Evaluation of anticancer potential of *Rubia cordifolia* L. aqueous extract against various gynecological human cancer cell lines: Cervix adenocarcinoma(HeLa), Breast adenocarcinoma(MCF7) and Ovarian teratocarcinoma(PA-1)

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Abstract
The modern technology has now shifted its focus on natural antioxidants to target against free radicals and to reduce the development of chronic diseases. Plant based investigation against various ailments have opened a new perspective in the field of biopharmaceutical research. Cancer being second leading cause for death globally has thrust it into the limelight of research. A substantial proportion of death caused by cancer could be prevented though advances in the field of biopharmaceutical research and its implementation.

The Rubia cordifolia L. also known as Manjishta (Indian Madder) is one of most valuable herbs in Ayurveda having enormous scope for drug development. The study reports the anticancer activity of manjishta against gynecological human cancer cell lines HeLa, MCF7and PA-1.

Keywords: *Rubia cordifolia* L, Cytotoxicity, Cervix adenocarcinoma (HeLa), Breast adenocarcinoma(MCF7), Ovarian teratocarcinoma (PA-1).

Introduction

Medicinal plants formulations are used in the treatment of various ailments since ancient times. Studies have been carried out globally to verify their efficiency and productivity. Some of the findings have led to the production of different types of plant-based medicines. Over 90% of conventional cure contains therapeutic plants and that have been implicated with preventive measures in the ailment control system[8]. In addition, herbal remedies have become more popular in the treatment of minor ailments and personal maintenance[24]. A good number of medicinal plants are mentioned in the classical Ayurvedic writings 'Charaka Samhita'. 'Susruta Samhita' and 'Astanga Hrdaya Samhita'[18].

Our concern is about the herbal extract of *Rubia cordifolia* L. belonging to the family Rubiaceae. It is also referred to as Manjishtha and is distributed throughout the hilly area of India. The traditional therapeutic use of the plant is for skin disorder and cancer treatment. The anthraquinones of the Rubiaceae family exhibit some interesting *in vivo* biological activities such as anti-inflammatory, urinary disorders, antistress, hepatoprotective, antimicrobial, hypotensive, analgesic, antimalarial, antioxidant, antileukemic and mutagenic functions and immune-modulatory[16]. In the olden days, Manjishta has been reputed as a good blood purifier and hence widely used against skin, blood and urinary ailments. Bioactive compounds such as anthraquinone, glycosides, naphthaquinones and terpenes are present in the root, stem, leaves and fruits of *Rubia cordifolia* L.[7]

Plants are used as a common alternative for cancer treatment in many countries and more than 3000 plants worldwide have been reported to have antitumor properties[25]. As per the view of the American Cancer Society Some of the cancers that most often affect women are breast, colorectal, endometrial, lung, cervical, skin and ovarian cancers. Gynecological cancers are among the most common cancers in women and hence an important public issue. Ovarian cancer has emerged as one of the most common malignancies affecting women in India and has shown an increase in the incidence rates over the years[3]. Breast and cervical cancer are the first and second most common cancers in women. In these, ovarian cancer is on a declining trend[13]. Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from this disease. Therefore, there is an increasing demand to develop new, effective and affordable anticancer drugs[5].Cell lines seem to be a key element for the molecular diagnosis of cancer as they can be widely used in many aspects of laboratory research, particularly as *in vitro* models in cancer research[3].

Material and Methods

Plant Material: *Rubia cordifolia* L. roots were collected from the hilly area of Kasargod district, Kerala.

Plant extract preparation: The root sample was allowed to shade dried until the evaporation of moisture content. The dried roots were finely powdered with the help of electric blender. 300g of fine powder was transferred to the conical flask containing 300 ml of distilled water and the entire contents were mixed using a magnetic stirrer for 48h. Afterward, dissolved contents were filtered with filter paper and transferred to sterile Petri plates. Filter the dissolved extract using fresh filter paper with the help of funnel and transfer the filtered contents to sterile Petri plates. Keep the Petri plates in a clean surface area to dry for 3-4 days at room

temperature. The collected crude extract was stored at 4° C for further use.

