

Full Length Research Paper

# Antioxidant activities and flavonoid contents of selected plants belonging to Family Loranthaceae

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The methanolic extract of three parasitic plants belonging to family Loranthaceae (*Plicosepalus acacia*, *Plicosepalus curviflorus* and *Phragmanthera austro arabica*) were investigated for their antioxidant activity. The free radical and nitrogen oxide scavenging abilities were evaluated using diphenyl-1-picrylhydrazyl (DPPH), and Griess reagent was used to determine the total antioxidant activity. The three extracts showed comparable activities. The three activities ranged from 18 to 56% of the activity of standard ascorbic. The total flavonoid contents were determined and calculated in terms of quercetin which was detected in all the examined extracts. The quercetin concentration was determined using high performance thin layer chromatography using  $\text{CHCl}_3$  : MeOH : glacial acetic acid (8.5: 1.5: 0.1) for development. The maximum sensitivity was obtained when the plates were scanned at 360 nm. The concentration of quercetin varied from 0.157 (*P. austro arabica*) to 0.062 g% (*P. acacia*) and *P. curviflorus* contained 0.115 g% w/w quercetin. All validation parameters were found to be satisfactory regarding accuracy, precision, limits of detection and quantitation.

**Key words:** Loranthaceae, antioxidant, spectrophotometry, high performance thin layer chromatography (HPTLC), flavonoids, quercetin, ascorbic acid.

## INTRODUCTION

Antioxidants are compounds that inhibit or delay the oxidation process which results in generation of free radicals and reactive oxygen species. Strong and consistent evidence indicates that certain antioxidants could be effective agents for the prevention of cancer incidence and mortality (Saxena et al., 2010; Chen et al., 2004). Many researches focused on the efficacy of antioxidants as hepatoprotective (Sasidharan et al., 2010), neuroprotective (Kelsey et al., 2010), anti-diabetic (Sadi and Guray, 2009) and protective against cardiovascular diseases (Subramanian et al., 2010).

Many flavonoides are well known for their significant

antioxidant activity (Pietta, 2000; Dai and Mumper, 2010), Nakayama, 1994; Ozen et al., 2011), among them, quercetin has been subjected to extensive studies as being powerful antioxidant (Zielinska et al., 2008; Schmalhausen et al., 2007). Generally, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent than vitamin C and E and carotenoids (Rice-Evans et al., 1995, 1996). Previous investigation on different species of family Loranthaceae indicated their accumulation of flavonoides as major active constituents (Lin and Lin, 1999; Kim et al., 2004). Moreover, these plants were proved to possess antidiabetic, antilipidemic and anticancer effects which coincide with their traditional use (Kim et al., 2004; Osadebe et al., 2010; Peter and Obi, 2010). In the present work, *Plicosepalus acacia*, *Plicosepalus curviflorus* and *Phragmanthera austro arabica*, three

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parasitic plants belonging to family Loranthaceae and collected from Saudi Arabia are evaluated for the first time for their antioxidant activity.

Also, the total flavonoid contents were determined and the major flavonoid in *P. austro arabica* was isolated and identified as quercetin which was also detected in the two other plants. Finally, the quercetin amount was estimated in the three plants using high performance thin layer chromatography.

## MATERIALS AND METHODS

### General experimental procedures

Spectrophotometric measurements were performed using a Hitachi 300 Double Beam Spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Joel spectrometer; at 500 MHz for  $^1\text{H}$ -NMR and 125 MHz for  $^{13}\text{C}$ -NMR. The high performance thin layer chromatography (HPTLC) system consisted of a Camag (Wilmington, NC) Linomat IV sample applicator was used to dispense samples in the form of bands. The plates were saturated in a twin trough chamber. The densitometric scanning was performed at 360 nm with a rate of 10 mm/s. For the high performance thin layer chromatography (HPTLC) analysis, pre-coated silica gel (Merck 60,  $F_{254}$ , 0.25 mm), methanol and chloroform (Merck) were used. For spectrophotometric analysis, diphenyl-1-picrylhydrazyl (DPPH), (Sigma-Aldrich) was prepared as  $6 \times 10^{-5}$  M solution. Phosphomolybdic acid (Aldrich Chem. Co) was prepared as 10% w/v aqueous solution. Sodium nitroprusside reagent was prepared immediately before the experiment by dissolving 10 mM sodium nitroprusside (Sigma Chem. Co., USA) in 20 mM phosphate buffer, pH 7.4, previously bubbled with nitrogen. Griess reagent was obtained by preparing solution A, containing 2% w/v sulphanilamide (Fluka) and 4% w/v  $\text{H}_3\text{PO}_4$ , and a solution B containing 0.2% w/v naphthylethylenediamine (Sigma-Aldrich). Immediately before the assay, equal volumes of solutions A and B were mixed. Sodium nitrite (Sigma-Aldrich) was prepared in a concentration of 5%, and aluminium chloride (Sigma-Aldrich) 10% and sodium hydroxide (Sigma-Aldrich) was prepared as 1 M.

