



## Article

# *Malus baccata* var. *gracilis* and *Malus toringoides* Bark Polyphenol Studies and Antioxidant, Antimicrobial and Anticancer Activities

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**Abstract:** Exploring new sources of polyphenols with biological activities that work against human diseases is the target of natural product studies. This study determined the polyphenol composition of the bark of *Malus* species *M. baccata* var. *gracilis* (Rehder) T.C.Ku and *M. toringoides* (Rehder) Hughes, using high-performance liquid chromatography with a diode-array detector (HPLC-DAD) analysis. The antiproliferative, cytotoxic, antioxidant and antimicrobial applications of these extracts, as well as the identified phenol, were studied. The HPLC-DAD analysis confirmed three polyphenols in the extracts out of the 21 screened compounds: protocatechuic acid, gallic acid, and catechin. The major constituents in *M. baccata* and *M. toringoides* were protocatechuic acid, at 3.16 and 7.15 mg 100 g<sup>-1</sup> dry weight (DW), respectively, and catechin, at 5.55 and 6.80 mg 100 g<sup>-1</sup> DW, respectively. *M. baccata* and *M. toringoides* bark extracts showed antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH),  $\beta$ -carotene bleaching, and ferric-reducing antioxidant power (FRAP) assays, which were attributed to the dominance of protocatechuic acid. The highest antiproliferative and cytotoxic effects were against Jurkat cells. Against MCF-7 and Hela cells, there was necrotic cell accumulation in the early apoptotic as well as the late apoptotic phase. The bark extracts showed noticeable antibacterial effects against *Listeria monocytogenes*, *Bacillus cereus*, and *Escherichia coli*. Protocatechuic acid showed comparable results to bark extracts. There were antifungal effects against *Aspergillus ochraceus*, *A. niger*, and *Candida albicans*, and the activities were higher than the commercial reagent. *M. baccata* and *M. toringoides* could be considered as a new source of phenolic acids, including protocatechuic acid with anticancer, antibacterial antifungal, and antioxidant-promising effects.

**Keywords:** *Malus*; bark extract; phenolic acids; catechin; antiproliferative; antioxidant; antibacterial; antifungal; cytotoxicity

## 1. Introduction

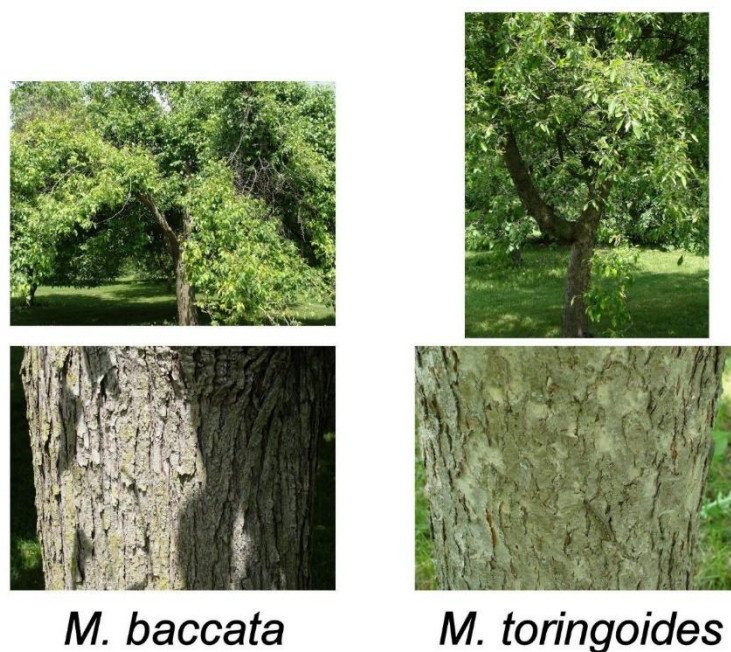
Polyphenol groups (e.g., phenolic acids) are important secondary plant metabolites that contain a large group of compounds with extremely important pharmacological applications.

They are widely used for the control of neurodegenerative diseases [1], cancers [2–5], inflammation cardiovascular health [6,7], type 2 diabetes [8], obesity [9], insect control [10], antibacterial agents [11,12], antioxidants [13], food processing, and the production of functional foods [14]. Polyphenols are abundant in plants, and specific phenols have strong anticancer activities [15]. Recent trends indicate that a balanced lifestyle with a polyphenol-rich diet can control cancers such as prostate cancer, and prevent the future occurrence of the disease [16]. Polyphenols act on tumors by different mechanisms, including the induction of detoxification enzymes and the activation of the antioxidant mechanism, antiproliferation, apoptosis through cell-cycle arrest, and molecular regulation of genes associated with cancer [2,17–20]. Tree barks are promising sources of these polyphenols; they contain phenolic acids, flavonoids, and catechins, as well as sugars [2,12,19,21,22]. Phenolic acids are an important group of phenols as they act as antioxidant agents and have, for example, anticancer and antimicrobial properties [3,23–27].

