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Antioxidant, Anticancer and Antimicrobial, Effects of *Rubia cordifolia* Aqueous Root Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PSN and ROA jointly designed the study and wrote the protocol. Authors RB, DB and AC performed all experiments and data analysis. Author RB wrote the 1st draft. All authors read and approved the final manuscript

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Short Research Article

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ABSTRACT

Aims: To evaluate the total antioxidant capacity (TAC) of *Rubia cordifolia* root extracts, to test anticancer activity against MDA-MB-231 breast cancer cell lines, and to evaluate antimicrobial activity of the same extract versus six Gram-positive and negative bacteria.

Study Design: *In vitro*.

Place of Study and Duration: School of Biomedical Sciences, Ulster University, July 2014-Sept 2015.

Methodology: TAC was tested using ABTS, DPPH, FRAP and Folin assays and values were expressed as mg-gallic acid equivalents per 100 g (GAE/100 g) of sample. Anticancer properties were examined against MDA-MB-231 breast cancer cell lines using Sulforhodamine B assay. Antimicrobial activity was examined using a disk diffusion assay with three Gram-positive (*Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus cereus*) and three Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*) bacteria.

Results: TAC of dry extracts of *Rubia cordifolia* ranged from 523±43 to 4513±208 (mg GAE mg/100 g) depending on the method of analysis, ABTS> FRAP> Folin > DPPH methods.

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R. cordifolia dry extract showed cytotoxicity against MDA-MB-231 with $IC_{50} = 44 \mu\text{g/ml}$ or $5.1 \mu\text{M}$ GAE. No antimicrobial activity was observed against the three Gram-positive, or three Gram-negative bacterial species using the water extract or *R. cordifolia*.

Conclusion: *R. cordifolia* aqueous extract possess high total antioxidant capacity but values depend on the method of analysis. *R. cordifolia* extract inhibits MDA-MB-231 breast cancer cells proliferation but nil anti-bacterial activity was observed for three Gram-positive and three Gram-negative bacterial strains tested.

Keywords: *Manjistha*; *Rubia cordifolia*; antioxidant; antimicrobial; MDA-MB-231; breast cancer; ABTS; DPPH; FRAP.

1. INTRODUCTION

It is a focus of the biotechnology industry to find solutions for hard to treat conditions like cancer and multiple-drug resistant bacteria. Cancer drugs include alkylating agents, platinum compounds, and antimetabolites many of which can cause cytotoxic damage to patients. There is a need for treatments, which are less harmful to the patient, or are beneficial when used in combination with established drugs [1]. The World Health Organisation (WHO) branded antibiotic resistance a major problem from 1994 owing to a lack of new classes of antibiotics [2].

Rubia cordifolia (Manjistha, Indian madder) is a plant within the *Rubiaceae* coffee family found in the lower Himalayas, India, Japan, Indonesia and Sri Lanka [3]. Originally used as a red pigment, *R. cordifolia* is also used in Ayurvedic medicine to treat jaundice, joint inflammation, and cough [3]. *R. cordifolia* is gaining popularity in western culture as alternative therapy for skin conditions such as eczema, psoriasis and dermatitis [3]. Past investigations demonstrated that *R. cordifolia* has significant antioxidant effects *in-vivo* [4-5] and *in-vitro* [6-8]. Preliminary screening studies identified *R. cordifolia* as a promising inhibitory agent breast cancer cell proliferation [9,10]. The aims of this investigation were to evaluate the antioxidant, anticancer and antimicrobial activity of aqueous extracts from *R. cordifolia* within a single study.

2. MATERIALS AND METHODS

2.1 Preparation of the *R. cordifolia* Extracts

R. cordifolia root powder (2.5 or 5 g) was stirred with 95ml of distilled water and the mixture centrifuged at 11,000 rpm for 20 minutes. The resulting supernatant was analysed as "whole

powder extract". To produce freeze-dried extract, the supernatant was frozen at -80°C overnight and then subjected to freeze drying for 72 hours. The freeze-dried powder was stored in a dry state at room temperature until use.

