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Study the Effect of *Rubia Cordifolia* Extract on Different Type of Cancer Cell Lines and Different Microbial

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Abstract

Rubia cordifolia (Manjistha, Indian madder) is a plant in the Rubiaceae coffee family distributed in the lower Himalayas, India, Indonesia, Sri Lanka and Japan. It was mainly used as a red pigment, R. Cordifolia can be used for the management of jaundice in Ayurvedic medicine., inflammation of the joints, and cough. R. Cordifolia is becoming increasingly popular in western culture as an alternative treatment for skin disease such as psoriasis, eczema and dermatitis. Past studies have also shown R. Cordifolia is a promising regulator of the spread of breast cancer cells.This research aims to find a solution to conditions such as cancer and multi-drug resistant bacteria that are difficult to treat and fungi by using *R.Cordifolia* aqueous root extract. Methods used in this inquiry to assess the antimicrobial and anticancer effects of low concentration *R. cordifolia* aqueous extracts were MTT assay on three cancer cell lines (HepG2, BxPC-3 and MCF-7) and the minimum inhibitory concentration MIC for antimicrobial susceptibility against six microorganisms,three are bacteria *(P.aeruginosa* (*Pseudomonas aeruginosa*),*E. coli* (*Escherichia coli*) and *B.subtilis* (Bacillus subtilis) and three are antibiotic resistant fungi (F. oxysporum, T. basicola and T. phaseolina). The findings indicate that R. Cordifolia In addition to its function as an antimicrobial and antifungal agent, it may have a potential use as an adjunct therapy to pancreatic, liver and breast cancer., as demonstrated in this study against *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *F. oxysporum, T. basicola* and *T. phaseolina* strains.

Keywords: *R. cordifolia*, MTT assay, MIC assay, cytotoxicity.

INTRODUCTION

Cancer is the most destructive illness and main cause leading to death in the world [1]. Natural drugs are under investigation for their selective cytotoxicity to cancer cells [2]. Cancer death rate has been dramatically increased. However, the strategy of anticancer therapy is progressing. Over the years, the burden has shifted to the developing countries, which currently account for about 57% of cases and 65% of cancer deaths worldwide [3]. People tend to use phytochemical compounds to kill cancer cell or/and to avoid the cytotoxicity effect of therapeutic approaches, such as multidrug resistance [4, 5]. According to the literature review of cytotoxic effect, many studies concern investigating herbal source compounds, which have potential antitumor activities and eliminate cancer [6, 7]. Medicinal herbs are a good source of synthetic medication. In addition, it is used as raw extracts in the supermarket, such as Holland and Barrett. Herbs have a crucial role to play in the prevention and treatment of cancer, bioactive substances derived from various medicinal plants and other therapeutic uses. In this race, phytochemical exploration of these herbs has led to some significant extent to the evolution of new anticancer drugs[8]. In recent years, people tend to use natural plant extracts for cancer treatment due to fear of side effects of chemotherapy. Previously, phytochemistry products are more popular and immense progress for example *Rubia cordifolia* [Rubiaceae] is known as Manjishtha, Indian madder which is found in Iraqi herbal market, it is a prickly climber with a stem, growing up to 12 m long. Leaves are highly variable, ovate lanceolate, occurring in whorls of 4-6. Flowers are fragrant, minute, whitish or greenish yellow. Fruit is minute, glabrous, dark purplish or blackish when mature. During August-October the plant carries flowers and fruit. Roots are perennial, long, cylindrical, and rusty brown in colour [9], and used as anti-inflammatory activity and anti-toxins role [10, 11], anti-cancer [12], antimicrobial and antifungal [13], hepatoprotective activity [14], anti-diabetic property [15]. Many studies have reported that *R. cordifolia* contains a variety of bioactive compounds such as anthraquinones and glycosides, iridoids, terpenes, saccharides and carboxylic acids, these compounds were isolated from different parts of *Rubia* [16, 17]. Additionally, *R. cordifolia* contains pseudoparpurins, alizarin, rubuadin, purpurin, lucidine, and manjisthin [18]. Furthermore, it contains bicyclic hexapeptides, which have a role anti-tumour activity. The root colouring feature of *R. cordifolia* is due to the presence of mixture of purpurin (trihydroxyanthraquinone) and manjistin [xanthopurpurin-2-carboxylic acid] [19, 20, 21]. This study revealed the potential role of *R.Cordifolia*

aqueous root extract on liver, pancreatic and breast cancer chemoprevention and also chemotherapy. However, there is no proof of the impact of R. Cordifolia aqueous root extract on these forms of cancer. This research first provides proof of cytotoxicity for R. Cordifolia aqueous root extract due to inhibition of cell proliferation in HepG2, BxPC-3 and MCF-7 cancer cells. In addition to research on the anticarcinogenic effect of R. Cordifolia aqueous root extract, this work included evaluating the antibacterial and antifungal activity of *R.Cordifolia* aqueous root extract toward six microorganisms of bacteria and fungi.