Microbial infections with *Staphylococcus aureus* cause serious human diseases such as soft tissue infection, pneumonia, sepsis, and peritonitis [28,29]. Other serious diseases, such as listeriosis (which leads to preterm birth and miscarriage) and mild gastroenteritis of immunocompetent individuals, are caused by the bacterial foodborne pathogen *Listeria monocytogenes* [30]. The outbreak of such diseases may cause a high number of deaths, especially with the rising issue of greater resistance to antibiotics [31]. These facts are propelling the search for natural alternative sources of antimicrobial agents, such as those found in tree barks. Recent studies have described the antifungal effects of many polyphenols against plant pathogenic fungi such as *Botrytis cinerea* and *Fusarium oxysporum* [32,33], as well as human pathogenic fungi such as *Candida albicans*, and others [34,35].

The genus *Malus* (Rosaceae) consists of 30 species, including the cultivated apple (*Malus pumila* Mill). *Malus baccata* and *Malus toringoides* (Figure 1) are wild taxa that are considered as sources of variation for apple breeding [36]. *M. baccata* is used as rootstock for commercial apple cultivars and is distributed in Northern America and Eurasia [37]. Few reports have described the chemical composition and biological activities of the fruits [38,39]. Furthermore, no reports have investigated the biological activities of bark extracts. *M. toringoides* is a drought-resistant wild species [40], and no previous investigations have been performed on the chemical composition of the bark.

*Malus baccata* and *Malus toringoides* are widely distributed species; however, experimental studies regarding the chemical composition and biological activities of bark extracts are limited. In this regard, the polyphenol profiles were evaluated qualitatively and quantitatively using the high-performance liquid chromatography with a diode-array detector (HPLC-DAD) method for the first time. Furthermore, the antiproliferative, cytotoxic, antioxidant, antibacterial, and antifungal effects of the bark were explored.

