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Proceeding Paper

Assessment of Phytochemicals and Antioxidant Properties of Root Extracts of *Rubia cordifolia* L. in Different Solvent Systems †

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Abstract: *Rubia cordifolia* L. is an important plant used in Ayurvedic and Siddha medicinal systems of India for treatment of blood disorders. Of all the plant parts, roots of *R. cordifolia* are the most suitable source of effective secondary metabolites. The present work investigated phytochemical content and antioxidant potential of *R. cordifolia* root powder extracted in different solvents. Total polyphenols and flavonoids content were estimated. High antioxidant activity was corroborated with DPPH, hydrogen peroxide, nitric oxide, reducing power and total antioxidant assays. Obtained results showed that ethanol extracts were most potent over methanol, aqueous, and PBS extracts for DPPH, hydrogen peroxide, and reducing power assays. In contrast, methanol and aqueous extracts had higher potency in nitric oxide and total antioxidant assays. Encouraging results were obtained for antioxidant activity even upon PVPP treatment that removed the polyphenols from the extracts. The results suggest a potential of ethanol and methanol extracts for cancer cytotoxicity.

Keywords: root extract; *Rubia cordifolia* L.; secondary metabolites; multiple solvents

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1. Introduction

Rubia cordifolia L. belongs to the family Rubiaceae and is largely distributed from Africa to tropical Asia, China, Japan, and Australia. The active compounds of *R. cordifolia* are 1-hydroxy,2-methoxy anthraquinone,3-dimethoxy 2 carboxy anthraquinone, rubiadin, mangistin, alizarin, garancin, rubiprasin A,B,C, ruiearbonls, mollugin and furomollugin [1]. The roots of *R. cordifolia* are used for the preparation of aqueous, ethanol, methanol, chloroform, and dichloromethane extracts. Some of their therapeutic activities are listed in Table 1.

Different extracts have shown varied potential and a comparative account among the extracts is missing. We hereby determined antioxidant activities of these extracts. We identified ethanol and methanol extracts of the root as the most suitable solvent for antioxidant activities.

Table 1. Known potential/activities of *R. cordifolia*.

Solvent	Plant Part	Effect on Cell Line/Animal Model	Reference
Ethanol	Root	Anti-thrombotic and anti-angiogenic effects in zebra fish	[2]
Ethanol	Root	Antioxidant activity which prevents the ethanol-induced immunosuppression in rats	[3]
Methanol	Root	Cardio protective effect in Wister rat	[4]
Methanol	Root	Cytotoxic activity in HEp-2	[5]
Methanol	Root	Anticancer and anti-inflammatory activities in carrageenan-induced rat-paw oedema model	[6]
Aqueous	Aerial	Anti-diarrheal and anti-inflammatory activities in male Swiss albino mice	[7]
Aqueous	Whole plant	Inhibits the multiplication of rotavirus by promoting virus induced apoptosis in rhesus monkey kidney cell line MA-104 cells	[8]
Chloroform	Whole plant	Anti-tumor activity on ascites leukemia, lung cancer, melanoma, and sarcoma cell lines	[9]

2. Experiments

2.1. Plant Collection

Powder of *R. cordifolia* (root) was collected from Maharashtra Arogya Mandal, Hadapsar Pune, Maharashtra, India. They were stored in airtight containers for future use.

2.2. Preparation of Extracts

Extracts of powders were prepared in ethanol (HiMedia, Mumbai, India), methanol (HiMedia, India) or distilled water as described. In brief, powder of *R. cordifolia* were extracted with solvent (ethanol, methanol or aqueous) by conventional Soxhlet apparatus (4951, Goel Scientific, Vadodara, India) extraction at the temperature of 60 °C. After the exhaustive extraction, each extract was evaporated to dryness by rotary evaporator (Aditya Scientific, Hyderabad, India) and if it was not dried then the extract was further concentrated using concentrator (5305000304, Eppendorf India Pvt. Ltd, Chennai, India) and stored at room temperature for future use. Phosphate Buffer Saline (PBS) extracts of *R. cordifolia* were made by mixing the powder of the root in PBS or media (50 mg/mL).

2.3. Phytochemical Screening

The presence of secondary metabolites *viz*. alkaloids, saponins, tannins, phenols, glycosides, terpenes, carotenoids and quinones was detected using the standard tests [10].