METHODS

Plant Material

The plant materials were obtained from Iraqi local vendors and authenticated. The roots part of *Rubia cordifolia* were thoroughly washed using tap water followed by sterile distilled water and sun dried for one week. They were then separately grinded into coarse powder using pestle and mortar.

Extraction of *R. cordifolia*

The coarse powders of roots *R. cordifolia* (20 g) were subjected to extraction in a Soxhlet apparatus using 100 ml of 70% ethanol at 60°C for 18 hrs. The extracted material was evaporated to dryness under reduced pressure at 45°C. The extract was stored in an airtight container at 40°C [22].

Cell Culture

Children liver hepatocellular carcinoma cell line (HepG2) and biopsy xenograft of pancreatic carcinoma line-3 (BxPC-3) were purchased from American Type Culture Collection ATCC (Middlesex, UK), human breast adenocarcinoma cell line (MCF-7) was gained from cell bank unite/ The Tissue Culture Research Centre (TCRC), College of Pharmacy, University of Al-Mustansiriyah. HepG2, BxPC-3 and MCF-7 Cell lines are being used as a pattern cancer cells for this study.

Cell Maintenance

HepG2 and BxPC-3 cells were preserved in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Merelbeke, Belgium) complemented by 0.5 per cent FBS (Fisher Science, USA) bovine fetal serum and 1 per cent L-Glutamine (Lonza, England) as well as 1 per cent Penicillin-Streptomycin-Amphotericin B 100X (Lonza, England) as antimicrobial. MCF-7 cells were kept in the DMEM medium (Lonza, UK) complemented by 0.5 per cent fetal bovine serum FBS and 1 per cent L-Glutamine (Lonza, England) as well as 1 per cent Penicillin-Streptomycin-Amphotericin B 100X (Lonza, England) as anti - microbial. Cells were grown in 75 cm2 flasks and incubated at 37 ° C in 5% CO2/95% humidified air. Once the cells had achieved a confluence of 90%, flasks usually contains HepG2, BxPC-3 or MCF-7 cells had passed under controlled circumstances. The cells were then washed with 5 ml of phosphate buffered saline solution (PBS) and incubated at 37 ° C for 2 min in trypsin solution to enable the cells to release from the bottom of the flask. Equal volume of full growth media was placed and the cell suspension was moved to a 50 ml conical tube. The cells were then centrifuged for 3min at 1200 rpm. The supernatant was removed, and the pellet of cell resuspended in freshly complemented growth media. Cells were then counted on a haemocytometer under a microscope and used as required[23].

Storage and Resuscitation of Cell Lines

After trypsinization of a confluent flask of 75 cm2, the cell suspension was centrifuged at 1200 rpm for 3 min. The cell pellet was then resuspended in 4 ml of freezing medium (Life Technologies) and 1 ml of aliquot was applied to the cryoviva (Thermo Fisher Scientific, Loughborough, UK). The cells were stored at-80 ° C for 24 hours and stored under liquid nitrogen for long-term storage. Cells stored under liquid nitrogen were rapidly detached at 37 ° C and added to 10 ml of fresh growth media.Cells were extracted by centrifugation and resuspended in 25 ml of fresh medium and transferred to a 75 cm2 flask.