Maintenance of cell cultures: PA-1, MCF-7 and HeLa cells were obtained from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in the logarithmic phase of growth in a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS).

MTT assay: MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity**.** Three different cell lines were used for the cytotoxicity study on the root extract of *Rubia cordifolia* L. In this study, aqueous root extract was evaluated to check the cytotoxicity study on the 3 cell lines namely, PA-1, HeLa and MCF7 at different concentrations (25,50,100,200 and 400). Seed 200µl cell suspension in a 96-well plate and the cell count was adjusted to 20,000 cells per well. To each well of 96 well micro titre plates, diluted cell suspension was added. The cell lines were grown in DMEM media containing 10% FBS. Plates were incubated for 24hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, plates were taken out from the incubator and spent media was removed. MTT reagent was added to all wells with the final concentration of 0.5mg/ml of total volume.

Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. To account for this reduction, it is important to use the same medium in control as well as test wells. Plates were wrapped with aluminum foil to avoid exposure to light. Return the plates to the incubator and incubate for 3 hours at 37°C. Dimethylsulfoxide (DMSO) was added. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures. Read the absorbance on a spectrophotometer at 570nm and 630nm used as the reference wavelength.

The reference wavelength should be more than 650 nm. By plotting a graph of Log concentration of compound in X-axis and % of cell inhibition in Y- axis, the concentration of compound required to inhibit 50 % (IC_{50}) cell growth was determined. The survival curves of each cell line were established based on extract concentration after the specified period. Results were expressed in the percentage of cell viability.

The IC50 value was determined by using the linear regression equation: Y =Mx+C.

Here, Y = 50, M and C values were derived from the viability graph.

Formula used for the study:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance of sample}}{\text{Mean absorbance of untreated}}$$

In vitro cytotoxicity was evaluated by MTT assay and cell morphological changes were observed by using a microscope and MICAM Software.

Results

Extract preparation: The identification of medicinal plant with anti-cancerous activity is very useful for the cancer treatment. 70mg pasty form of crude aqueous root extract of *Rubia cordifolia* L. was collected into vials **(**Figure1) and stored at 4 °C for further use. Here the cytotoxicity activity of *Rubia cordifolia* L. root extract was studied using MTT assay. The results were summarized below tables and graphs.

MTT cytotoxicity study of the test *Rubia cordifolia* L. root against PA-1 cell line: Cytotoxic potential of aqueous extract of *Rubia cordifolia* L. root against the proliferation of gynecological human cancer cell lines: Cervix adenocarcinoma (HeLa), Breast adenocarcinoma (MCF7) and Ovarian teratocarcinoma (PA-1) were evaluated by MTT Assay. The observations (Table 1) showed that cytotoxic activity of aqueous extract of *Rubia cordifolia* L. root was promisingly high against PA-1 cell line (ovarian cancer) when compared to standard drug doxorubicin with an IC_{50} value of 145.9µg/ml.

The aqueous root extract is more toxic to PA-1 cells. The percentage of cell viability is more (92.06%) in the concentration 25µg. It shows less cell viability (25.07%) at 400µg/ml (Figure 2). Cell viability gradually decreases with increasing concentration of the aqueous root extract (Graph 1). The results are suggesting us that the root extract of *Rubia cordifolia* L. has possessed significant cytotoxic and anticancer potential against human ovarian cancer cells. So this plant has a therapeutic proposition in the most life threatening disease like cancer and further studies are required.

