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Research Article

Antioxidant Activities and Phenolic Compounds of Various Extracts of *Rhus typhina* Fruits and Leaves

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Abstract: The antioxidant activities of various extracts (methanol, hexane, dichloro-methane, ethyl acetate, n-butanol water) of *Rhus typhina* fruits and leaves were investigated using different methods and the main phenolic compounds were analyzed by LC-MS. The ethyl acetate extracts from fruits and leaves of R. typhina exhibited the highest DPPH, hydroxyl radical and nitrite scavenging activity, reducing potential and protein protection ability. The phenolic and flavonoïd contents were highest in the ethyl acetate fraction. The LC-MS analysis showed that the contents of luteolin and luteolin-7-O-glucuronide in leaves are little higher (34.49 and 32.69%, respectively) than that (32.49 and 27.89%, respectively) in the fruits, the content of rutin in fruits (16.73%) is higher than that (7.79%) in the leaves. These results implied that the leaves of *R. typhina* might serve as a natural source of antioxidant using as the food additive for its good nutrition as well as the fruits of *Rhus typhina*.

Keywords: Antioxidant, free radical scavenging ability, LC-MS, *Rhus typhina* fruits and leaves

INTRODUCTION

Rhus typhina (Staghorn sumac) is used to make a beverage termed "sumac-ade" or "Rhus juice" prepared from its fruits and serves also as a traditional medicine having pharmacological functions such as antihaemorrhoidal, antiseptic, diuretic, stomachic and tonic (Foster and Duke, 1990; Moerman, 1998). Some phenolic compounds including gallic acid and gallotannin have been identified in the leaves (Frohlich *et al*., 2002; Werner *et al*., 2004). The fruits of *R. typhina* were rich in polyphenols (Kossah *et al*., 2010) and also found to abound in oleic and linoleic acids, vitamins (B1, B2, B6 and Vc), minerals as well as organic acids (Kossah *et al*., 2009). However, study of the antioxidant of *R. typhina* leaves is limited The present work focused on comparing the antioxidant activities of extract. including various fraction (n-hexane, dichloromethane, ethyl acetate, n-butanol and water) of methanol extracts from *R. typhina* fruits and leaves using different in vitro assay, such as the 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radicals scavenging ability, hydroxyl radical scavenging ability, nitrite scavenging ability, reductive potential, protection protein damage. Furthermore, the main compositions of polyphenolic compounds were analysis by analytical HPLC-MS.

The objectives of this study was to find the difference of chemical composition and antioxidant activity of *R. typhina* fruits and leaves extract and providing the proofs to use the leaves as functional food source as well the fruits.

MATERIALS AND METHODS

Preparation of plant extraction: R. typhina fruits and leaves were harvested from Xinxiang city in Henan province, China. The fruits and leaves were dried in the shade for 15 d and 10 d, respectively before solvent extraction. The dried R. typhina fruits and leaves were extracted three times with 70, 85 and 95% methanol sequencing at 45°C for 12 h respectively and then filtered through filter paper (100 mm; Whatman, Maidstone, UK). The methanol extract (RT-M) were concentrated under reduced pressure by a rotary evaporator machine (CCA-1110; EYELA, Tokyo, Japan). The RT-M extract were suspended in water and then partitioned with n-hexane, dichloromethane, ethyl acetate and n-butanol, repeat three times with each solvent. Removal of the solvents afforded the n-hexane (RT-M-H), dichloromethane (RT-M-D), ethyl acetate (RT-M-E), n-butanol (RT-M-B) and water fractions (RT-M-W), respectively.

Determination of total phenolic contents: Total Phenolic Content (TPC) was estimated using Folin-Ciocalteu according to Ragazzi and Veronese (1973). Briefly, 1.0 mL of extract solution containing 1.0 mg extracts was dilution with 5 mL deionixed water, 0.5

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mL of 50% Folin-Ciocalteu reagent was added. Five min later, 1 mL of 5% Na_2CO_3 was added, then mixed thoroughly and stand for 1 h in the dark. The absorbance was measured at 725 nm. The concentration of total phenolic compounds was expressed as mg Gallic Acid Equivalents (GAE)/g of extract.