Cell Viability and Inhibitory Concentration (IC50) by MTT method

The MTT assay was used to determine the impact of R. Cordifolia aqueous root extract on the viability of cancer cells. A 100 µl of all cell suspensions (HepG2, BxPC-3 and MCF-7) were administered to 96-well flat-bottom tissue culture plates (Falcon, USA) at concentrations of 5 x 103 cells per well and incubated 24 hours under normal conditions; 4 x 103 cells / well at 48h incubation and 3 x 103 cells / well at 72h incubation.. Cells were handled with R (0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM) after 24h. Extract cordifolia. After a recovery time of 24h, 48h and 72h, the cell culture medium was removed and the culture medium containing 30 µl of MTT solution (3 mg / ml MTT in PBS) (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) was incubated for 4h at 37 ° C.. After 4h, this medium was eliminated by a gentle inversion and tapped onto paper. Control wells got just 100 µl of growth media. Added 100 µl of dimethyl sulfoxide (DMSO) to each well, the plates were then held at room temperature in the dark for around 15-20 min. The absorbance of each well was measured by a multiscan reader at a wavelength of 540 nm and corrected by a wavelength of 650 nm for background absorbance. The viability of the cells was calculated by the optical density (OD) of the wells which contained no R. Extract cordifolia. The 50 percent inhibitory concentration (IC50) was identified as the minimum concentration of R. Cordifolia extract that reduced the viability of the incubated cells by 50% after 72 h[24, 25].

Antimicrobial resistance research in vitro

Preparation of the microorganism

Six phytopathogenic microorganisms were selected to screen antimicrobial activity against the selected *R. cordifolia* extract, of these six microorganisms, three are bacteria (*P.aeruginosa (Pseudomonas aeruginosa), E. coli (Escherichia coli)* and *B.subtilis were (Bacillus subtilis))* and three are fungi *(F. oxysporum, T. basicola and T. phaseolina).* All the bacteria microorganisms tested were collected from microbiology laboratory in Al-Yarmouk Teaching Hospital, Iraq while the fungi were collected from microorganism's bank in the biological resource centre (IBRC), Iran. All the pure cultures obtained in lyophilized or freeze-dried form are reconstituted in sterile water and produce a suspension of the microbial cells. Inoculation was done with a sterile inoculating loop to liquid broth medium. Liquid cultures are then incubated to allow cell replication and adequate growth of the culture. Following incubation, liquid cultures are refrigerated to store for further use. Typically, 24h provided sufficient growth to allow visibly thick spread of the microbes for bioassay. The bacterial strains are

maintained and tested on Nutrient agar (NA) and Potato Dextrose agar (PDA) for fungi.

Minimum inhibitory concentration MIC

The MIC was calculated by a ratio of 1:2 serial dilution in a microtiter plate assay of 96 microtiter plate wells[26]. This test was conducted on sterile 96-well microtiter plates. In order to test the active R. Cordifolia extract, dilution 1:2 was prepared at 100 mg / ml. The final concentrations were therefore 25, 12.5, 6.25, 3.125, 1.5625, 0.7813, 0.3906 and 0.195 mg / ml. Microdilution was conducted on 96-well microtiter plates with U-shaped wells. In simple, the cultures were diluted in Peptone water at a density calibrated to 0.5 McFarland turbidity.

The final inoculum was 5 x 105 CFU / ml bacterial colony. Controls of 0.5 ml of a standard culture medium as a negative control and antibiotic powdered dilution of Chloramphenicol and Penicillin have been used in the experiments. he wells were loaded with 50 µl of absolute ethanol and 100 µl of R. Cordifolia extract was applied to the wells by two-fold serial dilution of the suspension of R. Cordifolia extracts a stock solution. Every well was inoculated with 50 µl of 0.5 McFarland standard bacterial suspension, as each well received 5 x 105 CFU / ml. The plates were sealed, placed in plastic bags, and incubated at 37°C for 24 hours. In this work, the lowest concentration of R. Cordifolia represents the MIC of its extract, which showed no growth of the organism in the wells by visual reading sensitivity to extracts of R. cordifolia.

Statistical analysis

Data was analyzed using the Microsoft Office Excel (2007) SPSS software. Multiple comparisons were made using one-way ANOVA. The difference was found to be of importance at $p<0.05$. The data is viewed as a mean ± standard deviation of three replicates. Experimental findings are shown as mean ± SEM. MTT test were replicated three times. The IC_{50} values were calculated from linear regression analysis.

RESULTS

Percentage of cell death of Human biopsy xenograft of pancreatic carcinoma line-3 cell line (BxPC-3) by *R. cordifolia* extract.

To estimate the effect of the *R. cordifolia* extract on BxPC-3 cells viability, BxPC-3 cells were treated with 0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM *R. cordifolia* extract at 24, 48 and 72h (Figure 1) $p < 0.0001$. *R. cordifolia* extract was significantly increased the cell death of BxPC-3 at 2.5 µM (51, 79 and 85%) at 24, 48 and 72hours respectively $p< 0.001$ vs. other concentrations (Figure1). BxPC-3 cell line treated with *R. cordifolia* extract in concentration 2.5 µM had a close effect on cell death aafter 72h with Cisplatin (control) as shown in figure 2.