Figure 1: Aqueous extraction of *Rubia cordifolia* L. root

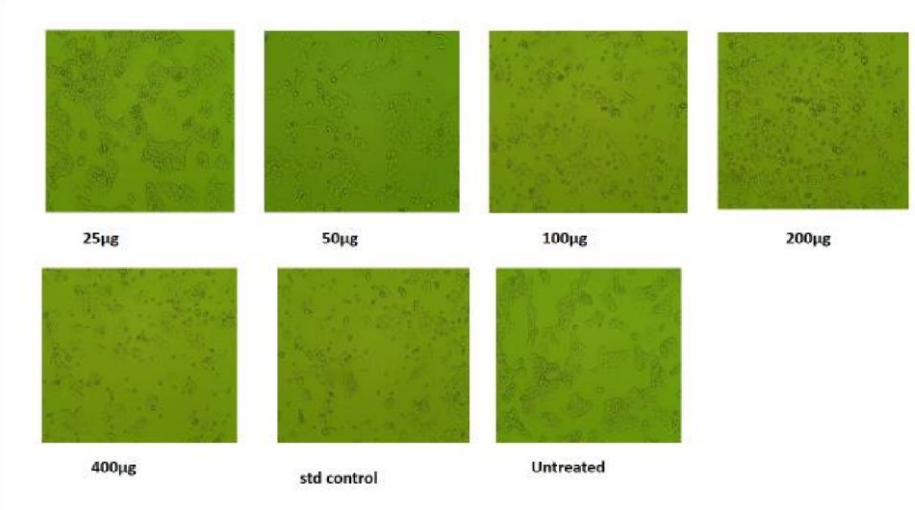
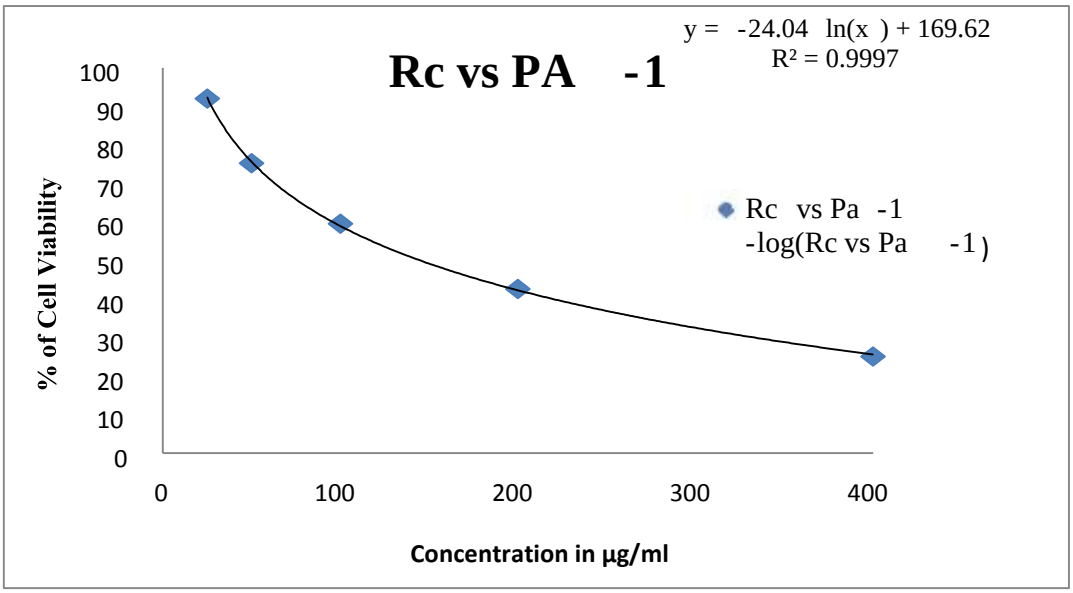