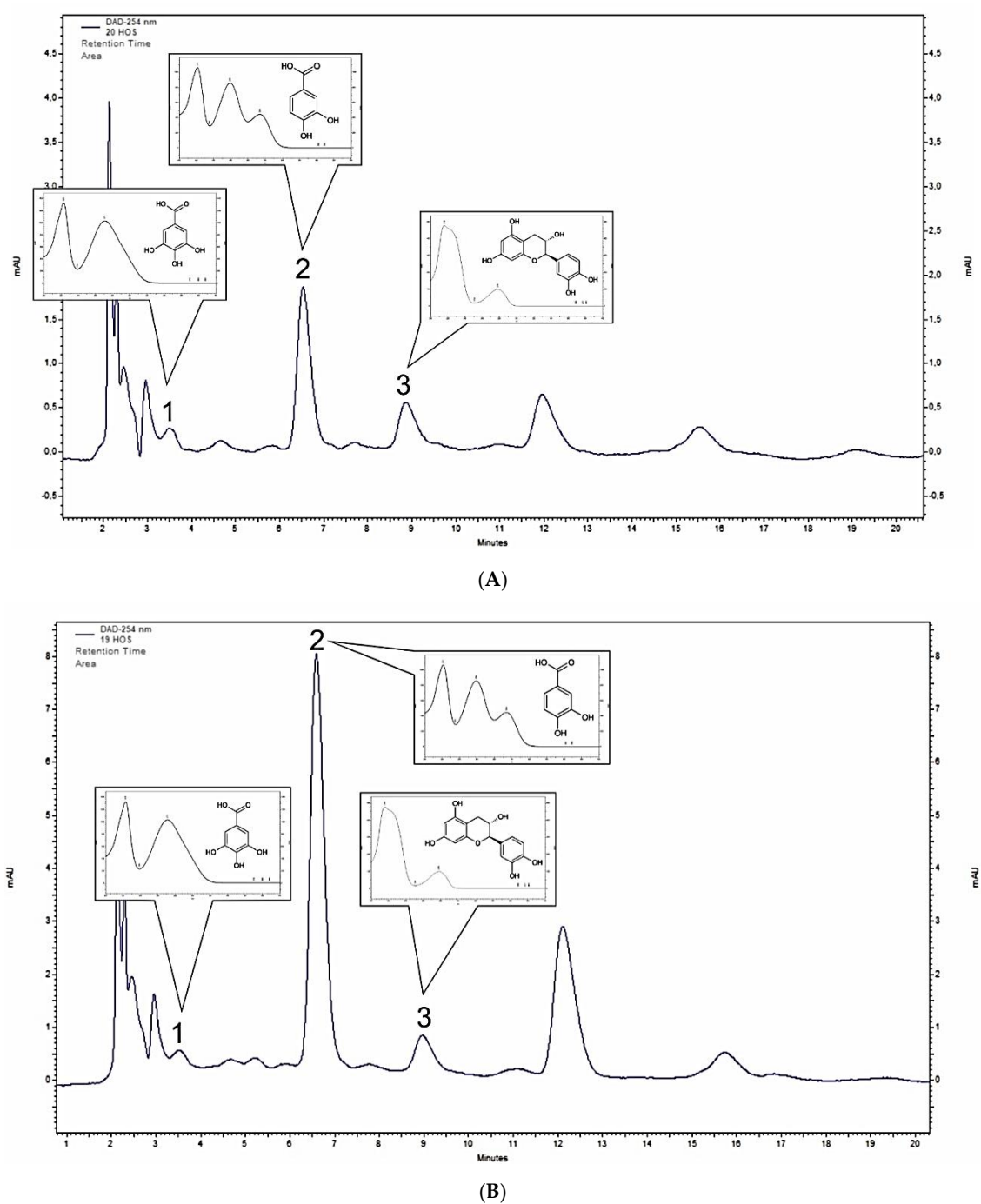


**Figure 1.** The morphological appearance of *Malus baccata* and *Malus toringoides* bark.

## 2. Results

### 2.1. Polyphenol Profiling of *M. baccata* and *M. toringoides* Bark

*M. baccata* and *M. toringoides* bark methanolic extracts were studied using the high-performance liquid chromatography with a diode-array detector (DAD-HPLC) method (Figure 2). Three polyphenols out of the 21 screened were determined in both bark extracts: protocatechuic acid, gallic acid, and catechin (see the Materials and Methods section) (Table 1). The amount of protocatechuic acid for *M. baccata* and *M. toringoides* was 3.16 and 7.15 mg 100 g<sup>-1</sup> (dry weight) DW, respectively. The lower amounts of gallic acid were found in both species: 0.29 mg 100 g<sup>-1</sup> DW and 0.44 mg 100 g<sup>-1</sup> DW, respectively. Catechin was estimated in considerable amounts: 5.55 and 6.80 mg 100 g<sup>-1</sup> DW, respectively (Table 1).



**Figure 2.** The chromatographic separation of methanolic bark extracts of *Malus baccata* (A) and *Malus toringoides* (B) by the high-performance liquid chromatography with a diode-array detector (HPLC-DAD) method; 1—gallic acid, 2—protocatechuic acid, 3—catechin.

**Table 1.** The quantitative estimations of phenols in *Malus baccata* and *Malus toringoides* bark extracts (mg 100 g<sup>−1</sup> (dry weight) DW ± SD).

	Protocatechuic Acid	Gallic Acid	Catechin
<i>M. baccata</i>	3.16 ± 0.44	0.29 ± 0.07	5.55 ± 0.91
<i>M. toringoides</i>	7.15 ± 0.40	0.44 ± 0.13	6.80 ± 1.12

## 2.2. Antioxidant Effects

The *M. baccata* and *M. toringoides* bark extracts showed antioxidant activities by several methods (Table 2). *M. toringoides* showed higher antioxidant effects than *M. baccata* using  $\beta$ -carotene bleaching, DPPH, and ferric-reducing antioxidant power (FRAP) assays. A Butylated hydroxytoluene (BHT) standard showed the lowest IC<sub>50</sub> value compared to the two examined species and the phenolic standard (protocatechuic acid).

**Table 2.**  $\beta$ -carotene bleaching, DPPH, and FRAP of *Malus baccata* and *Malus toringoides* bark extracts and protocatechuic acid.

	$\beta$ -Carotene-Bleaching Assay (IC <sub>50</sub> , $\mu\text{g mL}^{-1}$ )	DPPH (IC <sub>50</sub> , $\mu\text{g mL}^{-1}$ )	FRAP (IC <sub>50</sub> , mM TEAC/g Extract)
<i>M. baccata</i>	7.5 $\pm$ 0.3c	5.8 $\pm$ 0.2c	9.3 $\pm$ 0.1e
<i>M. toringoides</i>	6.2 $\pm$ 0.2d	5.2 $\pm$ 0.1cd	7.5 $\pm$ 0.3e
protocatechuic acid	9.1 $\pm$ 0.3d	7.3 $\pm$ 0.1d	11.5 $\pm$ 0.7f
BHT	3.3 $\pm$ 0.1e	2.7 $\pm$ 0.1e	-
Trolox	-	-	3.3 $\pm$ 0.1g

Different letters within a column indicate significant differences ( $p \leq 0.05$ ). TEAC: Trolox equivalents antioxidant.