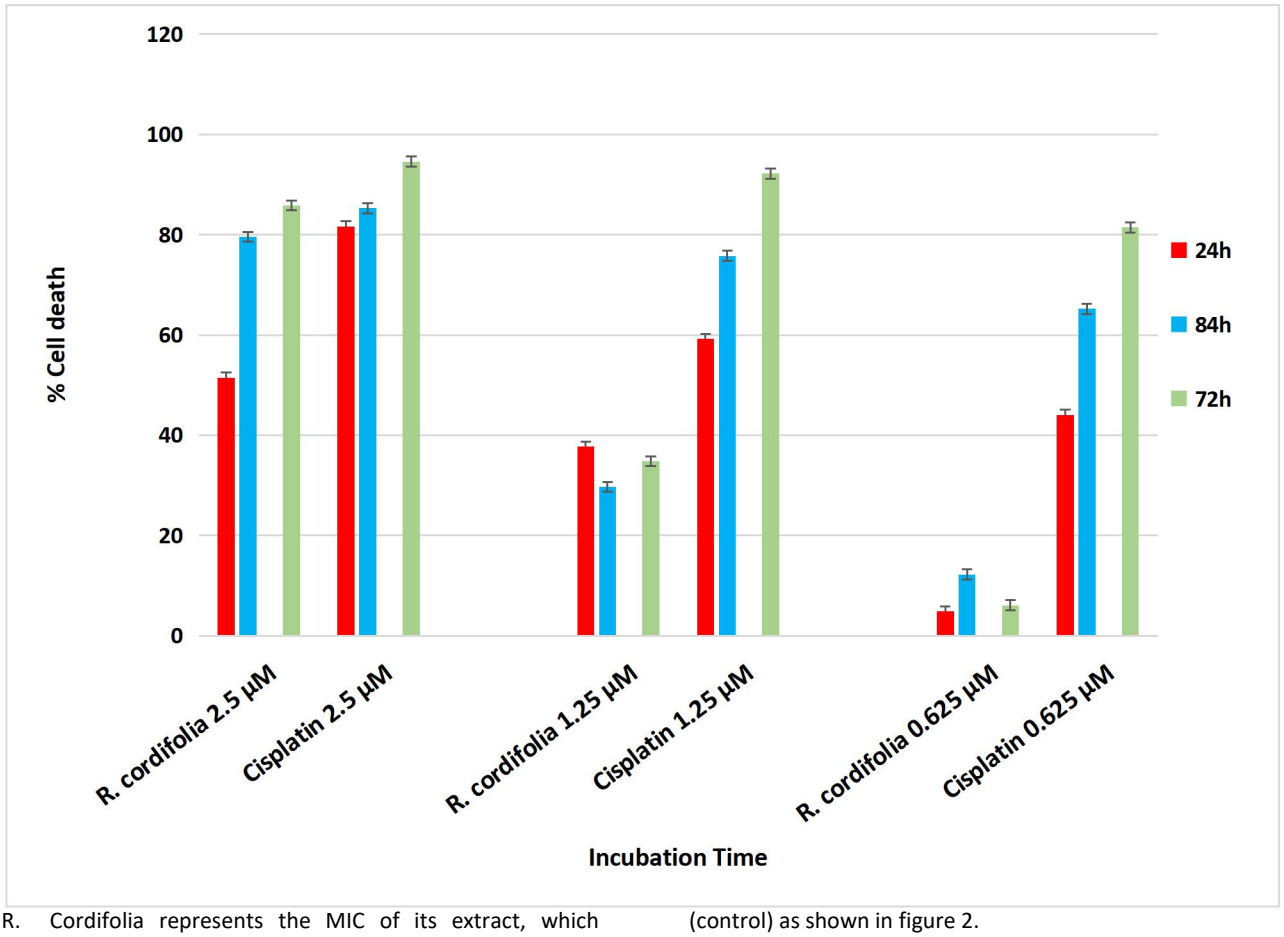

Figure 1. *In vitro* cell death percentage of the Human biopsy xenograft of pancreatic carcinoma line-3 cell line (BxPC-3) was calculated by MTT method in 96-well plates at 0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM of *R. cordifolia* extract for 24, 48 and 72h exposure to these concentration. Data is shown as % mean ± SEM of cell death for three separate experiments. Treated were substantially different from the untreated controls $p < 0.0001$.