2.2 Determination of the Total Antioxidant Capacity

2.2.1 Total antioxidant capacity by the FRAP method

The Ferric Reducing Ability of Plasma (FRAP) assay was used to calculate the antioxidant power of the sample as previously described by Benzie and Strain [11] and further modified by [12]. Gallic acid solutions (1000, 500, 250, 125, 62.5 and 0 μM) were used as calibration standards. *R. cordifolia* extract or different concentration of gallic acid (75 μl) and 1425 μl of FRAP solution were added to microcentrifuge tubes and the mixture stored in the dark at 37°C for 30 minutes. Thereafter, 200 μl of solutions were transferred to a 96-well microplates and absorbance was then recorded at 593 nm using a microplate reader (VERSAmax; Molecular devices, Sunnydale, California, USA).

2.2.2 Total phenols by the Folin method

The Folin method was used to determine total phenols as described by Singleton et al. [13]. Gallic acid (3000 μM , 1500 μM , 750 μM , 375 μM , 187.5 μM , 0 M) solutions were used as reference antioxidant. First 50 μl of *R. cordifolia* extract or gallic acid was added to microcentrifuge tubes followed with 100 μl of Folin Reagent and 850 μl of sodium carbonate (3.5%) solution. The mixture was stirred and incubated at 20°C for 20 minutes then 200 μl of solutions were transferred to a 96-well microplates and absorbance was then recorded at 760nm using a microplate reader as above.

2.2.3 Total antioxidant capacity by the DPPH method

DPPH was used to measure the antioxidant capacity of *R. cordifolia* as previously described by Thaipong et al. [14]. DPPH (24 mg / 100 ml methanol) was stored at -20°C until required. For a working solution 10 ml of stock solution was added to 45 ml of methanol and initial absorbance was adjusted to 0.7. Gallic Acid (1000, 500, 250, 125, 62.5 and 0 µM) was used as standard; 75 µl of each concentration of gallic acid was added to an Eppendorf followed by 1425µl of the DPPH working solution and the mixture was stored in the dark at 37°C for 30 minutes. Thereafter, 200 µl of solutions were transferred to a 96-well microplates and absorbance was then recorded at 515 nm using a micro plate reader as above.

2.2.4 Total antioxidant capacity by the ABTS method

The modification ABTS method described by Walker et al. [15] was used. ABTS stock solution (5.00 x 10⁻⁴ M) was created by adding 27.4 mg of ABTS to 90 ml of Phosphate Buffered Saline (PBS buffer). Sodium persulfate stock solution (7.4 x 10⁻³ M) was created by dissolving 2 mg sodium persulfate in 10 ml of PBS buffer. ABTS working solution was created by adding 90ml of ABTS stock solution to 10 ml of sodium persulfate stock solution and storing in the dark for 16 hours. The initial ABTS solution was adjusted to give an absorbance value of 0.85 at 734 nm using 1 cm cuvette. The ABTS assays was calibrated using gallic acid (1000, 500, 250, 125, 62.5 and 0 µM) as reference. *R. cordifolia* extract or gallic acid (20 µl) was added to an Eppendorf followed by 1480 µl of the ABTS working solution and the mixture was stored in the dark at 37°C for 30 minutes. Thereafter 6x 200 µl of mixtures were transferred to microcentrifuge tubes and absorbance recorded at 734 nm using a micro plate reader.

2.2.5 Total antioxidant capacity calculation

The total antioxidant capacity (TAC) for *R. cordifolia* extracts determined by different methods (section 2.2.1-2.2.4) were expressed as milligram gallic acid equivalents per 100 g dry weight (mg-GAE/100 g) using the relation;

$$\text{TAC (mg-GAE/ 100 g)} = \frac{\text{Abs}}{\epsilon'} * Av * \left(\frac{V_{ex}}{Sv}\right) * DF * FwW * 105$$

where, ϵ' (abs/M) = slope from the calibration graph, Av = Assay volume, 1500 (10⁻⁶litre), Sv = sample sip volume assayed, 75 (10⁻⁶ l), D_F = Dilution factor for sample before antioxidant assay (DF= 1 if undiluted), V_{ex} = original volume of sample extract 0.02 (l), F_w = Formula weight of gallic acid; g/mole), W = dry weight *R. cordifolia* sample (g).