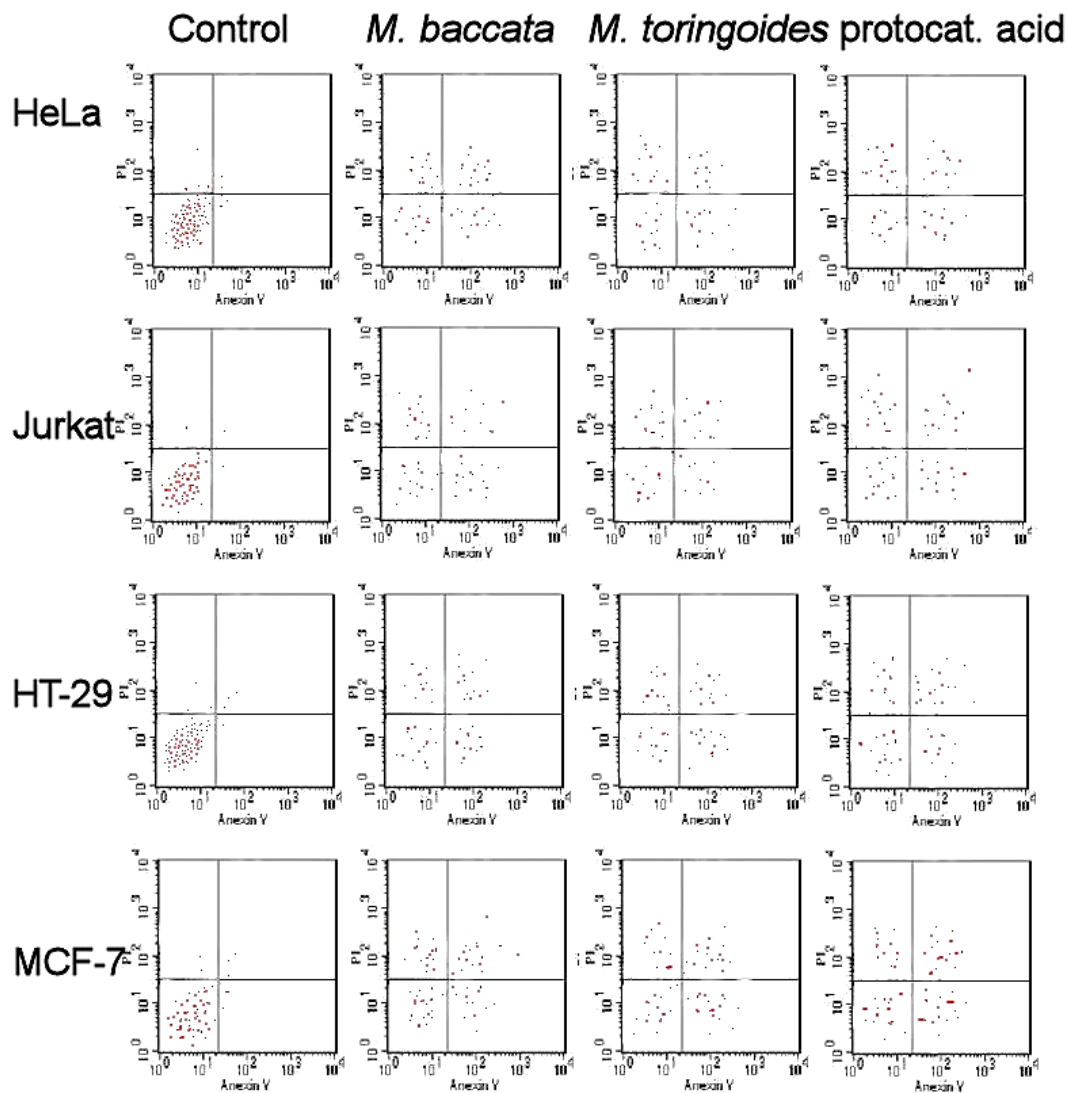
## 2.3. Anticancer Effects

The bark extracts of *M. baccata* and *M. toringoides* had noticeable antiproliferative activities against cancer cells, as shown in Table 3. The highest effects were against Jurkat cells. MCF-7 and Hela cells were affected moderately by bark extracts. The lowest anticancer effects were against HT-29 cells, using both extracts as well as the phenolic standard.

The apoptotic assay of *M. baccata* and *M. toringoides* is shown in Figure 3. Necrotic cell accumulation was found in the early and late apoptotic phases.