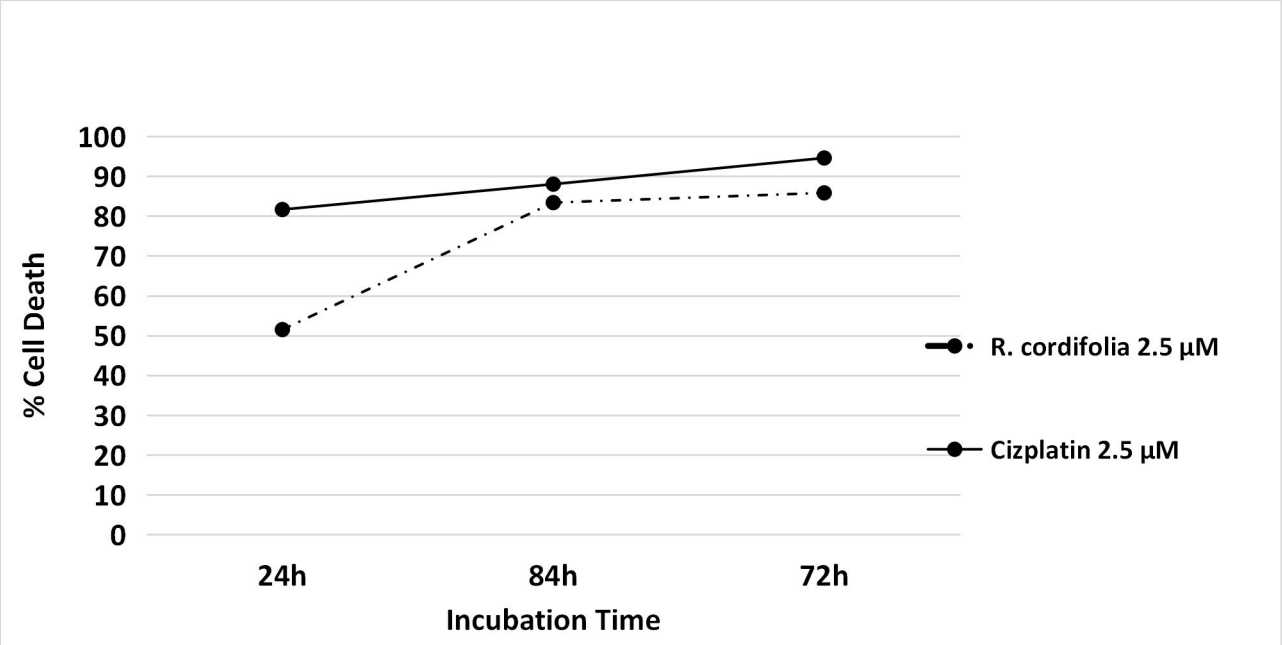

Figure 2. In vitro, a comparison of the percentage of cell death of human biopsy xenograft of pancreatic carcinoma line-3 cell line (BxPC-3) was performed with 2.5 µM R. Cordifolia extract and 2.5 µM Cisplatin (control). The absorbance was read nm (650 nm wavelength reference) using a microplate reader. The findings are the mean ± SEM of three independent experiments.

Percentage of cell death of Human Children liver hepatocellular carcinoma cell line (HepG2) by *R. cordifolia* extract.

HepG2 cell line had highly cytotoxicity effect at concentrations 2.5 and 1.25 µM (69, 78, 94% and 60, 69, 71%) respectively at 24h, 48h and 72h as compared to other concentrations which showed low death percentage as shown in Figure 3 p< 0.005.