Determination of total flavonoids contents: Total Flavonoids Content (TFC) was measured using the method described by Park *et al*. (1997) with a slight modification. An aliquot of 0.5 mL of the solution containing 1 mg extracts was added to test tubes containing 0.1 mL of 10% aluminiumchloride hexahydrate, 0.1 mL of 1 M potassium acetate, 2.8 mL of deionized water and 1.5 mL 95% ethanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The concentration of flavonoid compounds was expressed as mg Quercetin Equivalents (QUE)/g extract.

DPPH radical scavenging activity: A 2.0 mL aliquot of extract was added to 2.0 mL of 0.2 mM DPPH methanolic solution. The mixture left to stand at room temperature for 30 min and read the absorbance at 517 nm. The ability to scavenge the DPPH radical was calculated using the follow equation:

$$\text{Scavenging effect (\%)} = 1-(A_{sample}-A_{blank})/A_{control}\times 100\%$$

Synthetic antioxidants L-Ascorbic acid was used as positive control. The values of scavenging effect were expression as EC_{50} (μg/mL), which means the concentration of the sample antioxidant required to scavenge 50% of the DPPH radical in the mixture.

Hydroxyl radical (•OH) scavenging activity: The Fenton reaction mixture consisted of 200 μL of each $FeSO_4$ (10 mM), Ethylenediaminetetraacetic acid (EDTA, 10 mM) and 2-deoxyribose (10 mM). 200 μL of the samples and 1 mL of 0.1 M phosphate buffer (pH 7.4) were added to a total volume of 1.8 mL. Subsequently, 200 μL of H_2O_2 was added and the reaction mixture was incubated for 4 h for 37°C. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and the mixture was placed in a boiling water bath for 10 min, the mixture was then centrifuged (5 min, 3000 rpm) and the absorbance was measured at 532 nm. The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = 1-(A_{sample}-A_{blank})/A_{control}\times 100\%$$

Synthetic antioxidants, BHT were used as positive control. The values of scavenging effect were calculated for the various concentrations of extract. Tests were carried out in triplicate.

Reducing power assay: The reducing power of extracts was determined as described by Singh and Rajini (2004). Total 1 mL aliquot of the extract, 2.5 mL of 0.2 M phosphate buffer pH 6.6 and 2.5 mL of 1% (w/v) $K_3Fe(CN)_6$ were added. The mixture was incubated at 50°C for 30 min. Ten percent (10%, w/v) trichloroacetic acid (2.5 mL) was added and the resulting mixture was centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was added 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) $FeCl_3$ solution. The absorbance was measured at 700 nm using a spectrophotometer. Assays were performed in triplicate. The reducing power of Vc was also determined as the positive control.

Measurement of nitrite scavenging ability: The nitrite scavenging ability of the extracts was determined according to a method using Griess reagent (Kato *et al*., 1987). Briefly, 1 mL extract was mixed with 1 mL of 1 mM nitrite sodium. Then the mixture was added to 8 mL of 0.2 M citrate buffer (pH 1.2) and incubated for 1 h at 37°C. After incubation, 1 mL of solution/supernatant was withdrawn and added to 2 mL of 2% acetic acid and 0.4 mL of Griess reagent (1% sulfanilic acid and 1% naphthylamine in a methanol solution containing 30% acetic acid). After vigorous mixing, the mixture was placed at room temperature for 15 min and measured the absorbance at 520 nm. Quercetin was used as positive control.

Protein protection assay: Protein oxidation was assayed as described by Kwon *et al*. (2000) with minor modifications. Oxidation of Bovine Serum Albumin (BSA) in PBS was initiated by AAPH and incubated with various concentrations of the extract or galic acid (positive standard). After incubation for 24 h at 37°C, 0.02% BHT was added to prevent the formation of further peroxyl radical. The proteins were then assayed with normal SDS-PAGE.

