Phytochemical Characterization, Antioxidant and Anti-Proliferative Properties of *Rubia cordifolia* L. Extracts Prepared with Improved Extraction Conditions

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**Abstract:** *Rubia cordifolia* L. (Rubiaceae) is an important plant in Indian and Chinese medical systems. Extracts prepared from the root, stem and leaf have been used traditionally for the management of various diseases. Some of the known effects are anti-inflammation, neuroprotection, anti-proliferation, immunomodulation and anti-tumor. A comparative account of the extracts derived from different organs that lead to the identification of the most suitable solvent is lacking. We explored the presence of phytochemicals, antioxidant activity and anti-proliferative properties of a variety of solvent-based extracts of root, and methanol extracts of stem and leaf of *R. cordifolia* L. The antioxidant potential was determined by DPPH, hydrogen peroxide, nitric oxide and total antioxidant assays. The anti-proliferative nature was evaluated by MTT assay on HeLa, ME-180 and HepG2 cells. The composition of the extracts was determined by UPLC-UV-MS. We found that the root extracts had the presence of higher amounts of antioxidants over the stem and leaf extracts. The root extracts prepared in methanol exhibited the highest cytotoxicity in HepG2 cells. The main compounds identified through UPLC-UV-MS of the methanol extract give credibility to the previous results. Our comprehensive study corroborates the preference given to the root over the stem and leaf for extract preparation. In conclusion, we identified the methanol extract of the root to be the most suited to have bioactivity with anti-cancer potential.

**Keywords:** secondary metabolites; anti-cancer; alternative medicine; polyvinylpolypyrrolidone (PVPP); phenol quenching; cell lines; metabolic profiling; multiple solvents

1. Introduction

Various chemotherapeutic drugs inherently induce side effects due to a lack of non-specificity towards cancer cells. The search for newer molecules has led to a refreshed look at complementary and alternative medicinal practices [1,2]. A plant-derived anti-cancer molecule is expected to provide a solution owing to its natural source. Hence, many plants are being investigated in view of this vital necessity. Extracts from traditional medicinal plants, such as *Rubia cordifolia* L. may be one of the alternatives available to fill this lacuna.
Geographically, *R. cordifolia* L. is a widely distributed member of the Rubiaceae family. It is traditionally referred to as Indian Madder or Manjith or Manjistha in India and Qiancao in China. The inherent red color of the root is used as a food coloring agent and dye for fiber. Its usage as a phytomedicine has been documented in the traditional Indian medicine systems of Ayurveda and Siddha and traditional Chinese medicine. Broadly, the extracts have been used for the treatment of blood-related conditions, such as hematemesis, epistaxis, spotting, traumatic bleeding and amenorrhea [3]. The extract preparation may be water-based (aqueous) and organic-solvent-based (such as methanol, ethanol, chloroform and dichloromethane). The aqueous extract of the aerial parts effectively controls diarrhea and inflammation in male Swiss albino mice [4]. The aqueous extract of the whole plant limits the rotavirus multiplication in MA-104 cells [5]. Methanol extracts prepared from the root have cardioprotective [6] and anti-cancer activities, as determined in the human epidermoid laryngeal carcinoma cell line (HEp-2) [7], anti-human lymphoma cells (U937) and malignant skin melanoma (A375) [8]. The ethanol extract of the root has been evaluated to be anti-thrombotic and pro-angiogenic [9]. Animal studies using chloroform extract of the whole plant did not disclose any significant anti-tumor activity [10].

Many compounds have been identified from *R. cordifolia* that may be responsible for such therapeutic actions [9,11]. The compounds present in the *R. cordifolia* are reviewed by Shan et al. [3], and those with a PubChem ID are consolidated in Table 1.