Figure 2: Drug treated microscopic images of PA-1 cell line



Graph 1: Cytotoxicity of *Rubia cordifolia* L.extract against PA-1 cell line

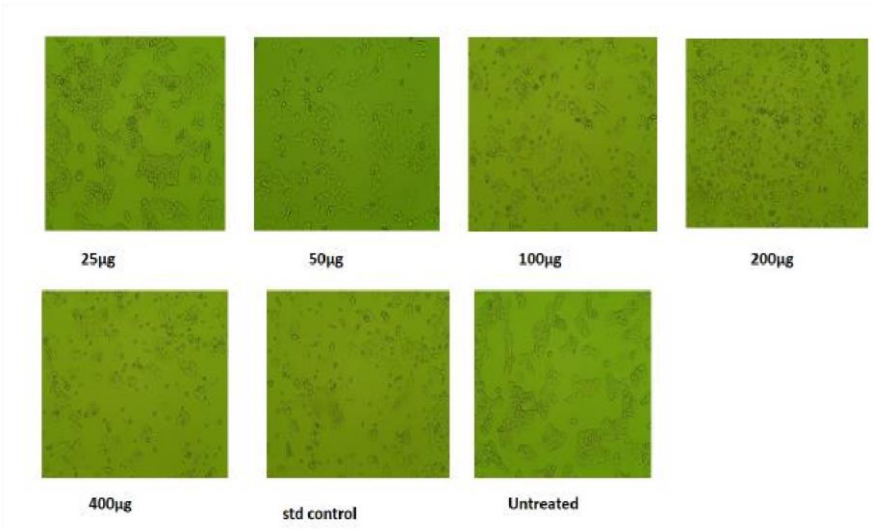


Figure 3: Drug treated microscopic images of MCF7 cell line

Cytotoxicity study of the *Rubia cordifolia* L. root against MCF7 cell line: The observations in table 1 showed cell cytotoxicity study of aqueous extract of *Rubia cordifolia* L. with an IC_{50} value of 302.27µg/ml. While comparing the cytotoxic potential properties of *Rubia cordifolia* L. and standard drug Camptothecin with a concentration of 12.5µM, the aqueous root extract is more cytotoxic against the MCF-7 cells. The percentage of cell viability is more (96.82%) in the concentration 25µg, which shows less cell viability (42.41%) at 400µg (Figure 3). Cell viability decreases with increasing concentration of the aqueous root extract (Graph 2). The results suggest that the *Rubia cordifolia* L. root is having satisfactory cytotoxic and anticancer potential against human breast cancer cells.

MTT cytotoxicity study of *Rubiacordifolia* L. root against HeLa cell line: The observations in table 1 showed cell cytotoxicity study of aqueous extract of *Rubia cordifolia* L. root being moderately cytotoxic against HeLa cell line (cervical cancer) with an IC_{50} value of 212.68µg/ml. While comparing the cytotoxic potential properties of *Rubia cordifolia* L. and the standard drug camptothecin with the concentration of 12.5µM, the aqueous root extract is more cytotoxic against the HeLa cells**.** The percentage of cell viability is more (95.15%) in the concentration 25µg. It shows less cell viability (35.12%). at 400µg (Figure 4). Cell viability decreases with increasing concentration of the aqueous root extract (Graph 3). The results reveal us that root extract of *Rubia cordifolia* L. has moderate cytotoxic and anticancer potential against human cervical cancer cells.

