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In vitro antiproliferative activity of ethanolic extract of Sida cordifolia Linn against various cancer cell lines

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Abstract: *Background:* Sida cordifolia of family Malvaceae is traditionally, used in treating the various ailments of respiratory and urinary system. It possesses astringent and emollient properties. *Aim and objectives:* Present study was aimed to assess in vitro antioxidant and antiproliferative potential of aerial parts of Sida cordifolia. *Material and Methods:* Serial extraction of aerial parts of Sida cordifolia was done by using Soxhlet apparatus. In vitro antioxidant potential was evaluated by DPPH, H_2O_2 and NO radical scavenging ability. Antiproliferative activity was assessed by MTT assay on human breast cancer (MCF7), ovarian cancer (PA1), colon cancer (HT29), melanoma (A375), liver cancer (HepG2) and normal mouse embryonic fibroblast (NIH-3T3) cell lines by treating them with increasing concentration of the extract for 24 hours. *Results:* Ethanolic extract of S. cordifolia showed good DPPH, H_2O_2 and NO radical scavenging activity (IC50 value 20.93µg/mL, 85.14µg/mL and 320.99µg/mL respectively). Extract also showed dose dependent cytotoxicity against all cancer cell lines used in the study. Among cancer cell lines, A375 and HT29 were more sensitive (IC50 16.51 and 49.86µg/mL respectively) than the other cancer cell lines. *Conclusion:* Further work to study mechanism of its action on these cell lines can be helpful in drug discovery program.

Keywords: Anticancer, Colon cancer, DPPH assay, MTT assay, Melanoma.

Introduction

Since ancient time plant and plant-based products are used as remedy for various ailments. According to the updated survey report by WHO, all over the world about 88% of the population rely on this traditional plant-based medicines [1]. These medicines are used not only for common conditions like fever or infections but also for chronic diseases like diabetes or cancers.

Cancer is a pathological condition which involves uncontrolled proliferation of abnormal cells that invade the adjacent tissues and cause their destruction and sometimes show metastasis [2]. International Agency for Research on Cancer (IARC) has estimated 48.4% incidence rate of all types of cancer in Asia [3]. At present, chemotherapy and radiotherapy are the most common therapeutic strategies to treat the cancer but they are associated with high systemic toxicity. Also, drug resistance to chemotherapeutic agents is the main challenge in the long-term cancer therapy [4]. As a result, there is constant search for new chemotherapeutic agents for safe and effective drug development [5-6]. In this regard, many plants which are used in traditional medicinal systems are studied extensively to obtain new sources of modern drugs.

Sida cordifolia belongs to family Malvaceae and extensively used in Indian traditional medicine. It is known as country mallow or Indian ephedra in English. It is called as Bala in Sanskrit, Baryal in Hindi and Arivalmukkan in Tamil. It grows as weed in open, dry places and has emollient and astringent properties. In folklore medicine it is used as diuretic, analgesic, cardiac tonic, bronchodilator and antimicrobial agent and to treat neural diseases like paralysis [7-8]. Medicinal properties of any plant are due to presence of variety of secondary metabolites in them. All plants produce secondary metabolites like phenols, flavonoids, steroids,

terpenoids, alkaloids etc. to protect themselves from adverse conditions [9]. Concentration and chemical structure of these compounds depend on habitat, climatic conditions and season of harvest. This, in turn, decides efficacy and potency of particular compound for their medicinal use [9].

Scientific studies done on this plant have revealed presence of alkaloids, steroids, reducing sugars and saponins. An important alkaloid present in all the parts of S. cordifolia is ephedrine [10]. It is known that alkaloids and phenols are important secondary metabolites which impart antioxidant properties to the plants [11]. Saponins play major role in anti-inflammatory effects [12-13]. Previous studies have reported that S. cordifolia possesses good antioxidant activity [10].

Antitumor activity of S. cordifolia was tested on hepatocellular carcinoma and HeLa cell lines and extract was found to be effective [14-15]. Taka-Aki Matsui et. al. isolated an alkaloid, cryptolepine from S. cordifolia and tested on human osteosarcoma (MG63) cells, and human colon adenocarcinoma wild-type HCT116 and $p21^{WAF1/CIP1}$ deleted HCT116 cells. They demonstrated that cryptolepine produces cell cycle arrest by promoting activity of p21 gene in both these cell lines [16]. Since very few studies were conducted on antiproliferative potential of S. cordifolia, present study was undertaken to analyse in vitro antiproliferative potential of ethanolic extract of S. cordifolia. This study also aimed to evaluate in vitro antioxidant activity of S. cordifolia.