| No. | Chemical Compounds | PubChem ID | Molecular Formula | Molecular Weight (g/mol) | Isolated from |
|-----|--------------------|------------|-------------------|--------------------------|--------------|
| 1   | 6-methoxygeniposidic acid | 50998059 | C_{17}H_{24}O_{11} | 404.4 | Root |
| 2   | Rubiprasin A       | 21594201 | C_{32}H_{35}O_{5} | 516.799 | Root |
| 3   | Rubiprasin B       | 21594133 | C_{32}H_{35}O_{4} | 500.8 | Root |
| 4   | Rubiarbonol A      | 12019473 | C_{30}H_{30}O_{4} | 474.7 | Root |
| 5   | Rubiarbonol B      | 12019474 | C_{30}H_{30}O_{3} | 458.7 | Root |
| 6   | Rubiarbonol C      | 21672545 | C_{32}H_{35}O_{5} | 516.799 | Root |
| 7   | Rubiarbonol D      | 21672546 | C_{32}H_{35}O_{4} | 516.799 | Root |
| 8   | Rubiarbonol E      | 21582934 | C_{30}H_{30}O_{4} | 474.7 | Root |
| 9   | Rubiarbonol F      | 21582935 | C_{30}H_{30}O_{3} | 490.7 | Root |
| 10  | 1,8-dihydroxyanthraquinone | 2950 | C_{14}H_{8}O_{4} | 240.21 | Root |
| 11  | 1-hydroxy 2-methoxy anthraquinone | 80103 | C_{15}H_{10}O_{4} | 254.24 | Root |
| 12  | 1,3-dimethoxy 2-carboxy anthraquinone | 129670266 | C_{17}H_{12}O_{6} | 312.27 | Root |
| 13  | 1,5-dihydroxy 2-methylanthraquinone | 182449 | C_{15}H_{10}O_{4} | 254.24 | Root |
| 14  | Pseudopurpurin     | 442765   | C_{15}H_{16}O_{7} | 300.22 | Root |
| 15  | Dihydromollugin     | 10779560 | C_{17}H_{16}O_{4} | 286.32 | Root |
| 16  | Munjistin          | 160476   | C_{15}H_{8}O_{6} | 284.22 | Root |
| 17  | 1-hydroxy-2-hydroxymethyl-9,10-anthraquinone | 32209 | C_{15}H_{10}O_{4} | 254.24 | Root |
| 18  | Mollugin           | 124219   | C_{17}H_{16}O_{4} | 284.31 | Root |
| 19  | 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone | 5319801 | C_{15}H_{10}O_{5} | 270.24 | Root |
| 20  | Rubioncolin B      | 14777446 | C_{31}H_{24}O_{10} | 556.5 | Root |
| 21  | Rubilactone        | 132415   | C_{15}H_{10}O_{5} | 270.24 | Root |
| 22  | 1-hydroxy-2-carboxy 3-methoxyanthraquinone | 129670276 | C_{16}H_{10}O_{6} | 298.25 | Root |
| 23  | Oleanolic acid acetate | 6708573 | C_{32}H_{35}O_{4} | 498.7 | Root |
| 24  | Hederagenin        | 73299    | C_{30}H_{46}O_{4} | 472.7 | Root |
| No.  | Chemical Compounds                               | PubChem ID   | Molecular Formula  | Molecular Weight (g/mol) | Isolated from         |
|------|--------------------------------------------------|--------------|--------------------|--------------------------|-----------------------|
| 25   | B-sitosterol                                      | 222284       | C_{29}H_{50}O      | 414.7                    | Root                  |
| 26   | Rubiasin A                                       | 101064500    | C_{15}H_{16}O_{2}  | 228.29                   | Root, Stem Root       |
| 27   | Rubiasin B                                       | 101064501    | C_{15}H_{16}O_{2}  | 228.29                   | Stem                  |
| 28   | Rubiasin C                                       | 101064502    | C_{15}H_{16}O_{2}  | 228.29                   | Root                  |
| 29   | 1-hydroxy-2-methylanthraquinone, 1,4-dihydroxy-2-methylanthraquinone | 160817 | C_{15}H_{16}O_{3}  | 238.24                   | Root                  |
| 30   | 2-methylanthraquinone                            | 6773         | C_{15}H_{16}O_{2}  | 222.24                   | Root                  |
| 31   | Alicantin                                       | 6293         | C_{14}H_{8}O_{4}   | 240.21                   | Root                  |
| 32   | Rubiarin                                         | 124062       | C_{15}H_{16}O_{3}  | 254.24                   | Root                  |
| 33   | Purpurin                                         | 6683         | C_{14}H_{8}O_{5}   | 256.209                  | Root                  |
| 34   | 1,4-dihydroxy-2-methyl-5-methoxyanthraquinone    | 12714658     | C_{16}H_{12}O_{5}  | 284.26                   | Root                  |
| 35   | Ruberythric acid                                 | 92101        | C_{25}H_{32}O_{13} | 534.5                    | Root                  |
| 36   | Physcion                                         | 10639        | C_{16}H_{12}O_{5}  | 284.26                   | Root                  |
| 37   | Nordamcanthal                                    | 160712       | C_{15}H_{8}O_{5}   | 268.22                   | Root                  |
| 38   | Quinizarin (1,4-dihydroxy-6-methyl-anthaquinone) | 6688         | C_{14}H_{8}O_{4}   | 240.21                   | Root                  |
| 39   | 1,4-dihydroxy-2-naphthoic acid                   | 671          | C_{11}H_{8}O_{4}   | 204.18                   | Root                  |
| 40   | Furomollugin                                     | 10354359     | C_{14}H_{10}O_{4}  | 242.23                   | Root                  |
| 41   | Xanthopurpurin                                   | 196978       | C_{14}H_{8}O_{4}   | 240.21                   | Root                  |
| 42   | Methyl 1,4-bisglycosyloxy-3-prenyl-2-naphthoate  | 10031663     | C_{29}H_{38}O_{14} | 610.6                    | Root                  |
| 43   | Physcion                                         | 10639        | C_{16}H_{12}O_{5}  | 284.26                   | Root                  |
| 44   | Nordamcanthal                                    | 160712       | C_{15}H_{8}O_{5}   | 268.22                   | Root                  |
| 45   | Quinizarin (1,4-dihydroxy-6-methyl-anthaquinone) | 6688         | C_{14}H_{8}O_{4}   | 240.21                   | Root                  |
| 46   | 1,4-dihydroxy-2-naphthoic acid                   | 671          | C_{11}H_{8}O_{4}   | 204.18                   | Root                  |
| 47   | Furomollugin                                     | 10354359     | C_{14}H_{10}O_{4}  | 242.23                   | Root                  |
| 48   | 2-methyl-1, 3, 6-trihydroxy-9, 10-anthaquinone   | 5319801      | C_{15}H_{10}O_{5}  | 270.24                   | Root                  |
| 49   | RA-I                                             | 14390137     | C_{40}H_{48}N_{6}O_{10} | 772.8            | Root                  |
| 50   | [Gly]-1ra-vii                                    | 10440996     | C_{26}H_{48}N_{6}O_{9} | 756.8            | Root                  |
| 51   | [Gly]-2ra-vii                                    | 12098468     | C_{26}H_{48}N_{6}O_{9} | 756.8            | Root                  |
| 52   | RA-III                                           | 14390141     | C_{41}H_{50}O_{10}  | 786.9                    | Root                  |
| 53   | RA-V                                             | 13361282     | C_{41}H_{50}O_{10}  | 786.9                    | Root                  |
| 54   | RA-XXIV                                          | 24881308     | C_{42}H_{51}O_{10}  | 813.9                    | Root                  |
| 55   | RA-VIII                                          | 152727187    | C_{42}H_{51}O_{10}  | 786.9                    | Root                  |
| 56   | RA-X                                             | 6444175      | C_{42}H_{52}N_{6}O_{11} | 828.9            | Root                  |
| 57   | RA-XI                                            | 131676023    | C_{42}H_{52}N_{6}O_{11} | 814.9            | Root                  |
| 58   | RA-XII                                           | 10373581     | C_{46}H_{56}N_{6}O_{14} | 919             | Root                  |
| 59   | RA-XIII                                          | 14999350     | C_{46}H_{56}N_{6}O_{16} | 977             | Root                  |
| 60   | RA-XVI                                           | 5320896      | C_{46}H_{56}N_{6}O_{16} | 977             | Root                  |
| 61   | RA-XVII                                          | 102355358    | C_{41}H_{50}N_{6}O_{9} | 770.9            | Root                  |
| 62   | RA-XVIII                                         | 25033039     | C_{41}H_{50}O_{10}  | 786.9                    | Root                  |
| 63   | RA-XIX                                           | 24829365     | C_{44}H_{56}N_{6}O_{9} | 812.9            | Root                  |
| 64   | RA-XX                                            | 24829366     | C_{42}H_{52}N_{6}O_{9} | 784.9            | Root                  |
| 65   | RA-XXI                                           | 24861920     | C_{41}H_{50}N_{6}O_{9} | 770.9            | Root                  |
| 66   | RA-XXII                                          | 24862183     | C_{41}H_{50}O_{10}  | 786.9                    | Root                  |
| 67   | Rubicoumaric acid                                | 5377693      | C_{39}H_{54}O_{6}  | 618.8                    | Whole Plant           |
| 68   | Rubifolic acid                                   | 91895456     | C_{39}H_{48}O_{4}  | 472.7                    | Whole Plant           |
| 69   | 1-hydroxy-9,10-anthaquinone                      | 8512         | C_{14}H_{8}O_{3}   | 224.21                   | Root                  |
| 70   | 2-carbamoil-3-methoxy-1,4-naphthoquinone         | 91825839     | C_{11}H_{7}NO_{4}  | 217.18                   | Root                  |
| 71   | N-nonadecane                                     | 12401        | C_{16}H_{40}       | 268.5                    | Root                  |
| 72   | 2,6-dihydroxyantraquinone                        | 6776         | C_{14}H_{8}O_{4}   | 240.21                   | Root                  |
| No. | Chemical Compounds                  | PubChem ID | Molecular Formula  | Molecular Weight (g/mol) | Isolated from |
|-----|------------------------------------|------------|--------------------|--------------------------|---------------|
| 73  | N-heptadecane                      | 12398      | C₁₇H₃₆             | 240.5                    | Root          |
| 74  | Rubiatriol                         | 21582929   | C₃₀H₃₀O₃           | 458.7                    | Root          |
| 75  | Epoxymollugin                      | 24814354   | C₁₇H₁₆O₅           | 300.3                    | Root          |
| 76  | 1,6-dihydroxy-2-methyl-9,10-       | 124063     | C₁₅H₁₀O₄           | 254.24                   | Root          |
|     | anthraquinone                      |            |                    |                          |               |
| 77  | Citric acid                        | 311        | C₆H₈O₇             | 192.12                   | Root          |
| 78  | Malic acid                         | 525        | C₄H₆O₇             | 134.09                   | Root          |
| 79  | Palmitic acid                      | 985        | C₁₆H₃₂O₂           | 256.42                   | Root          |
| 80  | 1-hydroxy-2,7-dimethylnaphthaquinone| 1382      | C₁₆H₁₂O₃           | 252.26                   | Root          |
| 81  | Emodin                             | 3220       | C₁₅H₁₀O₅           | 270.24                   | Root          |
| 82  | Eugenol                            | 3314       | C₁₀H₁₂O₂           | 164.2                    | Root          |
| 83  | Alizarin                           | 6293       | C₁₄H₄O₄           | 240.21                   | Root          |
| 84  | Quinic acid                        | 6508       | C₇H₁₂O₆           | 192.17                   | Root          |
| 85  | 2-methyl anthraquinone             | 6773       | C₁₅H₁₀O₂           | 222.24                   | Root          |
| 86  | Vanillic acid                      | 8468       | C₄H₄O₄           | 168.15                   | Root          |
| 87  | 1-hydroxyanthraquinone             | 8512       | C₁₄H₄O₃           | 224.21                   | Root          |
| 88  | Lucidin                            | 10163      | C₁₅H₁₀O₅           | 270.24                   | Root          |
| 89  | Naphthohydroquinone                | 11305      | C₁₀H₈O₂           | 160.17                   | Root          |
| 90  | Tricosanoic acid                   | 17085      | C₂₃H₄₂O₂           | 354.6                    | Root          |
| 91  | Ursolic acid                       | 64945      | C₃₀H₄₈O₃           | 456.7                    | Root          |
| 92  | Atraric acid                       | 78435      | C₁₀H₁₂O₄           | 196.2                    | Root          |
| 93  | Friedelinol                        | 101341     | C₃₀H₵₂O          | 428.7                    | Root          |
| 94  | Soranjioli                         | 124063     | C₁₅H₁₀O₄           | 254.24                   | Root          |
| 95  | Lariciresinol                      | 332427     | C₂₀H₂₄O₆           | 360.4                    | Root          |
| 96  | Naphthaquinone                     | 377214     | C₁₃H₁₁NO₄         | 245.23                   | Root          |
| 97  | Anethole                           | 637563     | C₁₀H₁₂O₂           | 148.2                    | Root          |
| 98  | Geraniol                           | 637566     | C₁₀H₁₆O₂           | 154.25                   | Root          |
| 99  | Geranyl acetate                    | 1549026    | C₁₂H₂₆O₂           | 196.29                   | Root          |
| 100 | Scopoletol                         | 5280460    | C₁₀H₄O₄           | 192.17                   | Root          |
| 101 | Rosmarinic acid                    | 5281792    | C₁₈H₁₆O₆           | 360.3                    | Root          |
| 102 | Daucosterol                        | 5742590    | C₃₅H₆₀O₆           | 576.8                    | Root          |
| 103 | 1-hydroxy-2-methyl anthraquinone   | 10250776   | C₂₅H₂₆O₅           | 406.5                    | Root          |
| 104 | Rubicordifolin                     | 11786393   | C₃₃H₂₉O₉           | 568.6                    | Root          |
| 105 | Oleanic acid                       | 12313704   | C₃₀H₄₀O₃           | 454.7                    | Root          |
| 106 | 1, 4-dihydroxy-2-methyl-           | 12488527   | C₁₆H₁₂O₅           | 284.26                   | Root          |
|     | 3-methylnaphthaquinone             |            |                    |                          |               |
| 107 | 1-Hydroxy-2-(methoxycarbonyl)-     | 13793380   | C₁₉H₁₄O₇           | 354.3                    | Root          |
|     | 3-[(methoxycarbonyl)methyl]-       |            |                    |                          |               |
|     | 9,10-anthaquinone                  |            |                    |                          |               |
| 108 | Rubiatriol                         | 21582929   | C₃₀H₃₀O₃           | 458.7                    | Root          |
| 109 | Rubiprasin B                       | 21594133   | C₃₂H₃₂O₄           | 500.8                    | Root          |
| 110 | Rubiprasin A                       | 21594201   | C₃₂H₃₂O₅           | 516.8                    | Root          |
| 111 | Rubiarbonol C                      | 21672545   | C₃₂H₃₂O₅           | 516.8                    | Root          |
| 112 | 1, 4-dihydroxy-2-methyl-           | 23626543   | C₂₀H₁₆O₇           | 368.3                    | Root          |
|     | 5-methoxy anthraquinone            |            |                    |                          |               |
| 113 | 2′-hydroxymollugin                 | 46187192   | C₁₇H₁₆O₅           | 300.3                    | Root          |
| 114 | Methyl 6-hydroxy-3-methoxy-2,2,2-  | 5319476    | C₁₈H₁₈O₅           | 316.3                    | Root          |
|     | dimethyl-3,4-dihydrobenzo[h]chromene-5-carboxylate | | | | |
| 115 | Methyl 3,6-dihydroxy-4-methoxy- 2,2-dimethyl-3,4-dihydrobenzo[h]chromene-5-carboxylate | 5319446 | C₁₈H₂₀O₆ | 332.3 | Root |
Table 1. Cont.

