, 1,8-cineole, and germacrene D [24] or borneol (10.2%) [25]. In addition, some polyphenols have been identified in the leaf extracts of Romanian populations such as sinapic acid, rutoside, quercetin, ferulic acid, and patuletin [26]. Species and subspecies belonging to *Artemisia* are subject to high genetic diversity both among and within populations (e.g., *A. absinthium* and *A. abrotanum*) according to their geographical origins [27–29], and polyploidy is common in this genus, with different cytotypes exhibiting different morphologies and phytochemical contents [30]. Furthermore, the phytochemical content of the leaves and stems depends on the ecotype, geographical origin, and environmental conditions.

In this study, we explored the polyphenol contents of natural populations of *R. graveolens* and *A. abrotanum* growing in northern Saudi Arabia by performing high-performance liquid chromatography-diode array detection (HPLC-DAD) on their leaf extracts and related these to their antioxidant, antiproliferative, cytotoxic, antibacterial, and antifungal activities to determine whether these natural populations are rich sources of secondary metabolites.

## 2. Materials and Methods

### 2.1. Collection and Preparation of Plant Materials

Leaves were obtained from natural populations of *R. graveolens* and *A. abrotanum* growing in the Riyadh region, northern Saudi Arabia, in the beginning of flowering stage of the plants. Plants were identified by Hosam Elansary and vouchered at the College of Food and Agricultural Sciences, King Saud University, Riyadh (Hosam0002213–101). The leaves were lyophilized, powdered, and extracted twice with methanol (0.2 g dry weight (DW) in 10 mL methanol) by sonication for 30 min at 30 °C. The extract was purified using Whatman paper; then, the residues were dried at room temperature to remove remaining methanol and frozen at –80 °C. Regarding HPLC analyses, the residues were dissolved in methanol (1 mL; Merck), whereas for bioassays, methanol was totally removed using a rotary evaporator [31]. All experiments were approved by the Animal Committee of the College of Agriculture, Damietta University, Damietta, Egypt (2018-2019-75365). Bacteria, fungi, and cancer cell lines (American Type Culture Collection) were obtained from the Faculty of Agriculture, Alexandria, Egypt.

## 2.2. Phenolic Compound Analysis

Chromatographic analyses were conducted using a Merck-Hitachi liquid chromatograph (LaChrom Elite) fitted with a diode array detector (L-2455). The Purospher® RP-18e column (250 × 4 mm; 5 µm; Merck (Berlin, Germany) was used as the stationary phase, whereas two solvents made up the mobile phase: solvent A—methanol, solvent B—methanol and 0.5% acetic acid 1:4 (v/v). These solvents were mixed using the following gradient program at a flow rate of 1 mL/min: 0–20 min, 0–20' 100% B; 20–35' 100–80% B; 35–55' 80–60% B; 55–70' 60–0% B; 70–75' 0% B; 75–80' 0–100% B; 80–90' 100% B. The sample injection volume was 20 µL, and analyses were conducted at a temperature of 25 °C and wavelength of 254 nm. Validation of HPLC has previously been conducted by our group [32,33].

Retention time and ultraviolet spectra were compared with standards to identify the compounds in leaf extracts, and calibration curves were used to quantify the identified compounds. This analysis focused on searching for a pool of compounds for which commercial patterns are available, which included both phenolic acids and flavonoids. All 38 standards that were used in the analysis were produced by Sigma-Aldrich (HPLC purity ≥ 95.0%, Berlin, Germany). The following compounds were screened for: 22 phenolic acids [cinnamic acid and its derivatives [caffeic acid ( $y = 598.118x - 1.456$ ,  $R^2 = 0.999$ ), *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid ( $y = 311.434x + 0.376$ ,  $R^2 = 0.999$ ), ferulic acid, hydrocaffeic acid, isoferulic acid, and sinapic acid), benzoic acid and its derivatives (3,4-dihydroxyphenylacetic acid, ellagic acid, gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid ( $y = 1357.761x - 2.599$ ,  $R^2 = 0.999$ ), salicylic acid, syringic acid ( $y = 628.954x + 0.574$ ,  $R^2 = 0.999$ ), and vanillic acid ( $y = 1276.874x - 1.692$ ,  $R^2 = 0.990$ )), and depsides (chlorogenic acid ( $y = 447.904x - 0.452$ ,  $R^2 = 0.999$ ), isochlorogenic acid ( $y = 394.361x + 2.389$ ,  $R^2 = 0.999$ ), neochlorogenic acid, and rosmarinic acid ( $y = 362.955x - 2.320$ ,  $R^2 = 0.999$ ); 7 flavonoids (cynaroside, myricetin, naringin, quercetin ( $y = 672.699x - 8.133$ ,  $R^2 = 0.999$ ), kaempferol, rhamnetin, and luteolin); and nine flavonoid glycosides (apigetrin, cynaroside, hyperoside, quercitrin, robinin, rutoside ( $y = 594.208x + 0.666$ ,  $R^2 = 0.999$ ), isoquercetin, trifolin, and vitexin).

## 2.3. Antioxidant Activity

*R. graveolens* and *A. abrotanum* leaf extracts antioxidant activities were investigated using β-carotene bleaching, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [5,34–38]. The IC<sub>50</sub> (µg/mL) was defined as the amount of extracts required to scavenge 50% of β-carotene bleaching/DPPH solution/FRAP reagent. This values (IC<sub>50</sub>) was determined by plotting the inhibition percent against extract concentration.

In the DPPH assay, serial concentrations of leaf extracts were added to 5 mL of 0.004% methanolic DPPH solution freshly prepared. After incubation for 30 mins in the dark at room temperature, the absorbance was measured at 517 nm. The free radical inhibition by DPPH was calculated as follows:

The percentage inhibition of antiproliferative activity (IAA) was calculated in triplicates:

$$IAA = \frac{(AB_{517nm})_C - (AB_{517nm})_S}{(AB_{517nm})_C} \times 100 \quad (1)$$

where  $(AB_{517nm})_C$  and  $(AB_{517nm})_S$  are Abs.517 nm of control and sample, respectively.

A standard antioxidant was used (butylated hydroxytoluene, BHT) as a positive control and the inhibition concentration of each sample was compared with that of the BHT and blank. In the β-carotene-bleaching assay the mixture was prepared by dissolving the β-carotene (0.5 mg) in chloroform (1 mL), then adding 25 µL linoleic acid and 200 mg Tween 40. The chloroform was removed by vacuum evaporation, and the distilled water was added (100 mL), followed by vigorous shaking. A 2.5 mL of the mixture were mixed with serial concentrations of leaf extracts, incubated for 48 h at room temperature and the absorbance was measured at 470 nm.