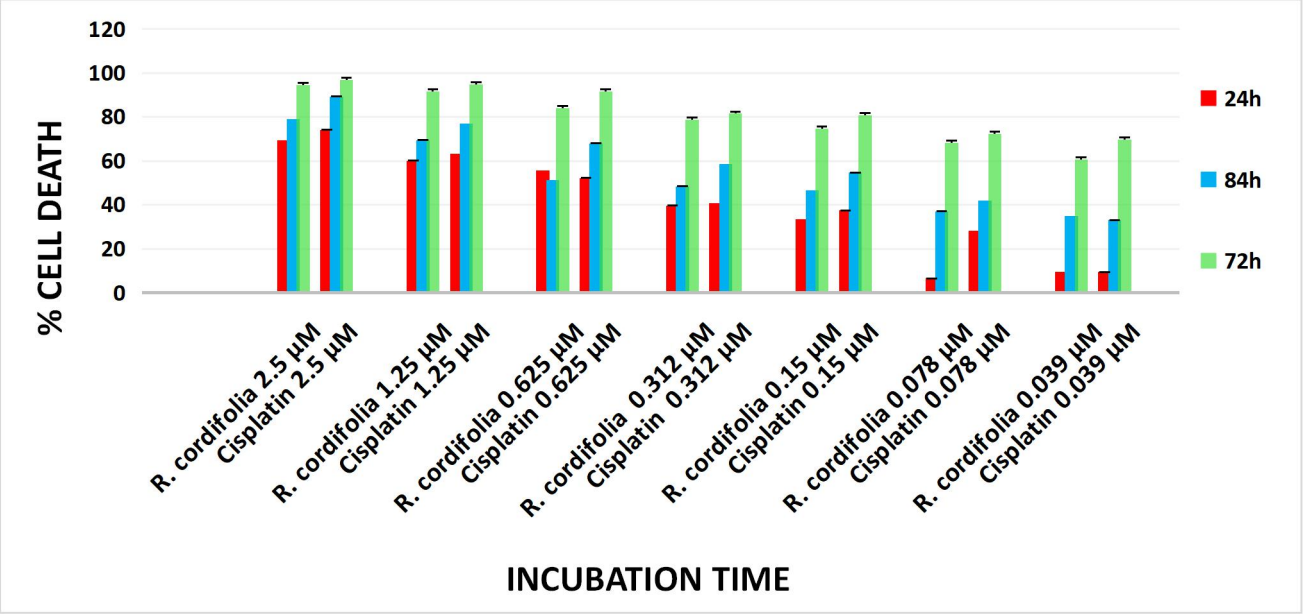

Figure 3. *In vitro* cell death percentage of the Human Children liver hepatocellular carcinoma cell line (HepG2), was measured by MTT assay in 96-well plates following 24, 48 and 72h exposure to 0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM *R. cordifolia* extract. Data is shown as % mean ± SEM of cell death for 3 separate experiments. Treatments were substantially different from the untreated controls $p < 0.005$.

Percentage of cell death of children human breast cancer cell line MCF-7 by *R. cordifolia* extract.

In order to assess the impact of R. Cordifolia extract on MCF-7 cell viability, MTT assay was performed. The results of the MTT assay showed 2.5 µM R. Cordifolia extract was obviously capable of reducing cell viability after 24, 48 and 72 h, p 0.00001 (Figure 4). MCF-7 cell line treated with R. cordifolia extract in concentration 2.5, 1.25 and 0.265 µM compared to the Cisplatin (control) showed high significant difference $p < 0.001$.