### Plant materials and sample preparation

The investigated plants were collected from different regions in Saudi Arabia; *P. acacia* from Alola (at the North), *P. curviflorus* and *P. austro arabica* from South Abha (at the South Western part). The three plants were kindly identified by Dr. Nahed Wally, Faculty of Science, King Abdulaziz University and voucher samples were deposited at Natural Products Department, Faculty of Pharmacy, King Abdulaziz University. Each plant was air-dried and finely powdered. For spectrophotometric analysis, twenty grams from each plant were extracted by sonication with methanol ( $3 \times 50$  ml) for 10 min, allowed to stand for 10 minutes and filtered. The combined methanolic extracts were concentrated under vacuum. From each extract, 50 mg were accurately weighed and dissolved in 100 ml methanol. Aliquot from each solution was ten fold diluted with the same solvent. For HPTLC analysis, 5 g of each of the three different plants were mixed with 20 ml 5%  $\text{Na}_2\text{CO}_3$  aqueous solution and left for one hour. Each mixture was filtered into 100 ml volumetric flask; the original flask and the residue were washed with successive portions of distilled water ( $3 \times 10$  ml) till complete extraction and volume adjusted with distilled water. The combined solution was neutralized with HCl and extracted with  $\text{CHCl}_3$  ( $3 \times 10$  ml). The  $\text{CHCl}_3$  was distilled off under reduced pressure and the

residue was dissolved in MeOH and transferred quantitatively to a 10 ml volumetric flask then completed to volume with MeOH.

### Extraction and isolation

A 500 g of the air dried powdered plant *P. austro arabica* were macerated at room temperature in 90% methyl alcohol and extracted till exhaustion. The combined alcohol extracts were concentrated under vacuum and then successively partitioned between petroleum ether, chloroform and ethyl acetate. The chloroform extract was fractionated on silica gel using chloroform: methanol gradient. The fractions eluted by 10 to 12% methanol in chloroform were combined, thin layer chromatography (TLC) examination of these fractions showed a major yellow spot which colour intensified upon exposure to ammonia vapours indicating its flavonoid nature. These fractions were purified by preparative thin layer chromatography using developing system chloroform: methanol (9:1). The compound was finally purified by repeated crystallization from methanol: chloroform (1:1). Extensive TLC examinations of the two other plants *P. curviflorus* and *P. acacia* revealed the presence of the same compound. NMR data were recorded;  $^1\text{H}$  NMR (measured in  $\text{CD}_3\text{OD}$ , ppm): H-6 (6.18, 1H, d,  $J=2.1$  Hz), H-8 (6.38, 1H, d,  $J=2.1$  Hz), H-2' (7.74, 1H, d,  $J=2.2$  Hz), H-5' (6.88, 1H, d,  $J=8.6$  Hz), H-6' (7.62, 1H, dd,  $J=8.6, 2.2$  Hz).  $^{13}\text{C}$  NMR (measured in  $\text{CD}_3\text{OD}$ , ppm): C-2 (146.8), C-3 (135.8), C-4 (175.9), C-5 (160.8), C-6 (98.3), C-7 (164.1), C-8 (93.5), C-9 (156.2), C-10 (103.1), C-1' (122.1), C-2' (115.2), C-3' (145.1), C-4' (147.7), C-5' (115.7), C-6' (120.1).

### Standard solutions preparation

Standard solution of ascorbic acid (Sigma-Aldrich) was prepared as  $0.1 \text{ mg ml}^{-1}$  in methanol, protected from light and kept in refrigerator. Standard solution of quercetin (isolated from *P. austro arabica* extract) was prepared as 0.5 and  $1 \text{ mg ml}^{-1}$  in methanol for spectrophotometric and HPTLC assays, respectively, protected from light and kept refrigerated.