In the FRAP assay, aliquots (100  $\mu\text{L}$ ) of leaf extracts/Trolox (Sigma-Aldrich, Berlin, Germany) were added to the FRAP reagent (3 mL), then mixed and incubated for 30 min at 37 °C. The calibration procedure of FRAP was conducted by applying serial dilutions of Trolox (0–0.5 mmol/L), as standard. The absorbance was measured at 593 nm for FRAP. All antioxidant experiments were conducted in triplicates and repeated thrice.

## 2.4. Anticancer Activities

### 2.4.1. Antiproliferative Assay

The antiproliferative and cytotoxic activities of *R. graveolens* and *A. abrotanum* leaf extracts were tested against the following cell lines: T-cell lymphoblast like (Jurkat), breast adenocarcinoma (MCF-7), cervical adenocarcinoma (HeLa), colon adenocarcinoma (HT-29), and normal human embryonic kidney (HEK-293) [4,5]. To determine changes in cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed. The leaf extracts were dissolved in 1% dimethyl sulfoxide (DMSO), and the resulting solutions were added to 75-cm<sup>2</sup> flasks containing standard minimum essential medium (comprising 10% fetal bovine serum, 0.1 mM nonessential amino acids, 17.8 mM NaHCO<sub>3</sub>, and 1 mM sodium pyruvate) and cancer cells. The cancer cells were prepared in microtiter plates using  $4 \times 10^{-4}$  cells per  $\mu\text{L}$  in 270  $\mu\text{L}$  medium for 48 h (37 °C, 5% CO<sub>2</sub>). Serial concentrations of the leaf extracts were used to obtain final concentrations of 50, 100, 200, 300, and 400  $\mu\text{g/mL}$ .

A washing procedure was performed using phosphate-buffered saline (PBS), following which MTT dissolved in PBS (12 mM) was added to the medium and isopropanol (0.04 N HCl) was mixed. Then, the mixture was left for 40 min, after which absorbance was measured at a wavelength of 570 nm. Positive control (vinblastine sulfate and taxol) and negative control (no treatment) were also prepared. Inhibition activity amount was calculated using the following equation:

$$\text{IAA} = \frac{(AB_{570\text{nm}})_C - (AB_{570\text{nm}})_S}{(AB_{570\text{nm}})_C} \times 100 \quad (2)$$

where  $(AB_{570\text{nm}})_C$  and  $(AB_{570\text{nm}})_S$  are the absorbances of the control and sample, respectively.

IC<sub>50</sub> was determined by plotting the percentage of viable cells against the extract concentration in  $\mu\text{g/mL}$ .

### 2.4.2. Flow Cytometry

The IC<sub>50</sub> of MTT was then applied to flow cytometry for investigating the cytotoxic activities of the leaf extracts of *R. graveolens* and *A. abrotanum*, following which the apoptotic cell populations were determined (FAC Scan, New York, NY, USA) [4,5]. Briefly, cultured cancer cells in six-well plates were subjected the IC<sub>50</sub> of leaf extracts as well as identified polyphenols (quercetin and isochlorogenic acid) for 48 h. Untreated cells were considered as control. trypsin (0.25%) was used to detach cells in Hank's balanced salt solution (Thermo Fisher Scientific, Berlin, Germany). The cells were stained using the Annexin V apoptosis detection kit (Sigma, St. Louis, MO, USA). By incubation in the dark at 37 °C for 15 min then washing with PBS. The data of flow cytometer is presented in quadrants as percentage: lower left (viable cells), upper left (necrotic cells), lower right (early apoptotic cells), and upper right (late apoptotic cells).

## 2.5. Antibacterial Activities

The antibacterial activities of *R. graveolens* and *A. abrotanum* leaf extracts were examined using isolates of *Listeria monocytogenes* (clinical isolate), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 35210) *ria*, *Bacillus cereus* (ATCC 14579), *Pseudomonas aeruginosa* (ATCC 27853), and *Micrococcus flavus* (ATCC 10240). A microtiter plate-based protocol (microdilution) was used as described previously [7,39–42], wherein serial concentrations of the extracts and pure standards of

identified polyphenols that were mixed with bacterial inoculum of  $1.0 \times 10^4$  colony-forming units and 100  $\mu$ L tryptic soy broth in each well and incubated for 1 day at 37 °C on a rotary shaker. Minimum inhibitory concentration (MIC) was defined as the lowest concentration that caused no visible growth under a binocular microscope, and minimum bactericidal concentration (MBC) was defined as the minimal concentration that eliminated 99.5% of each inoculum as determined using serial subculturing of the leaf extracts (2  $\mu$ L). Optical density was determined at a wavelength of 655 nm. Streptomycin (0.01–10 mg/mL) and DMSO (1%) were used as positive and negative controls, respectively. Experiments were conducted in triplicate and repeated thrice.

## 2.6. Antifungal Activities

The antifungal activities of *R. graveolens* and *A. abrotanum* leaf extracts were determined against *Penicillium ochrochloron* (ATCC 48663), *Aspergillus ochraceus* (ATCC 12066), *Candida albicans* (ATCC 12066), *As. niger* (ATCC 6275), *As. flavus* (ATCC 9643), and *Pe. funiculosum* (ATCC 56755) using microdilution [33,40,41]. MIC was determined under a stereomicroscope, whereas minimum fungicidal concentration (MFC) was determined by preparing serial dilutions of 2  $\mu$ L of each leaf extract and pure standards of identified polyphenols in subcultures of fungi for 72 h at 28 °C in microtiter plates that contained 100  $\mu$ L of broth medium. Ketoconazole (1–3500  $\mu$ g/mL) and DMSO (1%) were used as positive and negative controls, respectively. Experiments were conducted in triplicate and repeated thrice.

## 2.7. Statistical Analyses

Differences among treatments were expressed as the least significant differences (LSD) in SPSS software. The mean and standard deviation (SD) were calculated from the three replicates (of each assay).