LC-MS identifies the individual phenolic compounds in the EAF of R. typhina fruits and leaves: Identification the individual phenolic compounds in the EAF of R. typhina fruits and leaves were carried out using a Waters Alliance HPLC system (Waters, Milford, MA, USA), equipped with a PDA 2996 photo diode array detector (Waters, Milford, MA, USA), a Micromass Quattro microTM API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK). Sample solutions were separated on a C18 column (4.6 mm×250 mm, i.d. 5 μm). The chromatographic conditions for the determination of individual phenolic compounds were according to Enayat and Banerjee (2009) with some modifications. The detection wavelength was set at 280 nm for gallic acid, catechin and epigallocatechin gallate (EGCG), 320 nm for caffeic acid and coumaric acid and

Table 1: Total polyphenols (mg/g GAE dry extract) and flavonoid contents (mg/g QUE dry extract) of *R. typhina* leaves and fruits extracts

	Leaves		Fruits	
Sample	TPC mg/g GAE	TFC mg/g QUE	TPC mg/g GAE	TFC mg/g QUE
RT-M	163.02±1.2	41.91±1.24	183.42±2.29	54.53±1.34
RT-M-H	106.53±3.14	29.04±0.54	128.04±1.73	28.89±1.39
RT-M-D	180.15±1.98	47.53±1.03	181.66±1.38	51.77±1.27
RT-M-E	184.26±2.17	62.53±2.01	184.42±1.65	71.46±0.97
RT-M-B	134.71±1.54	38.28±1.78	168.05±0.97	12.53±1.03
RT-M-W	19.57±1.31	15.71±1.21	26.42±1.79	11.31±0.81

RT-M: methanol extract of *R. typhina*; RT-M-H: n-hexane fraction of RT-M; RT-M-D: dichloromethane fraction of RT-M; RT-M-E: ethyl acetate fraction of RT-M; RT-M-B: *n*-butanol fraction of RT-M; RT-M-W: water fractions of RT-M. The results are presented as the mean±SD of three independent experiments in triplicate. A p-value<0.05 was considered significant

360 nm for rutin and quercetin. Column temperature was set at 35°C. The effluent was introduced into a PDA detector and subsequently into an electrospray source (desolvation temperature 400°C, capillary voltage 3.0 kV, cone voltage 30 V). The split ratio of HPLC flow between PAD detector and MS detector was 2:1.

Statistical analysis: Statistical analysis software (SPSS version 11.50; SPSS Inc., Chicago, IL, USA) was used for all analyses. The data was subjected to ANOVA and significant differences were reported at the level of $p<0.05$ by the SPSS software. All experiments were performed with 3 replications. One-way ANOVA followed by Tukey's HSD test or paired Student t-test was used to assess the statistical significance of changes in all indices with the level of significant difference set at $p<0.05$.

RESULTS

Total polyphenol and flavonoid contents: Among the six different solvent fruits and leaves extracts, the TPC were significant differences ($p<0.05$), ranging from 19.57 to 184.26 mg GAE/g extract. In fruits, the TPC of RT-M-E was highest at 184.42±1.65 mg GAE/g extract, the TPC of RT-M (183.42±2.29) was a little bit lower than RT-M-E, then followed by RT-M-D at 181.66±1.38 mg GAE/g extract and RT-M-B at 168.05±0.97 mg GAE/g extract.

However, in same solvent extract the TPC of R. typhina leaves were lower than that of the fruits. In leaves, the TPC of RT-M-E was highest at 184.26±2.17 mg GAE/g extract, followed by RT-M-D at 180.15±1.98 mg GAE/g extract and RT-M at 163.02±1.2 mg GAE/g extract, the lowest content is RT-M-W at 19.57±1.31. A similar content tendency was found in fruits. The TFC in R. typhina leaves, range from 15.71 to 62.53 mg of QUE/g extract, in R. typhina fruits which range from 11.31 to 71.46 mg of QUE/g extract. The two RT-M-E extracts in leaves and fruits possess the highest polyphenol for its polarity compared to other fractions (Table 1).

DPPH radical scavenging activity: DPPH is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al*., 1997). Various concentration of extract was taken as a measure of antiradical activity, the lower the EC_{50}, the higher the antioxidant potential (Brand *et al*., 1995). L-ascorbic acid was the reagent used as standard. The EC_{50} of all extracts of R. typhina leaves and fruits and L-ascorbic acid against the DPPH radical were in Table 2. The lowest EC_{50} (7.85 and 8.6 µg/mL) were found in RT-M-E of R. typhina leaves and fruits respectively.