2.3 Determination of Anticancer Activity Using Sulforhodamine B Assay

MDA-MB-231 cells (American Type Cell Culture (ATCC) Middlesex UK) were grown in 75 cm³ flasks till ~70-80% confluent, rinsed three times using sterile PBS and trypsinized for 5 minutes at 37°C to detach cells from the flask. Cells were transferred to microplate (10,000 cells/50 µl/well) and incubated for 24 hours at 37°C to allow adherence. Prior to cytotoxicity testing, 20 mg *Rubia cordifolia* extract was dissolved in 4 ml DMEM media and passed through a 0.2 micron syringe filter. Sterile *R. cordifolia* extract was serially diluted (5, 1, 0.2, 0.04, 0.008 mg/ml) and 50 µl added to cells followed by incubation at 37°C for 72 hours. The negative control involved adding 50µl of DMEM to cells. Tests were repeated on three separate occasions with 6 treatments at each concentration.

Sulforhodamine (SRB) was performed according to the method described in [16], and absorbance was read at 525 nm. Cell viability (CV%) was calculated from the expression CV(%) = (100*[(A-B)/(A₀ -B)]) where A=absorbance of cells treated with extract, A₀ = absence from cells treated with vehicle/ media, B= absorbance readings for blank microplate wells lacking cells or vehicle treatment. The 50% inhibitory concentration for extract (IC50) was determined by linear regression of CV(%) plotted versus log. DrugConc (Y = mx + C). From graphing CV(%) = m. (logDrugConc) + C, then log (IC50) = (50 - C)/ m. In addition IC50 (µg/ml) was translated to total phenols equivalent (mol-GAE / l) using the relation: IC50 (mol-GAE / l) = IC50(g/l)* Total-phenols (mgGAE/g) *(1/170.12).

2.4 Determination of the Antimicrobial Activity by Disk Diffusion

The disk diffusion method was performed as outlined by Fiebelkorn et al. [17] with slight modifications. Three Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus cereus*) and three Gram-negative (*Escherichia coli*,

Pseudomonas aeruginosa and *Salmonella typhi*) bacteria were grown on agar plates and confirmed by Gram staining. A few colonies were removed from the agar plate using a sterilised loop and inoculated into 15 ml nutrient broth and incubated at 37°C for 24 hours to allow growth. After 24 hours 200 µl of the inoculated broth was pipetted onto an agar plate and spread evenly using a sterile spreader and briefly allowed to dry. Antibiotic disks (streptomycin, penicillin G and amoxicillin used as positive controls) and two blank paper disks were added to each agar plate. *R. cordifolia* dry extract solubilised in PBS was added to one of the blank disks in concentrations from 62.5 µg per disk to 1000 µg per disk to determine minimum inhibitory Concentration. 20 µl of PBS was added to the final blank disk as a negative control. Disks were added the agar plates followed by incubation at 37°C for 24 hours. The diameters of the zones of inhibition in mm were measured using a ruler. This procedure was carried out under sterile conditions via Bunsen burner or sterilised laminar flow cabinet.

2.5 Statistical Analysis

All methods were conducted in triplicate and the average of these results is displayed in the relevant figures/tables with ± Standard Deviation (SD). To assess the statistical significance between the whole plant powder and dry extract values the independent t-test and Mann-Whitney-U test was performed. To compare the statistical significance of the means between each concentration used in the Sulforhodamine B assay to that of the negative control, the One-Way ANOVA test was used. For these tests an alpha value of ($P=0.05$) was considered significant. IBM SPSS statistics software, version 22, was used to conduct the statistical analysis.