## 3. Results

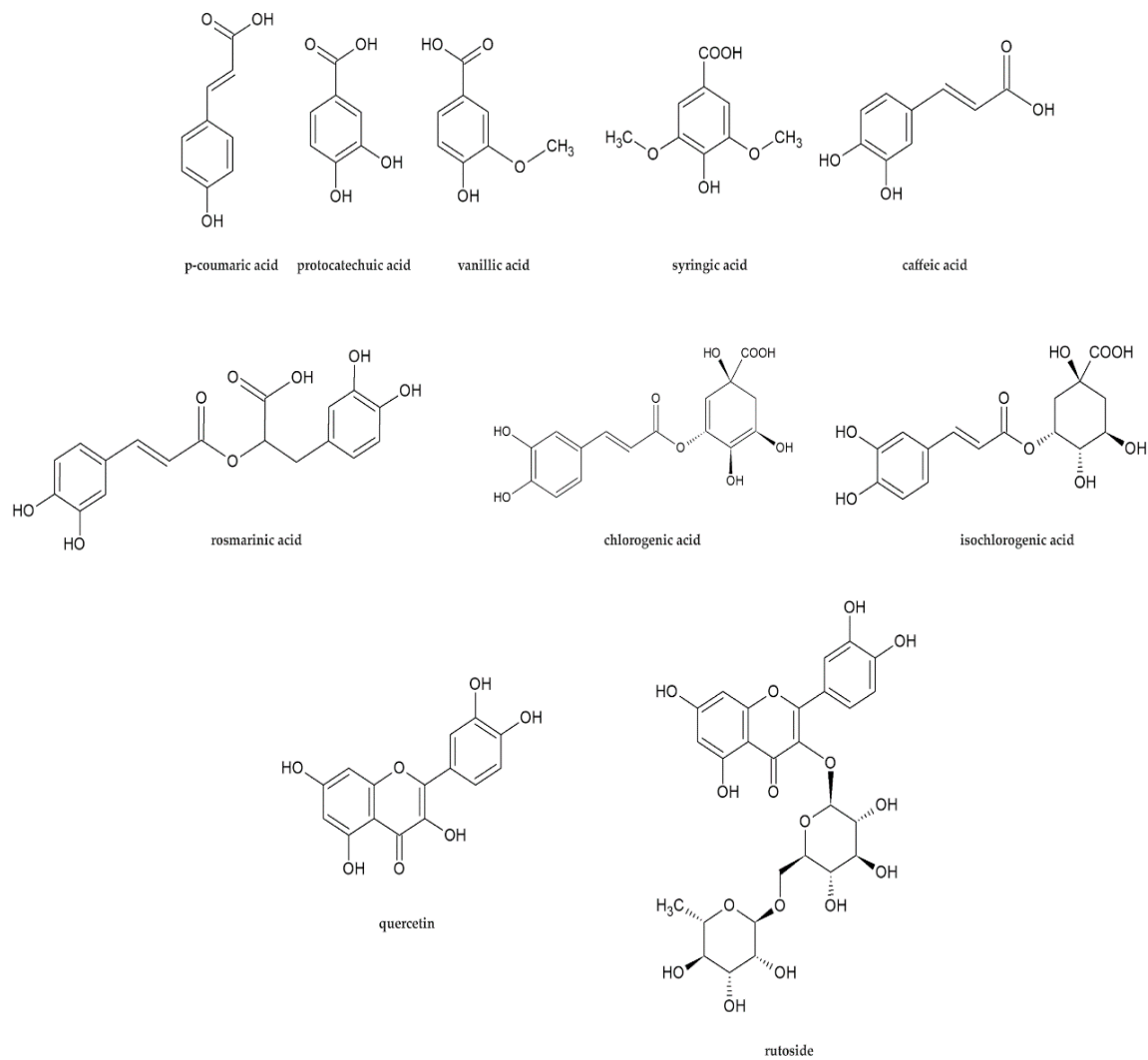
### 3.1. Polyphenol Profiles of the Leaf Extracts

The methanolic leaf extracts of *R. graveolens* were found to contain four phenolic acids and two flavonoids (Table 1, Figures 1 and 2A). The major phenolic acids were chlorogenic acid and p-coumaric acid, with protocatechuic acid and caffeic acid present at lower concentrations. In addition, high concentrations of the flavonoids rutoside and quercetin were detected.

**Table 1.** Polyphenol contents of *Ruta graveolens* and *Artemisia abrotanum* leaf extracts (mg/100 g dry weight).

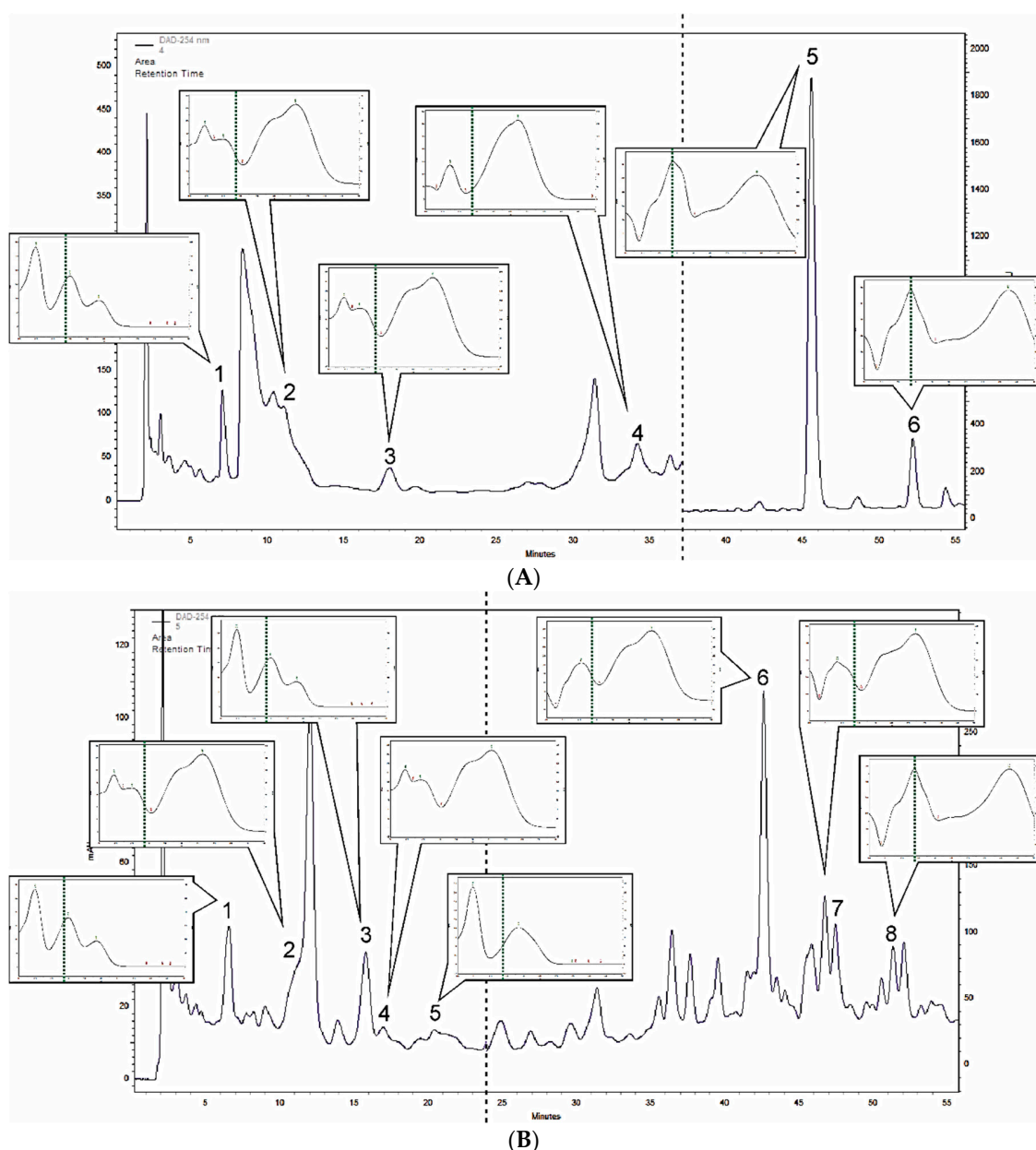
Compound	<i>R. graveolens</i>	<i>A. abrotanum</i>
Caffeic acid	18.2 $\pm$ 0.5	11.2 $\pm$ 1.0
Chlorogenic acid	356.0 $\pm$ 25.2	40.0 $\pm$ 3.6
p-Coumaric acid	158.6 $\pm$ 18.2	nd
Isochlorogenic acid	nd	496.3 $\pm$ 72.5
Protocatechuic acid	49.2 $\pm$ 4.8	25.2 $\pm$ 3.7
Syringic acid	nd	1.9 $\pm$ 0.3
Rosmarinic acid	nd	241.1 $\pm$ 9.2
Vanillic acid	nd	17.9 $\pm$ 1.0
Quercetin	375.4 $\pm$ 17.2	nd
Quercitrin	nd	106.6 $\pm$ 7.2
Rutoside	1010.1 $\pm$ 70.1	nd

