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HPLC Polyphenolics Profile and H_2O_2 Induced Cytoprotective Effect of *Salacia oblonga* Extracts on Human Lymphocytes

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Abstract

Salacia oblonga (Wall.) belonging to the family _Celastraceae_ is an important medicinal plant available in India and Sri Lanka. It possesses an array of pharmacological and therapeutic properties. In the present study phenolic compounds of _S. oblonga_ methanolic extracts were estimated by high performance liquid chromatography (HPLC) indicating the presence of catechin, quercetin, synapic acid and syringic acid. The study involved evaluation of _S. oblonga_ extracts for cytotoxic and cytoprotective effects on human lymphocytes. Aerial extracts have significant protective activity against hydrogen peroxide (H_2O_2) induced cytotoxic activity in comparison to the root extracts. The results relate the cytoprotective effect of _S. oblonga_ due to the presence of poly phenols and other phytochemicals, as they have significant ability to remove free radicals or reactive species and might induce the expression of antioxidant enzymes inside the cells.

Keywords

***Salacia oblonga*, Poly Phenol, Cytoprotective, Lymphocytes, H_2O_2**

1. Introduction

Plants produce an array of natural products possessing antioxidant properties which include polyphenols and flavonoids, that are believed to modulate oxidative stress and work for impeding diseases [1]. Poly phenolic compounds present in various medicinal plants have many applications in food, pharmaceuticals and cosmetic industries [2]. Phenolics are the most abundant secondary metabolites in plants, exceptionally rich in polyphenols with antioxidant activity as well as cytoprotectivity [3]. Poly phenols such as catechin, quercetin and gallic

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acid exhibit good antioxidant and cytoprotective activites [4] [5]. In addition to their antioxidant properties, polyphenols also exhibit a wide range of medicinal properties like antimicrobial, anticancer, antiinflammatory, cardioprotective and vasodilatory effects [6]. Therefore evaluation of crude plant extracts with potential therapeutic properties and *in vitro* screening methods are the most important step in identification of the compounds with chemical elucidation and pharmacological investigation.

Salacia oblonga, an inhabitant of tropical regions, has been used in ayurveda and traditional Indian system of medicine. The bark and roots of *S. oblonga* have been used in the treatment of diabetes, polyuria, gonorrhea, rheumatism, asthma and fever. Presence of various phytochemicals *viz*, salcinol, kotanol and mangiferin from *S. obloga* extracts has proven biological activities [7] [8]. *S. oblonga* possesses significant medicinal properties *viz* antibacterial, antioxidant, anticancer and anti-inflammatory [9]-[12]. The present study reports the poly phenolics profile of the aerial and roots extracts of *S. oblonga* and their cytoprotective effect by H_2O_2 induced cytotoxicity on human lymphocytes.

2. Materials and Methods

2.1. Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical/HPLC grade and purchased from Merck and HiMedia (Mumbai, India). Lymphocyte isolation medium (Histopaque), RPMI-1640 media, fetal bovine serum and other cell culture related chemicals were procured from Sigma-Aldrich (USA).

2.2. Extract Preparation

S. oblonga plants were collected from the Western Ghats, India. The shade dried plants were separated into aerial and root parts before grinding into a fine powder using an electric blender. The phytochemicals were extracted in methanol and concentrated using a rotavapor (IKA RV 10). The extracts were stored at −20˚C for further use.

2.3. Total Phenolic Content

Total phenolic content of methanolic extracts was determined using Folin-Ciocalteu's reagent [13]. 250 µg of extracts was dissolved in distilled water and the final volume was adjusted to 2 ml and a control was maintained with distilled water without extracts (2 ml). 1 ml of 10% Folin-Ciocalteu's reagent was added to all the tubes including control. After a brief vortex, 1 ml of 10% sodium carbonate was added and the mixture was incubated at room temperature for 1 hour, the absorbance was measured at 760 nm using a UV-Visible spectrophotometer (Shimadzu). The phenolic content was expressed as Gallic acid equivalence (GAE) in terms of mg/g of dry weight obtained from the standard graph of Gallic acid.