3. RESULTS AND DISCUSSION

3.1 Total Antioxidant Capacity

Aqueous extracts from *R. cordifolia* 2.5-5 g of powder per 100 g of solvent were prepared with a yield of 13% w/v. From Table 1 the total phenols content for the freshly prepared and freeze dried water extract from *R. cordifolia* were 0.23% (w/w) and 1.95% (w/w) by comparison with 2.11 (w/w) reported previously for a dried extract [6-7]. The total phenol values for water extracts from *R. cordifolia* were lower than some total phenols values for reported for common teas and similar to others [12]; green tea (11.9%

w/w), black tea (9.2% w/w), white tea (8.5% w/w), rooibos tea (2.8% w/w) and apple tea (1.76% w/w). Previous investigations suggested that the TAC of *R. cordifolia* root extracts were determined by hydroxyanthraquinones [3,7].

The total phenols values for *R. cordifolia* produced with a variety of solvents are shown in Fig. 1. Dimethyl sulfoxide (DMSO) was an efficient extracting agent and isopropanol, ethyl acetate and cyclohexane were less effective. Currently, it is not certain whether the composition of extracts produced using different solvents are similar or otherwise. In this preliminary study, the focus was on samples produced using water extraction, either freshly prepared or freeze dried.

3.2 Anticancer Properties

R. cordifolia water extract showed cytotoxicity towards breast cancer cell line MDA-MB-231 compared to a negative control treated with DMEM media (Figs. 2 and 3). From Fig. 2, the concentration of extract leading to 50% inhibition of cells (IC50) was 286 µg/ml and 43 µg/ml for 24 hrs or 72 hrs treatment with freshly prepared *R. cordifolia* extract. Therefore, *R. cordifolia* was more cytotoxic after 72 hrs treatment as compared to a 24 hr treatment. Close examination of Fig. 2 shows cell numbers had increased by 2-fold during 72 hr compared to 24hr treatment and *R. cordifolia* could reduce cell growth as well as causing cell death (reductions in cell numbers). From Fig. 3, the cytotoxicity of freeze dried *R. cordifolia* aqueous extracts yielded an IC50 value of 45 µg/ml, which is similar to results using freshly prepared water extracts and 72 hrs treatment (Fig. 2). In summary these 72 hr tests suggest an average IC50 value of 44 µg/ml for *R. cordifolia* extract; the corresponding total phenols concentration is 5.1×10^{-6} M GAE. The findings of the present study support previous *R. cordifolia* screening results for four human breast cancer cells lines [9,10] as well as other cell lines [17,18]. Campbell et al. [9] examined the effect of 71 Chinese medicinal herbs on four human breast cancer lines and identified *R. cordifolia* as one of the promising agents for future study also confirmed by Shoemaker et al. [10].

Previous screening studies did not report dose-response profiles or IC50 values for *R. cordifolia* applied to MDA-MB-231 cells [9,10]. Interestingly, the IC50 was 100 µg/ml for MCF-7 cells treated with *R. cordifolia* extract [9] and

486-1000 µg/ml for other herbal agents [10]. *R. cordifolia* root extracts prepared with methanol, petroleum ether or dichloromethane yielded IC₅₀ values of 12-29 µg/ml for Hep 2G cells and 23-49 µg/ml for Hela cells [17]. Anthraquinones from *R. cordifolia* are believed to be responsible for anticancer activity but IC₅₀ values seem not yet available. A major anthraquinones from rhubarb roots tested with MDA-MB-231 cells produced an IC₅₀ value of 16 µg/ml (60 µM) [18]. *R. cordifolia* extracts are thought to promote cell apoptosis via a caspase dependent route as well as causing cell cycle arrest [9,19].

3.3 Antimicrobial Activity

In this study, the antimicrobial activity of *R. cordifolia* aqueous freeze dried extract was analysed using the Kirby-Bauer disk diffusion test. Table 2 shows that *R. cordifolia* extract (62.5 µg – 1 mg per disk) did not produce a zone of clearance with any bacterial strain examined. PBS vehicle for *R. cordifolia* extract produced nil results. By contrast, streptomycin, amoxicillin and penicillin G used as positive controls showed antibacterial effects with streptomycin having a strong effect against each bacterial strain.