Material and Methods

Study protocol was cleared by institutional ethical committee (VMKVMC/ICE/17/70 dated 28/12/2017)

Plant material and extraction: Plant material, S. cordifolia, was collected from the area in and around Salem, Tamil Nadu. It was authenticated and voucher specimen was deposited in the department (PHY/002/2018). Collected material was cleaned manually to remove other unwanted materials. It was air dried in shed and coarsely powdered using electrical grinder. Serial extraction was performed by using petroleum ether (60-80^{o}C), chloroform, acetone and ethanol 95% v/v (75-78^{o}C). Four hundred gram of coarsely powdered plant material was evenly packed in Soxhlet apparatus and was extracted using petroleum ether for 72 hours. Extract was filtered by using Whatmann filter paper (no.10) and was concentrated by vacuum distillation to $1/10^{th}$ volume. Remaining solvent from this concentrated extract was removed by evaporation by using hot water bath and stored in desiccater to remove moisture. Dried extract was weighed and then stored in airtight container till further use.

Marc left after petroleum ether extraction was dried completely; weighed and packed in Soxhlet apparatus for extraction with chloroform. Above process was repeated for extraction with acetone and then with ethanol. Marc left after ethanol extraction was dried and soaked in 2L distilled water for 72 hours and was stirred occasionally. Contents were filtered and solvent was removed by using hot water bath. Dried extract was weighed and stored in airtight container. Thus, aqueous extract was obtained by cold maceration.

Phytochemical analysis: Preliminary phytochemical analysis was carried out by chemical methods as described in earlier literature [17]. Alkaloids were detected by Dragendorff's test, Hager's test and Wagner's test. For flavonoids, Shinoda's test and concentrated sulphuric acid test were used. Presence of glycosides and phenols were confirmed by Molisch's test and foam test respectively. Steroids were detected by Liebermann-Burchard's test and Salkowski Reaction. Phytochemical analysis revealed presence of flavonoids, phenolic compounds, glycosides, saponins and carbohydrates in ethanolic and aqueous extracts. As ethanolic extract has the polarity in between, it was selected for further evaluation.

Antioxidant analysis

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay: Hydrogen donating ability of the extract was assessed by modified method as described by Perumal *et al*., 2018 by using DPPH [18]. In brief, 0.135 mM DPPH was prepared in methanol. Extract in concentrations of 5, 10, 20, 40, 80, 160 and 320 µg/mL was mixed with 2.5 mL of DPPH solution. Reaction mixture was vortexed thoroughly and kept at room temperature for

30 min. Absorbance of the mixture was measured at 517 nm. Ascorbic acid was used as reference standard. The ability the extract to scavenge DPPH radical was calculated as: % DPPH inhibition = [(Absorbance of control - Absorbance of test) / (Absorbance of control)] × 100.

Hydrogen Peroxide Scavenging Capacity: Ability of extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989) [19]. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Extract in concentrations of 5, 10, 20, 40, 80, 160 and 320 µg/mL distilled water was added to hydrogen peroxide solution (0.6 mL, 40mM). After 10 minutes, absorbance of hydrogen peroxide was measured at 230 nm against a blank solution containing the phosphate buffer without hydrogen peroxide. Percentage of hydrogen peroxide scavenging by the extract and standard compound (alpha-Tocopherol) were calculated as % H_2O_2 scavenged = [(AC– AS)/AC] x 100, where AC is the absorbance of the control and AS is the absorbance of sample or standard. Amount of extract required to reduce the absorbance by 50% (IC_{50}) was calculated.

Nitric Oxide Radical Scavenging Assay: Nitric oxide radical scavenging capacity was determined using slightly modified method as described previously by F. Boora et al in 2014 [20]. We used extract in concentrations of 100, 200, 400, 800, and 1600µg/mL. Sodium nitroprusside (0.5 mL, 10 mM) in phosphate buffered saline was added to the extract or standard and incubated at 25°C for 180 minutes. Equal volume of freshly prepared Griess reagent was added in the tubes (Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use).