Values are expressed as mean  $\pm$  standard deviation. nd, not detected.



**Figure 1.** The chemical structures of the identified polyphenols.

The methanolic leaf extracts of *A. abrotanum* were found to contain seven phenolic acids and one flavonoid (Table 1, Figures 1 and 2B). The major phenolic acids were isochlorogenic acid and rosmarinic acid, with chlorogenic acid, protocatechuic acid, vanillic acid, caffeic acid, and syringic acid present at lower concentrations. Furthermore, quercetin was identified.



**Figure 2.** Examples of high-performance liquid chromatography-diode array detection-based separation ( $\lambda = 254$  nm) of leaf extracts of (A) *Ruta graveolens* (1, protocatechuic acid; 2, chlorogenic acid; 3, caffeic acid; 4, p-coumaric acid; 5, rutoside; 6, quercetin) and (B) *Artemisia abrotanum* (1, protocatechuic acid; 2, chlorogenic acid; 3, vanillic acid; 4, caffeic acid; 5, syringic acid; 6, isochlorogenic acid; 7, rosmarinic acid; 8, quercetin).

### 3.2. Antioxidant Effects

The methanolic leaf extracts of both *R. graveolens* and *A. abrotanum* and the major polyphenols showed strong antioxidant activities (Table 2). However, the methanolic leaf extracts of *R. graveolens* showed higher antioxidant activity than those of *A. abrotanum* according to DPPH,  $\beta$ -carotene bleaching, and FRAP assays. Quercetin, chlorogenic acid, and p-coumaric acid, which were identified in *R. graveolens*, showed strong antioxidant activities, with p-coumaric acid having the lowest  $IC_{50}$  value. In addition, isochlorogenic acid, rosmarinic acid, and quercitrin, which were identified in *A. abrotanum*, showed strong antioxidant activities. Indeed, the antioxidant activities of rosmarinic acid and p-coumaric acid were comparable to those of the BHT and Trolox antioxidant standards.



**Table 2.** Antioxidant activities of *Ruta graveolens* and *Artemisia abrotanum* leaf extracts and the identified polyphenols according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH),  $\beta$ -carotene bleaching, and ferric-reducing antioxidant power (FRAP) assays.

	DPPH (IC <sub>50</sub> , $\mu$ g/mL)	$\beta$ -Carotene-Bleaching Assay (IC <sub>50</sub> , $\mu$ g/mL)	FRAP (IC <sub>50</sub> , mM TEAC/g Extract)
<i>R. graveolens</i>	21.3 $\pm$ 1.5c	26.7 $\pm$ 2.7c	32.8 $\pm$ 3.1c
<i>A. abrotanum</i>	27.1 $\pm$ 2.3a	35.4 $\pm$ 0.3a	39.1 $\pm$ 4.1a
Quercetin	5.6 $\pm$ 0.1d	6.3 $\pm$ 0.1d	7.9 $\pm$ 0.3d
Chlorogenic acid	4.2 $\pm$ 0.3e	5.1 $\pm$ 0.3d	6.9 $\pm$ 0.2d
Isochlorogenic acid	5.17 $\pm$ 0.1d	6.2 $\pm$ 0.1d	7.3 $\pm$ 0.1d
Rosmarinic acid (ok)	2.7 $\pm$ 0.3f	3.1 $\pm$ 0.2e	3.5 $\pm$ 0.2e
p-Coumaric acid	3.4 $\pm$ 0.1e	3.9 $\pm$ 0.1e	4.3 $\pm$ 0.1e
Quercitrin	23.1 $\pm$ 2.1b	28.2 $\pm$ 1.5b	34.9 $\pm$ 2.6b
BHT	2.7 $\pm$ 0.1f	3.3 $\pm$ 0.1e	–
Trolox	–	–	3.2 $\pm$ 0.3e

Butylated hydroxytoluene (BHT) and Trolox were used as standards. Values are expressed as mean  $\pm$  standard deviation. TEAC: Trolox equivalent antioxidant capacity, IC<sub>50</sub>: half maximal inhibitory concentration ( $\mu$ g/mL). Different letters within a column indicate significant differences ( $p \leq 0.05$ ).