2.4. Removal of Polyphenols from Plant Extracts

The plant extracts were treated with 10% Polyvinylpolypyrrolidone (PVPP) (HiMedia, India) made in respective solvents for the removal of polyphenols. The extracts (5 mL) were treated with PVPP (5 mL) in respective solvents and kept on a shaking incubator at 37 °C overnight. The supernatant was used for further experiments [11].

2.5. Quantification of Phenols

The phenolic content was determined according to the method given earlier [12]. A total of 1 mL of 1 mg/mL extract or gallic acid (HiMedia, India) with the concentration of 20, 40, 60, 80 and 100 µg/mL was mixed with 0.5 mL of 1N Folin–Ciocalteu reagent. Mixture was kept for 5 min, followed by addition of 1 mL of 20% sodium carbonate (HiMedia, India). After 10 min incubation at room temperature, absorbance was measured at 730 nm using UV-Vis spectrophotometer. Gallic acid was used as the standard.

2.6. Quantification of Flavonoids

Flavonoid content in the extract was determined according to the method given earlier [13]. A total of 1 mL of extract or quercetin (HiMedia, India) with the concentration of 100, 200, 300, 400 and 500 µg/mL was mixed with 1.25 mL of distilled water and 75 µL

of 5% of sodium nitrite (HiMedia, India). This solution was incubated for 5 min, and then 150 µL of 10% aluminum chloride (Sigma-Aldrich, St. Louis, MO, USA) solution was added. After incubation of 6 min, 500 µL of 1 M sodium hydroxide (HiMedia, India) and 275 µL of distilled water were added to prepare the final mixture. The absorbance was read at 510 nm using UV-Vis spectrophotometer. Quercetin was used as the standard.

2.7. Antioxidant Assays

2.7.1. DPPH Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was measured with spectrophotometer method described previously [14]. To the 0.5 mL extract solution with or without PVPP, made in respective solvents of concentration ranging from 20–100 µg, 1 mL of 0.2 mM DPPH (HiMedia, India) made in ethanol was added and volume was made up to 2 mL with methanol, and incubated for 30 min at room temperature. The absorbance was measured at 517 nm against blank. Ascorbic acid (HiMedia, India) was used as the standard. The percentage of inhibition of DPPH was calculated as follows:

$$Percent\ scavenging = \frac{((Acontrol - Asample) \times 100)}{Acontrol},$$

2.7.2. Hydrogen Peroxide Assay

The scavenging effect of hydrogen peroxide was determined as described previously [15]. A total of 1 mL of extract solution treated with or without PVPP, of concentration ranging from 20–100 µg was treated with 0.6 mL, 40 mM of hydrogen peroxide (Thermo Fisher, Concord, NH, USA) prepared in phosphate buffer (pH 7.4) for 10 min. The absorbance was read at 230 nm against blank. Ascorbic acid was used as standard.

$$Percent\ scavenging = \frac{((Acontrol - Asample) \times 100)}{Acontrol},$$

2.7.3. Scavenging Activity of Nitric Oxide

Nitric oxide was generated from sodium nitroprusside and its scavenging effect was determined as per [16]. Different concentration from 20–100 µg of 1 mL of extract solution with or without PVPP, phosphate buffer 1 mL (pH 7.4) was used to prepare sodium nitroprusside (HiMedia, India) 0.5 mL, 10 mM, and then incubated for 5 h at 25 °C. After 5 h of incubation, 0.5 mL of supernatant liquid was removed and 0.5 mL of Griess reagent (Thermo Fisher, USA) (1 mM) prepared in distilled water was added. The absorbance was read at 546 nm. Ascorbic acid was used as standard.

$$Percent\ scavenging = \frac{((Acontrol - Asample) \times 100)}{Acontrol},$$

2.7.4. Total Antioxidant Capacity

The total antioxidant capacity was determined by phosphomolybdate assay [17]. A total of 1 mL of extract with or without PVPP, of concentration prepared in respective solvents, ranging from 20–100 µg was taken in centrifuge tube and 1 mL of reagent containing 0.6 M sulfuric acid (HiMedia, India), 28 mM sodium phosphate (Thermo Fisher, USA) and 4 mM ammonium molybdate (Thermo Fisher, USA) was added. The tubes were incubated at 95 °C for 90 min, and were cooled to room temperature, and absorbance was read at 695 nm. Ascorbic acid was used as standard.