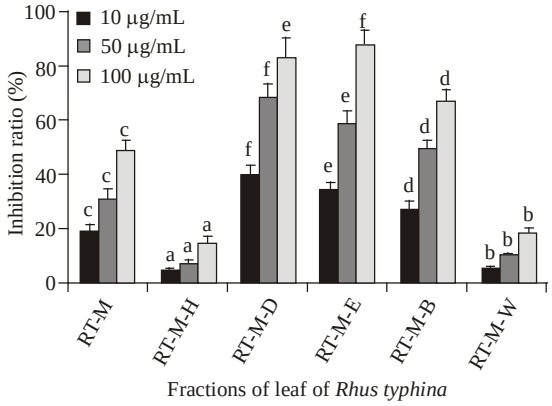
Table 2: DPPH radical scavenging activities of *R. typhina* leaves and fruits extracts, expressed as EC_{50} (µg/mL)

Sample name	Leaves	Fruits
RT-M	18.57±0.56	13.57±1.24
RT-M-H	74.56±2.01	83.19±1.95
RT-M-D	20.49±0.86	9.78±1.07
RT-M-E	8.64±0.93	7.85±1.34
RT-M-B	39.07±1.48	29.76±0.68
RT-M-W	228.72±2.31	275.48±1.67
L-ascorbic acid BHA	11.10±0.43	
	18.57±1.37	

RT-M: methanol extract of *R.typhina*; RT-M-H: n-hexane fraction of RT-M; RT-M-D: dichloromethane fraction of RT-M; RT-M-E: ethyl acetate fraction of RT-M; RT-M-B: n-butanol fraction of RT-M; RT-M-W: water fractions of RT-M. The results are presented as the mean±SD of three independent experiments in triplicate. A p-value<0.05 was considered significant

It was observed that the RT-M-E of *R. typhina* leaves and fruits exhibited the best DPPH radical scavenging activity for the highest levels of TPC and TFC, the RT-M-W showed relatively weak DPPH scavenging ability, because it is extracted polyphenolic compounds difficultly from the non-polarity solvent. Compared with the same solvent fraction of leaves and fruits, all the fruits extract exhibited better DPPH radical scavenging activity than leaves extract because the fruits contain a bit more TPC and TFC than that of the leaves, all these suggested that the polyphenols and flavonoids may be the main constituents responsible for the DPPH radical scavenging activity.

Hydroxyl radical scavenging activity: The active hydrogen peroxide can be toxic to cell, therefore, removing H_2O_2 as well as $O_2^{\cdot-}$ is very important for antioxidant defense. The hydroxyl radical scavenging ability of all extracts was shown in Fig. 1. It was noticed that most of the extract were capable of scavenging hydrogen radical in a dose-depend manner except the RT-M-W. The results exhibited that RT-M-E



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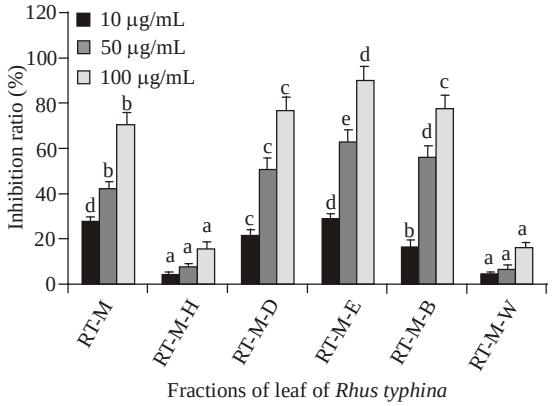
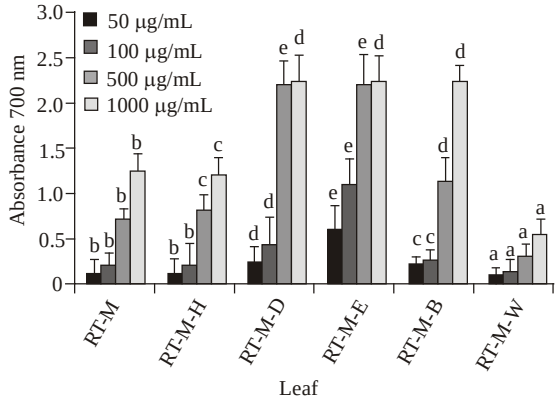