### Spectrophotometric analysis

#### Total flavonoid contents

Aliquots from the extracts or standard quercetin solutions within the linearity range (Table 1) were pipetted into series of 10 ml volumetric flask. To each flask, 0.3 ml  $\text{NaNO}_2$  solution was added. After 5 minutes, 0.3 ml  $\text{AlCl}_3$  solution was added. At the 6<sup>th</sup> min, 2 ml NaOH were added and the volume was adjusted at 10 ml with distilled water and mixed well. The absorbance was measured against blank at 510 nm and total flavonoid contents were expressed as quercetin (Marinova et al., 2005).

#### Total antioxidant activity

Aliquots from extracts or ascorbic acid solutions were pipetted into a series of 10 ml volumetric flasks. To each flask, 1.7 ml phosphomolybdic acid solution was added followed by 1.5 ml of conc.  $\text{H}_2\text{SO}_4$  (added drop wise with shaking), left for 10 minutes at room temperature. The volume was completed to 10 ml with 50%  $\text{H}_2\text{SO}_4$ . The absorbance of the resultant blue colour was measured at 667 nm against similarly treated blank. A calibration curve was constructed representing concentration of ascorbic acid in  $\text{mg ml}^{-1}$  (x axis) and absorbance (y axis). The total antioxidant activities of the examined extracts were expressed in terms of ascorbic acid

**Table 1.** Different validation parameters of spectrophotometric methods used in determination of antioxidant activities and total flavonoid contents of the examined plant extracts.

Method	Validation parameter*				
	Regression equation	Correlation coefficient	Linearity Range ( $\mu\text{g ml}^{-1}$ )	Precision (RSD %)	Accuracy (%recovery)
Total antioxidant activity	$y=0.0622x + 0.0032$	0.9994	0.5 - 0.9	0.72	98.07
Free radical scavenging activity	$y=0.1522x + 0.0084$	0.9982	0.6 - 1.6	1.58	97.56
Nitric oxide scavenging activity	$y=0.0654x + 0.0662$	0.9970	0.2 - 0.6	1.92	97.41
Total flavonoids content	$y=0.0038x + 0.3044$	0.9998	50 - 125	1.47	98.71

\*All measurements are performed in triplicates.

equivalents.

#### Free radical scavenging activity

Aliquots from extracts or standard ascorbic acid solutions within the linearity range (Table 1) were pipetted into a series of 5 ml volumetric flasks. To each flask, 3 ml of DPPH solution was added, mixed with the solution and flasks were allowed to stand in dark at room temperature for 10 minutes (Williams et al., 1995). The absorbance of each of the resulted solutions was measured at 516 nm against similarly treated blank.

#### Nitrogen oxide scavenging assay

Aliquots from different extracts and ascorbic acid standard solutions were pipetted into separate sets of 10 ml volumetric flasks. To each flask 0.5 ml of freshly prepared sodium nitroprusside solution was added. The flasks were incubated at 25°C for 150 min. After incubation period, 1 ml of Griess reagent was added to each flask and the volume was completed with distilled water (Kang et al., 2006). Absorbance was measured at 542 nm and results were expressed as  $\mu\text{g}$  ascorbic acid equivalents per  $\mu\text{g}$  of extract.

#### High performance thin layer chromatography (HPTLC) analysis

A volume of 1  $\mu\text{l}$  from extracts or standard quercetin solutions covering the range of 0.02 to 2  $\text{mg ml}^{-1}$  was applied in triplicates (6 mm band length), onto a 20 x 10 cm plate, developed to a distance of 8 cm using  $\text{CHCl}_3$ : MeOH : glacial acetic acid (8.5 : 1.5 : 0.1). The development time was 15 min, the plates were air-dried for 15 min and zones were scanned at wavelength 360 nm. Peak areas were recorded for the tracks, calibration curve was constructed for quercetin, by plotting the peak area (y axis) against the amount of the standard in  $\mu\text{g}$  (x axis).

## RESULTS AND DISCUSSION

Comparison of the obtained NMR data of the isolated flavonoid with the previously reported confirmed its identity as quercetin (Agrawal, 1989) Figure 1.