### 3.3. MTT Assay and Flow Cytometry

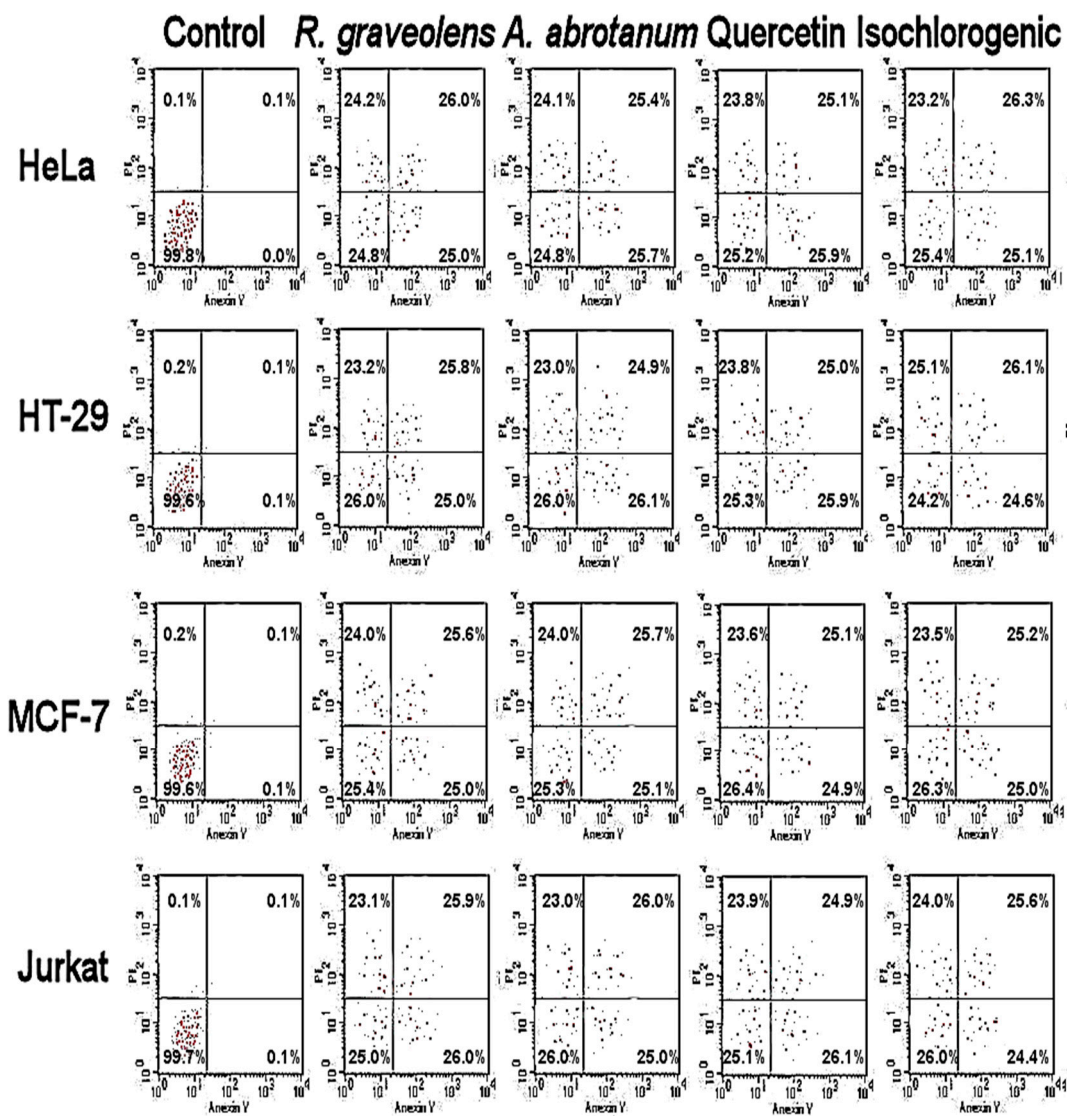
The antiproliferative activities of the methanolic leaf extracts of *R. graveolens* and *A. abrotanum* and the identified polyphenols against different cancer cells were measured using MTT assay. It was found that the leaf extracts of both species and the identified polyphenols had strong antiproliferative activities against all cells, except normal HEK-293 cells (Table 3). In particular, quercetin, chlorogenic acid, isochlorogenic acid, and p-coumaric acid showed strong antiproliferative activities against most cancer cells, with Jurkat being the most resistant cell line.

**Table 3.** Antiproliferative activities of methanolic leaf extracts of *Ruta graveolens* and *Artemisia abrotanum* and the identified polyphenols against different cancer cell lines.

	HeLa *	HT-29	MCF-7	Jurkat	HEK-293
<i>R. graveolens</i>	42.12 $\pm$ 3.8b	47.17 $\pm$ 3.2b	64.86 $\pm$ 4.3b	73.33 $\pm$ 4.6b	>400
<i>A. abrotanum</i>	49.97 $\pm$ 5.1a	54.75 $\pm$ 2.9a	71.04 $\pm$ 5.5a	82.64 $\pm$ 4.1a	>400
Quercetin	4.9 $\pm$ 1.3d	7.45 $\pm$ 1.8f	21.11 $\pm$ 2.6e	38.31 $\pm$ 3.1e	>400
Chlorogenic acid	4.35 $\pm$ 0.5d	15.65 $\pm$ 2.5e	38.65 $\pm$ 3.4d	41.65 $\pm$ 4.6e	>400
Isochlorogenic acid	5.6 $\pm$ 1.7d	18.53 $\pm$ 1.1d	46.63 $\pm$ 2.1c	52.73 $\pm$ 3.2c	>400
Rosmarinic acid	35.30 $\pm$ 3.1c	25.26 $\pm$ 1.7c	24.26 $\pm$ 1.5e	45.75 $\pm$ 3.7d	>400
p-Coumaric acid	6.2 $\pm$ 0.3d	7.8 $\pm$ 0.3f	16.8 $\pm$ 1.3f	33.7 $\pm$ 2.6f	>400
Vinblastine sulfate	2.0 $\pm$ 0.04e	15.8 $\pm$ 0.5e	–	0.12 $\pm$ 0.02g	43.2 $\pm$ 2.4
Taxol	–	–	0.06 $\pm$ 0.005g	–	–

The following cell lines were used: cervical adenocarcinoma (HeLa), colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), T-cell lymphoblast like (Jurkat), and normal human embryonic kidney (HEK-293). Vinblastine sulfate and taxol were used as positive controls. Values are expressed as mean  $\pm$  standard deviation; IC<sub>50</sub>: half maximal inhibitory concentrations ( $\mu$ g/mL). \* Different letters within a column indicate significant differences ( $p \leq 0.05$ ). Values are means of three replicates.

Investigation of the cytotoxic activities of the leaf extracts as well as quercetin and isochlorogenic acid using flow cytometry showed apoptotic cell accumulation in the upper and lower right quadrants following 48 h of exposure (Figure 3).



**Figure 3.** Cytotoxicity of methanolic leaf extracts of *Ruta graveolens* and *Artemisia abrotanum*, quercetin, and isochlorogenic acid against different cancer cell lines as estimated with flow cytometry.

### 3.4. Antibacterial Activities of Leaf Extracts and Identified Polyphenols

The methanolic leaf extracts of both *R. graveolens* and *A. abrotanum* showed antibacterial activities against a range of bacteria, but *R. graveolens* showed higher antibacterial activities against all bacteria studied (Table 4). The most sensitive bacterium (i.e., with the lowest  $IC_{50}$ ) was *S. aureus*, whereas the most resistant was *M. flavus*. Quercitrin, isochlorogenic acid, and p-coumaric acid showed strong antibacterial activities against all bacteria, with the former two having comparable activity to the antibiotic streptomycin. By contrast, rosmarinic acid and quercetin showed moderate-to-low antibacterial activities.

**Table 4.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic leaf extracts of *Ruta graveolens* and *Artemisia abrotanum* and the identified polyphenols (pure standards) against a range of bacterial species (mg/mL). Values are means of three replicates.