| No. | Chemical Compounds | PubChem ID | Molecular Formula | Molecular Weight (g/mol) | Isolated from |
|-----|--------------------|------------|-------------------|--------------------------|---------------|
| 116 | 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone | 70698136 | C_{29}H_{32}O_{15} | 620.6 | |
| 117 | Rubifolic acid | 72994727 | C_{30}H_{48}O_{4} | 472.7 | |
| 118 | 2-Acetoxy-1,5-dihydroxy-7-methylantraquinone | 100994924 | C_{17}H_{12}O_{6} | 312.27 | |
| 119 | 1,3-dimethoxy 2-carboxy antraquinone | 129670266 | C_{17}H_{12}O_{6} | 312.27 | |
| 120 | Rubicordin A | 132553188 | C_{46}H_{60}N_{6}O_{14} | 921 | |
| 121 | Rubicordin B | 132553189 | C_{47}H_{62}N_{6}O_{14} | 935 | |
| 122 | Rubicordin C | 132553190 | C_{42}H_{54}N_{6}O_{9} | 766.9 | |
| 123 | 2,6-methylanthraquinone | 155490709 | C_{25}H_{32}O_{6} | 424.5 | |
| 124 | Sitosteryl acetate | 348285530 | C_{29}H_{30}O | 414.71 | |
| 125 | Sitostenone | 60123241 | C_{29}H_{46}O | 412.7 | |

Quinones, terpenoids, alkaloids and their derivatives form a major class of compounds with considerable bioactivities. These components are responsible for the various antioxidant, anti-inflammation and anti-proliferative bioactivities, among others. Mollugin (derivative of anthraquinone) inhibits pro-inflammatory chemokine production [12]. Purpurin is another anthraquinone that gives *R. cordifolia* L antioxidant properties [13]. Alizarin, 6-hydroxyrubiadin, purpurin and rubiadin are expected to be key constituents responsible for analgesic and anti-inflammatory properties [14]. The mode of action of the exhaustive list of compounds has not been elucidated completely as many compounds are solvent-specific and are not available in large quantities.

Quantified research that directs to the therapeutic usage of specific extraction solvents for different plant organs is still lacking. Further, a comparison among the different extracts prepared from different *R. cordifolia* plant organs remains unattempted so far. Within this frame of reference, we have focused our attention on the antioxidant and anti-proliferative activities of various extracts prepared from *R. cordifolia* and have identified methanol as the most suitable solvent [15]. An in vitro analysis on the cancer cell lines confirmed the methanol extract of the root as the most suitable for pertinent pre-clinical studies.

2. Materials and Methods

2.1. Plant Collection

The stems and leaves of *R. cordifolia* were freshly collected from Torna fort (18°16′33.86″ N 73°37′21.78″ E) and Mahabaleshwar Forest (17°55′51″ N 73°38′52″ E) located in Maharashtra State, India. Air-dried leaves and stems were separated. The dried samples were pulverized into a coarse powder and stored for further use. The plant was authenticated at Botanical Survey of India, Pune, India center with specimen number mgJRC-1 and a voucher specimen is deposited at the BSI herbarium.

2.2. Preparation of Extracts

All solvents, reagents and standards used were of analytical grade (HiMedia, Mumbai, India). Extracts of powders were prepared in methanol, ethanol or distilled water as described previously [16]. In brief, powders of different plant parts of *R. cordifolia* were extracted with solvent individually by conventional Soxhlet apparatus (Goel Scientific, Vadodara, India) extraction procedure. After the exhaustive extraction, each extract was evaporated to dryness by rotary evaporator (Aditya Scientific, Hyderabad, India). We quenched the polyphenols using polyvinylpolypyrrolidone (PVPP) to determine if antioxidant activity is exclusive to the polyphenols present in the extract. To remove polyphenols from the extracts, they were treated with 10% (w/v) PVPP made in respective solvents and kept on a shaking incubator (238019, Thermo Fisher, Waltham, MA, USA) at 37 °C.
overnight. The polyphenols bind with PVPP and settle at the bottom, while the supernatant contains the polyphenol-free extract [17].