2.4. HPLC-DAD Analysis

Polyphenolics estimation of *S. oblonga* aerial and root extracts was done by HPLC Diode array detector (DAD) equipped with luna C_{18} column (250 mm and 4.6 mm id; particle size 5 µm). The mobile phase consisted of 6% acetic acid in 2 mM sodium acetate (solvent A) and acetonitrile (solvent B). Different proportions of solvent gradient was maintained from A to B with a linear gradient 0% - 15% of B for 45 min, 15% - 30% of B for 15 min, 30% - 50% of B for 5 min and 50% - 100% B for 5 min.

2.5. Isolation of Lymphocytes

Human blood (5 ml) was collected from a healthy donor under aseptic conditions. Lymphocytes were isolated with the Histopaque 1077 by density gradient centrifugation. The blood was spread over Histopaque 1077, following centrifugation at 4000 g for 30 min at room temperature, the upper layer was discarded and the opaque interface containing mononuclear cells at the top of the Histopaque 1077 was transferred into a fresh sterile centrifuge tube. Isolated lymphocytes were washed many times with the RPMI 1640 media and once again subjected to centrifugation at 2509 g for 10 min. The resulting pellet was resuspended in 0.5 ml of RPMI 1640 media. The lymphocytes were counted using a Neubauer type haemocytometer. Trypan blue exclusion assay was used to evaluate the viability of isolated lymphocytes.

2.6. Effect of *S. oblonga* Extracts on the Viability of Lymphocytes

Lymphocytes were incubated with the *S. oblonga* extracts (10 - 120 µg/ml) for 24 h and equivalent concentration of methanol was used as control. After incubation period, the lymphocytes were collected by centrifugation at 8000 g for 10 min and their viability was evaluated by MTT assay [14]. The lymphocytes were resuspended in 1.0 ml of 0.4 mg/ml MTT in RPMI 1640 and incubated in the dark for 4 h. Samples were again centrifuged and formazan crystals were dissolved in DMSO. Amount of formazan was determined by measuring the absorbance at 540 nm using an ELISA plate reader. The data were presented as percent post treatment recovery (% live cells), whereas the absorbance from untreated control cells was defined as 100% live cells.

2.7. Protective Effect of *S. oblonga* Extracts on H_2O_2 Induced Cytotoxicity on Lymphocytes

Lymphocytes were exposed to H_2O_2 in the range of concentration 0 - 500 µM (Dissolved in ice cold PBS) at 37˚C for 10 min in dark incubator to determine concentration of H_2O_2 for significant cytotoxicity on lymphocytes. The lymphocytes were harvested and the viability was assessed by MTT assay [14] [15]. Lymphocytes were incubated with *S. oblonga* extracts (50 µg/ml) for 3 h at 37˚C before exposure to H_2O_2 (250 µM) to determine the protection against H_2O_2 induced cytotoxicity. Cells without treatment (H_2O_2 and *S. oblonga* extracts) served as a negative control and cells treated with only H_2O_2 (250 µM) acted as positive control. Following incubation viability of the lymphocytes was determined by MTT assay.

2.8. Hydrogen Peroxide Scavenging Assay

S. oblonga extracts (250 µg/ml) and quercetin/BHT (100 - 500 µg/ml) were incubated with 0.6 ml of H_2O_2 (40 mM in a 0.1 M (pH 7.4) phosphate buffer) in the dark for 10 min. A negative control was set up in parallel with entire reagent excluding extract or standard. After incubation, residual H_2O_2 was allowed to react with 1.0 ml of dichromate in acetic acid (5% potassium dichromate and glacial acetic acid in the ratio of 1:3) in a boiling water bath for 10 min. Green colour developed was measured at 620 nm absorbance. All experiments were performed in triplicate. H_2O_2 percentage scavenging activity of *S. oblonga* extracts, quercetin and BHT were calculated using following equation:

$$H_2O_2 \text{ scavenging activity } (\%) = [A_0 - A_1/A_0] \times 100$$

where A_0 was absorbance of negative control and A_1 was absorbance of the extracts or standards. H_2O_2 scavenging activity of extracts and standards was expressed as IC_{50}, which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

3. Results and Discussion

3.1. Total Phenolic Content

Plants produce remarkable diversity of secondary metabolites, among which phenolic compounds with diverse functions in plants are considered important [16] [17]. In the human body, dietary phenolic compounds inhibit the oxidation of low density lipoproteins (LDL) induced by H_2O_2 [18]. Total phenolic content of *S. oblonga* methanolic aerial and root extracts was carried out by Folin-Ciocalteu (FC) method and expressed as gallic acid equivalent [19]. Total phenol content of the aerial and root extracts were found to be 297 ± 0.005 GAE/gdw and 275 ± 0.006 µg GAE/gdw of the extracts respectively.