Table 1. Characteristics of antioxidant assays and the total antioxidant capacity for *R. cordifolia* aqueous extract

Assay ^a	LDR (µM)	ϵ (M cm ⁻¹)*	R ²	TAC ‡ (RCFE) ^b	TAC ‡ (RCFD)
FRAP	0-25	195014	0.9829	159±11	2508±95
DPPH	0-25	92886	0.999	63±4	523±43
Folin	0-75	25836	0.9987	235±59	1954±417
ABTS	0-20	50642	0.995	601±13	4513±208

^aResults from 3-experiments, n_>48 data points, RCFE = *R. cordifolia* fresh extract, RCFD = *R. cordifolia* freeze dried extract, *LDR = Range of concentrations or which calibration graph is straight, ϵ (M cm⁻¹) = molar absorptivity, R² = correlation coefficient. ‡Total antioxidant capacity (TAC) expressed as mg-GAE/100g dry weight), ^bWithin each column all TAC are significantly different at (P=0.05). *Calibration graph gradient corrected for microplate reader pathlength of 0.5 cm [12]

Table 2. Summary of the disk diffusion results

	Streptomycin (2.5 µg)	Amoxicillin (1 µg)	Penicillin G (1 µg)	RCGF (62.5 µg)	RCFD (1 mg)	PBS
<i>B. cereus</i>	24±1	13±0.4	6	6	6	6
<i>P. aeruginosa</i>	19±1.1	6	6	6	6	6
<i>S. typhi</i>	28±1	22±1.2	16±2.8	6	6	6
<i>E. coli</i>	24±0.8	13±0.8	6	6	6	6
<i>S. epidermidis</i>	23±1.4	6	6	6	6	6
<i>S. aureus</i>	20±1	12±0.8	6	6	6	6

Measurements are average zones of Inhibition from three replicates ± SD and include the size of the paper disk (6mm). RCFD = Freeze dried *R. cordifolia* extract, Phosphate Buffered Saline (PBS) was used as a negative control. Antibiotics were used as a positive control. Treatment time was 24 hours

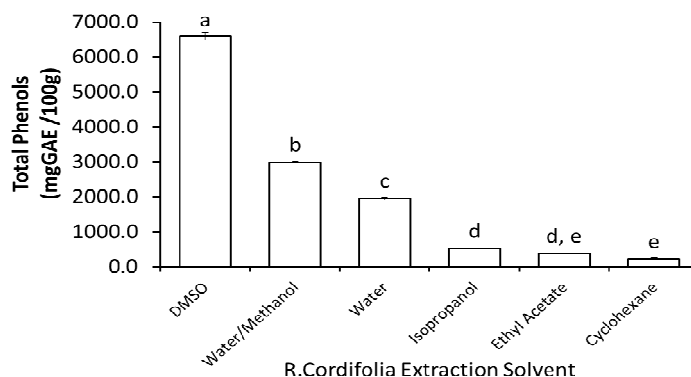


Fig. 1. Total phenol content for *R. cordifolia* extracts using different solvents
Antioxidant capacity expressed as mg-GAE per 100 g of dried extract

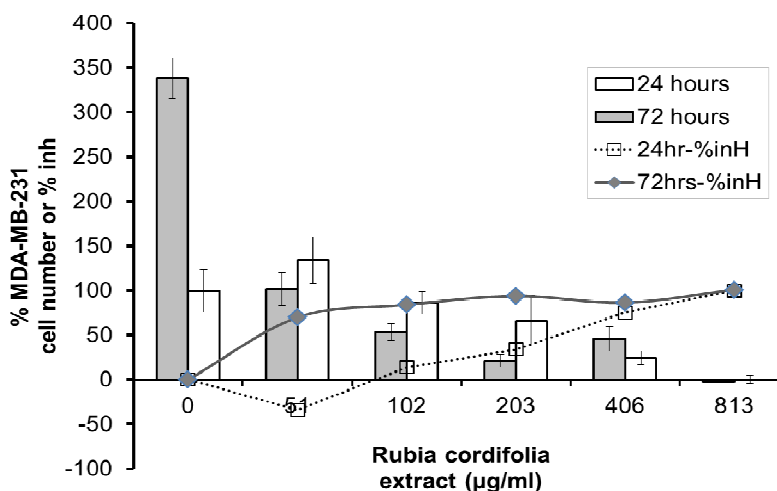


Fig. 2. Anticancer activity of fresh aqueous *R. cordifolia* root extract measured by the Sulforhodamine B assay