2.3. Qualitative Phytochemical Screening of R. cordifolia Constituents

The preliminary screening of different classes of natural plant constituents was performed. The presence of secondary metabolites viz. alkaloids, saponins, tannins, phenols, glycosides, terpenes, carotenoids and quinones was detected using the standard tests as described below [16,18].

2.3.1. Alkaloid Detection

Mayer’s test for alkaloids was performed by treating equivalent volumes of extract with Mayer’s reagent (in-house prepared by dissolving 1.36 g of mercuric chloride (GRM1067, HiMedia, Mumbai, India) and 5 g of potassium iodide (GRM252, HiMedia, Mumbai, India) in 100 mL distilled water), and the subsequent development of cream-colored precipitate implied existence of alkaloid. Dragenforff’s reagent was prepared by dissolving 8 g of bismuth nitrate (RM1221, HiMedia, Mumbai, India) in 20 mL of concentrated nitric acid (GRM6105, HiMedia, Mumbai, India) and 27.2 g of potassium iodide (KI) in 50 mL of distilled water. Both the solutions were kept standing till KIO₃ crystallized out. The supernatant was decanted, and final volume was adjusted to 100 mL with distilled water. Dragendorff’s test for alkaloids was accomplished by treating equivalent volumes of extract with Dragendorff’s reagent. Subsequent generation of red-colored precipitate suggested presence of alkaloid. Wagner’s reagent was prepared by dissolving 2 g of iodine (GRM1064, HiMedia, Mumbai, India) and 6 g of potassium iodide in 100 mL of distilled water. Wagner’s test for alkaloids was performed by treating equivalent volumes of extract with Wagner’s reagent. Subsequent development of reddish-brown-colored precipitate indicated existence of alkaloid. Hager’s reagent was prepared by dissolving 1 g of picric acid (S026, HiMedia Mumbai, India) in 100 mL of distilled water. Hager’s test for alkaloids was performed by treating equivalent volumes of extract with Hager’s reagent. Subsequent development of yellow-colored precipitate suggested presence of alkaloid.

2.3.2. Saponin Detection

Saponin was detected by dissolving equivalent quantity of extract in water followed by vigorous shaking. Formation of honeycomb-shaped persistent froth indicated the existence of saponins in the sample.

2.3.3. Tannin Detection

Tannins were determined by mixing extract with 0.5% aqueous ferric chloride (GRM165-500G, HiMedia, Mumbai, India), and dark green/bluish-green coloration of the sample indicated presence of tannins.

2.3.4. Phenol Detection

Phenols were determined by adding equivalent volumes of extract to Folin–Ciocalteu reagent (RM10822, HiMedia, Mumbai, India), and blue coloration of sample indicated presence of phenols.

2.3.5. Glycoside Detection

Glycosides were identified by treating equivalent volumes of extract with glacial acetic acid (AS001, HiMedia, Mumbai, India) and some drops of 5% aqueous ferric chloride (FeCl₃) and concentrated sulphuric acid (H₂SO₄) (AS016-500ML, HiMedia, Mumbai, India). This is known as Keller–Kiliani test. Reddish-brown coloration at the confluence and bluish-green color in top layer solution indicated presence of glycosides.
2.3.6. Flavonoids Detection

Flavonoids were detected by Shinoda test when to 1 mL of extract, few mg turnings were added followed by a few drops of concentrated hydrochloric acid (HCl). Development of reddish pink coloration indicated presence of flavonoids.

2.3.7. Terpene Detection

Terpenes were detected by mixing equivalent volumes of extract with chloroform and concentrated sulphuric acid. Reddish-brown coloration at the junction of two solutions suggested the occurrence of terpenes.

2.3.8. Steroid Detection

Steroids were detected by formation of orange color in solution consisting of equivalent volumes of extract with chloroform, glacial acetic acid and concentrated sulphuric acid.

2.3.9. Quinone Detection

Presence of quinone was determined by formation of green color upon addition of concentrated hydrogen chloride (RM5955-500ML, HiMedia, Mumbai, India) to the extract [19].

2.3.10. Carotenoids Detection

Carotenoids were detected by formation of deep blue color in solution consisting of equivalent volumes of extract with concentrated sulphuric acid (H₂SO₄) and a few crystals of iodine.

2.4. Quantification of Phenols

Phenolic content was determined according to the method reported earlier [17]. Briefly, 1 mL of 1 mg/mL extract and gallic acid with the concentrations of 20, 40, 60, 80 and 100 µg/mL was mixed with 0.5 mL of 1N Folin–Ciocalteu reagent and incubated for 5 min, followed by addition of 1 mL of 20% sodium carbonate. After 10 min incubation at room temperature, absorbance was measured at 730 nm. Gallic acid was used as the standard and the phenolic content was expressed as gallic acid equivalent (GAE). The equation of the curve: \( y = mx + c \) with \( R^2 > 0.99 \). The limit of detection (LOD) and limit of quantification (LOQ) were based on the standard deviation of the blank and calculated using following equations:

\[
LOD = 3.3 \times \frac{\sigma}{S} \quad (1)
\]

\[
LOQ = 10 \times \frac{\sigma}{S} \quad (2)
\]

where \( \sigma \) is the standard deviation of \( y \)-intercepts of the regression line, and \( S \) is the slope of the calibration curve.

2.5. Quantification of Flavonoids

Flavonoid content in the extract was determined in accordance with the reported method [20]. In brief, 1 mL of extract and quercetin 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 solution incubated for 5 min; subsequently, 150 µL of 10% aluminum chloride (Sigma-Aldrich, Burlington, MA, USA) solution was added. After incubation for 6 min, 500 µL of 1 M sodium hydroxide and 275 µL of distilled water were added to prepare the mixture. The absorbance was recorded at 510 nm. Quercetin was used as the standard, and the flavonoid content is expressed as quercetin equivalent (QE). The equation of the curve: \( y = mx + c \) with \( R^2 > 0.99 \). The LOD and LOQ were based on the standard deviation of the blank and calculated as described by equations 1 and 2, respectively.
2.6. Antioxidant Assays

2.6.1. DPPH Free Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was measured with spectrophotometric method as described previously [21]. To 0.5 mL extract solution made in respective solvents of concentration ranging from 20 to 100 µg/mL, 1 mL of 0.2 mM DPPH (RM2798, HiMedia, Mumbai, India) made in methanol 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 was used as the standard control. The antioxidant activity was presented as IC$_{50}$ value (µg/mL) based on percentage of inhibition of DPPH as calculated in accordance with Equation (3).

$$\text{Percent scavenging activity} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}}$$ (3)

2.6.2. Hydrogen Peroxide Scavenging Assay

The scavenging effect of hydrogen peroxide was determined as described earlier [22]. Briefly, 1 mL of extract solution of concentration ranging from 20 to 100 µg/mL was treated with 0.6 mL, 40 mM of hydrogen peroxide (Fisher Scientific, Pittsburgh, PA, USA) prepared in phosphate buffer (pH 7.4) for 10 min. The absorbance was read at 230 nm against blank of hydrogen peroxide. Ascorbic acid was used as standard, and the antioxidant activity was presented as IC$_{50}$ value (µg/mL) based on percentage of inhibition of hydrogen peroxide (Equation (3)).