3.2. Phenolics Profile by HPLC DAD Analysis

HPLC-DAD analysis provides specific information about the individual components of polyphenols unlike the total phenolic content estimated by the FC reagent.

S. oblonga aerial and root extracts have shown the presence of specific polyphenols after the HPLC-DAD analysis. Polyphenols were identified from the aerial and root parts of the *S. oblonga* extracts, which were identified as catechin, quercetin, syringic acid and vanillic acid based on the retention time of the standards (**Table 1** and **Figures S1-S3**). Aerial part extract have displayed higher quantity of polyphenols in comparison to the root part extract. All the components were predominant in aerial parts. Seven phenolic standards were used for HPLC analysis, however, four phenolic compounds have been detected from the *S. oblonga* extracts. Aerial

Table 1. Polyphenol content of *S. oblonga* extracts aerial and root extracts after the HPLC analysis.

Poly phenols	Aerial extracts[a]	Root extracts[a]
Catechin	16.64 ± 0.02	ND[b]
Quercetin	19.548 ± 0.09	1.310 ± 0.006
Gallic acid	ND[b]	ND[b]
Synapic acid	ND[b]	0.0188
Vanillic acid	ND[b]	ND[b]
Rutin	ND[b]	ND[b]
Syringic acid	6.53 ± 0.04	0.019 ± 0.0005

[a]Concentration µg/gdw; [b]Not detected.

extracts contain natural phenolic compounds like catechin (16.64 ± 0.02 µg/gdw) and quercetin (19.548 ± 0.09 µg/gdw) associate with an array of biomedical applications on human health *viz*, antioxidant, anticancer, antibacterial [20] anti-inflammatory, antiviral, bronchodilator and hepatoprotectivity [21]. In addition the presence of syringic acid (6.53 ± 0.04 µg/gdw) in the aerial extracts of *S. oblonga* attribute to medicinal properties like antioxidant, antibacterial, antidiabetic and cytoprotective [22]-[25] making it an ideal candidate for drug development. Root extracts contain lesser quantity of quercitin, syringic acid and sinapic acid (**Table 1**). Yoshikawa *et al* (2001) [26] reported the presence of polyphenols *viz* mangiferin, catechin and catechin dimers in *Salacia reticulate* root extracts. Poly phenols *viz*, Catechin, quercetin and gallic acid present in medicinal plant, *Gardenia jasminoides* have shown free radical scavenging activity [27]. Our study have also corroborates with the reports from other medicinal plant species shown similar polyphenolic profile and displaying cytoprotective activity [28] [29].

3.3. Effect of *S. oblonga* Extracts on the Viability of Lymphocytes

The effect of *S. oblonga* aerial and root extracts was evaluated based on the viability of lymphocytes by MTT assay. The viability of lymphocytes was shown to be greater than 90%, when the extracts were added at a range of 10 - 120 µg/ml concentrations. In case of aerial extracts, no cytotoxicity was observed at the tested concentration but on other hand, the root extracts were cytotoxic towards the lymphocytes with increasing concentration (80 - 120 µg). This indicated that the root extracts were cytotoxic towards human lymphocytes only at higher concentrations (**Figure 1**). Hence the tested concentrations of extracts which have shown greater than 90% of cell viability were used for further analysis.

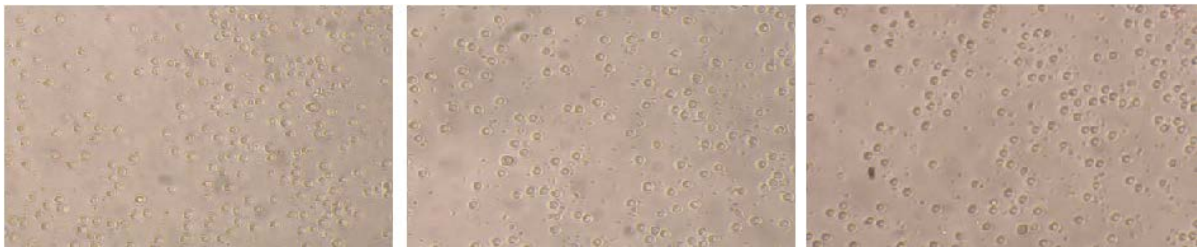
3.4. Protective Effect of *S. oblonga* Extracts on H_2O_2 Induced Cytotoxicity on Lymphocytes