**Table 3.** Antiproliferative activity [IC<sub>50</sub> ( $\mu\text{g mL}^{-1}$ )] of *Malus baccata* and *Malus toringoides* bark extracts, ( $\mu\text{g mL}^{-1}$ ) as well as protocatechuic acid, on cancer cells. Values are the means of 3 replicates.

	HeLa	HT-29	MCF-7	Jurkat	HEK-293
<i>M. baccata</i>	64.2 $\pm$ 0.2	104.62 $\pm$ 4.8	41.3 $\pm$ 2.1	38.3 $\pm$ 1.1	>400
<i>M. toringoides</i>	55.31 $\pm$ 1.3	89.59 $\pm$ 2.5	30.35 $\pm$ 1.2	31.25 $\pm$ 1.4	>400
protocatechuic acid	40.58 $\pm$ 2.3	97.54 $\pm$ 4.9	19.65 $\pm$ 1.7	45.32 $\pm$ 3.1	>400
vinblastine sulfate	2.3 $\pm$ 0.08	18.23 $\pm$ 0.7	-	0.1 $\pm$ 0.01	45.5 $\pm$ 0.9
taxol	-	-	0.07 $\pm$ 0.009	-	-



**Figure 3.** Flow cytometry of bark extracts after 48 h. Necrotic cell accumulation was found in the early and late apoptotic phases using IC<sub>50</sub> concentration of bark extracts and protocatechuic acid. Protocat. acid: protocatechuic acid.

#### 2.4. Antibacterial Activities

The bark extracts of *M. baccata* and *M. toringoides* had noticeable antibacterial activities against some bacteria such as *Bacillus cereus*, *Escherichia coli* and *Listeria monocytogenes*. Other bacteria, such as *Micrococcus flavus* and *Staphylococcus aureus*, were more resistant (Table 4).

The phenolic standard protocatechuic acid showed comparable results to the bark extracts. The highest effects of bark extracts were against *Bacillus cereus*.

#### 2.5. Antifungal Effects

The bark extracts of *M. baccata* and *M. toringoides* had noticeable antifungal effects against fungi (Table 5). The lowest IC<sub>50</sub> values were against *Aspergillus ochraceus*, *A. niger*, and *Candida albicans*. However, there was low activity against *Penicillium funiculosum* and *P. ochrochloron*, which were more resistant. The activities of the extracts were higher than the commercial reagent ketoconazole (KTZ). The antifungal effects of protocatechuic acid were comparable to those of *M. baccata* and *M. toringoides*.

**Table 4.** *Malus baccata* and *Malus toringoides* bark extracts and protocatechuic acid antibacterial activities by means of minimum inhibitory (MIC) and bactericidal concentration (MBC). Values are means of 3 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>M. baccata</i>	0.21 ± 0.03 0.56 ± 0.05	0.36 ± 0.02 0.87 ± 0.03	0.32 ± 0.03 0.92 ± 0.05	0.41 ± 0.05 1.29 ± 0.11	0.79 ± 0.05 2.13 ± 0.27	0.47 ± 0.03 1.12 ± 0.17
<i>M. toringoides</i>	0.15 ± 0.03 0.43 ± 0.05	0.24 ± 0.03 0.73 ± 0.05	0.30 ± 0.03 0.83 ± 0.05	0.37 ± 0.03 1.13 ± 0.15	0.98 ± 0.07 2.01 ± 0.31	0.31 ± 0.03 1.03 ± 0.13
protocatechuic acid	0.23 ± 0.02 0.65 ± 0.05	0.35 ± 0.03 0.83 ± 0.04	0.25 ± 0.01 0.67 ± 0.05	0.27 ± 0.01 0.71 ± 0.03	0.55 ± 0.03 1.37 ± 0.13	0.26 ± 0.03 0.79 ± 0.05
streptomycin	0.09 ± 0.01 0.19 ± 0.02	0.08 ± 0.01 0.17 ± 0.01	0.12 ± 0.02 0.27 ± 0.03	0.10 ± 0.01 0.21 ± 0.02	0.11 ± 0.01 0.21 ± 0.01	0.16 ± 0.02 0.31 ± 0.03

**Table 5.** Minimum inhibitory (MIC) and fungicidal concentration (MFC) of *Malus baccata* and *Malus toringoides* bark extracts as well as protocatechuic acid. Values are means of 3 replicates.