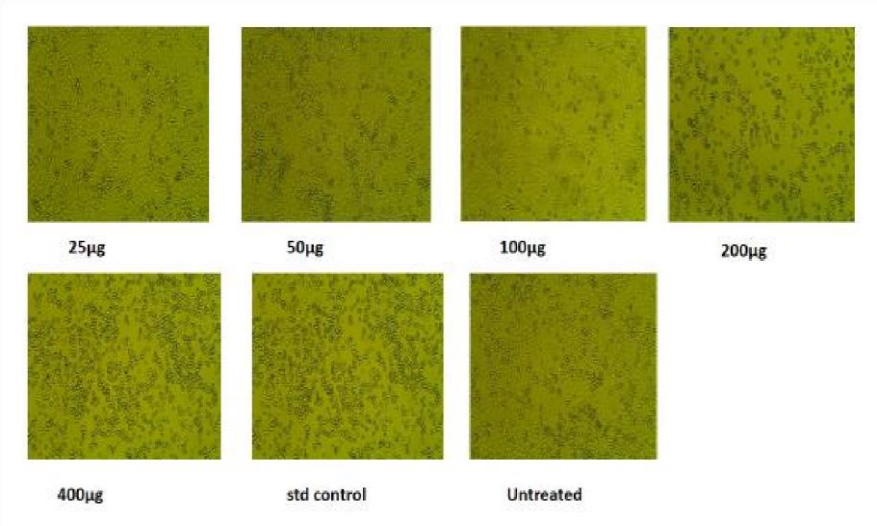
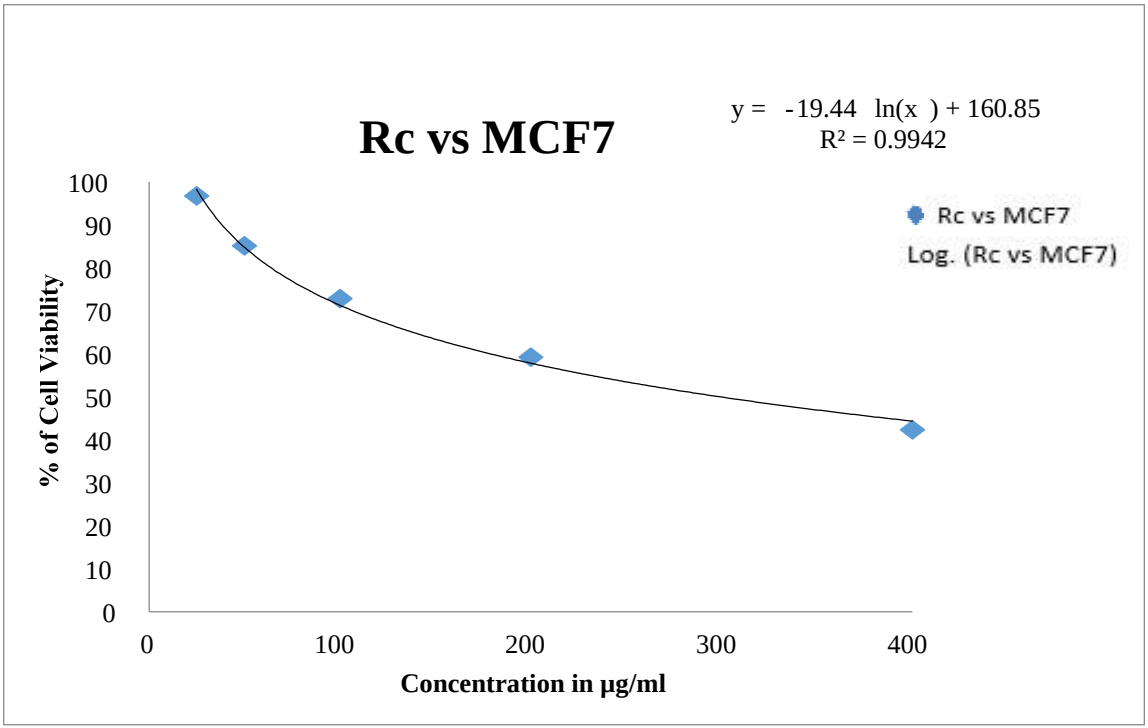


