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In Vitro Antioxidant Activity and Polyphenolic Contents of *Rauvolfia tetraphylla* L., *Rhinacanthus nasutus* Kurz. and *Solena amplexicaulis* (Lam.)

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ABSTRACT

Methanol extracts of the leaves of *Rauvolfia tetraphylla* L., *Rhinacanthus nasutus* Kurz. and *Solena amplexicaulis* (Lam.) were evaluated for their antioxidant activity. The extracts were investigated for total phenolic and flavonoid contents, antihemolytic activity, reducing power capacity and radical scavenging activity towards DPPH and ABTS radicals. The total phenol content was in the range of 25-41.7 mg gallic acid equivalent/g dry weight (DW) of sample and the total flavonoid content varied from 10.2-25.8 mg quercetin equivalent/g of DW sample. The antioxidant activity was expressed as IC_{50} values. The IC_{50} values of DPPH activity for *R. tetraphylla*, *R. nasutus* and *S. amplexicaulis* extracts were 170, 230 and 190 µg extract/ml, respectively. The IC_{50} values of ABTS scavenging (80-120 µg extract/ml) and antihemolytic activity (135-157 µg extract/ml) were calculated. The IC_{50} value of reducing power activity for *R. tetraphylla* extract was 200 µg extract/ml while *R. nasutus* and *S. amplexicaulis* extracts at a concentration of 250 µg/ml did not reach 50% of reducing capacity. In the present study, linear regression analysis of antihemolytic, DPPH and reducing power activities with total phenol and flavonoid contents were evaluated and showed a positive correlation ($R^2 > 0.735$). However, no correlation was found between ABTS activity and polyphenol content. The results confirm that polyphenols in methanol extracts of plants are largely responsible for their antioxidant activities.

Keywords: $ABTS^{\cdot+}$ cation scavenging activity, antihemolytic activity, DPPH-RSA, total flavonoid content, total phenolics content

INTRODUCTION

In living organisms reactive oxygen species (ROS) and reactive nitrogen species (RNS) can form in different ways (Halliwell 1994). ROS and RNS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and cancer (Duh 1998; Samak *et al*. 2009). When produced in excess, ROS can cause tissue injury, whilst, tissue injury can itself cause ROS generation (Tepe *et al*. 2005). Nevertheless, all aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damage and numerous damage removal and repair enzymes to remove or repair damaged molecules (Sun *et al.* 1998; Ridha *et al*. 2007). However, the natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important (Duh 1998; Espin *et al.* 2000). Recently there has been a surge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury (Pourmorad *et al.* 2006). Investigations for phenolic compounds in medicinal herbs have gained importance due to their high antioxidative activity and large number of reports has demonstrated that these compounds are of great value in preventing the onset or progression of many human diseases (Chang *et al*. 2007). Several plants have been investigated in the search for novel antioxidants. Therefore, research into the determination of natural antioxidant potential from medicinal plant is warranted.

Medicinal plants which were studied in the present investigation, *Rauvolfia tetraphylla* L. (Apocyanaceae), *Rhinacantus nasutus* Kurz. (Acanthaceae) and *Solena amplexicaulis* (Lam.) Gandhi (Cucurbitaceae). *R. nasutus*, are widely distributed in South China and India, and have been cultivated for use in the treatment of hepatitis, diabetes, hypertension, cancer (Siripong *et al.* 2006) skin diseases (Sendl *et al*. 1996; Wu *et al*. 1998) and immunomodulatory effect (Punturee *et al*. 2005). *Rhinacanthus* plant is well known as the sources of flavonoids, steroids, triterpenoids, anthraquinones, lignans and especially naphthoquinone analoges (Kupradinun *et al*. 2009). The methanolic extract of aerial parts showed hepatoprotective and free radical scavenging activities (Suja *et al*. 2004). Naphthoquinone and rhinacanthone compounds have been reported to possess in vitro antiproliferative activity towards various cancer cells and activity against Dalton's lymphoma ascitic cells (Thirumrugan *et al*. 1994; Gotoh *et al*. 2004; Siripong *et al*. 2006). *R. tetraphylla* is medicinally important in the treatment of cardiovascular diseases, hypertension, psychiatric diseases, cholera and intestinal disorders (Anonymous 1969; Anitha and Ranjithakumari 2006). Leaves and callus extracts of *R. tetraphylla* are found to possess antimicrobial activity (Shariff *et al*. 2006) *S. amplexicaulis* is useful in anorexia, dyspepsia, flatulence, asthma, cardiac disorder, bronchitis, vomiting, hepatosplenomegaly, hemorrhoids, constipation, gonorrhoea and spermatorrhoea (Warrier *et al*. 1996). A detailed review of the literature afforded no information on the antioxidant potential of these plants. Therefore, the present study aims to assess the antioxidant activity and to measure crude polyphenolic contents of the aforesaid plants.