	<i>A. Flavus</i> MIC MFC	<i>A. Ochraceus</i> MIC MFC	<i>A. Niger</i> MIC MFC	<i>C. Albicans</i> MIC MFC	<i>P. Funiculosum</i> MIC MFC	<i>P. Ochrochloron</i> MIC MFC
<i>M. baccata</i>	0.43 ± 0.03 0.95 ± 0.05	0.37 ± 0.03 0.69 ± 0.03	0.39 ± 0.03 0.75 ± 0.05	0.47 ± 0.05 1.34 ± 0.21	0.51 ± 0.01 1.23 ± 0.23	1.89 ± 0.13 N.D.
<i>M. toringoides</i>	0.29 ± 0.03 0.57 ± 0.05	0.25 ± 0.02 0.49 ± 0.03	0.29 ± 0.05 0.69 ± 0.03	0.34 ± 0.05 1.23 ± 0.13	0.45 ± 0.05 1.11 ± 0.09	1.13 ± 0.02 N.D.
protocatechuic acid	0.37 ± 0.03 0.86 ± 0.05	0.29 ± 0.03 0.53 ± 0.03	0.33 ± 0.02 0.61 ± 0.03	0.59 ± 0.7 1.11 ± 0.16	0.59 ± 0.01 1.73 ± 0.31	1.37 ± 0.15 N.D.
KTZ	0.19 ± 0.01 0.41 ± 0.03	0.20 ± 0.01 0.43 ± 0.03	0.11 ± 0.01 0.21 ± 0.03	0.21 ± 0.01 0.42 ± 0.03	2.04 ± 0.15 3.73 ± 0.09	0.20 ± 0.03 0.41 ± 0.03

N.D.: Not detected.



### 3. Discussion

In our study, *M. baccata* and *M. toringoides* bark extracts were shown to contain three polyphenols: protocatechuic acid, gallic acid, and catechin (Table 1 and Figure 2). Protocatechuic acid and catechin were the most quantitatively dominant compounds. A previous investigation on the fruit pulps and seeds of *M. baccata* found amino acids, fructose, sucrose, and fatty acids, and suggested that the fruits are a good source of phenolic compounds [38]. Further, the analyses of the fruit skin and flesh of different apple cultivars revealed the presence of several triterpenes, including ursolic and oleanolic [39], and showed anti-inflammatory activities. A preliminary study using two-dimensional chromatograms on Whatman No. 1 paper indicated the presence of few flavonoids (e.g., quercetin) in some *Malus* cultivars in the United Kingdom, and the results have been used in the classification of this species [41]. However, to our knowledge, this is the first report exploring the phenolic content and biological activities of the tree bark of *M. baccata* and *M. toringoides* extracts. Protocatechuic acid and catechin were detected in low quantities, but they are essential antioxidant and anticancer compounds [42–44]. Undoubtedly, a more thorough chemical composition analysis will be needed, for example, one using more modern methods, in order to obtain more detailed knowledge about the composition of *M. baccata* and *M. toringoides* bark extracts.

*M. baccata* and *M. toringoides* bark extracts showed antiradical activities, and *M. toringoides* was higher as an antioxidant. These activities could be attributed to the phenolic profile detected. The major compound detected was protocatechuic acid, which was strongly associated with antioxidant activities in all assays and was relatively comparable to standard antioxidants. Protocatechuic acid is a strong antioxidant, as several studies previous studies have found [42,45]. Other tree bark extracts showed noticeable antiradical activities which were attributed to several phenols including protocatechuic acid [2].

*M. baccata* and *M. toringoides* had noticeable antiproliferative activities against cancer, and the highest effects were against the Jurkat cells MCF 7 and Hela. The apoptotic assay of *M. baccata* and *M. toringoides* showed necrotic cells increase in the early and late apoptotic phases in the flow cytometry assay. Such effects are associated with high concentrations of protocatechuic acid. Previous investigations revealed that protocatechuic acid inhibited the chemical carcinogenesis and showed proapoptotic and antiproliferative activities [42]. Protocatechuic acid inhibits the metastasis of B16/F10 cancer cells by the expression and control of Ras/Akt/NF- $\kappa$ B and MMP-2 production [46]. It also has toxic effects against gastric epithelial cancer cells, and works as a natural urease inhibitor [47].

The extracts of *M. baccata* and *M. toringoides* bark had antibacterial activities against some bacteria such as *B. cereus*, *E. coli*, and *L. monocytogenes*. Further, protocatechuic acid showed comparable results to bark extracts. The antibacterial activities of bark extracts are attributed to the availability of phenolic acids such as protocatechuic acid. A previous investigation revealed that protocatechuic acid revealed antibacterial activities against *S. aureus* [48]. Further, protocatechuic acid and other phenols in mushrooms were responsible for the antimicrobial and antifungal activities against several organisms [49].

The bark extracts of *M. baccata* and *M. toringoides* had noticeable antifungal activities, and the lowest IC<sub>50</sub> values were against *A. ochraceus*, *A. niger*, and *C. albicans*. The antifungal activities of protocatechuic acid were comparable to those of *M. baccata* and *M. toringoides*. The antifungal activities of these bark extracts are attributed to their phenolic profile, including protocatechuic acid. Protocatechuic acid has known antifungal activities against several fungi [50], and had a strong antifungal effect against *Botrytis cinerea* [51].