$$Percent\ scavenging = \frac{((Acontrol - Asample) \times 100)}{Acontrol},$$

2.7.5. Assay of Reducing Power

The reducing power assay was determined by Spectrophotometric method of Oyaizu (1986) [18]. 2.5 mL of extract solution made in respective solvents, of various concentrations

ranging from 20–100 µg was treated with 2.5 mL of 0.2 M phosphate buffer (pH 6.6), 2.5 mL of 1% potassium ferricyanide (Thermo Fisher, USA), incubated at 50 °C for 20 min, cooled, 2.5 mL of 10% trichloro acetic acid (HiMedia, India) was added and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) of the solution was removed and 2.5 mL of methanol and 0.5 mL of 0.1% ferric chloride (HiMedia, India) solutions were added, the absorbance of the resulting solution was read at 700 nm. Ascorbic acid was used as standard.

$$Percent\ scavenging = \frac{((Acontrol - Asample) \times 100)}{Acontrol}$$

2.8. Statistical Analysis

All experiments were performed in triplicate. All the values were expressed as mean ± standard error of mean. The data were analyzed by Student–Newman–Keuls test using Sigma Plot version 14 (Systat software Inc., San Jose, CA, USA) and IC50 values calculated using Origin software version 8.1 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Extracts of Plant Powders Show Presence of Several Phytochemicals

3.1.1. Qualitative Analysis of Secondary Metabolites

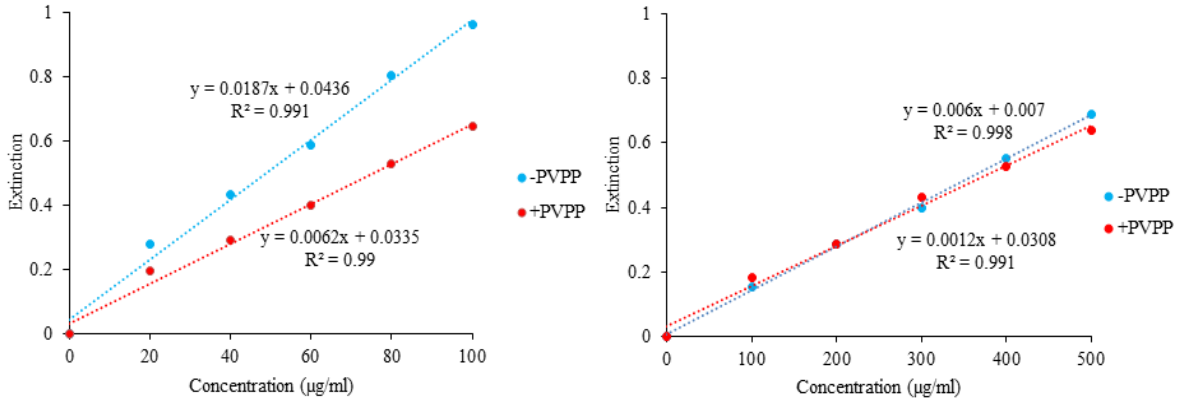
The ethanol extract of the *R. cordifolia* powder showed the presence of alkaloids, flavonoids, glycoside, phenols and terpenes while the methanol extract showed the presence of alkaloids, tannins, phenols, flavonoids, and terpenes. The aqueous extract of plant powder showed the presence of alkaloids, saponins, tannins, phenols, flavonoids, and terpenes. The PBS extract of powder showed the presence of alkaloids, flavonoids, terpenes and phenols (Table 2).

Table 2. Phytochemical screening of *R. cordifolia* extracts in different solvents—Qualitative Assay.

	Assays	Ethanol Extract	Methanol Extract	Aqueous Extract	PBS
1	Mayer's	–	–	–	–
2	Dragendorff's	+	+	+	+
3	Wagner's	–	–	–	–
4	Hager's	–	–	–	–
5	Saponins	–	–	+	–
6	Tannins	–	+	+	–
7	Phenols	+	+	+	+
8	Glycosides	+	–	+	–
9	Flavonoids	+	+	+	+
10	Terpenes	+	+	+	+
11	Steroids	–	–	–	–
12	Quinones	–	–	–	–
13	Carotenoids	–	–	–	–

3.1.2. Quantification of Phenols and Flavonoids in Plant Extracts

Standard curves were generated for phenols (with gallic acid) and flavonoids (with quercetin) to quantify phenols and flavonoids in the extracts. These assays were repeated for presence and absence of PVPP (Figure 1). Ethanol and methanol extracts of powders had 75% more phenol and flavonoid content compared to aqueous and PBS extracts for 1 mg/mL concentration of extracts (Tables 3 and 4).



(**a**) (**b**)

Figure 1. (**a**) Standard curve of gallic acid without (blue) and with (red) PVPP for Phenols; (**b**) Standard curve of quercetin without (blue) and with (red) PVPP for Flavonoids.