2.6.3. Scavenging Activity of Nitric Oxide

Nitric oxide was generated from sodium nitroprusside, and its scavenging effect was determined as described previously [16]. Briefly, different concentrations ranging from 20 to 100 µg/mL of 1 mL of extract solution and 1 mL (pH 7.4) phosphate buffer were used to prepare 0.5 mL of 10 mM sodium nitroprusside. After incubation for 5 h at 25 °C, 0.5 mL of supernatant liquid was removed and 0.5 mL of Griess reagent (G7921, Thermo Fisher, Waltham, MA, USA) (1 mM) prepared in distilled water was added. The absorbance of the chromophore formed during diazotization of nitric oxide with sulphanilamide and its subsequent coupling with N-(1-naphthyl) ethylene–diamine was determined at 546 nm. Ascorbic acid was used as standard, and the antioxidant activity was presented as IC$_{50}$ value (µg/mL) based on percentage of inhibition of nitric oxide (Equation (3)).

2.6.4. Determination of Total Antioxidant Capacity

The total antioxidant capacity was determined by phosphomolybdate assay [23]. In brief, 1 mL of extract of concentrations ranging from 20 to 100 µg/mL prepared in respective solvents was taken and mixed with 1 mL of reagent containing 0.6 M sulphuric acid, 28 mM sodium phosphate (MB047-250G, Thermo Fisher, Waltham, MA, USA) and 4 mM ammonium molybdate (A7302-100G, Sigma-Aldrich, Burlington, MA, USA). The solution formed was incubated at 95 °C for 90 min, cooled to room temperature and absorbance was noted at 695 nm. Ascorbic acid was used as the standard, and the total antioxidant capacity was calculated as percentage scavenging activity (refer Equation (3)).

2.7. Principal Component Analysis

Principal component analysis (PCA) was performed to point out the clustering of data into two separated groups, namely PVPP untreated (−PVPP) and treated (+PVPP) extracts. The PCA is a procedure aiming at reducing the dimensionality of the data and allowing the visualization of a large number of variables in a two-dimensional plot [24]. The input data were obtained from quantification of phenol and flavonoid and antioxidant activity (phenol content expressed as mg GAE/g of plant extract; flavonoid content expressed as mg QE/g of plant extract; antioxidant potential by DPPH free radical scavenging expressed as IC$_{50}$; antioxidant potential by hydrogen peroxide scavenging expressed as IC$_{50}$; antioxidant
potential by nitric oxide scavenging assay expressed as IC$_{50}$ and total antioxidant capacity expressed as IC$_{50}$) in root-methanol, root-ethanol, root-aqueous, leaf-methanol and stem-methanol extracts. A diagram of the values obtained from each treatment condition was plotted in the bidimensional space, defined by the 1st and 2nd principal component functions (PC1 and PC2, respectively).

2.8. Cell Culture and Cytotoxicity

Authenticated cell lines ME-180, HeLa and HepG2 were procured from National Centre for Cell Science, Pune, India. The cells were grown in Roswell Park Memorial Institute-1640, Eagle’s minimal Essential Medium and Dulbecco’s Modified Eagle Medium media, respectively, and 10% FBS (16000044, Thermo Fisher, Waltham, MA, USA) and 1% antibiotic solution were used for supplementation. Cells were grown in T-25 flasks and were passaged upon confluence using trypsin-EDTA [16]. Nearly 5000 cells were seeded per well in 96-well plate and incubated at 37 °C in 5% CO$_2$ incubator and left overnight to enable surface attachment. Cells were treated with extracts (methanol, ethanol and aqueous) with concentrations of 50, 25, 10, 5, 1, 0.5, 0.1, 0.05 mg/mL and left overnight in incubator. 5 mg/mL of MTT per well was added and incubated for 2 h at 37 °C. Formazan crystals were solubilized with 100 µL DMSO and incubated for 10 min. The absorbance was measured at 570 nm and reference at 630 nm.

2.9. UPLC-UV-MS Analysis

UPLC-UV-MS phytochemical profiling of root methanol extract (1 mg/ml) was performed on an Agilent 6540 UHD Accurate Mass QTOF MS system (Agilent Technologies, Santa Clara, CA, USA). The separation was performed using a Zorbax 2.1 × 50 mM 1.8 µm column. The gradient applied was: 0.1% formic acid in water (A), acetonitrile (B); 0 min 95% B; 5 min 95% B; 6 min 5% B; 8 min 5% B. Injection volume was 10 µL; flow-rate was 0.2 mL/min. ESI-Q-TOF-MS analysis was performed in the positive and negative ionization modes using the following parameters: mass range 70–1600 m/z; gas temperature 270 °C; nitrogen flow 11 L/min; nebulizer pressure 45 psig; skimmer 45 V; capillary voltage 4000 V; fragmentor 150 V, fixed collision energy 40 V. Data were processed with Agilent MassHunter 6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0) (Agilent Technologies, Santa Clara, CA, USA).

2.10. Statistical Analysis

All experiments were performed in triplicate and the values were expressed as mean ± standard error of mean (SEM). The data were analyzed by Student–Newman–Keuls test using Sigma Plot version 14 (Systat Software Inc., Palo Alto, CA, USA), and IC$_{50}$ values were calculated using OriginPro, version 2021 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Qualitative Analysis of Secondary Metabolites of R. cordifolia Extracts

The methanol extract of R. cordifolia root had alkaloids, tannins, phenols, flavonoids and terpenes (Table 2). In comparison, while the ethanol extract lacked tannins, the aqueous extract had saponins and glycosides. Considering the maximally reported usage of methanol extracts for roots, we evaluated methanol extracts of leaves and stems in the same way. In contrast to the methanol extracts of roots, the methanol extracts of leaves had glycosides and quinones, while the stem-methanol extracts had quinones and carotenoids.
Table 2. Phytochemical screening of root, leaf and stem extracts of *R. cordifolia*.

| S.No. | Detection | Assays                          | Root                  | Leaf                  | Stem                  |
|------|-----------|---------------------------------|-----------------------|-----------------------|-----------------------|
|      |           |                                 | Methanol Extract     | Ethanol Extract       | Aqueous Extract       |
| 1    | Alkaloids | Mayer’s test                     | –                     | –                     | +                     |
| 2    | Alkaloids | Dragendorff’s test               | +                     | +                     | +                     |
| 3    | Alkaloids | Wagner’s test                    | +                     | –                     | +                     |
| 4    | Alkaloids | Hager’s test                     | –                     | –                     | +                     |
| 5    | Saponins  | Foam test                        | –                     | –                     | +                     |
| 6    | Tannins   | Ferric chloride test             | +                     | –                     | +                     |
| 7    | Phenols   | Folin–Ciocalteu reagent test     | +                     | +                     | +                     |
| 8    | Glycosides| Keller–Kiliani test              | –                     | –                     | +                     |
| 9    | Flavonoids| Shinoda test                     | +                     | +                     | +                     |
| 10   | Terpenes  | Chloroform-Sulphuric acid test   | +                     | +                     | +                     |
| 11   | Steroids  | Liebermann–Burchard test         | –                     | –                     | –                     |
| 12   | Quinones  | Hydrochloride test               | –                     | –                     | +                     |
| 13   | Carotenoids| Iodine crystal test             | –                     | –                     | +                     |

+ Present; − absent.

3.2. Quantification of Phenols and Flavonoids in Extracts

Standard calibration curves were plotted for the quantification of phenols in extracts. The plot for standard gallic acid for both PVPP-untreated and -treated was linear, with correlation coefficients \( R^2 \) equal to 0.9916 and 0.99, respectively. The regression equations for PVPP-untreated and -treated were \( y = 0.0093x + 0.0436 \) and \( y = 0.0062x + 0.0335 \), respectively, with an LOD under 10 mg/g and LOQ under 30 mg/g for both. Similarly, standard quercetin calibration plots were obtained as linear with \( R^2 \) of 0.9986 and 0.991, and regression equations of \( y = 0.0014x + 0.0067 \) and \( y = 0.0012x + 0.0308 \) for PVPP-untreated and -treated, respectively. The LODs were under 20 mg/g and LOQs were under 40 mg/g for both.

