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# Phenolic constituents from apple tree leaves and their *in vitro* biological activity

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ABSTRACT

Apple (*Malus domestica* Borkh.) leaves are good source of polyphenols. Considering the increasing demand of such phytochemicals, particularly in healthcare sector, the objective of this study was to evaluate the bioactivity of apple leaves phenolics. Different solvent mediated extracts obtained from the apple leaves were assessed for presence of phenolic compounds. Among different extracts, the highest phenolic content of 30.38 ± 0.50 mg/g were recorded in 70% aqueous ethanol (ALE-7) with subsequent high antioxidant value ($IC_{50}$ 49.16 μg/mL) by ABTS assay. RP-HPLC-DAD phenolic profiling of leaves extract, irrespective of solvent used for extraction, revealed presence of five major compounds with maximum yield of phloridzin (24.43 ± 0.05 mg/g), followed by quercitrin (2.06 ± 0.05 mg/g), quercetin-3-*O*-glucoside (1.55 ± 0.001 mg/g), epicatechin (0.37 ± 0.07 mg/g) and phloretin (0.15 ± 0.05 mg/g). ALE-7 extract was further fractionated with hexane (ALH) and ethyl acetate (ALEA), which were evaluated for their *in vitro* biological activities. ALEA extract exhibited higher nitric oxide (NO) scavenging activity (63.3%) at 200 μg/mL. This fraction also showed maximum lymphocyte proliferation (34%) at 25 μg/mL after 48 h. The antimicrobial testing of isolated fractions revealed that ALH fraction (MIC value ranging from 1.18–2.37 μg/mL) could be a good candidate, especially for controlling food borne pathogen. Furthermore, the *in vitro* cytotoxicity assessment of different apple leaves fractions was also performed against human cancer cell lines (KB, SiHa and A-549), but none of the fraction was found cytotoxic against selected cell line. In conclusion, the presence of biologically active phenolics in apple leaves makes it a feasible renewable bioresource for extraction of such phytochemicals for the development of nutraceuticals particularly against inflammation and microbial infections.



## 1. Introduction

In addition to dietary constituents, fruits and vegetables also enriched human diet with specific non-nutritious biologically active constituents like carotenoids, flavonoids, isoflavonoids and phenolic acids. These phytochemicals hold a range of activities and provide array of health benefits through prevention of oxidative burst, hypolipidemic and inflammatory damage, regulation of immune response and protection against various chronic diseases (Alissa and Ferns, 2012; George et al., 2009; Liu, 2003). The potential as well as demand of such phytochemicals and plant extracts can be gauzed from their overall global market worth, valued at around $2.5 billion in previous year (M&M, 2014). However, there is an unprecedented gap between the demand and supply of these molecules, particularly in healthcare sector. This deficit is mainly owned by dwindling natural resources, which drives the scientific predilection to look for alternative renewable resources. Among different phytochemicals, polyphenols hold prime position among various health promoting natural ingredients owing to their protective role during oxidative damage of cellular tissues (Pandey and Rizvi, 2009). These compounds play significant role in regulating metabolic disorders like coronary heart disease

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(CHD)/Cardiovascular disease (CVD), a topmost killer disease of previous decade (WHO, 2014). Recently, few reports on exploring compounds from the leaves of various plants are published using different extraction solvents and techniques (Dahmoune et al., 2014; Kazan et al., 2014; Melguizo-Melguizo et al., 2014). Similarly, apple (*Malus domestica* Borkh.) leaves can be a potential source for extraction of valuable bioactives. Surprisingly, except fruits and pomace, limited information is available on leaves and stem of apple plants. A study on scab (*Venturia inaequalis*) resistance in different apple cultivars revealed that apple leaves contain variety of polyphenolic compounds (Piccinelli et al., 1995). In a similar study, higher content of hydroxycinnamic acid, flavanols and phloridzin were reported from *V. inaequalis* infected leaves as compared to healthy leaves of Golden delicious cultivar (Mayr et al., 1997; Petkovsek et al., 2011). It was revealed that ethanolic extracts of apple leaves possess comparable antioxidant activity to that of the fruit, as evident from the degree of lipid peroxidation (Bonarska-Kujawa et al., 2011).

A number of reports are available exhibiting numerous biological activities such as antioxidant, anti-inflammatory, antimicrobial, anticancer and cardioprotective effects of the phenolic compounds extracted from apple fruits (Barreca et al., 2014; Chang et al., 2012; Olson et al., 2007; Rezik et al., 2002; Wu et al., 2009). However, information pertaining to such diverse activities from extracts of apple leaves is lacking. Hence, the efforts were made in this work to evaluate mature apple leaves as a source of polyphenols along with their possible *in vitro* biological activities.