MATERIALS AND METHODS

Chemicals and supplies

All chemicals used, including the solvents, were of analytical grade. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2, 2'-azinobis-3-

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ethylbenzothiozoline-6-sulfonic acid (ABTS), quercetin and butylated hydroxy toluene (BHT) were purchased from Merck (Mumbai, India). Potassium ferricyanide, Folin-Ciocalteu reagent, methanol, gallic acid, trichloroacetic acid (TCA), ferric chloride, sodium carbonate, hydrogen peroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, aluminium chloride, potassium persulfate, were purchased from SD Fine Chemicals (Mumbai, India).

Plant material

R. tetraphylla, *R. nasutus* and *S. amplexicaulis* were taxonomically identified by Dr. R. Gopalan, taxonomist, Department of Botany, Karpagam University, and voucher specimens were deposited in the herbarium of Karpagam University, Coimbatore. The parts of plants studied were collected from Eachanari, Coimbatore, Tamil Nadu, India.

Sample preparation

Leaves of the selected plants were dried at room temperature (RT), finely powdered and the powder (100 g) was exhaustively extracted with methanol (500 ml). Then, the extracts were centrifuged (3000 × *g*) three times and the clear supernatants were combined. The combined supernatants were filtered over Whatman No. 1 filter paper. The extracts were then evaporated to dryness by a rotary flash evaporator (Buchi type Rotavapor, Flawil, Switzerland). Different concentrations of extracts were prepared from the resultant methanol extract to determine *in vitro* antioxidant assays.

Determination of total phenol content

Total phenol content of the methanolic extract was determined using the Folin-Ciocalteu reagent method with a slight modification (Singleton *et al*. 1999). Briefly, the methanolic extract was dissolved in methanol/water (30: 70, v/v) solution; a 100-mL aliquot of the sample solution was transferred to a 10 mL volumetric flask containing 6.0 mL distilled water, to which 500 mL undiluted Folin-Ciocalteu reagent was subsequently added. After 3 min, 2 mL of Na_2CO_3 (15 g/100 mL) was added and the volume was made up to 10 mL with distilled water. After a 2-h incubation period at RT, the absorbance was measured at 760 nm. Gallic acid was used as an analytical standard for total phenol quantification. The total phenol content was determined as mg of gallic acid equivalents (GAE)/g dry weight (DW).

Determination of total flavonoid content

The total flavonoid content (TFC) of methanolic extract was quantified based on the method of Ordonez *et al*. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample solution. After 1 h at RT, the absorbance was measured at 420 nm with a UV-Visible spec (Elico, India). Yellow indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. The results were expressed as mg quercetin equivalents (QE)/g DW.

In vitro antioxidant studies

The methods followed to determine the antioxidant activity of methanol extract of the leaves were the DPPH radical scavenging assay (DPPH-RSA), ferric reducing capacity (FRC), scavenging of ABTS radical cation and antihemolytic activity of H_2O_2-induced red blood cells (RBCs).

1. DPPH˙ radical scavenging activity (DPPH-RSA)

DPPH˙ radical scavenging activity was adopted from a previously described method with slight modifications (Blois 1958). Various concentrations (50-250 µg/ml) of samples and BHT were taken in different test tubes. The volume was adjusted to 100 µl by adding methanol. 5 ml of 0.1 mM methanol solution of DPPH was added to these test tubes and vortexed. The tubes were allowed to stand at room temperature for 20 min. The control was prepared as above without any extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. The inhibition percentage was calculated using the following formula:

% Radical scavenging activity = [(Absorbance $_{control}$ − Absorbance $_{sample}$)]/ (Absorbance $_{control}$)] × 100.