#### Determination of total flavonoids content

The method depends on formation of aluminium complex which exhibited maximum absorption at 510 nm. The

flavonoids content in the three plants were found to be 5.39, 5.82, 6.2 g /100 g of dry plant weight for *P. acacia*, *P. curviflorus* and *P. austro Arabica*, respectively. The results are expressed in terms of quercetin.

#### Total antioxidant activity

Reaction with phosphomolybdic acid reagent provides sensitive and fast preliminary test for reducing organic compounds; the test is useful for neutral, acidic and basic ones (Fiegl and Anger, 1966). Reducing reagents under suitable conditions convert phosphomolybdic acid to molybdenum blue. The formed blue colour is a lot of "genotoxic" compounds, in which the main oxidative state of molybdenum is between 6+ and 5+. The investigated extracts reacted with phosphomolybdic acid in acidic medium indicating their antioxidant abilities. The reaction product (molybdenum blue) exhibited maximum absorption at 667 nm. Standard methanolic solution of ascorbic acid was used to determine the optimum reaction conditions and to validate the method. The optimum volumes of phosphomolybdic acid (10%) solution and conc. sulphuric acid were found to be 1.7 and 1.5 ml, respectively. A period of 10 min. at room temperature was found to be necessary for full colour development. The formed colour was stable when the reaction mixture was completed to the mark with 50% sulphuric acid; dilution with water lead to colour vanishing. Validation parameters are presented in Table 1. Results are expressed in term of ascorbic acid equivalent (Table 2). *P. austro arabica* extract exhibited slightly higher antioxidant activity than the other 2 extracts which were of equal activities.

#### Free radical scavenging activity

The method is based on the reduction of a methanol solution of DPPH in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H (Williams et al., 1995). This transformation results in a change in colour from purple to yellow, which was measured spectrophotometrically at 516 nm. The

**Table 2.** Results of antioxidant activities of investigated plants extracts.

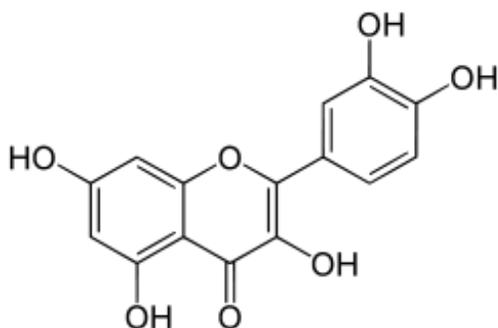
Plant extract	Antioxidant activity in terms of ascorbic acid equivalent ( $\mu\text{g}/\mu\text{g}$ of extract)		
	Total antioxidant*	Free radical scavenging*	Nitric oxide scavenging*
<i>Plicosepalus curviflorus</i>	$0.37 \pm 1.78$	$0.5 \pm 1.47$	$0.19 \pm 0.93$
<i>Plicosepalus acacia</i>	$0.37 \pm 1.29$	$0.59 \pm 1.24$	$0.19 \pm 0.19$
<i>Phragmanthera austro arabica</i>	$0.39 \pm 1.35$	$0.49 \pm 1.54$	$0.18 \pm 0.79$

\*Mean of five measurements  $\pm$  RSD.

**Table 3.** Validation parameters of the HPTLC method for the determination of quercetin in the examined plant extracts.

Validation parameter	Result*
Regression equation	$y = 9085x + 1622$
correlation coefficient	0.9978
Range of linearity	0.2 to 1.4 $\text{mg ml}^{-1}$
Precision	1.7
Accuracy	2
Limit of detection	0.01 $\text{mg ml}^{-1}$
Limit of quantitation	0.04 $\text{mg ml}^{-1}$

\*All measurements are performed in triplicates.

**Figure 1.** Quercetin structure.

method was validated and different validation parameters are recorded in Table 1. The percentage inhibition of the purple colour of the DPPH as a measure of the free radical scavenging activity is shown in Figure 2. The free radical scavenging ability was expressed relative to standard ascorbic acid (Table 2).