	<i>B. cereus</i> MIC MBC	<i>P. aeruginosa</i> MIC MBC	<i>L. monocytogenes</i> MIC MBC	<i>E. coli</i> MIC MBC	<i>M. flavus</i> MIC MBC	<i>S. aureus</i> MIC MBC
<i>R. graveolens</i>	0.37 ± 0.02c 0.75 ± 0.05	0.39 ± 0.01c 0.98 ± 0.04	0.43 ± 0.03c 0.89 ± 0.05	0.36 ± 0.05c 0.96 ± 0.03	0.49 ± 0.05c 0.99 ± 0.07	0.31 ± 0.05c 0.84 ± 0.03
<i>A. abrotanum</i>	0.41 ± 0.03c 0.91 ± 0.03	0.47 ± 0.02c 1.75 ± 0.03	0.45 ± 0.03c 0.99 ± 0.04	0.39 ± 0.03c 1.02 ± 0.05	0.57 ± 0.03c 1.08 ± 0.04	0.38 ± 0.05c 0.97 ± 0.05
Quercitrin	0.07 ± 0.01c 0.13 ± 0.02	0.12 ± 0.01c 0.25 ± 0.03	0.14 ± 0.01c 0.31 ± 0.03	0.13 ± 0.01c 0.28 ± 0.03	0.12 ± 0.01c 0.30 ± 0.03	0.15 ± 0.01c 0.31 ± 0.03
Isochlorogenic acid	0.14 ± 0.01c 0.35 ± 0.03	0.13 ± 0.01c 0.34 ± 0.03	0.13 ± 0.01c 0.35 ± 0.03	0.11 ± 0.01c 0.30 ± 0.03	0.11 ± 0.01c 0.31 ± 0.03	0.11 ± 0.01c 0.31 ± 0.03
p-Coumaric acid	0.13 ± 0.01c 0.30 ± 0.01	0.07 ± 0.01c 0.23 ± 0.03	0.25 ± 0.01c 0.57 ± 0.03	0.13 ± 0.01c 0.26 ± 0.03	0.15 ± 0.02c 0.39 ± 0.03	0.24 ± 0.02c 0.48 ± 0.03
Rosmarinic acid	38.42 ± 2.53a >500	36.4 ± 1.46a >500	45.42 ± 2.75a >500	40.4 ± 2.67a >500	30.53 ± 2.53a >500	21.53 ± 1.53a >500
Quercetin	31.37 ± 1.86b >500	32.1 ± 1.15b >500	41.42 ± 2.75b >500	37.8 ± 1.42b >500	27.21 ± 3.98b >500	20.75 ± 0.86b >500
Streptomycin	0.06 ± 0.01c 0.15 ± 0.02	0.10 ± 0.01c 0.20 ± 0.01	0.11 ± 0.01c 0.23 ± 0.02	0.10 ± 0.01c 0.21 ± 0.02	0.11 ± 0.01c 0.20 ± 0.03	0.14 ± 0.01c 0.32 ± 0.03

Different letters within a column (MIC) indicate significant differences ( $p \leq 0.05$ ). Streptomycin was used as a positive control. Values are expressed as mean ± standard deviation.

### 3.5. Antifungal Effects

The methanolic leaf extracts of both *R. graveolens* and *A. abrotanum* showed moderate antifungal activities against most fungal species studied, but *R. graveolens* showed higher activity (i.e., lower IC<sub>50</sub> values) (Table 5). Quercetin, isochlorogenic acid, p-coumaric acid, quercitrin, and rosmarinic acid also showed antifungal activities, with isochlorogenic acid and quercetin having the highest activities among the studied polyphenols.

**Table 5.** Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the methanolic leaf extracts of *Ruta graveolens* and *Artemisia abrotanum* (and the identified polyphenols pure standards) against a range of fungal species (mg/mL). Values are means of three replicates in (mg/mL).

	<i>Aspergillus flavus</i> MIC MFC	<i>Aspergillus ochraceus</i> MIC MFC	<i>Aspergillus niger</i> MIC MFC	<i>Candida albicans</i> MIC MFC	<i>Penicillium funiculosum</i> MIC MFC	<i>Penicillium ochrochloron</i> MIC MFC
<i>R. graveolens</i>	0.33 ± 0.03b 0.84 ± 0.03	0.47 ± 0.05b 1.02 ± 0.7	0.61 ± 0.06b 1.12 ± 0.09	0.78 ± 0.07b 1.77 ± 0.13	0.53 ± 0.03d 1.05 ± 0.05	0.64 ± 0.05c 1.54 ± 0.23
<i>A. abrotanum</i>	0.39 ± 0.05b 0.91 ± 0.05	0.55 ± 0.05b 1.23 ± 0.09	0.78 ± 0.08b 1.32 ± 0.11	0.86 ± 0.09b 1.96 ± 0.17	0.85 ± 0.07d 1.75 ± 0.09	0.86 ± 0.07c 1.89 ± 0.15
Quercetin	0.30 ± 0.02b 0.61 ± 0.03	0.21 ± 0.01b 0.77 ± 0.05	0.23 ± 0.03b 0.79 ± 0.04	0.05 ± 0.01b 0.35 ± 0.03	0.25 ± 0.02d 0.71 ± 0.05	0.28 ± 0.01c 0.61 ± 0.05
Isochlorogenic acid	4.13 ± 0.2b 24.23 ± 1.97	5.34 ± 0.75b 32.42 ± 1.64	6.12 ± 0.76b 38.33 ± 3.1	10.33 ± 0.87b 51.23 ± 3.53	23.21 ± 2.42b 61.45 ± 3.89	30.32 ± 1.97b 82.76 ± 3.53
p-Coumaric acid	0.20 ± 0.02b 0.41 ± 0.05	0.21 ± 0.01b 0.43 ± 0.03	0.23 ± 0.02b 0.42 ± 0.01	0.30 ± 0.01b 0.62 ± 0.03	0.23 ± 0.02d 0.58 ± 0.03	0.21 ± 0.01c 0.41 ± 0.03
Quercitrin	0.16 ± 0.01b 0.31 ± 0.03	0.19 ± 0.01b 0.43 ± 0.03	0.11 ± 0.01b 0.25 ± 0.02	0.26 ± 0.03b 0.57 ± 0.03	0.29 ± 0.01d 0.50 ± 0.01	0.21 ± 0.02c 0.45 ± 0.03
Rosmarinic acid	185.14 ± 9.86a >1000	221.53 ± 12.63a >1000	235.42 ± 23.53a >1000	381.3 ± 25.53a >1000	211.43 ± 13.71a >1000	353.2 ± 24.76a >1000
KTZ (Ketoconazole)	0.21 ± 0.01b 0.43 ± 0.05	0.21 ± 0.01b 0.45 ± 0.02	0.11 ± 0.01b 0.20 ± 0.02	0.21 ± 0.02b 0.41 ± 0.02	2.04 ± 0.11c 3.63 ± 0.12	0.23 ± 0.01c 0.45 ± 0.03

Ketoconazole was used as a positive control. Values are expressed as mean ± standard deviation. Different letters within a column (MIC) indicate significant differences ( $p \leq 0.05$ ).