(b)

Fig. 1: Hydrogen peroxide scavenging activities of *R. typhina* leaves (A) and fruits (B) extracts. RT-M: methanol extract of *R. typhina;* RT-M-H: *n*-hexane fraction of RT-M; RT-M-D: dichloromethane fraction of RT-M; RT-M-E: ethyl acetate fraction of RT-M; RT-M-B: *n*-butanol fraction of RT-M; RT-M-W: water fractions of RT-M



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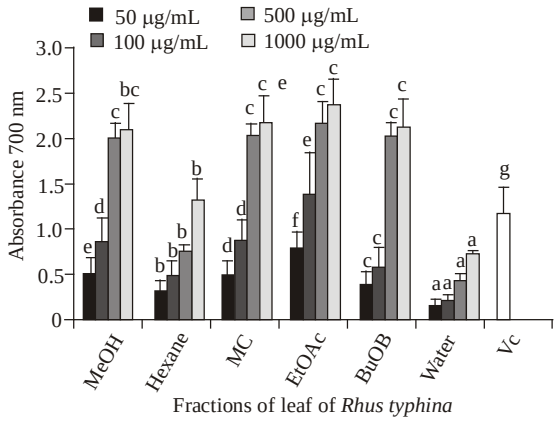

(b)

Fig. 2: Reducing power of *R. typhina* leaves (A) and fruits (B) extracts

of R. typhina leaves and fruits had the higher scavenging ability than other fraction; the RT-M-W of R. typhina leaves and fruits had the very weak scavenging capacity on deoxy-D-ribose degradation. There was significant correlation between the TPC and hydrogen radical scavenging activity (Fig. 1).

Reducing power assay: The reduction of an oxidant antioxidant molecule to regenerate the reduced antioxidant is another reaction pathway in electron donation. The reducing power of all samples showed a dose-dependent manner (Fig. 2) and followed the order:

RT-M-E>RT-M-D>RT-M-B>RT-M-M>RT-M-H>RT-M-W

Nitric oxide scavenging activity: Sodium nitroprusside spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions in physiological pH that can be estimated using Griess reagent. NO. react O^{2-} to produce reactive peroxynitrite (ONOO-), which causes serious damage to lipids, protein and nucleic acids (Moncada *et al*., 1991). Our data revealed that all the extract exhibited the better scavenged nitric oxide activity in pH 1.2 than those of at pH 4.6 and pH 6.0. (Fig. 3) Of the leaves extracts, RT-M-E exhibited the best nitric oxide scavenging activity than others, followed by RT-M. Of the fruits extracts, RT-M-E showed the best scavenging activity, followed by RT-M, RT-M-E, RT-M-B and RT-M-D. Both leaves and fruits water fraction showed the weak nitric oxide scavenging ability.

Protein protection ability: AAPH is a water-soluble initiator, which decomposes into alkyl radicals at physiological condition, then react to oxygen and produce alkyl peroxyl radicals to initiate DNA oxidative fragmentation (Cai *et al*., 2003). Under many pathological conditions cellular proteins get oxidized, the vulnerability of various amino acid residues of