Absorbance was measured at 546 nm using a Spectrophotometer. Gallic acid was used as the positive control. Percentage inhibition of the test sample and standard was calculated using the formula: Nitric Oxide Scavenged (%) = [(Absorbance of control - Absorbance of test) / Absorbance of control] x 100. IC_{50} value was calculated which gives concentration of extract to reduce the production of NO radical by 50%.

Cell viability studies by MTT assay: Principle: MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt. In viable cells, mitochondrial dehydrogenase converts it to insoluble purple formazan. This water insoluble formazan is solubilized using DMSO and resulting purple solution is measured by spectrophotometer. It is accurate, cost effective, quick and reproducible test for cell viability studies [21].

In the present study, 5 human cancer cell lines viz. breast cancer (MCF7), ovarian cancer (PA1), colon cancer (HT29), skin melanoma (A375) and liver carcinoma (HepG2) and one normal mouse embryonic fibroblast cell line-NIH-3T3 were used. All the cell lines were procured from National Centre for Cell Sciences (NCCS), Pune. All the chemicals were procured from Hi Media Laboratories. Cells were cultured in minimal essential media supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) in humidified atmosphere of 5% CO_2 at 37°C until confluent.

Each cell line was tested for Mycoplasma contamination and found to be negative. Cells were dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS) and their viability was checked. Further, 50,000 cells/well were seeded in a 96 well plate and incubated for 24 hrs at 37°C in 5% CO_2 incubator. After 24 hours, when partial monolayer was formed, supernatant was flicked off and monolayer was washed once with medium. To this monolayer, 100µL of the extract or standard drug in concentration of 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL was added and plates were incubated at 37°C in 5% CO_2. Cytotoxic potential of the extract was evaluated after 24 hours of treatment. Standard drug of cancer treatment was used as positive control. For MCF 7, PA1, A375 and HepG 2 cell lines, cisplatin was used whereas for HT 29 cell line, 5-Fluorouracil was used as positive control.

After 24 hours of incubation, solutions in the wells were discarded and 100µL of MTT (5 mg/10 mL of MTT in PBS) was added to each

well. Plates were incubated for 4 hours at 37°C in 5% CO_2 atmosphere. Supernatant was removed and 100µL of DMSO was added and plates were gently shaken to solubilize formazan.

Absorbance was read using a microplate reader at 570nm. Cell viability was calculated by using the formula- Cell viability (%) = [(Absorbance of sample − Absorbance of blank) / (Absorbance of control − Absorbance of blank)] × 100. Direct microscopic observations of cell lines treated with extract was done with inverted microscope.

Statistical analysis: All the tests were performed in triplicates and values were expressed as mean ± SD of the absorbance. IC_{50} (concentration of extract needed to inhibit cell growth or to scavenge free radicals by 50%) values were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve by using GraphPad Prism 8 software.

Results

Phytochemical analysis: We obtained maximum yield of 13% in aqueous extract followed by 10% yield in ethanolic extract. Phytochemical evaluation of the extracts showed presence of flavonoids, phenolic compounds, saponins and carbohydrates in ethanolic and aqueous extracts.

Antioxidant activity: Figure 1 demonstrates free radical scavenging ability of the extract and its comparison with standard antioxidant. Ethanolic extract of S. cordifolia showed gradual dose dependent increase in DPPH radical scavenging activity (IC50 value 50.93µg/mL). Standard antioxidant, ascorbic acid, was more effective at the concentration of 40µg/mL and above (IC50 value 20.93µg/mL). Such boost in the activity was not observed with the extract (figure 1A). As indicated in figure 1B, ethanolic extract is very effective in scavenging H_2O_2 radicals (IC50; 85.14µg/mL). It showed similar trend that of α tocopherol (IC50; 33.35µg/mL). Figure 1C reveals that NO radical scavenging (IC50; 320.99 µg/mL) by the extract is comparable with that of quercetin (IC50; 112.55), the standard antioxidant.

Fig-1: Dose dependent free radical scavenging ability of ethanolic extract of S. cordifolia. A: DPPH radical scavenging by extract and ascorbic acid, B: H_2O_2 radical scavenging by the extract and α tocopherol, C: NO scavenging by extract and quercetin.