## 4. Discussion

Qualitative and quantitative variation in bioactive compound content within populations of the same plant species but with different origins is a fairly well-known phenomenon that has been widely documented [39,43]. In the present study, we investigated the polyphenol contents of leaf extracts of

two quite well-known plants that are used as traditional medicine and grow naturally at sites that experience harsh weather conditions in northern Saudi Arabia.

We confirmed that methanolic leaf extracts of the Saudi-origin *R. graveolens* contained four phenolic acids and two flavonoids, among which rutoside (1010.1 mg/100 g DW), quercetin (375.4 mg/100 g DW), chlorogenic acid (356.0 mg/100 g DW), and p-coumaric acid (158.6 mg/100 g DW) were the main metabolites (Table 1). *R. graveolens* is generally recognized as a plant with a high content of furanocoumarin compounds [44,45], and there is evidence that rutoside and quercetin are the main active flavonoids in this species and may be responsible for its pharmacological functions, which include anti-inflammatory, analgesic, antiandrogenic, antihyperglycemia, antihyperlipidemia, antigout, and anticancer activities [46]. However, phenolic acids may also play an important role in the biological activities of this species. Meinhart et al. [47] previously investigated the availability of chlorogenic acid and its derivatives in various commercial plants in Brazil and confirmed that the leaf extracts of *R. graveolens* contained chlorogenic acid (103 mg/100 g DW) and 3,5-dicaffeoylquinic acid (132 mg/100 g DW), but not caffeic acid, which is one of the precursors of chlorogenic acid derivatives. By contrast, the Saudi-origin *R. graveolens* leaf extracts examined here had 3.5 times higher chlorogenic acid as the Brazilian-origin plants and contained caffeic acid (18.2 mg/100 g DW). In an investigation of leaf extracts of Polish-origin *R. graveolens*, Ekiert et al. [48] confirmed the presence of protocatechuic acid (114 mg/100 g DW) and p-coumaric acid (5 mg/100 g DW). By contrast, the Saudi-origin leaf extracts contained a 31.5 times higher amount of p-coumaric acid, but a 2.3 times lower amount of protocatechuic acid.

*A. abrotanum* is widely recognized as a typical raw material that contains essential oil. The main volatile constituents of this plant are 1,8-cineole, linalool, davanone, and thujyl alcohol [49]. While these compounds appear to play a major role in determining the biological properties of *A. abrotanum*, including spasmolytic and antimicrobial activities [50], polyphenolic compounds and other compounds that are present in the raw material may have considerable synergistic effects [51]. However, there have been few studies on the phenolic acid and flavonoid contents of this species. In the present study, we confirmed that the leaf extracts of Saudi-origin *A. abrotanum* contained several phenolic acids, among which isochlorogenic acid (496.3 mg/100 g DW) and rosmarinic acid (241.1 mg/100 g DW) were the main metabolites (Table 1). It has been reported that this species contains the flavonols 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (casticin), 5,7,3'-trihydroxy-3,6,4'-trimethoxyflavone (centaureidin), 3,5,7-trihydroxy-3,4'-dimethoxyflavone (quercetin 3,4'-dimethyl ether), and 3',4',5-trihydroxy-3,7-dimethoxyflavone (quercetin 3,7-dimethyl ether) [52]. However, the only flavonoid we detected in the extracts of Saudi-origin *A. abrotanum* was quercitrin (3-rhamnosyl quercetin; 106.6 mg/100 g DW). We confirmed, for the first time, that *A. abrotanum* leaf extracts contain several phenolic acids including chlorogenic acid, caffeic acid, isochlorogenic acid, and rosmarinic acid and other phenolic acids such as protocatechuic acid, syringic acid, and vanillic acid (Table 1).

The methanolic leaf extracts of *R. graveolens* and *A. abrotanum* showed noticeable antioxidant activities in DPPH, FRAP, and  $\beta$ -carotene bleaching assays, with the former showing the highest antioxidant activity. Diwan and Malpathak [20] suggested that the phenolic compounds in the leaves of *R. graveolens* originating from central India serve as a good source of antioxidants that could offer potential protective effects against lipid oxidation. Supporting this, we found that the leaf extracts of Saudi-origin *R. graveolens* contained quercetin, chlorogenic acid, and p-coumaric acid, all of which also showed strong antioxidant activities. Quercetin, which is a flavonoid glycoside that is common in plants, can reduce reactive oxygen species production and activate the acute monocytic leukemia cell line THP-1 in vitro [53] and has strong antioxidant and antiproliferative activities against RAW264.7 cancer cells [54]. Chlorogenic acid, which is considered an ester between caffeic and quinic acids, has the capacity to control oxidative and inflammatory stresses and is strongly recommended for consumption in beverages due to its ability to modulate the number of metabolic pathways, thus reducing oxidative stress [55]. p-Coumaric acid, which is a derivative of cinnamic acid, has strong antioxidant activities

that are comparable to standard antioxidants [56,57]. Diwan and Malpathak [20] previously showed that in vitro shoot culture extracts from Indian-origin *R. graveolens* had DPPH IC<sub>50</sub> values ranging from 33 to 60 µg/mL. However, in the present study, we found that Saudi-origin plants had higher antioxidant activities as revealed by DPPH assay, and similarly Pavić et al. [58] found that choline chloride leaf extracts of *R. graveolens* originating from Bosnia and Herzegovina had strong antioxidant activities.

The strong antioxidant activity of the *A. abrotanum* leaf extracts was also attributed to the polyphenols present, which included isochlorogenic acid, rosmarinic acid, chlorogenic acid, and quercitrin. Rosmarinic acid was found to have a strong antioxidant effect, which was comparable to that of the antioxidant standards BHT and Trolox, supporting previous findings [59]. Furthermore, both isochlorogenic acid, which was the major compound in *A. abrotanum* extracts, and chlorogenic acid showed strong antioxidant activities. Previous studies have also observed strong antioxidant activities of both isochlorogenic acid (3,5-dicaffeoylquinic acid), which has the same structure as chlorogenic acid but with >1 caffeic acid group [60], and chlorogenic acid, which is commonly found in fruits and vegetables [7,61].