BHT was used as the reference standard (50-250 µg/ml). Mean values were obtained from triplicate experiments. The percentage inhibition versus concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as the IC_{50} (inhibitory concentration of 50%) value.

2. Ferric reducing capacity (FRC)

The reducing power of the extracts was quantified according to the method of Oyaizu (1986). Various concentrations of the extracts and BHT (50-250 µg/ml) were prepared. To all the extracts in test tubes 2.5 ml of sodium phosphate buffer followed by 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution was added. The contents were vortexed well and then incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% TCA was added to all the tubes and centrifugation was carried out at 3000 × *g* for 10 min. Afterwards, to 5 ml of the supernatant, 5 ml of deionized water was added. To this about 1 ml of 1% ferric chloride was added to each test tube and incubated at 35°C for 10 min. The absorbance was read at 700 nm. Mean values were obtained from triplicate experiments. The reducing power of the extract was linearly proportional to the concentration of the sample. Increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as the reference standard.

3. $ABTS^{\bullet+}$ radical cation scavenging activity

Radical scavenging activity of the extract was assessed spectrometrically by the $ABTS^{\bullet+}$ cation decolorization assay (Re *et al*. 1999). The test was based on the relative activity of antioxidants to quench the radical cation $ABTS^{\bullet+}$. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 OD at 734 nm using a UV-VIS spectrophotometer. ABTS solution was freshly prepared for each assay. Plant extract and BHT of various concentrations (50-250 µg) were allowed to react with 1 ml of the ABTS solution and the absorbance was read at 734 nm after 7 min with the spectrophotometer. Percentage inhibition was calculated as:

$ABTS^{\bullet+}$ radical scavenging activity (%) = [(Absorbance $_{control}$ − Absorbance $_{sample}$)]/ (Absorbance $_{control}$)] × 100.

4. Antihemolytic activity of H_2O_2-induced RBCs

The antihemolytic activity of methanolic extracts was examined by the *in vitro* method of Naim *et al*. (1976). The RBCs from cow blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4), until the supernatant was colorless. The RBCs were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of sample (50-250 µg/ml) with saline or buffer were added to 2 ml of the suspension of RBCs and the volume was made up to 3.5 ml with saline or buffer. This mixture was pre incubated for 5 min and then 0.5-ml H_2O_2 solutions of appropriate concentration in saline or buffer were added. The concentration of H_2O_2 in the reaction mixture was adjusted so as to bring about 90% hemolysis of RBCs after 120-min incubation. Because of varying conditions the exact amount of H_2O_2 had to be determined in each instance by a preliminary experiment. In the case of cow blood, the concentration of H_2O_2 was between 0.1 and 0.2% was used. About 80-90% hemolysis of cow RBCs was obtained after 4-6 h. Incubation was concluded after these time intervals by centrifugation during 5 min at l000 × *g* and the extent of hemolysis was determined by measurement of the absorbance (at 540 nm) corresponding to hemo-

globin liberation. Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula:

% Antihemolytic activity = [(Absorbance $_{control}$ − Absorbance $_{sample}$)]/ (Absorbance $_{control}$)] × 100.

Statistical analysis

The experimental results concerning the study were mean and standard deviation (±) of three parallel measurements. Linear regression analysis was performed, quoting the correlation coefficient by Microsoft Excel program. One-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were carried out to determine significant differences ($P < 0.05$) between the means of assays by SPSS (version 10 for Windows 98, SPSS Inc.).