Anticancer activity evaluated using MDA-MB-231 breast cancer cells. Treatment time was 24 hrs or 72 hrs. Left Y-axis shows bar-chart for cell number (%) referenced at 24 hr results. Right Y-axis shows (line drawing) % inhibition (% inh) for 24 hr or 72 hrs. Mean results are for three experiments (n=18) for each treatment with \pm SEM. The 50% inhibitory concentration (IC50) occurs at 43 µg/ml (72 hr treatment) or 286 µg/ml (24 hr treatment)

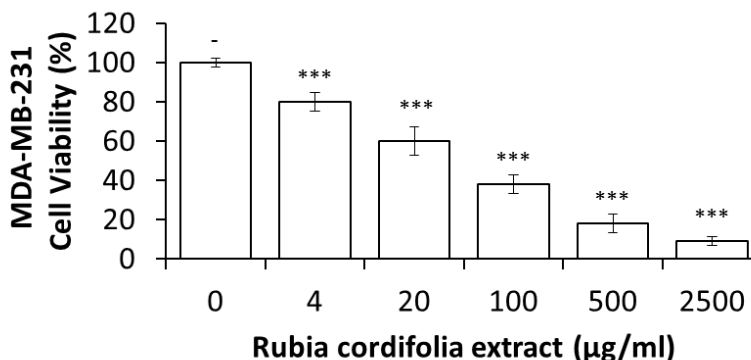


Fig. 3. Anticancer activity of freeze dried *R. cordifolia* root extract measured by the Sulforhodamine B assay

Anticancer activity evaluated using MDA-MB-231 breast cancer cells. Treatment time was 72 hours. Viability was determined by Sulforhodamine B staining. Data shows mean results from three experiments (n=18) for each treatment with \pm SEM. ***Show significant difference between treatments and vehicle control (P=0.05). The 50% inhibitory concentration (IC50) occurs at 45 µg/ml

Previous investigations by Basu et al. [20], found that methanol and chloroform extracts from *R. cordifolia* (1–10 mg/mL; 0.1-1 mg /disk) were effective against Gram-positive bacteria and inactive for Gram-negative strains, with exception of *P. aeruginosa*. In agreement with present findings, water extracts of *R. cordifolia* were inactive with most Gram-negative strains. The Gram-positive strains, *B. subtilis* and *S. aureus* were affected by water extract of *R. cordifolia* [20] but this result is not supported by this study.

The preliminary studies using extracts prepared with DMSO or isopropanol showed some activity for *P. aeruginosa* and *S. epidermis* (data not shown). It has been suggested that solvent choice has a large effect on the antimicrobial characteristic of *R. cordifolia* [20].

4. CONCLUSION

R. cordifolia aqueous extract shows significant antioxidant capacity as measured by four

common antioxidant assays but results depend on the assay used: ABTS > FRAP > Folin > DPPH method. Tests using MDA-MB-231 breast cancer cell line showed *R. cordifolia* produced a dose-dependent inhibition of cell proliferation with IC50 of 44 µg /ml compared with ~16 µg /ml reported for purified anthraquinones. However, the water extract from *R. cordifolia* showed no antimicrobial activity using a standardized disk diffusion assay at concentrations of 1 mg per disk and it may be concluded, that effective doses for *in-vitro* anticancer activity of *R. cordifolia* is lower compared to doses required for anti-microbial activity. Future studies are envisaged to explore the effect solvent choice on the characteristics of *R. cordifolia* extracts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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