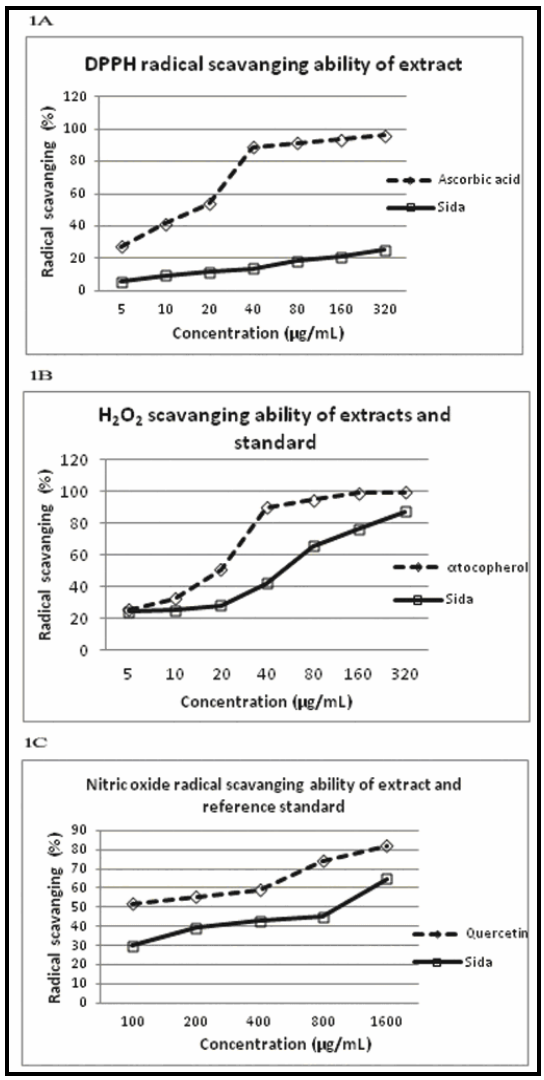


Fig-2: Comparison of H_2O_2 and DPPH radical scavenging ability of the extract.

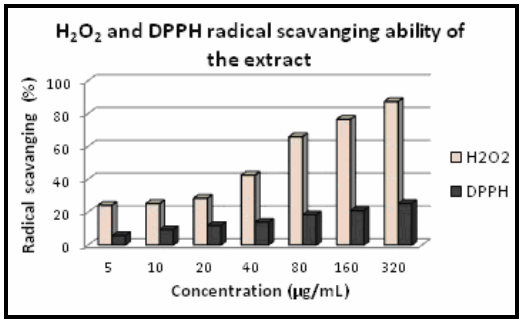


Figure 2 reveals that even though IC50 concentration of the ethanolic extract for DPPH scavenging is lower than that for H_2O_2 scavenging, at any given concentration, extract has better H_2O_2 radical scavenging ability.

Antiproliferative activity: Figure 3 demonstrates that ethanolic extract of S. cordifolia exhibited dose dependent cytotoxicity against all the cancer cell lines used in this study. It also indicates that A375 i.e. human skin melanoma cell line was maximally sensitive to ethanolic extract of S. cordifolia with IC 50 value of 16.51µg/mL. HT29 i.e. human colon cancer cell line was also moderately sensitive to the ethanolic extract of S. cordifolia (IC50-49.86µg/mL). Figure also indicates that cytotoxicity of ethanolic extract of S. cordifolia is minimal against normal human fibroblast (NIH-3T3) cell line (IC50 >100µg/mL). But still the extract is found to be more toxic to the fibroblast than to MCF7 cell line at all the concentrations used in this study. PA1 and HepG 2 cell lines showed better response at 100µg/mL concentration.

Figure 4 shows comparative response of cell lines when tested with increasing dosage of extract and the standard drug used in treatment of cancer. Figure 4A indicates that antiproliferative effect of ethanolic extract is very similar to that of cisplatin, the standard anticancer drug, in A375 cell line. Ethanolic extract of S. cordifolia also shows similar response to that of 5fluorouracil in HT29 cell line (figure 4B). Extract has moderate antiproliferative effect in HepG2 and PA1 cell line when compared with that of cisplatin (figure 4C and 4D respectively). It is clear from figure 4E that MCF7 cells show more viability than that of cisplatin. Thus, it requires higher concentration of the extract to be effective in killing the breast cancer cells (figure 4E).

Fig-3: Effect of 24-hour treatment by the ethanolic extract of S. cordifolia on cell viability of various cell lines. MCF7: Breast cancer; PA1: Ovarian cancer; HT29: Colon cancer; A375: skin melanoma; HepG2: Liver cancer and NIH 3T3: Mouse embryonic fibroblast.