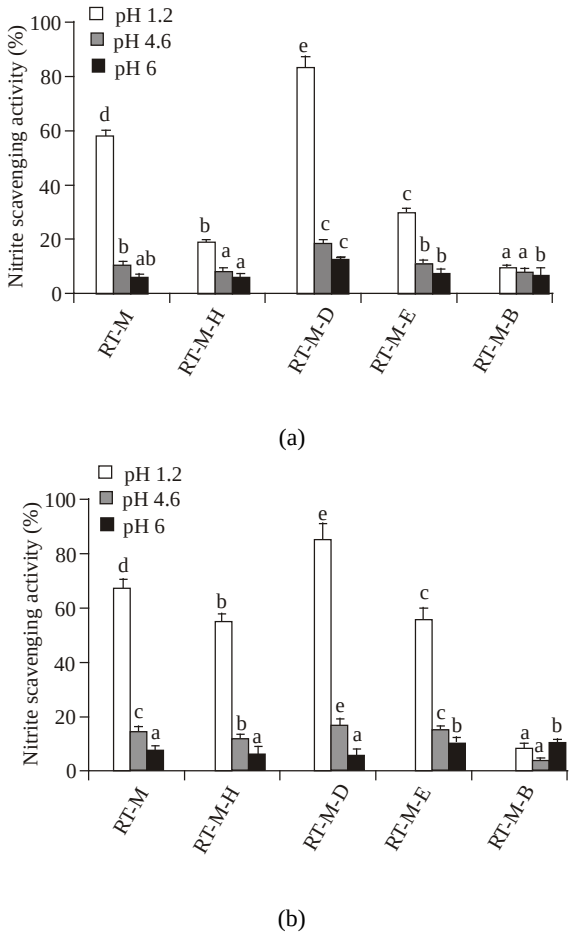

Fig. 3: Nitric oxide scavenging activities of *R. typhina* leaves (A) and fruits (B) extracts at 100 µg/mL

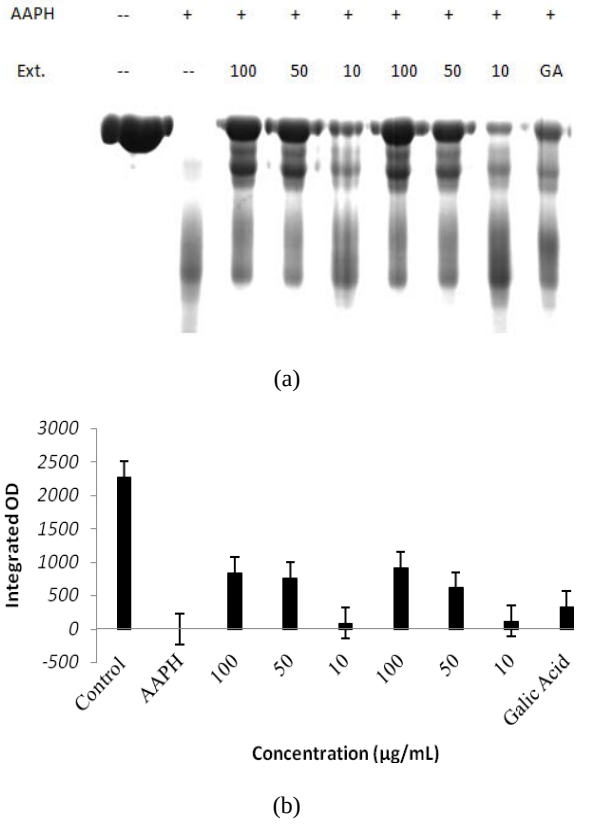

Fig. 4: Protective activity of EAF against oxidation of BSA; (A): A representative result of the SDS-PAGE is presented here. Lane 1; non-treated with AAPH (control), Lane 2; without antioxidant (negative control), Lane 3-5; 100-10 µg/mL EAF of *R. typhina* leaves, Lane 6-8; 100-10 µg/mL EAF of *R. typhina* fruits, Lane 9; 20 µg/mL Galic acid as positive control; (B): Quantification of the value of integrated OD from three independent experiments is shown and each value is expressed as the mean±S.E

proteins to oxidation varies with reactive oxygen species (Ames *et al*., 1993). The protection against protein oxidative damage was determined by the oxidation of BSA initiated by AAPH. The normal SDS-PAGE results demonstrated that, RT-M-E of leaves and fruits exhibited significant protective effect against oxidation of BSA in a concentration depend manner (Fig. 4). The effect of R. typhina fruits extracts at the same concentration is a bit better than that of leaves. The RT-M-D and RT-M also showed some extent protective effect against oxidation of BSA (data not shown).