H_2O_2 concentration was evaluated against lymphocytes for oxidative damage at a range of concentrations (0 - 500 µM). After treatment with H_2O_2 for 10 min no reduction was observed at lower concentration in cell viability however, at concentrations > 100 µM, significant reduction in cell viability was observed, when compared to the untreated cells. Lymphocytes were incubated with the extracts for 3 hours before being exposed to 200 µM H_2O_2. After the extract treatment no reduction in the number of lymphocytes was observed. However, H_2O_2 alone at higher concentration has shown significant reduction in cell viability compared to the extract treated cells. Among the *S. oblonga* extracts (50 µg/ml), methanolic aerial extracts have shown better cytoprotective activity against H_2O_2 induced cytotoxicity on lymphocytes than root extracts (**Figure 2**).

Polyphenolics and other phytochemicals were able to inhibit cell damage and protect from cell damage induced by oxidants and scavenge the free radicals, thereby reducing the free radical mediated oxidative damage. Polyphenols can also accept electron to form stable phenoxyle radicals thereby disrupting chain oxidation reactions in cellular components [30]. Hence the presence of rich polyphenolics from the extracts *S. oblonga* could play a major role in cytoprotective effect on H_2O_2 induced cytotoxicity on human lymphocytes.

3.5. H_2O_2 Radical Scavenging Activity

In the normal metabolism, H_2O_2 is formed in the human body as a result of the various cellular mechanisms,



(a) (b) (c)

Figure 1. Effect of *S. oblonga* extracts (50 µg/ml) on viability of human lymphocyte. (a) Treated with aerial extracts; (b) Treated with root extracts; (c) Untreated (control).