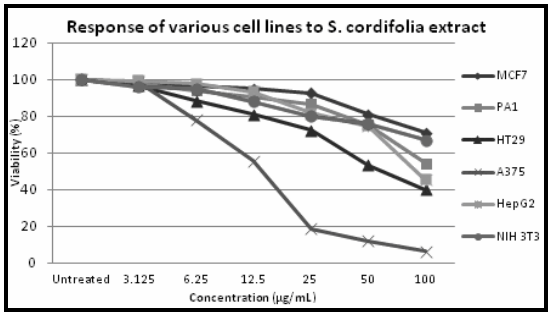
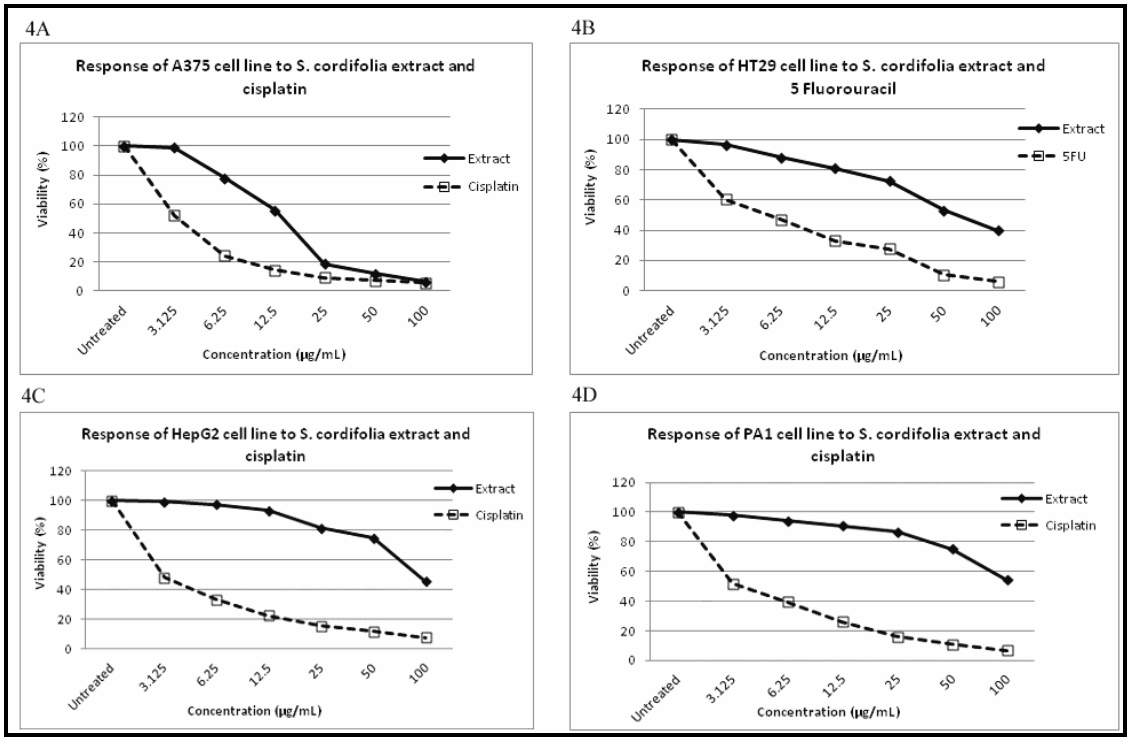


Fig-4: Comparison of the cancer cell line response to the increasing concentration of extract and standard drug. A: A375 (Melanoma) cell line; B: HT29 (Colon cancer) cell line; C: HepG2 (Hepatic cancer) cell line; D: PA1 (Ovarian cancer) cell line; E: MCF7 (Breast cancer) cell line. Note that A375 cell line response is very similar to that of cisplatin, the standard anticancer drug used in treatment.