### Nitrogen oxide scavenging activity

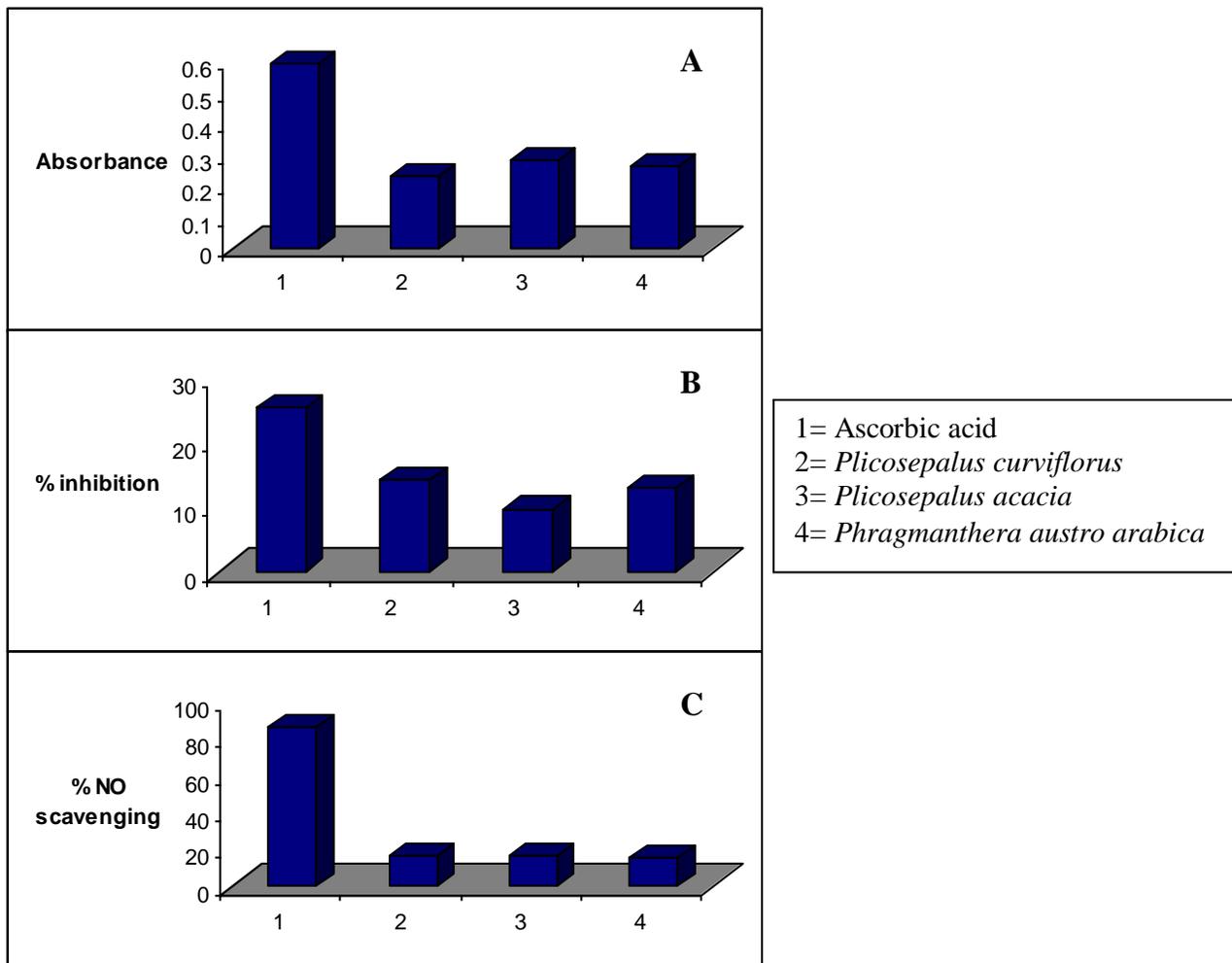
A specific target of antioxidants is to scavenge nitrogen oxide to prevent the formation of the carcinogenic nitrosamines and nitrosamides. These are produced in human body from amines or amides and nitrite derived from food or inhaled nitrogen oxides (Lijinsky et al., 1972). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite. In the applied

method, sodium nitroprusside was used as a source of nitrogen oxide. The investigated extracts as nitrogen oxide scavengers compete with oxygen leading to reduced production of nitrite, and subsequently a decrease in the formation of the coloured azodye due to reaction with Griess reagent. The different reaction conditions were optimized and the method has been validated (Table 1). Extracts of *P. curviflorus* and *P. acacia* exhibited slightly higher activity than *P. austro arabica* (Table 2).