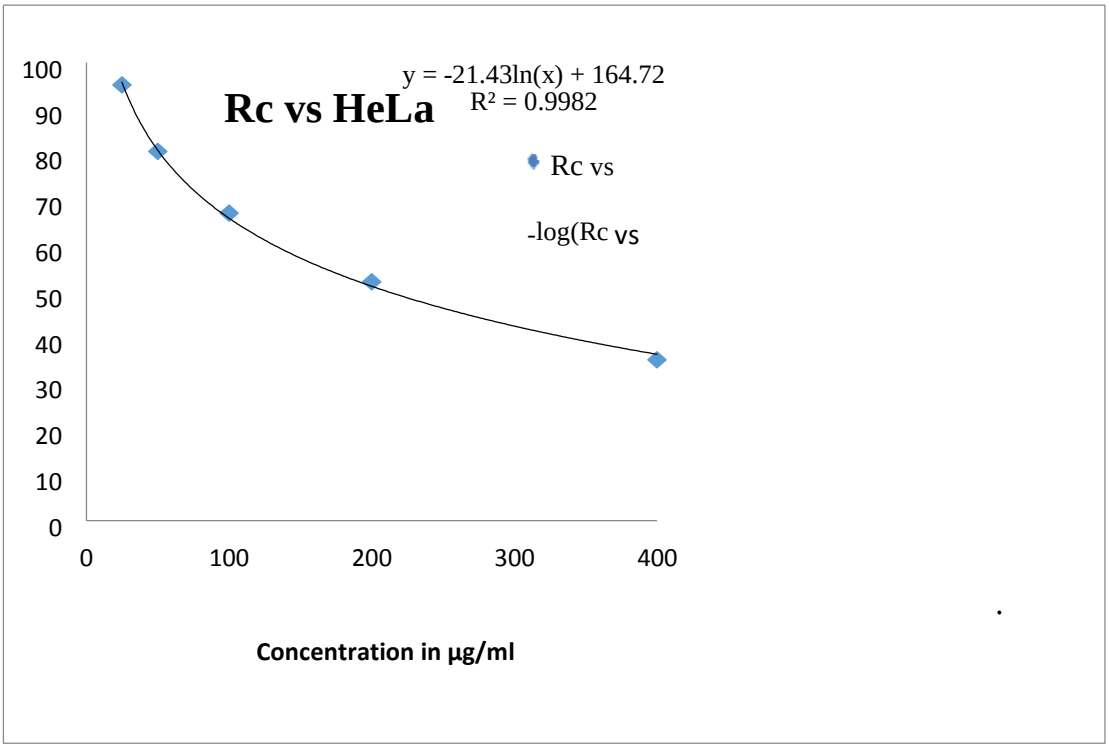
Figure 4: Drug Treated Microscopic Images of MCF7 Cell Line



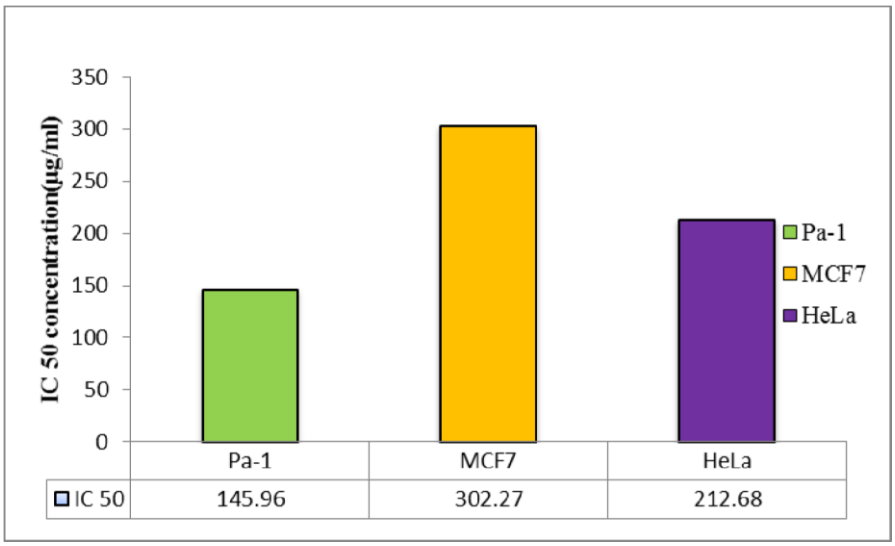
Graph 2: Cytotoxicity of extract against MCF 7 cell line

Table 1
Cytotoxicity of *Rubia cordifolia* L. extract against different cell lines