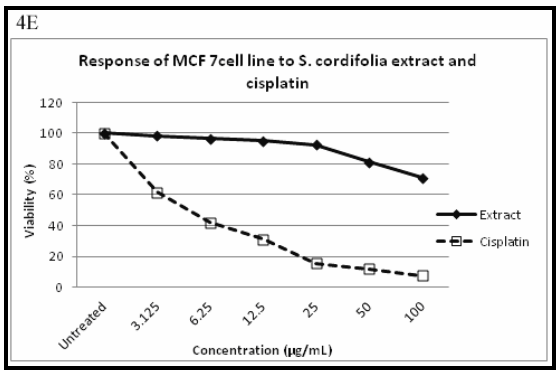


Table 1 gives IC50 values of extracts for various cell lines. Concentration required to inhibit cell growth by 50% (IC50) is very less in case of A375 cell line and moderate in HT29 cell line. For all other cell lines >100µg/mL concentration is necessary to inhibit proliferation by 50%.

Table-1: IC50 values of various cell lines when treated with increasing concentration of S. cordifolia extract

Cell Line	IC50 (µg/mL)
MCF7	>100
PA1	>100
HT29	49.86
A375	16.51
HepG2	>100
NIH-3T3	>100

Discussion

Genus Sida is well known for its medicinal properties and is one of the important plant sources in Indian traditional medicine [8]. Few studies have evaluated antioxidant activities of S. cordifolia [22- 23]. Dhawal et. al. have shown that ethanol extract of roots of S. cordifolia (1000µg/mL) possesses maximum DPPH radical scavenging activity whereas extract from the entire plant is less effective in scavenging DPPH radicals. But in our study, we found that IC50 value for DPPH radical scavenging ability of the extract was 50.93µg/mL.

Ethanolic extract of S. cordifolia showed dose dependent hydrogen peroxide radical scavenging ability with IC50 value of 85.14µg/mL. It is said that total phenolic content and flavonoid content of the extract determine antioxidant property of any extract [23]. Phytochemical analysis of the ethanolic extract has revealed presence of phenols and flavonoids. These compounds may be responsible be antioxidant property of the extract. Based on these findings, we can say that ethanolic extract can be the good source of natural antioxidants.

As shown in figure 3 and 4, extract possesses dose dependent cytotoxicity against all the cell lines used in this study. Amongst all the cancer cell lines, extract was most effective against A375 followed by HT29 cell line. One of the mechanisms of cancer development is oxidative stress to the cells. Free radicals cause DNA damage and mutations in the cells leading to cancerous growth of the cells. As S. cordifolia extract possesses good antioxidant activity this may be one of the reasons for its antiproliferative property. It is said that antioxidants modulate activity of protein kinases which in turn alter cell signalling pathways and prevent cytotoxicity [18].

Ethanolic extract of S. cordifolia contains polyphenols and flavonoids which are said to exhibit strong anticancer activity [18, 24]. According to Taka-Aki Matsui et al alkaloid, called cryptolepine from S. cordifolia, causes G2/M arrest by upregulating p21 signalling pathway, independent of p53 in osteosarcoma cell line. They have also showed that this cell cycle arrest was inhibited in HCT116 cell line with p21 gene knockout. Thus, activation of p21 may also be the reason for antiproliferative action seen in melanoma and HT29 cell lines in our study [16].

On microscopic examination, cells also demonstrated apoptotic changes in the form of swelling and presence of membrane blebbing. This may be due to the presence of active principles like cryptolepine in the extract. According to the guidelines of US National Cancer Institute (NCI) for plant screening program, extract with IC50 value of < 30 µg/mL after 72 hours of treatment is considered as a promising source for purification of a crude extract [25-26].

Since IC50 value of the ethanolic extract of S. cordifolia after 24 hours of treatment is

16.51µg/mL in melanoma cell line and 49.86µg/mL in colon cancer cell line, it can be the promising candidate for further studies.

Limitation and future scope: This is the preliminary study done to assess whether S. cordifolia extract is effective against cancer cell growth. As it demonstrated dose dependent inhibition of viability of cancer cell lines, further studies can be planned to understand mechanism of action of extract. Future in vivo studies on melanoma and colon cancer can throw light on efficacy of the extract in natural environment in presence of various bodily reactions.

Conclusion

Our results revealed that ethanolic extract of S. cordifolia possesses good antioxidant activity hence it can be a good source of natural antioxidant. Present study also demonstrated that ethanolic extract of S. cordifolia is effective in preventing cancer cell proliferation in dose dependent manner. Extract is most effective against human melanoma and colon cancer cell lines and can be the potential source of anticancer agent.

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Conflicts of interest: There are no conflicts of interest.

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