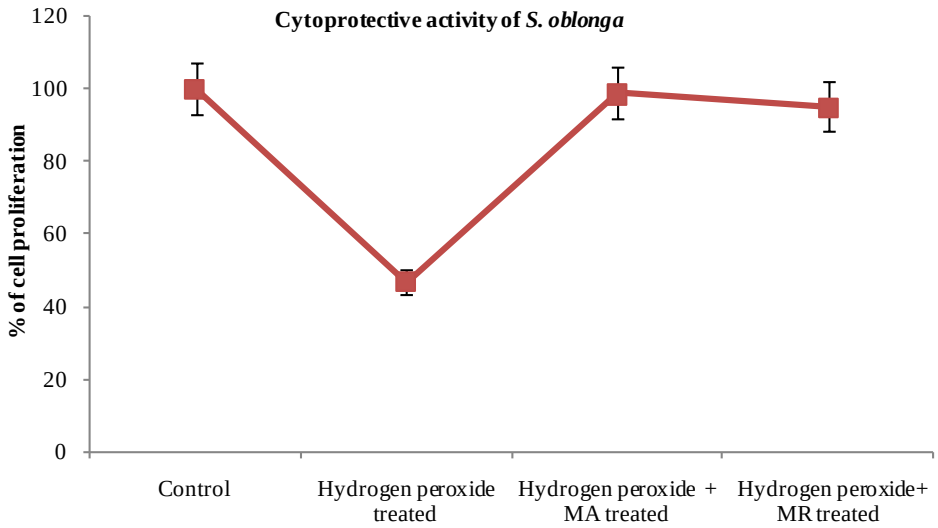


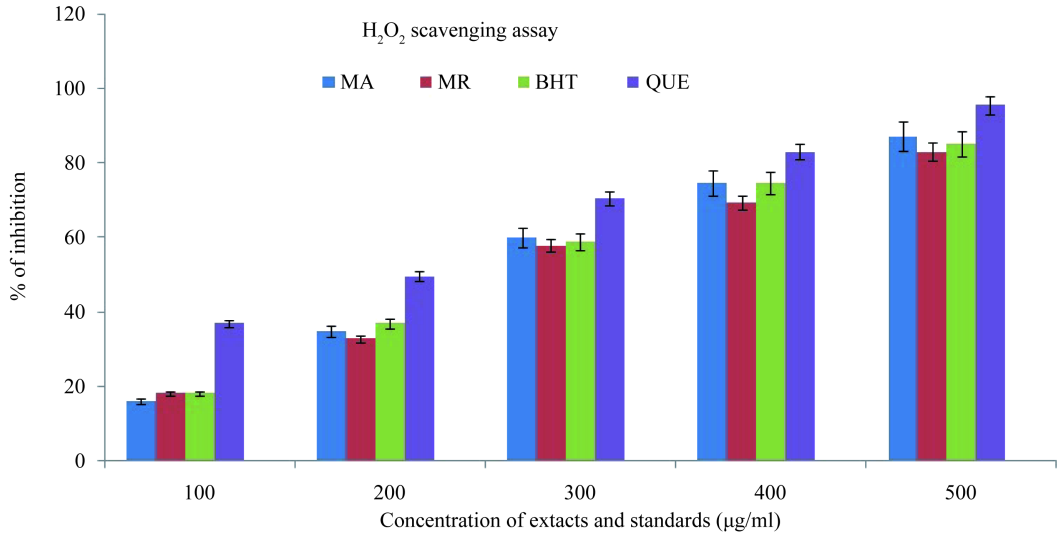
Figure 2. Cytoprotective effect of *S. oblonga* extracts (MA—methanolic aerial, MR—methanolic root) on H_2O_2 induced cytotoxicity on human lymphocytes.

Generally H_2O_2 is non reactive, but in living cells its get converted into free radicals called hydroxyl radical (·OH), which react with biomolecules and cause tissue damage leading to cell death [31]. *S. oblonga* aerial and root extracts (100 - 500 µg/ml) displayed scavenging activity on par with the positive control quercetin and BHT (100 - 500 µg/ml). Aerial extract, root extract, BHT and quercetin gave IC_{50} values of 276 µg/ml, 290 µg/ml, 238 µg/ml and 277 µg/ml respectively. The extracts have shown significant IC_{50} values in scavenging H_2O_2, however quercetin have shown greater scavenging activity compared to the plant extracts and BHT is almost equal to the plant aerial extracts. Aerial extracts exhibited better H_2O_2 scavenging activity in comparison to the root extracts.

Oxygen and peroxide ions are examples of reactive oxygen species formed as natural byproducts of normal metabolism and increase dramatically due to environmental stress conditions [32]. ROS react with membranes, lipids, nucleic acids, proteins, enzymes and other small molecule leading to cellular damage [33]. *S. oblonga* aerial and root extracts were efficient in scavenging the H_2O_2 (**Figure 3**), which might be due to the presence of phenolic groups and other phytochemicals in the extracts that might donate the electron to peroxide and neutralized into water molecules.

4. Conclusion

The present study unveils a spectrum of important polyphenols in the extracts of *S. oblonga* and displays significant cytoprotective effect of H_2O_2 induced cytotoxicity on human lymphocytes. Polyphenols and other phytochemicals of *S. oblonga* extracts could exhibit synergistic effect or act individually towards removing the free radical by stimulating the antioxidant enzymes in the system thus reducing the free radical mediated cytotoxicity on lymphocytes.



MA-Methanolic aerial, MR-Methanolic root, BHT-Bulylated hydroxy toluene, QUE-Quercetin

Figure 3. H_2O_2 scavenging activity of *S. oblonga* aerial and root extracts, BHT and Quercetin at various concentrations. Values are mean ± SD (n = 3).

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Conflict of Interest

Authors have no conflict of interest.