HPLC-MS identifies the individual phenolic compounds in the RT-M-E of R. typhina fruits and leaves: The nine compounds such as gallic acid, catechin, EGCG, caffeic acid, p-coumaric acid, luteolin-7-β-D-glucopyranoside, luteolin, rutin and quercetin were taken as standard substances to identify and quantify the phenolic compounds in the RT-M-E of R. typhina fruits and leaves. The RT-M-E of R. typhina fruits and leaves is rich in phenolic compounds, including 8 standards based on comparison of their retention time and mass with the authorized compounds. In the RT-M-E of R. typhina fruit, luteolin is the most abundant present with 32.493%, luteolin-7-β-D-glucopyranoside is the second abundant present with 27.899%, rutin is the third abundant present with 16.734%, gallic acid and p-coumaric acid is present with 1.496 and 0.792%, respectively, the other standards are present very weak (Table 3). In the RT-M-E of R. typhina leaves, luteolin, luteolin-7-β-D-glucopyranoside and rutin are the main abundant compounds. As shown in Fig. 5 and Table 3, we can find that the content of luteolin, luteolin-7-β-D-glucopyranoside and p-coumaric acid is higher in leaves than those in fruits, however the content of rutin and gallic acid is higher in fruits than those in leaves.

DISCUSSION

The antioxidant activity of methanol extract and its five fraction of leaves and fruits of *R. typhina* were

Table 3: LC-MS identify the polyphonic compounds in EAF of *R. typhina* fruits and leaves

		Fruits		Leaves		
Constituent	λ_{max} (nm)	Rt (min)	Area %	Rt (min)	Area %	Positive ions (m/z)
Gallic acid	280	3.694	1.496	3.692	3.118	$(M+H)^+$: 171
Luteolin-7-*β*-D-glucosiduronc acid		12.936	27.899	12.965	32.693	$(M+H)^+$: 463
Caffeic acid		19.222	0.035			$(M+H)^+$: 181
EGCG				23.902	0.001	$(M+H)^+$: 459
Rutin				36.196	1.274	$(M+H)^+$: 611, 303
Luteolin		37.864	32.493	37.901	34.495	$(M+H)^+$: 287
Luteolin-7-*β*-D-glucosiduronic acid	320	12.819	9.38	12.888	6.815	$(M+H)^+$: 463
Caffeic acid		19.169	0.510	19.189	0.175	$(M+H)^+$: 181
Coumaric acid		27.259	0.792	27.247	0.549	$(M+H)^+$: 165
Rutin		36.218	3.082	36.149	4.238	$(M+H)^+$: 611, 303
Luteolin		37.926	29.842	37.899	16.311	$(M+H)^+$: 287
Quercetin				54.799	0.062	$(M+H)^+$: 303
Caffeic acid	360			19.098	0.039	$(M+H)^+$: 181
Coumaric acid		26.974	0.016	27.245	0.008	$(M+H)^+$: 165
Rutin		36.063	16.734	36.197	7.796	$(M+H)^+$: 611, 303
Quercetin				54.300	0.013	$(M+H)^+$: 303