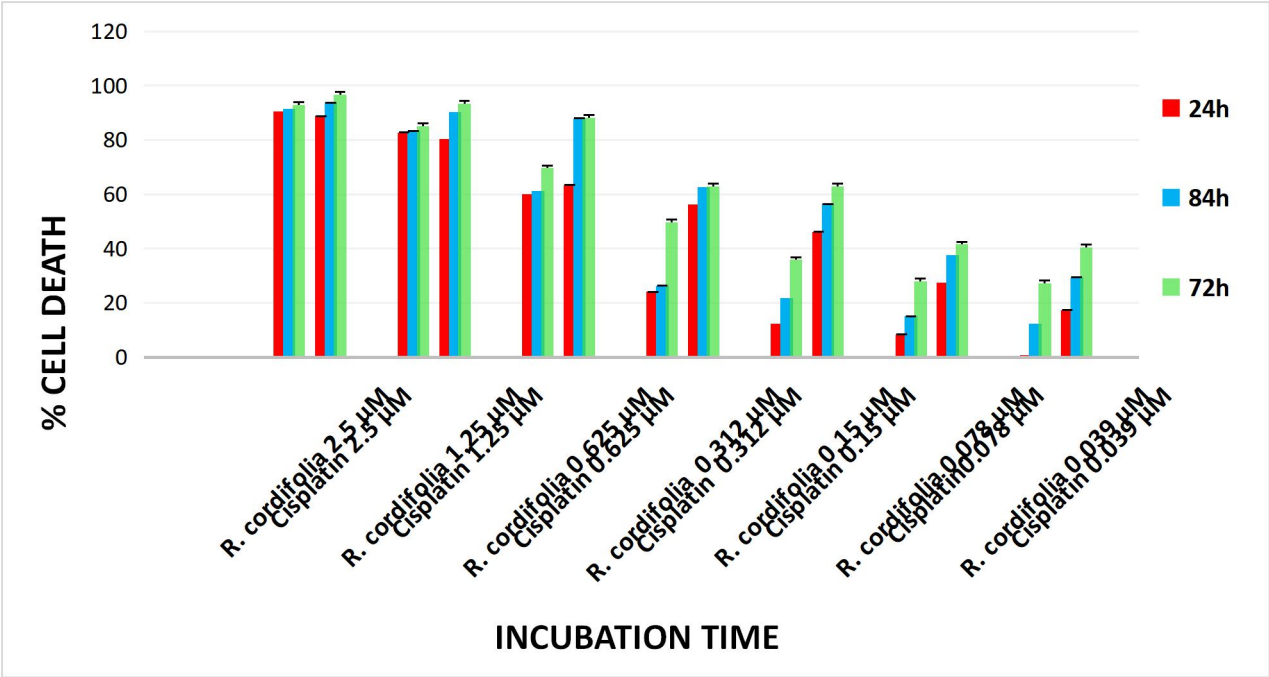

Figure 4. *In vitro* cell death percentage of the Human Breast carcinoma cell line (MCF-7), was estimated by MTT assay in 96-well plates following 24, 48 and 72h exposure to 0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM *R. cordifolia* extract. Data is shown as % mean ± SEM of cell death for three separate experiments. Treatments were substantially different from the untreated controls $p < 0.00001$.

Half Maximal Inhibitory Concentration (IC_{50}) Value of *R. cordifolia* extract

The dose-response curve created by Graph pad 2018 using nonlinear regression analysis for *R. cordifolia* extract in BxPC-3, HepG2 and MCF-7 cells. The IC_{50} values were achieved by a series of concentrations of *R. cordifolia* extracted from 0.039, 0.078, 0.15, 0.312, 0.625, 1.25 and 2.5 µM by MTT assay. The results of IC_{50} for *R. cordifolia* extract (1.73, 0.03, 0.57 µM) in BxPC-3, HepG2 and MCF-7 cells respectively.

Minimum inhibition concentration (MIC)

In the current work,MIC of *R. cordifolia* extract against six microorganisms, three are bacteria (*P.aeruginosa (Pseudomonas aeruginosa), E. coli (Escherichia coli)*and *B.subtilis were (Bacillus subtilis))* and three are fungi *(F. oxysporum, T. basicola and T. phaseolina).* by using a microtiter plate assay was variable. The MIC of Gram-negative bacterium was high against gram- positive bacteria as illustrated in Table 1.

Table 1. MIC of *R. cordifolia* extract against different bacterial isolates by using microliter plate technique

Bacterial isolates	Serial dilutions of *R. cordifolia* extract (mg/ml)								MIC
	25	12.5	6.25	3.125	1.56	0.78	0.39	0.19	(mg/ml)
***Escherichia coli* (Gram-negative bacterium)**	13*	13*	10*	9*	8*				**1.562**
Pseudomonas aeruginosa* (Gram-negative bacterium*)	10*	9*	8*						**6.25**
***Bacillus subtilis* (Gram-positive bacterium)**	14*	1*3	12*	11*	10*	9*			**0.78**
Fungi isolates	**Serial dilutions of *R. cordifolia* extract (mg/ml)**								**MIC**
	25	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**0.39**	**0.19**	**(mg/ml)**
F. oxysporum	18*	16*	14*	12*					**3.125**
T. basicola	9*	12*	10*	9*	11*				**1.562**
T. phaseolina	13*	19*	17*						**6.25**