Conc. of the samples (µg/ml)	Cell Viability %		
	PA-1	**MCF 7**	**HeLa**
25	92.062	96.828	95.15260
50	75.285	85.199	80.6104
100	59.53	72.885	67.145
200	42.6337	59.2661	52.0124
400	25.07	42.4129	35.128
Standard	50.39085	48.320	50.2094
Negative control	100	100	100
IC_{50}Value	**145.96µg/ml**	**302.27µg/ml**	**212. 68µg/ml**



Graph 3: Cytotoxicity of *Rubia cordifolia* L. extract against HeLa



Graph 4: Comparative IC_{50} values of *Rubia cordifolia* L. Root extract against the MCF7, PA-1 and HeLa cell lines and results plotted in bar graph

Discussion

For the last many years, plants have beneficial activity in different types of diseases produced in human beings[15]. As per WHO, about 80% of the world's inhabitants problem should be treated by medicinal herbal drugs for their primary care. Plants have long history in the treatment of cancer. The valuable part is root stocks which contain phytochemicals like anthraquinone, terpenes, glycosides, etc[26]. Phytochemical studies of *Rubia cordifolia* L. showed positive for alkaloids, cardiac glycosides, tannins, flavonoids and phenols and negative for saponins, volatile oils, anthraquinones and cyanogenic glycosides and very trace amount of steroids[12]. According to previous study, the cyclic hexapeptides and quinones of *Rubia cordifolia* L. exhibited a significant anticancer activity against various proliferating cells[21].

Rubia cordifolia L. is an ethnic plant where all parts are medicated. Many phytochemicals and pharmacological studies support its traditional use and have now proven as a source of several clinically important drug resources. The leaves of this plant were also studied for its antiviral and in-vitro free radical scavenging activity. Alizarin, a natural hydroxyl anthraquinone obtained from the root of *Rubia cordifolia* L. was assessed as an osteotropic drug for the treatment of bone tumors in light of its high affinity to the bone[2]. *Rubia cordifolia* L. is known to contain substantial amounts of anthraquinones, especially in the roots. Bicyclic hexapeptides and hexapeptides have been isolated from roots of *Rubia cordifolia* which were found to be cytotoxic[6]. The cyclic hexapeptides and quinones of *R. cordifolia* exhibited significant anticancer activity against various proliferating cells[10].

Our study was conducted to investigate the cytotoxic activities of crude aqueous root extracts of *Rubia cordifolia* L. PA-1 (Ovarian cancer), MCF7 (Breast cancer) and HeLa (Cervical cancer). These three cell lines were treated with the aqueous root extract of *Rubia cordifolia* L. However, *in vitro* cytotoxicity determinations could serve as one of the adjuncts in profiling justifications for clinical trials[23].

The method used for the study is MTT based on the metabolic reduction of the soluble MTT 3- (4, 5-dimethylthiazol -2-yl) -2 ,5 diphenyltetrazolium bromide which reflects the normal function of mitochondria dehydrogenase activity and cell viability into an insoluble colored formazan product which was measured spectrophotometrically[22]. PA-1, HeLa and MCF7 cell lines were treated with the root extract of *Rubia cordifolia* L. 25,50,100,200 and 400µg/ml concentration.

According to the result, the cell viability of three cell lines was different and cell viability decreased with increasing concentration. Here the aqueous root extracts of *Rubia cordifolia* L. showed an IC_{50} value of 302.27µg/ml against the MCF7 cell line. According to the previous study, the root extract of the *R. cordifolia* plant showed cytotoxicity activity against the MCF7 breast cancer cell line with an IC_{50} value of 400µg/ml[17]. Anthraquinone derivatives (Xanthopurpurin) from the roots of *Rubia philippinensis* showed significant cytotoxicity against MCF-7 cell line[11].

The bark and wood of *Hymenodictyonexcelsum*, belonging to the Rubiaceae family, were screened for cytotoxicity against Vero, NIH3T3, AGS HT-29, MCF-7 and MDA-MB-231 cell lines and significant cytotoxic effects of the bark were observed against all cell types (IC_{50} values were 230, 70, 90, 160, 80 and 440 µg/mL respectively[1].