RESULTS AND DISCUSSION

Total phenol and flavonoid contents

Results obtained in the present study revealed that the levels of the phenol, flavonoid and extract yield percentage were shown in **Table 1**. The phenolics and flavonoid contents of methanol extracts in *R. tetraphylla* was significantly higher than that of *R. nasutus*, *S. amplexicaulis*. The total phenolic content was differed significantly and ranged between 25 to 41.7 mg gallic acid/g DW. Total flavonoid content was raised from 10.2 to 25.8 mg quercetin/g DW. The extract yield percentage of *R. tetraphylla*, *R. nasutus* and *S. amplexicaulis* were found to be 35, 18 and 25%, respectively. Polyphenols are the major plant compounds with antioxidant activity. This activity is believed to be mainly due to their redox properties (Zheng and Wang 2001; Adedapo *et al.* 2008), which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The values obtained for methanolic leaf extracts indicate that *R. tetraphylla* is rich in phenolic compounds. Phenolic compounds and flavonoids have been reported to have antioxidant properties, they act as free radical scavengers, ROS and they also chelate metal ions (Zhang *et al.* 2001; Oboh and Akindahunsi 2004; Oboh 2006).

DPPH$^{\cdot}$ radical scavenging activity (DPPH-RSA)

DPPH-RSA has been extensively used for screening antioxidants such as polyphenols and flavonoids from medicinal plants. DPPH is scavenged by polyphenols and flavonoids through the donation of hydrogen, forming the reduced DPPH-H$^{\cdot}$. The colour changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm (Sun *et al.* 2009). From the result, we have observed that the IC_{50} values (**Table 2**), *R. tetraphylla* had highest activity (170 µg/ml) followed by *S. amplexicaulis* (190 µg/ml) and *R. nasutus* (230 µg/ml). However, based upon the values, scavenging activities of methanol extracts of all plants were relatively lower than BHT (70 µg/ml), a known antioxidant used as positive control. It was evident that the extracts did show the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants (Adedapo *et al.* 2008).

Table 1 Total phenolics and flavonoid contents of the methanol extracts of medicinal plants.

Plant species	Total phenolics (mg/g)[a]	Total flavonoid (mg/g)[b]	Yield (%)
R. tetraphylla	41.7 ± 0.006	25.8 ± 0.005	35
R. nasutus	25.0 ± 0.010	10.2 ± 0.008	18
S. amplexicaulis	33.3 ± 0.002	21.6 ± 0.003	25

(n=3), ± standard deviation.
[a] Expressed as mg gallic acid equivalent/g dry weight of sample.
[b] Expressed as mg quercetin equivalent/g dry weight of sample.

Ferric reducing capacity (FRC)

For the measurement of reducing capacity, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of methanolic extract using the method of Oyaizu (1986). The reducing power increased with increasing the phenolic content of extract and reducing power was positively correlated with phenolic content. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Sfahlan *et al.* 2009). In applied concentration range of 50-250 µg/ml, IC_{50} values (the effective concentration at which the absorbance was 0.5 for reducing capacity) for BHT and *R. tetraphylla* extracts were 165 and 200 µg/ml, respectively. While *R. nasutus* and *S. amplexicaulis* extracts did not reach 0.5 absorbance at the applied concentration range (**Table 2**). Antioxidants are strong reducing agents and this is principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure (Rice-Evans *et al.* 1997; Oboh and Rocha 2007). The free radical scavenging ability of the methanolic extracts is an indication that *R. tetraphylla* promises to be good source of antioxidant potential.

ABTS$^{\cdot+}$ radical scavenging activity

ABTS$^{\cdot+}$ is another synthetic radical and more versatile than DPPH$^{\cdot}$, because the ABTS$^{\cdot+}$ model can be assessed the scavenging activity for both the polar and non-polar samples and the spectral interference is lessened as the absorption maximum often used is a wavelength not normally encountered by natural products (Re *et al.* 1999). The methanolic extracts were also able to scavenge ABTS$^{\cdot+}$ radicals in a dose dependent manner. The IC_{50} values (**Table 2**) indicate that the methanolic extract of *R. tetraphylla* (80 µg/ml) does possess the higher ability to scavenge this reactive oxygen species than other two plants, but it is less efficient compared to the positive control BHT (45 µg/ml). The decolourization of ABTS$^{\cdot+}$ radical cation also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate these radical species (Pellegrini *et al.* 1999). The ABTS activities of the *R. tetraphylla* plant extracts showed higher and comparable activity to that of BHT. This may be due to variation in the types of phenolic compounds that differ significantly in their reactivity towards ABTS (Katalinic *et al.* 2006).