Significant levels of difference were observed in all the root extracts post-PVPP treatment for the phenols and flavonoids. The ethanol and methanol extracts of roots had the highest phenol and flavonoid content, respectively, compared to the other extracts for 1 mg/mL concentrations of extracts (Table 3). The roots had the highest phenol and flavonoid content among the methanol extracts of different organs of *R. cordifolia* L.

Table 3. Quantification of phenol and flavonoid contents in extracts of *R. cordifolia*.

| Extracts in Solvent | PVPP ‘+’ = presence of PVPP, ‘−’ = absence of PVPP | Phenol Content (mg GAE/g of Plant Extract) | Flavonoid Content (mg QE/g of Plant Extract) |
|---------------------|----------------------------------------------------|------------------------------------------|---------------------------------------------|
| Root-Methanol       | −                                                  | 43.34 ± 0.27 \( a,b,c \)                  | 369.69 ± 1.49 \( a,b,c \)                   |
|                     | +                                                  | 6.59 ± 0.73                              | 55.28 ± 2.7                                 |
| Root-Ethanol        | −                                                  | 74.31 ± 0.16 \( a,d \)                   | 334.9 ± 1.8 \( a,d \)                       |
|                     | +                                                  | 5.46 ± 0.25                              | 49.64 ± 3.11                                |
| Root-Aqueous        | −                                                  | 67.14 ± 0.11 \( a \)                     | 177.05 ± 3.6 \( a \)                       |
|                     | +                                                  | 6.80 ± 0.25                              | 37.08 ± 1.54                                |
| Leaf-Methanol       | −                                                  | 35.12 ± 0.32 \( a \)                     | 55.1 ± 0.46 \( a \)                        |
|                     | +                                                  | #                                        | #                                           |
| Stem-Methanol       | −                                                  | 26.87 ± 0.23 \( a \)                     | 49.19 ± 0.61 \( a \)                       |

Phenol content (gallic acid equivalent, GAE) and flavonoid content (quercetin equivalent, QE) is expressed as mean ± SEM \( (n = 3) \); \(^{a-d}\) column-wise values with different superscripts of this type indicate significant difference \( (p < 0.001) \). \(^{a}\) between −PVPP and +PVPP for same solvent; \(^{b-d}\) for −PVPP for different solvents, \(^a\) between methanol and ethanol, \(^a\) between methanol and aqueous and between ethanol and aqueous. # post-PVPP values were not detectable by spectrophotometer at the concentration tested.
3.3. Root Extracts Have Better Antioxidant Activity Than Leaf and Stem Extracts

The percentage of scavenging activity of the root-ethanol extract in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide assays is less in absence of the PVPP treatment, while higher IC\textsubscript{50} values were obtained in the presence of PVPP in nitric oxide and total antioxidant assays (Figure 1 and Table 4). With the post-treatment of root extracts by PVPP, the aqueous extract was found to be 84%, 81% and 84% more potent in DPPH, hydrogen peroxide and total antioxidant assay, respectively. The methanol extracts of leaf and stem showed higher IC\textsubscript{50} in all the assays. Considering the absence of significant levels of antioxidant activities in the methanol extracts of leaves and stems, we continued with the extracts of root for further assays.

**Figure 1.** In vitro antioxidant assays (A) DPPH assay, (B) hydrogen peroxide scavenging assay, (C) nitric oxide scavenging assay, (D) total antioxidant assay of \textit{R. cordifolia} root without (i) and with (ii) polyvinylpolypyrrolidone (PVPP), where: blue—ethanol extract, red—methanol extract, dark green—aqueous extract, black—ascorbic acid and (iii) leaf and stem, where: green—leaf-methanol extract (−PVPP), orange—methanol extract (+PVPP), red—stem-methanol extract (−PVPP), blue—stem-methanol extract (+PVPP).

| Extract       | DPPH IC\textsubscript{50} (µg/mL) | Hydrogen Peroxide IC\textsubscript{50} (µg/mL) | Nitric Oxide IC\textsubscript{50} (µg/mL) | Total Antioxidant IC\textsubscript{50} (µg/mL) |
|---------------|----------------------------------|-----------------------------------------------|----------------------------------------|-----------------------------------------------|
| PVPP -        | 79.1 ± 1.92                      | 89.47 ± 0.79                                  | 74.5 ± 1.38                            | 97.71 ± 1.69                                  |
| PVPP +        | 89.47 ± 0.79                     | 74.5 ± 1.38                                   | 97.71 ± 1.69                           | 89.47 ± 0.79                                  |
| Root-Methanol | 79.1 ± 1.92                      | 94.53 ± 1.84                                  | 97.71 ± 1.69                           | 89.47 ± 0.79                                  |
| Leaf-Methanol | 79.1 ± 1.92                      | 94.53 ± 1.84                                  | 89.47 ± 0.79                           | 79.1 ± 1.92                                   |
| Stem-Methanol | 89.47 ± 0.79                     | 97.71 ± 1.69                                  | 89.47 ± 0.79                           | 97.71 ± 1.69                                  |
| Experiments | DPPH | Hydrogen Peroxide | Nitric Oxide | Total Antioxidant Capacity |
|-------------|------|------------------|--------------|----------------------------|
|              | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) |
| PVPP        | -    | +    | -    | +    | -    | +    |
| Root-Methanol | 79.1 ± 1.92 ** | 89.47 ± 0.79 | 74.5 ± 1.38 *** | 97.71 ± 1.69 | 94.53 ± 1.84 ** | 78.46 ± 0.7 | 88.62 ± 1.05 ** | 97.52 ± 0.88 |
| Root-Ethanol | 85.5 ± 2.68 ** | 98.26 ± 0.73 | 61.2 ± 2.12 *** | 101.14 ± 1.52 | 95.11 ± 0.74 ** | 82.17 ± 0.51 | 101.15 ± 1.77 ** | 85.92 ± 0.74 |
| Root-Aqueous | 99.97 ± 2.09 ** | 85.53 ± 1.01 | 92.97 ± 2.31 ** | 80.85 ± 1.89 | 85.49 ± 0.82 | 84.23 ± 0.75 | 71.86 ± 0.3 ** | 85.14 ± 0.81 |
| Leaf-Methanol | 115.76 ± 0.85 * | 84.63 ± 0.03 | 96.35 ± 1.62 ** | 146.98 ± 7.13 | 126.86 ± 1.14 | 118.99 ± 2.16 | 91.84 ± 4.24 * | 117.95 ± 0.58 |
| Stem-Methanol | 153.12 ± 1.19 | 112.75 ± 0.09 | 109.02 ± 1.62 | 138.41 ± 0.69 | 111.16 ± 1.36 | 86.17 ± 0.53 | 134.83 ± 2.05 | 103.91 ± 0.78 |
| Ascorbic Acid | 159.34 ± 3.41 *** | 100.42 ± 1.25 | 64.49 ± 0.51 * | 99.12 ± 2.7 | 100.01 ± 0.6 | 86.35 ± 0.39 | 104.26 ± 0.62 * | 100.29 ± 1.4 |

Results were expressed as the mean ± SD of three independent experiments, test of significance among PVPP untreated and treated extracts by ANOVA, wherein * , ** and *** represent statistical significance of p < 0.05, p < 0.01 and p < 0.001, respectively.