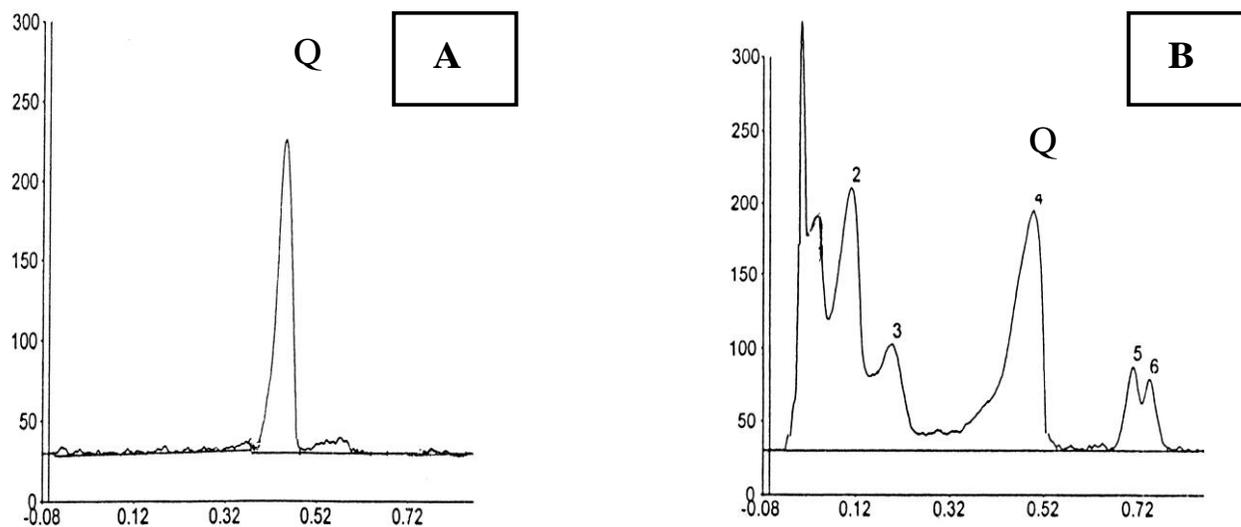
### HPTLC analysis

Different mobile phases have been tried in order to achieve good separation of quercetin from co-existing components. The optimum one was  $\text{CHCl}_3$  : MeOH : glacial acetic acid (8.5 : 1.5 : 0.1). The maximum sensitivity was obtained when the plates were scanned at 360 nm. Figure 3 represent the chromatograms of the analysis of standard quercetin and a representative sample (*P. austro arabica*). The suggested procedure was validated; the validation parameters are presented in Table 3. HPTLC determination procedure has the advantage of being rapid, easy and economic as minimal solvent amount could be used for the analytical process. Applying the proposed method, the concentration of quercetin varied from 0.157 (*P. austro arabica*) to 0.062 g% (*P. acacia*) and *P. curviflorus* contained 0.115 g% w/w quercetin. Quercetin was used to validate the HPTLC methods. A linear relationship was found between the response and standard concentrations over the applied ranges. The good linearity is evident from the values of the correlation coefficients. The accuracy of the proposed method was assessed by calculating the percentage recovery of standard spiked in the three investigated extracts. Good percentage recoveries were obtained. The precision as repeatability was calculated as relative standard deviation of the assay results of three different standard concentrations each in three replicates and was found to be less than 2%. Detection and quantitation limits were calculated on the basis of signal to noise ratio  $S/N = 3$  and  $S/N = 10$ , respectively) for HPTLC method.

Finally, it could be concluded that, the three investigated extracts exhibit comparable significant antioxidant activity. As antioxidant, they could be used for the



**Figure 2.** (A) Total antioxidant activities (10  $\mu\text{g mL}^{-1}$ ), (B) free radical scavenging activity (1  $\mu\text{g mL}^{-1}$ ) and (C) nitric oxide scavenging activity (10  $\mu\text{g mL}^{-1}$ ) of investigated plants extracts compared with ascorbic acid.



**Figure 3.** HPTLC densitogram of standard quercetin. A, Extract of a representative sample, *Phragmanthera austro Arabica*; B, scanned at wavelength 360 nm.

prevention and treatment of a number of diseases such as diabetes mellitus (Sadi and Guray, 2009) and in prevention of cancer (Saxena et al., 2010; Chen et al., 2004). This finding is in agreement with their use in folk medicine as antidiabetic and anticancer (Wahab et al., 2010). Further studies are needed to evaluate the bioavailability and safety of the three extracts.

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