### 4. Materials and Methods

#### 4.1. Plant Material and Preparation

The barks of *Malus* species *M. baccata* var. *gracilis* (Rehder) T.C.Ku and *M. toringoides* (Rehder) Hughes were obtained in the spring from the arboretum of the University of Guelph, Canada,



and vouchered in both Alexandria University, Egypt (Hosam0001050–1052) and the University of Guelph. Bark samples (3 replicates) were dried by lyophilization (Labconco, California, USA) and powdered [3,52]. Of the dried powder (DW), 0.2 g was sonicated in a methanol extraction (10 mL, Chempur, Lublin, Poland) for  $2 \times 30$  min at 30 °C (Sonic-2, POLSONIC, Poland). The filtered extracts (filtered by Whatman paper) were left at room temperature to lose the methanol (for about 24 h). The methanol (1 mL) was added to the residue and mixed until dissolved completely, and the samples were kept in deep freezing at −80 °C. Before using the samples for bioassays, the methanol was eliminated using a rotary evaporator (40 °C). The bacterial and fungal isolates were obtained from Alexandria University, Egypt. Breast adenocarcinoma cultures (MCF-7), T-cell lymphoblast like (Jurkat), colon adenocarcinoma (HT-29), cervical adenocarcinoma (HeLa), and HEK-293 (normal human cells) were obtained from an institutional collection of the American Type Culture Collection (ATCC).

#### 4.2. Chemicals

A collection of high-performance liquid chromatograph (HPLC) standards were used for the qualification and quantification of phenolics, including cinnamic acid and its derivatives (caffeic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, 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, salicylic acid, syringic acid, and vanillic acid); and depsides (chlorogenic acid, neochlorogenic acid, and rosmarinic acid). The flavonoids were quantified using the standards of aglycone (myricetin, quercetin, kaempferol, rhamnetin, and luteolin) and glycoside (apigenin, cynaroside, hyperoside, quercitrin, robinin, rutoside, isoquercetin, trifolin, and vitexin). The catechin and its derivatives (epigallocatechin gallate, epicatechin, epigallocatechin, and epicatechin gallate) were also screened. All chemicals were purchased from Sigma-Aldrich, Berlin, Germany.

#### 4.3. Analyses of Phenolic Compounds

Bark extracts were subjected to HPLC-DAD analysis [2,3,53,54] using a Merck-Hitachi liquid chromatograph (LaChrom Elite) containing a diode-array detection (DAD) detector (L-2455). A Purospher RP-18e column was used (5 µm, 250 × 4 mm; Merck, Berlin, Germany). A flow rate was set at 1 mL min<sup>−1</sup> at 25 °C. The extract volume of 20 µL was used for the injection. The compounds were detected at 254 nm by the validated HPLC method [53,54]. Compound identification was accomplished by comparing UV retention times and spectra of reference substances. Co-chromatography was also used for identification. The quantification was determined using calibration curves [53–55].

#### 4.4. Anticancer Activities

The antiproliferative and cytotoxic activities of each bark were examined against HeLa, MCF-7, Jurkat, and HT-29 [2,3,33]. HEK-293 (normal cells) were also used. The 3–2,5-diphenyltetrazolium bromide (MTT) assay was used. Briefly, the cells were grown in Minimum Essential Medium (MEM) medium with 10% FBS, 0.1 mM non-essential amino acids, 17.8 mM NaHCO<sub>3</sub>, and 1 mM sodium pyruvate in 75 cm<sup>2</sup> flasks. Then, microtiter plates (96-well) were used for seeding cells at  $4 \times 10^4$  cells per µL in 270 µL medium at 37 °C, 5% CO<sub>2</sub> for 48 h. Extracts were added to the culture media in the plates until reaching 50, 100, 200, 300, and 400 µg/mL final concentration. Then, the Phosphate-buffered saline (PBS) was used to wash the extract traces, and 12 mM MTT dissolved in PBS was added to the medium. Isopropanol (0.04 N HCl) was added to each well, and the mixture was left for 40 min. Vinblastine sulfate and taxol served as positive controls; a negative control was also used (untreated cells). A microplate reader was used at a 570 nm wavelength (Thermo Scientific, California, USA). The inhibition activity percentage was determined in triplicate:

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

where AB is the absorbance, and  $(AB_{570nm})_C$  and  $(AB_{570nm})_s$  are Abs.570 nm of control and sample, respectively.