Diameter of zone of inhibition in mm includes well diameter 6mm

* is the mean of three replicates

DISCUSSION

Rubia cordifolia is a perennial climbing herbaceous plant. It is also known as Indian madder, which is a flowering plant species in the coffee family, *Rubiaceae*. A red pigment is derived from its root hence it is cultivated. Genus Rubia has grown to about 70 species widely distributed around the world, a total of 36 species and two varieties from China have been reported. Extracts and phytochemicals of Rubia plants have attracted considerable attention due to their potent bioactivity.[27]. Leaves are arranged in four whorls whereas the stem is slender, rough and woody at the base. Flowers are in cymes, greenish white. Fruits are smooth, shining, and purplish black when ripe [28]. The root of the plant is commonly referred to as Manjistha and is sweet, bitter, and acrid. These roots, which are clustered in the soil, are aubergine or orange red. The elongated and rugged stems gently lignify at the root. The branches are formed to four edges. [29, 30]. The pharmacological action of the crude drug largely depends on the metabolites present in it [31]. *R. cordifolia* (Manjistha) basically known for its anthraquinones and naphthohydroquinones phytochemical constituents [32]. The major phytoconstituents of R. cordifolia reported include rubiadin, rubicordone A, rubiasins A-C, rubiatriol (triterpenoid), 6-

methoxygeniposidic acid an iridoid glycoside and two pentacyclic triterpenoidrubicoumaric acid, and rubifolic acid. Mollugin, furomollugin, and dehydro-alpha-lapachone are isolated from chloroform fraction [33-37]. Biologically active compounds are chemical in nature and they have the potential to cure various diseases. *R. cordifolia* also revealed important phytochemical compounds and evidenced that this plant was important for curing various diseases in traditional medicine. Anthraquinones were mainly found in root, stem, and leaf which have been shown to be antibacterial, antifungal, and laxative and were also used as natural dyes [31, 38]. Traditionally, herbal medicines have been extensively used to treat cancer and produced promising clinical results; however, the underlying mechanisms of action have not been systematically investigated. Roots of *Rubia cordifolia* were extracted in this study with 70 % aqueous ethanol. The dry extract was evaluated for anti-proliferative activity by MTT assays. It was found to have significant anti-proliferative effects against BxPC-3, HepG2 and MCF-7 cancer cells. *Rubia cordifolia* did not test cytotoxicity to these cancer cell lines previously. The antiproliferative property of *Rubia cordifolia* extract was tested on A-431 cells (epidermal carcinoma cells) and 3T3 fibroblast cells [39]. It was observed that a fraction of *Rubia cordifolia* significantly inhibited the incorporation of [3H]-thymidine, induced by fetal bovine serum, in a dose dependent manner. It also inhibited the PMA (phorbol 12-myristate 13-acetate) induced expression of c-fos genes in A431 cells. It appears that inhibition of DNA synthesis underlies the mechanism for its antiproliferative properties [40]. The results in current study showed that *R. cordifolia* extract has cytotoxicity towards children liver hepatocellular carcinoma cell line (HepG2), biopsy xenograft of pancreatic carcinoma line-3 (BxPC-3) and breast cancer cell line MCF-7 which compared to a negative control treated with DMEM media (Figures 1-4). The concentration of *R. cordifolia* extract contributing to 50% inhibition of cells (IC50) was 1.73, 0.03, and 0.57 µM in BxPC-3, HepG2 and MCF-7 cells respectively at hours of treatment with freshly prepared R. cordifolia extract. Therefore, *R. cordifolia* was more cytotoxic after 72 hours treatment compared to a 24 hours treatment and *R. cordifolia* can reduce cell growth as well as cause cell death (reduction in cell numbers). The results of this analysis confirm the previous R. Cordifolia screening findings for four human breast cancer cell lines [41,42] and other cell lines [43,44]. Campbell et al.[41] looked at the impact of 71 Chinese medicinal herbs on four human breast cancer lines and described R. Cordifolia as one of the promising agents for potential research has also been confirmed by Shoemaker et al. [42]. Interestingly, the IC50 was very low concentration as compared with the IC50 for other studies. R. Cordifolia was thought to facilitate cell apoptosis through a caspase-dependent route as well as to induce cell cycle arrest. [41, 45]. The results in current work show highly effective against fusarium oxysporum.which agree with other reports that found from the MIC assays of *Rubia cordifolia* extract Both antifungal and antibacterial activity is seen at very low concentrations ranging from 5 mg / ml to 10 mg / ml. (46).

CONCLUSION

The findings indicate that R. Cordifolia extract may have a impact role as an adjunct therapy for pancreatic, liver and breast cancer, in addition to its function as an antimicrobial agent as shown in this study against six microorganisms., three are bacteria (*P.aeruginosa (Pseudomonas aeruginosa)* , *E. coli (Escherichia coli)* and *B.subtilis were (Bacillus subtilis))* and three are fungi *(F. oxysporum, T. basicola and T. phaseolina*) and may be useful as natural fungicides.

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