3.4. Principal Component Analysis of R. cordifolia Phenol, Flavonoid and Antioxidant Levels in PVPP Untreated and Treated Extracts

The data obtained by the quantification of the phenols and flavonoids with antioxidant levels of R. cordifolia among PVPP-untreated and -treated extracts have been used to perform a principal components analysis (PCA) (Table 5).

| Experiments | PVPP | Phenol Content | Flavonoid Content | DPPH Free Radical Scavenging Activity | H₂O₂ Scavenging Activity | NO Scavenging Activity | Total Antioxidant Capacity |
|-------------|------|----------------|------------------|--------------------------------------|--------------------------|------------------------|---------------------------|
|              | mg GAE/g of Plant Extract | mg QE/g of Plant Extract | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) | IC₅₀ (µg/mL) |
| Root-Methanol | -    | 43.34 ± 0.27 | 369.69 ± 1.49 | 79.1 ± 1.92 | 74.5 ± 1.38 | 94.53 ± 1.84 | 88.62 ± 1.05 |
| Root-Ethanol | +    | 6.59 ± 0.73 | 55.28 ± 2.7 | 89.47 ± 0.79 | 97.71 ± 1.69 | 78.46 ± 0.7 | 97.52 ± 0.88 |
| Root-Aqueous | -    | 74.31 ± 0.16 | 334.9 ± 1.8 | 88.49 ± 2.68 | 61.2 ± 2.12 | 95.11 ± 0.74 | 101.15 ± 1.77 |
| Leaf-Methanol | +    | 5.46 ± 0.25 | 49.64 ± 3.11 | 98.26 ± 0.73 | 101.14 ± 1.52 | 82.17 ± 0.51 | 85.92 ± 0.74 |
| Stem-Methanol | -    | 67.14 ± 0.11 | 177.05 ± 3.6 | 99.976 ± 2.01 | 92.97 ± 2.31 | 85.49 ± 0.82 | 71.86 ± 0.3 |
| Ascorbic Acid | +    | 68.00 ± 0.25 | 37.08 ± 1.54 | 85.53 ± 1.01 | 80.85 ± 1.89 | 84.23 ± 0.75 | 85.14 ± 0.81 |

* post-PVPP values were not detectable by spectrophotometer at the concentration tested. NA—Not Applicable.
As shown in Table 6, the first principal component was highly correlated with flavonoid content and antioxidant levels by H$_2$O$_2$ scavenging activity (Antioxidant$_{H_2O_2}$) variables, while the second principal component was highly correlated with antioxidant levels by NO scavenging activity (Antioxidant$_{NO}$) variable (highest score coefficients in absolute value).

| Variables | 1st Principal Component (PC1) | 2nd Principal Component (PC2) |
|-----------|-------------------------------|-------------------------------|
| Phenol content | -0.779 | 0.517 |
| Flavonoid content | -0.812 | 0.447 |
| Antioxidant$_{DPPH}$ Assay | 0.706 | 0.435 |
| Antioxidant$_{H_2O_2}$ scavenging activity | 0.817 | -0.276 |
| Antioxidant$_{NO}$ scavenging activity | 0.430 | 0.692 |
| Antioxidant_Total antioxidant capacity | 0.735 | 0.526 |

The first and the second principal components explain together 77.44% of the total observed variance, which is a considerable value. The PCA showed a clear separation between the -PVPP and +PVPP data (Figure 2), as better evidenced by the dotted line added in the plot.

Negative PC1 values correlated to the flavonoid content and Antioxidant$_{H_2O_2}$ variables were mostly associated with the root samples (blue symbols). On the other hand, PVPP-untreated (empty symbols) and -treated (solid plain symbols) roots were markedly separated by the dotted line, indicating that the flavonoid content, H$_2$O$_2$ and NO antioxidant activities are different. The stem samples (green symbols) have different PC2 values correlated to Antioxidant$_{NO}$ scavenging activity, and leaf samples (red symbols) have different PC1 and PC2 values since they are separated by the dotted line.

![Figure 2. Principal components analysis (PCA) of R. cordifolia L antioxidant activity in PVPP untreated (–PVPP, empty symbols) and treated (+PVPP, solid plain symbols) extracts. The different samples and extractions conditions have been indicated with different shape and color symbols: methanol = circle; ethanol = triangle; aqueous = square; root = blue; leaf = red and stem = green.](image-url)
The methanol extracts (circle symbols) have mostly positive PC1 values but different PC2 values, indicating a difference in the Antioxidant_NO activity related to the PVPP treatment. The aqueous extracts (square symbols) have negative PC1 and similar PC2 values, indicating a similar Antioxidant_NO activity independent from the PVPP treatment. The ethanol extracts (triangle symbols) have both PC1 and PC2 values, indicating different flavonoid content, Antioxidant_H$_2$O$_2$ and NO activities related to the PVPP treatment.

3.5. Plant Extracts Are Cytotoxic to Cancer Cells

Cancer cell lines ME-180, HeLa and HepG2 were exposed to various concentrations of extracts and standard drug (5-Flurouracil) to determine the cell viability by MTT cell proliferation assay. HeLa and HepG2 cells were susceptible to any of the extracts at similar concentrations (Figures 3 and 4). The root-methanol extract was more potent than other extracts for HeLa (IC$_{50}$ of 0.29 ± 0.23 mg/mL) and HepG2 (IC$_{50}$ of 0.39 ± 0.26 mg/mL) (Table 7). 5-Flurouracil (5-FU) was most toxic to HepG2 cells (IC$_{50}$ of 1.51 ± 0.38µM), and the levels of toxicity were significantly lower than those in the other cell lines evaluated.

Figure 3. Comparative cell viability assay on three cell lines: HeLa (blue intermittent line with square marker), ME-180 (green continuous line with triangle marker) and HepG2 (red dotted line with diamond marker) using *R. cordifolia* extracts (A) methanol extract, (B) ethanol extract, (C) aqueous extract and (D) 5-Flurouracil. The cell viability is relative to the vehicle control (cells treated with solvent in equivalent amounts of respective extract). Results were expressed as the mean ± SD of three independent experiments.

|          | Methanol Extract (mg/mL) | Ethanol Extract (mg/mL) | Aqueous Extract (mg/mL) | 5-FU (µM)  |
|----------|--------------------------|-------------------------|-------------------------|------------|
| HeLa     | 0.29 ± 0.23              | 1.41 ± 0.37             | 0.51 ± 0.34              | 34.73 ± 10.02 |
| ME-180   | 1.68 ± 0.39              |                        |                         |            |
| HepG2    | 0.38 ± 0.26              | 0.45 ± 0.07             | 0.57 ± 0.31              | 1.51 ± 0.38µM|

For all the experiments, $n = 3$. $a$–$d$ Column-wise values with different superscripts of this type indicate significant difference as determined by Student–Newman–Keuls method ($p < 0.05$); $a$ between 5-FU and methanol extract for same solvent; $b$–$d$ for–PVPP for different solvents; $b$ between 5-FU and aqueous; $c$ between methanol and ethanol $d$ and between 5-FU and ethanol.
Figure 4. Comparison among the IC$_{50}$ of HeLa (blue), ME-180 (green) and HepG2 (red) cells upon treatment with *R. cordifolia* root extracts of methanol, ethanol, aqueous extract and 5-Flurouracil (5-FU). Results were expressed as the mean ± SD of three independent experiments, test of significance by ANOVA, wherein * and *** represent statistical significance of $p < 0.05$ and $p < 0.001$, respectively.

Table 7. IC$_{50}$ of *R. cordifolia* extracts on viability of HeLa, ME-180 and HepG2 cells.

|         | Methanol Extract (mg/mL) | Ethanol Extract (mg/mL) | Aqueous Extract (mg/mL) | 5-FU (µM) |
|---------|--------------------------|-------------------------|-------------------------|-----------|
| HeLa    | 0.29 ± 0.23$^{a,c}$      | 1.41 ± 0.37             | 0.51 ± 0.34$^{b}$       | 34.73 ± 10.02 |
| ME-180  | 1.68 ± 0.39$^{a}$        | 2.37 ± 0.96$^{d}$       | 1.78 ± 0.55$^{b}$       | 13.68 ± 2.04 |
| HepG2   | 0.38 ± 0.26              | 0.45 ± 0.07             | 0.57 ± 0.31             | 1.51 ± 0.38 |

For all the experiments, $n = 3$.$^{a-d}$ Column-wise values with different superscripts of this type indicate significant difference as determined by Student–Newman–Keuls method ($p < 0.05$);$^{a}$ between 5FU and methanol extract for same solvent; $^{b-d}$ for -PVPP for different solvents; $^{b}$ between 5-FU and aqueous; $^{c}$ between methanol and ethanol $^{d}$ and between 5-FU and ethanol.