By plotting the percentage of viable cells against extract concentration, the  $IC_{50}$  values were calculated in  $\mu\text{g mL}^{-1}$  using flow cytometry (FAC Scan, Becton Dickinson, Iowa, USA) as described in [2,33,56], and untreated cells were considered control.

#### 4.5. Antioxidant Activity

Bark extracts were subjected to  $\beta$ -carotene bleaching as well as FRAP and DPPH assays in triplicate replications [3,57–61]. A wavelength of 470 nm was used in the  $\beta$ -carotene bleaching experiment, while a 517 nm wavelength was used in the DPPH to measure absorbance. The bark extract amounts that scavenged 50% of the  $\beta$ -carotene or DPPH were considered as the  $IC_{50}$  value and expressed as  $\mu\text{g mL}^{-1}$ . This was determined by plotting the extract concentration against the inhibition percentage. The FRAP reagent was prepared in the experiment as in, e.g., [43]. The FRAP reagent (3 mL) was mixed with 100  $\mu\text{L}$  of bark extracts as well as Trolox. The mixture was incubated for 30 min at 37 °C and the absorbance was determined at 593 nm. The calibration procedure was accomplished using serial dilutions of Trolox (0–0.5 mmol/L).

#### 4.6. Antibacterial Activity

The bark extracts' bacterial control against *Listeria monocytogenes* (clinical isolate), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 35210), *Bacillus cereus* (ATCC 14579), *Pseudomonas aeruginosa* (ATCC 27853) and *Micrococcus flavus* (ATCC 10240), were tested using the micro-dilution protocol [5,34,62,63] in microtiter plates (96-well). Serial concentrations of bark extracts were mixed with  $1.0 \times 10^4$  colony forming unit (CFU) bacterial inoculum in each well, with 100  $\mu\text{L}$  tryptic soy broth per well, then incubated for one day at 37 °C on a rotary shaker. The lowest concentration of extracts that caused no visible growth (using a binocular microscope) was considered as the minimum inhibitory concentration (MIC). To determine the minimum bactericidal concentration (MBC), serial sub-culturing of bark extracts (2  $\mu\text{L}$ ) was used, and the lowest concentration causing elimination of 99.5% of the inoculum was considered as the MBC. The optical density was determined using 655 nm wavelength. Streptomycin (positive control) was used at 0.01–10 mg/mL, as well as DMSO (negative control, 1%).

#### 4.7. Antifungal Activity

The antifungal activities of *Malus* sp. bark extracts were determined against various fungi, including *Penicillium ochrochloron* (ATCC 48663), *A. ochraceus* (ATCC 12066), *Candida albicans* (ATCC 12066), *A. niger* (ATCC 6275), *Aspergillus flavus* (ATCC 9643), and *Penicillium funiculosum* (ATCC 56755). The microdilution method was employed in this assay [34,56,62]. The minimum inhibitory and fungicidal concentrations (MIC and MFC, respectively) were determined. The minimal concentration inhibiting fungi growth by stereomicroscope was considered as MIC. Extracts' serial dilution (of 2  $\mu\text{L}$ ) was used in sub-cultures (at 28 °C for 72 h) of the fungi in microtiter plates containing 100  $\mu\text{L}$  of broth medium and inoculum to determine the MFC. Positive and negative controls were used, including ketoconazole (KTZ, 1–3500  $\mu\text{g/mL}$ ) and DMSO (1%), respectively.

#### 4.8. Research Ethics

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Alexandria University (63663-937) on 1 December 2017.

#### 4.9. Statistical Analyses

The least significance difference (LSD) was determined using SPSS software.

## 5. Conclusions

This is the first report investigating the phenolic profile of *M. baccata* bark. In addition, this is the first report on the antiproliferative, cytotoxic, antioxidant, antibacterial, and antifungal effects of *M. baccata* and *M. toringoides* bark extracts. Out of the 21 screened, the HPLC study revealed the availability of three different polyphenols: protocatechuic acid, gallic acid, and catechin. The major compounds were protocatechuic acid and catechin. *M. baccata* and *M. toringoides* bark extracts showed antioxidant activities using  $\beta$ -carotene bleaching, DPPH, and FRAP assays, which were attributed to the dominance of protocatechuic acid. The highest antiproliferative and cytotoxic effects were against Jurkat cells. Against MCF-7 and Hela cells, there was an accumulation of necrotic cells in the apoptotic phases. The bark extracts showed noticeable antibacterial effects against *Listeria monocytogenes*, *Bacillus cereus*, and *Escherichia coli*. The protocatechuic acid showed comparable results to bark extracts. There were antifungal effects against *Aspergillus ochraceus*, *A. niger*, and *Candida albicans*, and the effects were higher than the commercial reagent. *M. baccata* can be considered as a newly explored source of phenols that have antioxidant and other biological activities.

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