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Supplement

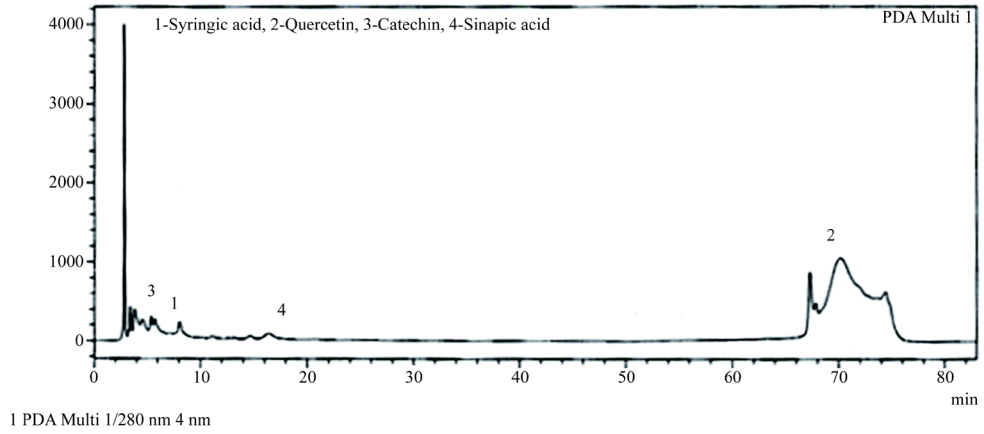

Figure S1. HPLC chromatograms of *S. oblonga* aerial extracts at 280 nm.

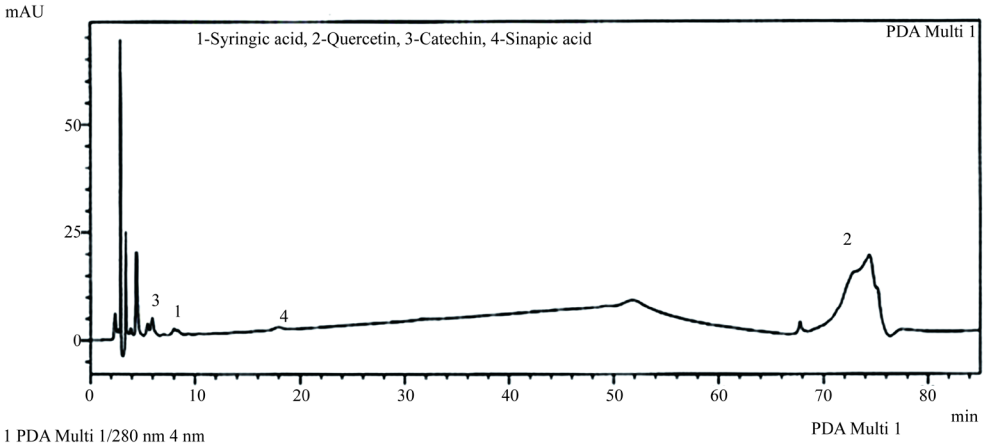

Figure S2. HPLC chromatograms of *S. oblonga* root extracts at 280 nm.

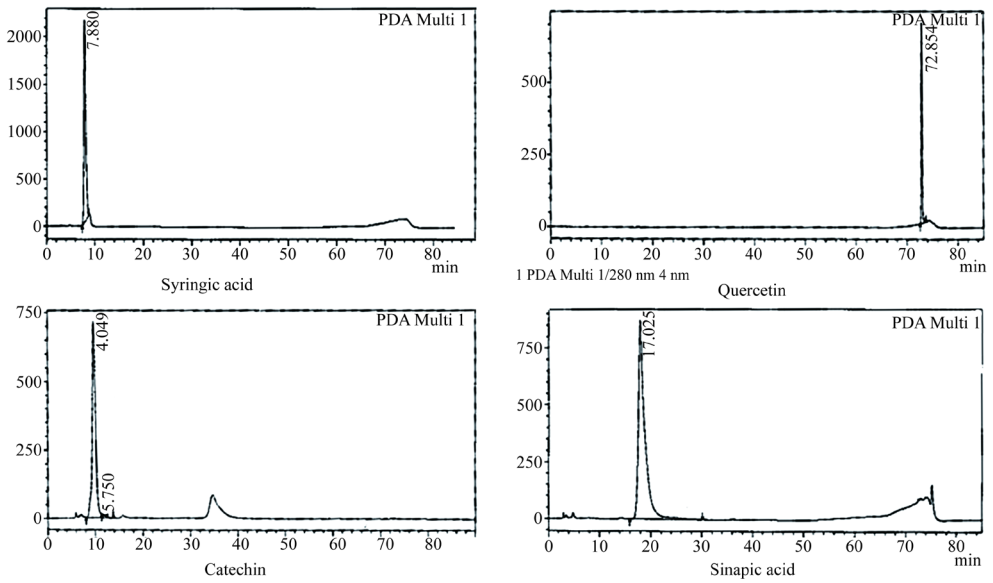

Figure S3. Standard poly phenolics after HPLC analysis at 280 nm.