Table 3. Quantification of phenol contents of root powder extracts of *R. cordifolia*.

Extracts in Solvent	PVPP	Phenol content (GAE) Mean Value
Ethanol	−	36.95 ± 0.08 ^{a,b,c,d}
	+	5.45 ± 0.24
Methanol	−	21.55 ± 0.13 ^{a,f,g}
	+	6.58 ± 0.72
Aqueous	−	33.39 ± 0.05 ^{a,e}
	+	6.80 ± 0.24
PBS	−	17.66 ± 0.16 ^{a}
	+	6.58 ± 0.81

Phenol content (GAE) in last column is expressed as mean ± SEM (n = 3), $^{a–g}$ Column wise values with different superscripts of this type indicate significant difference ($p < 0.001$), ^{a} between −PVPP and +PVPP for same solvent, $^{b–g}$ for −PVPP, ^{b} between Ethanol and PBS, ^{c} between Ethanol and Methanol, ^{d} between Ethanol and Aqueous, ^{e} between Aqueous and PBS, ^{f} between Aqueous and Methanol, ^{g} between Methanol and PBS.

Table 4. Quantification of flavonoid contents of powder extracts of *R. cordifolia*.

Extracts in Solvent	PVPP	Flavonoid Content (QE) Mean Value
Ethanol	−	78.27 ± 0.41 ^{a,d,e,f}
	+	35.44 ± 3.3
Methanol	−	86.44 ± 0.34 ^{a,b,c}
	+	41.55 ± 2.9
Aqueous	−	41.44 ± 0.83 ^{a,g}
	+	21.83 ± 1.6
PBS	−	36.83 ± 0.72 ^{a}
	+	27.38 ± 3.9

Flavonoid content (QE) in last column is expressed as mean ± SEM (n = 3), $^{a–g}$ Column wise values with different superscripts of this type indicate significant difference ($p < 0.001$), ^{a} between −PVPP and +PVPP for same solvent, $^{b–g}$ for −PVPP, ^{b} between Methanol and PBS, ^{c} between Methanol and Aqueous, ^{d} between Methanol and Ethanol, ^{e} between Ethanol and PBS, ^{f} between Ethanol and Aqueous, ^{g} between Aqueous and PBS.

3.2. Plant Extracts Have Antioxidant Activity

Ascorbic acid showed significant radical scavenging activity ($p \leq 0.05$) (Figures 2 and 3, Table 5). Prior to PVPP treatment, in antioxidant assays *viz.* DPPH, hydrogen peroxide, and reducing power assay, the ethanol extract had 0.55, 0.54 and 0.95 times scavenging activity as that of ascorbic acid, respectively. In nitric oxide and total antioxidant assay, methanol extract was 93% and 82% more potent as compared to standard.