## 2. Material and methods

### 2.1. Plant material

*Malus domestica* leaves (Red Chief) were collected from the orchard situated in the mid-hills of the Northwestern Himalayas at Bulsan, District Shimla, Himachal Pradesh, India. The mature leaves were collected from the orchard and dried at 55 °C until constant weight was attained in an oven (Macro scientific). The dried leaves were pulverized in Retsch cutting mill (1 mm size) and stored in an air tight polybags till further evaluation.

### 2.2. Chemical and reagents

Epicatechin, phloridzin, phloretin, quercitrin, quercetin-3-*O*-glucoside, quercetin, gallic acid and trolox were purchased from Sigma Aldrich. All other chemicals and solvents were of analytical grade (Merck, India).

### 2.3. Extraction of plant material

The extraction of phenolics from dried leaves powder was done using methanol and ethanol solvents along with their aqueous concentration (70 and 50%). In brief, 100 mg leaf powder was taken in 15 mL centrifuge tube, with addition of 2 mL of respective solvents. Tubes were vortexed for 2 min followed by centrifugation at 5000 rpm for 10 min at room temperature (RT). The extraction process was repeated twice with 1.5 mL solvent and supernatant was collected and pooled to make final volume 5 mL with respective solvent. The extracts were filtered using 0.45 μm filter and were labeled as ALE (ethanol), ALE-5 (50% Ethanol), ALE-7 (70% Ethanol), ALM (Methanol), ALM-5 (50% Methanol) and ALM-7 (70% Methanol).

### 2.4. Total phenolic content

The total phenolic contents of apple leaves extracts obtained using various solvents (ALE-7, ALE-5, ALE, ALM-7, ALM-5 and ALM) were determined by spectrophotometric method (Swain and Hillis, 1959; Rana et al., 2013). The absorbance of reaction mixture was measured at 735 nm using spectrophotometer (T 90$^+$, PG instruments Ltd). The total phenolics content was expressed as mg gallic acid equivalent (GAE)/g dry plant material. All measurements were done in triplicate.

### 2.5. Total flavonoid content

Total flavonoid content in different solvent extracts of apple leaves was measured spectrophotometrically (Kosalec et al., 2004). The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer (T 90$^+$, PG instruments Ltd). The total flavonoid content of samples was expressed as mg quercetin equivalent (QE)/g dry plant material. All measurements were done in triplicate.

### 2.6. In vitro *antioxidant activity*

#### 2.6.1. 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The antioxidant activity of apple leaves extracts was determined spectrophotometrically by previously reported method with some modifications (Re et al., 1999). Different concentration of apple leaves extracts were mixed with 2.0 mL of ABTS solution and absorbance was read at 734 nm using spectrophotometer (T 90$^+$, PG instruments Ltd.) exactly after 4 min. The radical scavenging activity (% inhibition) was calculated as:

% inhibition = [(AB − AA)/AB] × 100, where AA – absorption of respective extract and AB – absorption of blank sample. The percent inhibitions were plotted against respective concentrations and $IC_{50}$ values were calculated.

### 2.7. RP-HPLC-DAD profiling of phenolics

Profiling of phenolic constituents in apple leaf extracts was performed using previously reported Reverse Phase- High Performance Liquid Chromatography (RP-HPLC-DAD) method (Rana et al., 2014). The separation of phenolic constituents was done using Synergi MAX RP80, $C_{12}$ column (4.6 × 250 mm length, 4 μm particle size). Acetonitrile (A) and 0.01% trifluoroacetic acid (B) was used as mobile phase at a flow rate of 1.0 mL/min and phenolics spectral data was recorded at 280 nm. For the quantification of phenolics, five different concentrations (5–25 μg/mL) of each standard were prepared from respective stock solution (1 mg/mL in HPLC grade methanol).

### 2.8. Extract preparation for antimicrobial, cytotoxicity and anti-inflammatory potential

For determining the antimicrobial, *in vitro* cytotoxic activity and anti-inflammatory potential, aqueous ethanol (70%) was used as extraction solvent due to high polyphenolic yield obtained in previous experiments. Dried apple leaves powder (500 g) was extracted thrice with 70% aqueous ethanol (1 L × 24 h × 3) at room temperature. The obtained extract was pooled and concentrated in a rotary evaporator (Buchi 210). The aqueous portion was partitioned with hexane and ethyl acetate (3 × 200 mL). The hexane (ALH), ethyl acetate (ALEA) and aqueous (ALW) fractions were concentrated in a rotary evaporator and lyophilized until a constant weight was obtained. The lyophilized extract powder was stored at 4 °C for further analysis.