Antihemolytic activity of H_2O_2-induced RBCs

Inhibition of hemolysis of cow RBC by H_2O_2 induced peroxy radicals was also concentration dependent manner. Concentrations at which extracts decrease hemolysis by

Table 2 IC_{50} values of the methanol extracts in antioxidant properties of medicinal plants.

Sample	Antihemolytic activity (µg extract/ml)	ABTS (µg extract/ml)	DPPH (µg extract/ml)	Reducing capacity (µg extract/ml)
BHT	91 ± 1.57	45 ± 0.64	70 ± 0.37	165 ± 0.15
R. tetraphylla	135 ± 1.12	80 ± 1.22	170 ± 0.46	200 ± 0.11
R. nasutus	157 ± 1.06	93 ± 1.51	230 ± 1.83	>250
S. amplexicaulis	142 ± 1.33	120 ± 1.36	190 ± 1.15	>250

(n=3), ± standard deviation.
IC_{50} value: the effective concentration at which the inhibition of radicals by 50%; reducing power was the absorbance at 0.5.

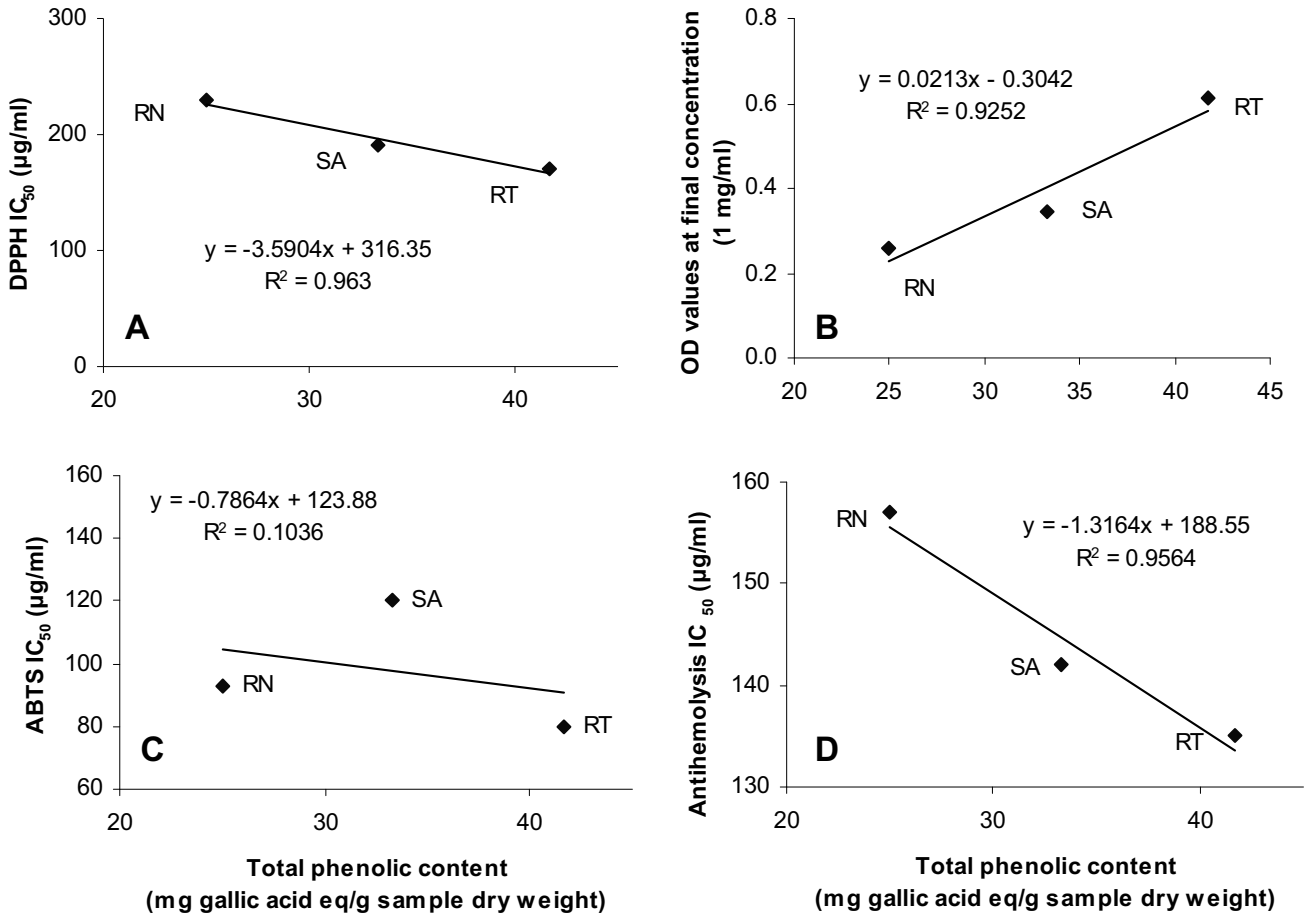

Fig. 1 A-D Linear regression analysis for the total phenolics content and antioxidant activities of methanol extracts. SA, *Solena amplexicaulis*; RN, *Rhinacanthus nasutus*; RT, *Rauvolfia tetraphylla.*

50% (IC_{50} values) were 142, 157, and 135 µg/ml for *S. amplexicaulis*, *R. nasutus* and *R. tetraphylla* extracts, respectively (**Table 2**). IC_{50} values for BHT, used as standard, was 91 µg/ml. The H_2O_2-induced hemolysis is inhibited by phenols of different structure (Younkin *et al.* 1971), tocopherols, and isoflavones. Another function of phenolic compounds acting against hemolysis may be a specific interaction of these inhibitors with membranous phospholipids and proteins leading to physicochemical stabilization of the membranes and reduction of their permeability (Lucy 1972; Naim *et al.* 1976). This concept is supported by the experimental evidence provided by Leibowitz and Johnson (1971) for the incorporation of the phenolic antioxidant BHT in membranes, which results in their stabilization. As outlined above the antihemolytic action of methanolic extracts of leaves on cow erythrocytes is at least partly due to the antioxidative property of the phenols.

Correlation between antioxidant activities, total phenolics and flavonoid contents