MTT assay and flow cytometry revealed that *R. graveolens* and *A. abrotanum* leaf extracts had antiproliferative and cytotoxic activities against different cancer cells, similar to quercetin, chlorogenic acid, isochlorogenic acid, and p-coumaric acid. In a previous study, American-origin *R. graveolens* leaf extracts showed anticancer activities against breast, colon, and prostate cancers [62]. However, the polyphenolic compounds responsible for these effects were not identified. In another study, furanoacridones (alkaloids) were identified in German-origin *R. graveolens* leaf extracts and were found to be associated with anticancer activities against MCF-7 cells, which included reduced cell proliferation and cell cycle disturbance [63]. In the present study, several polyphenols, including quercetin, isochlorogenic acid, chlorogenic acid, and p-coumaric acid, were identified for the first time in a natural population of *R. graveolens*, and their activities were associated with the antioxidant, antiproliferative, and cytotoxic activities of this plant. Quercetin is widely recognized as a strong antiproliferative factor against cancer cells and has been shown to suppress the activity of specificity protein 1 and reduces the proliferation of human hepatocellular carcinoma HepG2 cells [64]. It has also been associated with antiproliferative activities against prostate cancer cells as a synergistic factor to epigallocatechin (polyphenol) in green tea [65] and has recently been described as having cytotoxic activity against lung cancer cells [66]. To the best of our knowledge, the antiproliferative and cytotoxic activities of isochlorogenic acid have not been previously described, making this the first report on the activity of this compound against cancer cells. Chlorogenic acid showed moderate antiproliferative activities against cancer cells in this study, which is in agreement with the findings of previous studies [67,68]. Finally, the antiproliferative activities of p-coumaric acid have rarely been studied, with only two investigations reporting antiproliferative and, to a certain extent, cytotoxic activities of this polyphenol against colon cancer cells [69,70]. Therefore, this is the first report on the activity of this polyphenol against other types of cancer cells.

*A. abrotanum* leaf extracts also showed antiproliferative activities against cancer cells, which were attributed to the major polyphenols identified, particularly isochlorogenic acid and rosmarinic acid. Tayarani-Najaran et al. [71] found that CH<sub>2</sub>Cl<sub>2</sub> leaf extracts of wild Iranian-origin *A. biennis* had cytotoxic activities against K562 and HL-60 cancer cells, but they did not identify the responsible polyphenols. Similarly, methanolic flower, leaf, stem, and root extracts of other *Artemisia* species, including *A. absinthium*, *A. vulgaris*, and *A. incana*, showed cytotoxic activities against MCF-7 cells [72], but no specific polyphenols were associated with these activities.

The leaf extracts of *R. graveolens* and *A. abrotanum* had antibacterial activities against a range of bacteria. This antibacterial activity of *R. graveolens* is attributed to the high contents of specific polyphenols such as chlorogenic acid and p-coumaric acid, but particularly to quercetin. It has been shown that quercetin has strong antibacterial activities against [73], as found in this study, as well as against other bacteria including *E. coli* and *Ps. aeruginosa* [74]. Furthermore, chlorogenic acid has strong antibacterial activities [75,76] and p-coumaric acid is a strong antibacterial agent against *B. cereus* and

*Salmonella typhimurium*, acting synergistically with niacin [77]. However, this is the first comprehensive study to illustrate the wide antibacterial activities of p-coumaric acid. Pavić et al. [58] similarly found that choline chloride leaf extracts of *R. graveolens* originating from Bosnia and Herzegovina had strong antioxidant and antibacterial activities, with MIC values ranging from 62 to 125 µg/mL against different strains of *E. coli*, *Ps. aeruginosa*, *B. subtilis*, and *Staphylococcus aureus*. In the present study, we detected relatively lower MIC values, indicating that Saudi-origin *R. graveolens* have stronger antibacterial activities against microbes.

The leaf extracts of *R. graveolens* and *A. abrotanum* showed moderate antifungal activities against most of the fungal species studies. These antifungal activities are strongly associated with the identified polyphenols including quercetin, isochlorogenic acid, p-coumaric acid, quercitrin, and rosmarinic acid. It has previously been shown that quercetin has strong antifungal activities against *C. albicans* and *C. parapsilosis* [78]. However, only one study has demonstrated the antifungal activities of p-coumaric acid (against *Botrytis cinerea*) [79], which contrasts with the wide-spectrum effects observed in the present study, and this is the first report on the antifungal activities of isochlorogenic acid obtained from *R. graveolens* and *A. abrotanum*. Oliva et al. [80] reported on the antifungal activities of ethyl acetate leaf extracts of American-origin *R. graveolens* against *Colletotrichum* sp. and *Botrytis cinerea*, which they attributed to the presence of one quinoline alkaloid and four quinolone alkaloids. In the present study, we investigated a larger number of fungal species and found that *A. abrotanum* showed strong antifungal activities against these fungi, which was attributed to the specific polyphenols it contains. A recent report on *A. gmelinii* similarly found that ethanolic and chloroform leaf extracts showed antifungal activities against *Candida* spp. [81], which were attributed to flavonoid, coumarin, and chlorogenic acid contents.

## 5. Conclusions

To the best of our knowledge, this is the first study to investigate the polyphenol contents and biological activities of methanolic leaf extracts of natural populations of *R. graveolens* and *A. abrotanum* from the Riyadh region of northern Saudi Arabia. Using HPLC-DAD, we identified several polyphenols in the leaf extracts, among which quercetin and isochlorogenic acid were the major components in *R. graveolens* and *A. abrotanum*, respectively. More specifically, *R. graveolens* contained quercetin, chlorogenic acid, p-coumaric acid, protocatechuic acid, caffeic acid, and rutoside, whereas *A. abrotanum* contained isochlorogenic acid, rosmarinic acid, quercitrin, chlorogenic acid, protocatechuic acid, syringic acid, vanillic acid, and caffeic acid. The detected compounds were identified tentatively by HPLC-DAD method; further analyses, using more modern chromatographic methods, are needed to better understand the composition of studied plants. In addition, the phytochemical analyses conducted in this study was a partial analysis of selected compounds in the extract. For, e.g., fingerprinting purposes, a more sophisticated analysis should be conducted. Most of the polyphenols showed antioxidant activities and consequently were associated with the observed antioxidant activities of the leaf extracts. In addition, the leaf extracts of both species showed antiproliferative and cytotoxic activities against cancer cells, which may be attributed to necrotic cell accumulation during the early and late apoptotic periods. Antibacterial activities were also observed in the leaf extracts of both species and in the identified polyphenols, with the *R. graveolens* leaf extracts showing higher antibacterial activity, which was attributed to the high content of quercetin and other polyphenols. Similarly, the leaf extracts of both species exhibited antifungal effects, which were attributed to their polyphenol contents and associated with specific polyphenols. This is the first study to confirm the polyphenolic richness and wide spectrum of biological activities associated with natural populations of *R. graveolens* and *A. abrotanum* from northern Saudi Arabia.

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