3.6. UPLC-UV-MS Phytochemical Profiling of Methanol Extract of *R. cordifolia*

To identify the compounds responsible for anti-proliferative potential, the composition of the root methanol extract was evaluated by UPLC-UV-MS analysis. A number of secondary metabolites were detected (Supplementary Table S1). Out of them, the structures of two of the signature compounds from *R. cordifolia* L. were used to compare with the existing PubChem database (Figures 5 and 6). In addition, the formula, score, mass and CAS numbers with retention time are listed in Table 8.
The utility of secondary metabolites for human health has achieved high recognition owing to their promising usage in traditional knowledge-based medication for centuries. *Rubia cordifolia* L. produces a range of secondary metabolites that have been evaluated for various illnesses. In the present study, we have evaluated three solvent systems for roots and methanol as a solvent for stems and leaves to extract secondary metabolites from *R. cordifolia* L. The suitability of methanol extracts in antioxidant assays prompted us to evaluate methanol extracts of stems and leaves for phytochemical analysis. Nevertheless, the antioxidant levels of roots were noticed to be higher than stems and leaves.

**Table 8.** UPLC-UV-MS identification of major compounds from *Rubia cordifolia* L. root extract prepared in methanol. ID source: DBSearch.

| No. | Name               | Formula | Score | Mass    | CAS            | RT   |
|-----|--------------------|---------|-------|---------|----------------|------|
| 1   | Pseudopurpurin     | C_{15}H_{16}O_{7} | 97.4  | 300.0275 | 476-41-5       | 9.442 |
| 2   | Morindaparvin A    | C_{13}H_{14}O_{4} | 84.38 | 252.0421 | 41621-32-3     | 10.821 |

**4. Discussion**

The utility of secondary metabolites for human health has achieved high recognition owing to their promising usage in traditional knowledge-based medication for centuries. *R. cordifolia* L. The suitability of methanol extracts in antioxidant assays prompted us to evaluate methanol extracts of stems and leaves for phytochemical analysis. Nevertheless, the antioxidant levels of roots were noticed to be higher than stems and leaves.
Phenols are a major antioxidant group present in plants. We detected significant amounts of phenols in the ethanol extract of root, followed by the methanol extract of leaf. Flavonoids are the largest group of natural phenolics that possess tremendous free radical scavenging properties and, hence, antioxidant potential. Our method of Soxhlet extraction led to an increased release of phenols and flavonoids.

The presence of antioxidants in the extract is crucial for usage as an anti-proliferative agent. The results of the DPPH assay for the ethanol extract of root reported by Zhang et al. [25] were in the range of 23.88 to 65.23 µg/mL. They used an ultrasonic-assisted extraction process. These values are much lower than the presently reported values in the range of 88.5 to 98.26 µg/mL. We believe the suitability of the extraction method and the mother plant selection are the drivers of differential results. Basu and Hazra [26] reported a range of 153.7 to 310.3 µg/mL for methanol and aqueous extracts of root as evaluated by a nitric oxide assay. They used the filtrate of the directly solubilized extracts in the respective solvents. Our results have a different range, possibly due to our choice of method of the Soxhlet exhaustive extraction process.

The antioxidant activity of the plant extract is attributed to various secondary metabolites, including polyphenols. Studies pertaining to the significance of polyphenols have emphasized their influence on the antioxidant results [27–29]. We propose to present the case that the antioxidant activity observed in R. cordifolia L. is not entirely due to polyphenols. To prove that the determined antioxidant activity is not exclusive to the polyphenols present in the extract and is contributed to by other secondary metabolites as well, we quenched the polyphenols using PVPP. Rantunge et al., 2017 have demonstrated the quenching effect of PVPP on different polyphenols, and it clearly shows remarkable differences [17]. The precipitation allows the removal of any complex of PVPP-polyphenols. A comparison of the PVPP-untreated and -treated extracts by the same antioxidant assays proved that there are other compounds responsible for antioxidant properties as well. We are reporting for the first time the results pertaining to R. cordifolia root extracts (ethanol, methanol and aqueous) treated with PVPP for antioxidant assays. Even after the removal of phenols and flavonoids, the antioxidant activity of the extract is not hampered. This suggests the involvement of other non-phenolic secondary metabolites in bringing about the antioxidant potential. The PCA correlated the phenol, flavonoid and antioxidant levels, as evaluated by hydrogen peroxide and nitric oxide scavenging assays. Our evaluation of R. cordifolia leaves and stems demonstrates that the root is more suited to be used for antioxidant properties. The high prevalence of antioxidant compounds in root extracts may be utilized for the anti-proliferative process in certain cancers [30,31]. The anti-proliferative assay corroborated the suitability of the methanol extract of the root for anti-cancer activity. The sensitivity of HepG2 towards 5-Flurouracil as compared to other cell lines was not reflected for plant extracts as similar toxicity was observed in the ME180 and HeLa for cell lines, suggesting its usage for the management of multiple cancers. The cytotoxicity may be mediated by reactive oxygen species, as indicated in laryngeal squamous cell carcinoma HeP-2 cells [7]. However, the apoptotic pathway responsible for cell toxicity needs further elucidation. The isolation of suitable cancer-specific bioactive compounds is necessary, or else it may yield a negative result [10].

Our results of UPLC-UV-MS identified some previously reported compounds and some new compounds from Rubia plants. Pseudopurpurin (anthraquinone) is a characteristic natural red-color compound present in the roots of R. cordifolia and Rubiaceae family members. It is a derivative of purpurin (pseudopurpurin is purpurin 3-carboxylic acid). It improves bone geometry [32] and selectively exhibits tumor inhibitory potential [33]. Morindaparvin A is reported to be an antileukemic anthraquinone and is chemically derived from alizarin (1,2-methylenedioxyanthraquinone by synthesis from alizarin) [34]. We report its presence in R. cordifolia for the first time. It is possible that its presence was not detected previously or was not considered as it is a derivative of alizarin. These preliminary findings require a detailed supplemental study for verification before confirmation.
The presence of multiple compounds in the methanol extract that are established to be cytotoxic to cancer cells supports our results. However, the validation of cytotoxic activities requires independent assays.

5. Conclusions

*R. cordifolia* L. is a widely used plant for its significant medicinal value. This is attributed to the presence of unique secondary metabolites in *R. cordifolia* L. Exhaustive methods of extraction lead to an increase in the retrieval of secondary metabolites, as observed in our research endeavor. This work provides the initial steps required in selecting the suitable solvents for *R. cordifolia* extract preparations. Our study has revealed the presence of a high quantity of antioxidants in the root, stem and leaf extracts of *R. cordifolia*. The antioxidant levels in the root, stem and leaf provide a comparative benchmark for further exploration. The results obtained for the antiproliferative assay make the extracts valuable to medicinal practitioners. Identification of different compounds may help in determining a metabolite signature characteristic of *R. cordifolia*. The individual compounds need to be evaluated to verify the extent of the utility of the antioxidant nature for identifying a potential anti-cancer agent. In summary, the medicinal value imparted by the extracts is comprehensively documented for its usage in anti-cancer research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11051006/s1. The following supporting information: Table S1. UPLC-UV-MS identification of possible compounds from *Rubia cordifolia* L. root extract prepared in methanol. ID source: DBSearch.

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Abbreviations

DPPH 2,2-diphenyl-1-picrylhydrazyl
PVPP Polyvinylpolypyrrolidone
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