The total phenolics and flavonoid contents should correlate with DPPH, reducing capacity, ABTS and antihemolytic assays were shown in **Figs. 1** and **2**. A direct correlation between radical scavenging activity, total phenol and flavonoid content of the extracts was demonstrated by linear regression analysis. R^2 values for the total phenolic contents (methanolic extracts of three plants) and IC_{50} of DPPH, ABTS, antihemolytic activity and OD values (1 mg/ml) of reducing power assays were 0.963, 0.104, 0.956 and 0.925, respectively (**Fig. 1A-D**).

Similarly, the correlations of total flavonoid content against the antioxidant activity based on DPPH, ABTS, antihemolytic and reducing power assays (**Fig. 2A-D**) were 0.995, 0.004, 0.997 and 0.735, respectively. The results indicate that total phenols and flavonoid contents against ABTS were not correlated and the correlation with DPPH, antihemolytic and reducing power assays were satisfactory. The results confirm that polyphenols in methanol extracts of plants were largely responsible for the antioxidant activities. A strong correlation between the polyphenol contents and antioxidant activities, it implies that polyphenols in *S. amplexicaulis*, *R. nasutus* and *R. tetraphylla* were capable of scavenging free radicals. Some authors have reported similar correlations between polyphenols and antioxidant activity (Kukic *et al*. 2006; Chang *et al*. 2007; Liu *et al*. 2009).

CONCLUDING REMARKS

The methanolic extract of the plants studied were exhibited the antioxidant potent to some degree in *in-vitro* assays. Therefore, the potency of these plants due to compounds presents in it and provide some of the health benefits. Among all the plants studied, *R. tetraphylla* had the best antioxidant potential. Nevertheless, further studies are needed to isolate, identify, characterize and elucidate the structure of the bioactive compounds and also to investigate the physiological and pharmacological properties. This is of potential research and development value in the field of pharmaceutical and functional foods.