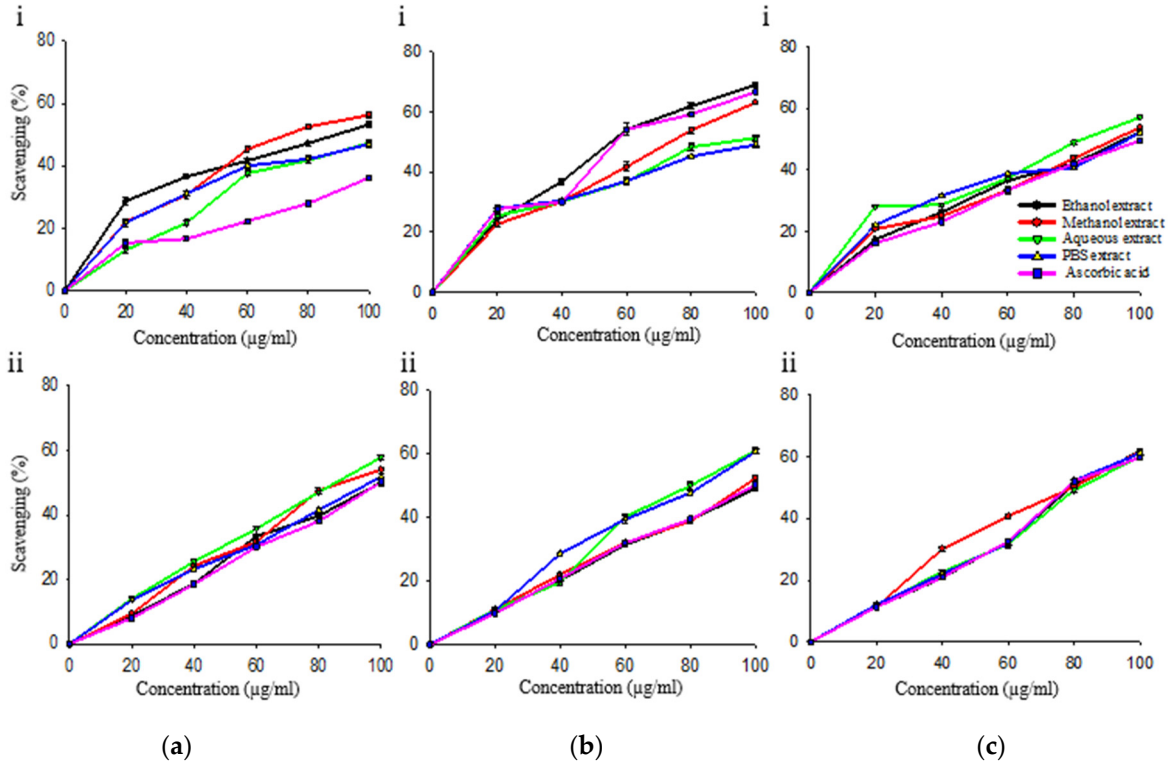


Figure 2. In vitro antioxidant assays of *R. cordifolia* without (i) and with (ii) PVPP for (**a**) DPPH assay; (**b**) hydrogen peroxide assay; (**c**) nitric oxide assay.

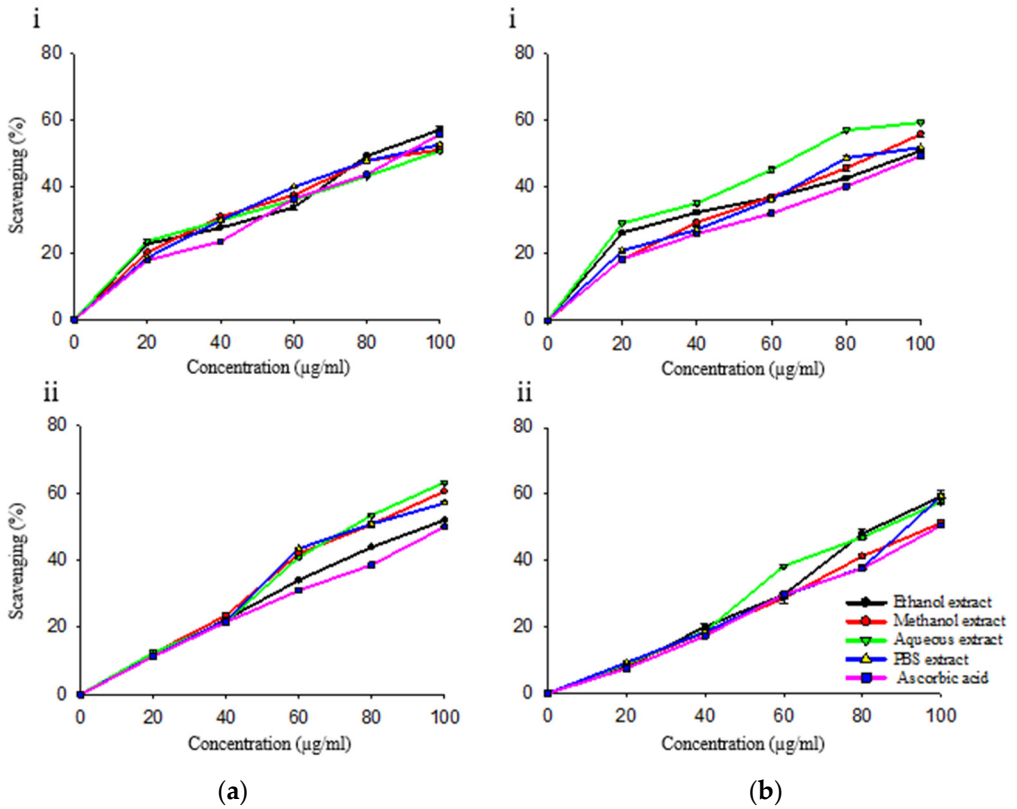


Figure 3. In vitro antioxidant assays of *R. cordifolia* without (i) and with (ii) PVPP for (**a**) Assay of reducing power; (**b**) Total antioxidant assay.

Table 5. IC_{50} values of DPPH, Hydrogen peroxide, Nitric oxide, Reducing power and Total antioxidant assay of *R. cordifolia*.