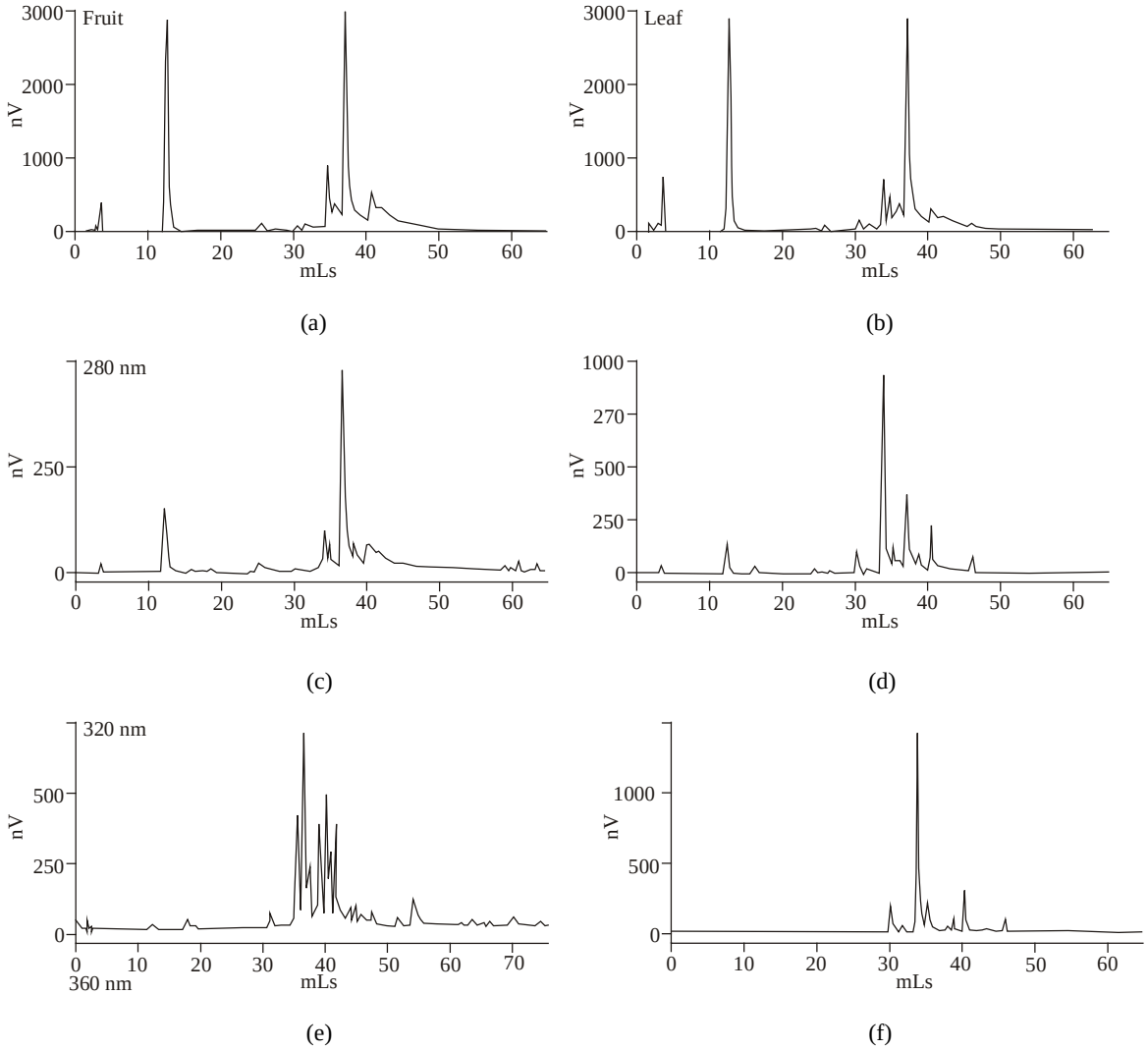

Fig. 5: The HPLC profile of EAF of R. typhina fruits (A) and leaves (B) at 280 nm, 320 nm and 360 nm

investigated using various analytical methods based on different mechanisms. As all the methods tested, the RT-M-E of *R. typhina* fruits had the highest TPC and TFC, which also exhibited the highest DPPH radical (EC_{50} is higher than the positive control, L-ascorbic acid and BHA), hydroxyl radical scavenging activity,

reducing power and protection protein damage activities, all these activities were positive correlated with the TPC and TFC. The RT-M-D of *R. typhina* leaves and fruits also exhibited the good antioxidant in many antioxidant assays, Their higher TPC and TFC contribute to these activities. The TPC, TFC in fruit is a bit higher than in leaves. The LC-MS analyzed the leaves and fruits of *R. typhina* possess large amounts of flavonoids including the luteolin, luteolin-7-O-glucuronide and rutin. Flavonoids can prevent some diseases such as cancers, diabetes and Alzheimer's disease through antioxidative action and/or the modulation of several protein functions. The higher concentrations of flavonoid derivatives enhance the neutraceutical value in terms of health-promoting effects. As a whole, all the results find that the leaves of *R. typhina* also could be used as effective functional foodstuff resource for its antioxidative and bioactive flavonoids as well the fruits. Now, a study on the effects on inhibition the human tumor cell proliferation with leaves and fruits of *R. typhina* is currently in progress.

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