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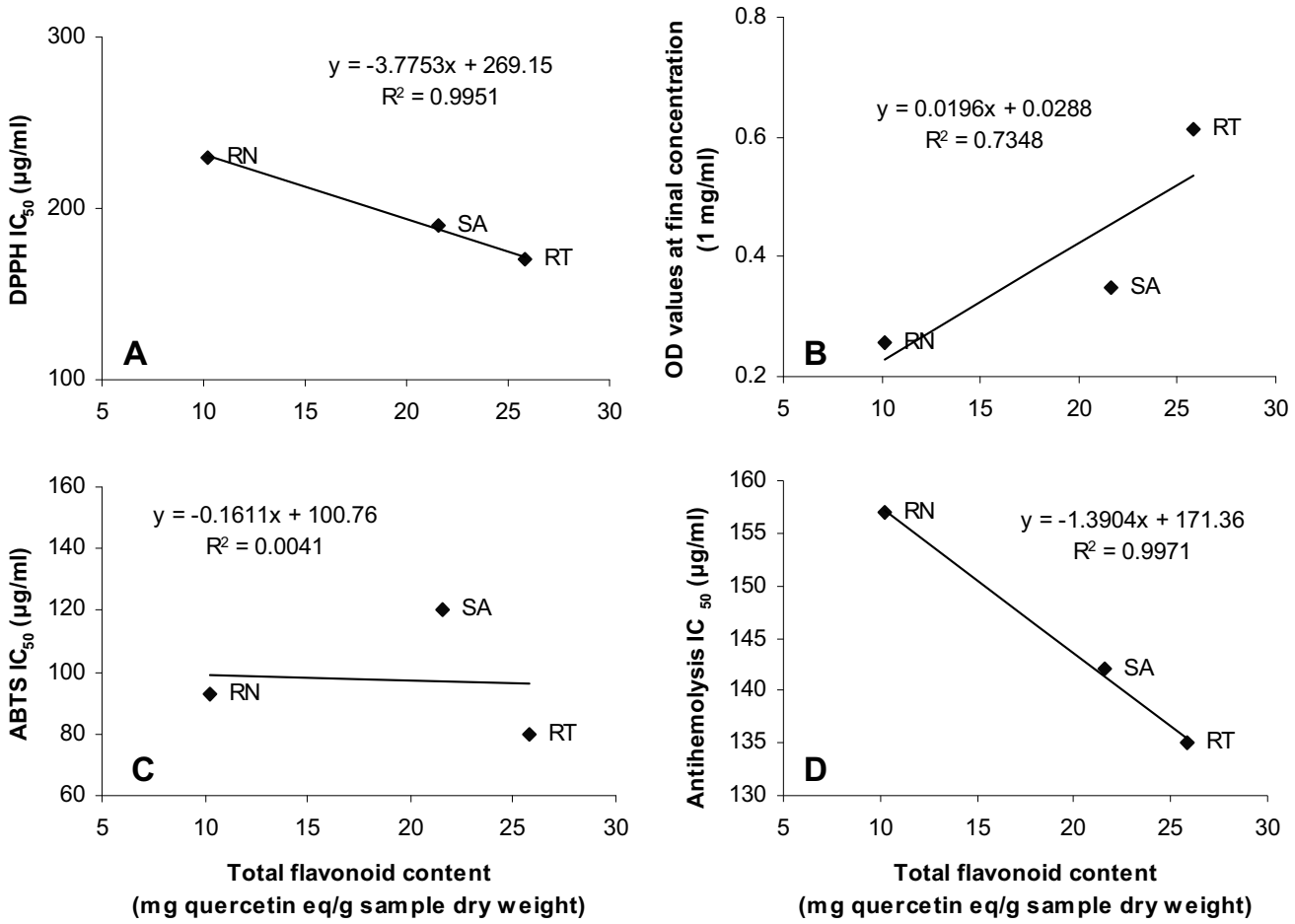

Fig. 2 A-D Linear regression analysis for the total flavonoid content and antioxidant activities of methanol extracts. SA, *Solena amplexicaulis*; RN, *Rhinacanthus nasutus*; RT, *Rauvolfia tetraphylla.*

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