	DPPH		**Hydrogen Peroxide**		**Nitric Oxide**		**Reducing Power**		**Total Antioxidant**	
Extracts	**IC_{50} (µg/mL) (−PVPP)**	**IC_{50} (µg/mL) (+PVPP)**	**IC_{50} (µg/mL) (−PVPP)**	**IC_{50} (µg/mL) (+PVPP)**	**IC_{50} (µg/mL) (−PVPP)**	**IC_{50} (µg/mL) (+PVPP)**	**IC_{50} (µg/mL) (−PVPP)**	**IC_{50} (µg/mL) (+PVPP)**	**IC_{50} (µg/mL) (−PVPP)**	**IC_{50} (µg/mL) (+PVPP)**
Ethanol	88.5	98.26	61.2	101.14	95.11	82.17	83.89	93.72	101.15	85.92
Methanol	79.1	89.47	74.5	97.71	94.53	78.46	85.69	79.79	88.62	97.52
Aqueous	99.97	85.53	92.97	80.85	85.23	84.23	106.36	77.62	71.86	85.14
PBS	104.39	97.55	102.05	81.05	97.35	85.61	88.72	81.81	91.36	91.92
Ascorbic acid	159.34	100.42	112.125	99.12	100.5	82.87	90.77	101.48	104.48	100.29

4. Discussion

R. cordifolia L. produces a variety of secondary metabolites. These metabolites are responsible for spatiotemporal and sustainable growth of the plant. They also act as important defense compounds. Utility of secondary metabolites for human health has achieved high recognition owing to the usage in traditional medication. *R. cordifolia* L. has been a less explored system and identification of suitable extract with maximal components is urgently required. In the present study, we have selected four solvent system for extracting secondary metabolites from roots of *R. cordifolia* L. Quantitative analysis indicated a good number of phenols and flavonoids in the root extracts. Our method of Soxhlet extraction, led to more release of phenols (in ethanol, and methanol extracts) from *R. cordifolia* root powder than that obtained from field [19].

Presence of antioxidant in the extract is crucial. Results of DPPH assay for ethanol extract reported by Zhang et al. [20] had EC_{50} in the range of 23.88 to 65.23 µg/mL. They used ultrasonic-assisted extraction process. These values are much lower than our range of 78.25–88.63 µg/mL. We believe suitability of extraction method is driver of differential results. Basu and Hazra [21] reported a range of 153.7–310.3 µg/mL for methanol and aqueous extracts as evaluated by nitric oxide assay. Here the authors used filtrate of direct solubilization of extracts in respective solvents. Our results have a better range (94.46–108.21 µg/mL), possibly due to our choice of method of Soxhlet exhaustive extraction process. We are also reporting for the first time, results of *R. cordifolia* extracts (ethanol, methanol, aqueous and PBS) treated with PVPP for antioxidant assays. The IC_{50} of *R. cordifolia*, extracts (ethanol, methanol, aqueous, PBS extracts), for DPPH assay (98.26, 89.47, 85.53 and 97.55 µg/mL), hydrogen peroxide free radical scavenging assay (101.34, 97.71, 80.85 and 81.05 µg/mL), nitric oxide assay (82.17, 78.46, 84.23 and 81.95 µg/mL), reducing power assay (93.72, 79.79, 77.62 and 81.81 µg/mL), total antioxidant assay (87.92, 97.52, 85.14 and 91.92 µg/mL). Hence, even after removal of phenols and flavonoids, antioxidant activity is not hampered. This suggests antioxidant potential for different classes of secondary metabolites. High presence of antioxidant may be used for anti-proliferative properties in certain cancers [1].

5. Conclusions

Our study has revealed presence of high antioxidants in root extracts of *R. cordifolia*. Methods of extraction is important as observed when compared reports. This work provides initial steps required in suitability of solvents for *R. cordifolia* extract preparations. Further work regarding anticancer potential needs to be evaluated to verify the extent of utility of antioxidant nature.

Author Contributions: S.C.K., A.A.K., and R.B.H. conceived and designed the experiments; R.B.H. performed the experiments; S.C.K., R.B.H., J.S. and A.A.K. analyzed the data; S.C.K. and A.A.K. contributed reagents/materials/analysis tools; S.C.K. and J.S. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All the relevant data is presented in this study itself.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DPPH	2,2-diphenyl-1-picrylhydrazyl
PBS	Phosphate Buffer Saline
PVPP	Polyvinylpolypyrrolidone

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