Hence according to our results, root extract of *Rubia cordifolia* L. showing moderate cytotoxic potential against MCF7 cells with IC_{50} concentration at 302.27µg/ml compared to the standard drug. Camptothecin with a concentration of 12.5µM was used for the study. The aqueous root extracts of *Rubia cordifolia* L. showed an IC_{50} value of 212.68µg/ml against HeLa cell line. Cytotoxicity property of extracts of roots of *Rubia Cordifolia* L. was carried out by XTT method against HeLa.

Based on this previous study root extract of *Rubia cordifolia* L. exhibited (IC_{50} values 23.12 µg/ml, 38.13 µg/ml, 48.87 µg/ml) cytotoxicity against HeLa cells[20]. Methanol fraction of *Rubia cordifolia* L. extracts exhibited potent inhibition of Human cervical cancer cell line and Human larynx carcinoma cell line while it was found to be less cytotoxic against normal human kidney cells displaying safety for normal cells[19].

Hence according to our results, root extract of *Rubia cordifolia* L. was showing significant cytotoxic potential properties against HeLa cells with IC_{50} concentration at 212.68µg/ml compared to the standard drug. The aqueous root extracts of *Rubia cordifolia* L. showed an IC_{50} value of 145.96µg/ml against the PA-1cell line. The previous study determined the combination of *Rubia cordifolia* L. and *Murraya koenigii* plant extract in different ratios showing a beneficial effect against ovarian cancer (PA-1cell line)[4]. The cytotoxic activity of the leaf extract of *Pergulariadaemia* was determined against PA-1 which showed an IC_{50} value of 30 mg/ml. The study showed that the leaf extract of *P. daemia* has a potent cytotoxic effect. Triterpenoids play a vital role as anticancer agent[14].

Hence according to our study, *Rubiacordifolia* L. showed significant cytotoxic potential against PA-1 cells with IC_{50} concentrations at 145.96µg/ml compared to the standard drug doxorubicin with a concentration of 15µM used for the study. These comparative studies and results suggest us that potential natural product of *Rubia cordifolia* L. root could be developed as an anticancer agent.

There is an increasing need of research of new compounds with cytotoxicity activity in the treatment of cancer with the available anticancer drugs which are often insufficient due to the problem of cytotoxicity to the normal cells. *R.*

cordifolia L. can be a source of potent pharmacophore for treatment of disease like cancer.

Thus, cancer patients who already got crippled with this disease, who are further burdened by drug-induced toxic side effects, have now turned to seek help from the complementary and alternative medicine hoping for a better cure naturally[26].

Therefore, our study successfully evaluated the comparison between the cytotoxicity of crude aqueous root extract of *Rubia cordifolia* L. against PA-1, MCF7 and HeLa cell lines. Here our investigations strongly suggest us that the crude aqueous root extract of *Rubia cordifolia* L. may have possible therapeutic potential against Human ovarian cancer cells.

Conclusion

The aqueous root extract of *Rubia cordifolia* L.is proved to contain effective concentration of the active compound that exhibited promising activity with (IC_{50}) values. IC_{50} values of three cell lines PA1, MCF7 and HeLa were 145.96, 302.27 and 212.68µg/ml respectively. Here according to our study, *Rubia cordifolia L.* root extract is more cytotoxic against ovarian cancer (PA-1) with IC_{50} value 145.9µg/ml. Gynecological cancer is among the most common cancers in women and hence is an important public health issue[13].*Rubia cordifolia* L. can be a source of potent pharmacophore for treatment of disease like cancer[18].

The observations of our study strongly indicates that the root extract of the *Rubiacordifolia* L. may possibly have therapeutic potential against Human ovarian cancer cells. Hence further studies need to be performed to evaluate the molecular mechanism of action behind the anticancer potential of the aqueous root extract of *Rubia cordifolia* L. against the human ovarian cancer cells in *in vitro* conditions.

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