International Journal of Pharmacy

www.ijpjournal.org

e-ISSN: 2249 – 7684
Print ISSN: 2249 – 7692

IN-VITRO ANTI BACTERIAL AND EX VIVO CYTOTOXICITY STUDIES OF AERIAL PARTS OF CYPERUS IRIA L

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ABSTRACT

Cyperus is the second largest genus of the sedges or cyperaceae family. Cyperus iria (also known as Rice Flat Sedge) is a smooth, tufted sedge found worldwide. It's an important and widespread weed in south and southeast Asia. The plant is astringent, stimulant, stomachic and tonic. It is used to treat amenorrhoea. The whole plant is used to treat rheumatism and to regulate menstruation. The rhizome is used as a diuretic. A decoction of the ground tubers is used for treating fevers. The objective of present study is pharmacognostical, phytochemical and pharmacological (invitro antimicrobial & exvivo cytotoxicity) studies on aerial parts of Cyperus iria. The extracts of Cyperus Iria.L showed the presence of alkaloids, phenols, steroids, glycosides, flavonoids, terpenoids, carbohydrates and saponins. The extracts obtained were utilized for ex-vivo cytotoxicity studies by Daltons Lymphoma Ascites method. The chloroform extract found to have significant cytotoxicity in mice carcinoma cells. Antimicrobial activity by Disc Diffusion method was performed against both gram positive and gram-negative organisms. The methanolic extract shows significant antimicrobial properties. The phytochemical studies gave conformation of the above said results. The pharmacological studies gave supportive results to affirm the above said activities.

Keywords: Cyperus iria, Cyperaceae, Sedges, Weed, Cytotoxicity, Anti-microbial.

INTRODUCTION

The objective of each research is to produce a potent, inexpensive, safe drug moiety from herbs can be met to some extend by promoting isolation of the active ingredients or synthesising a new entity from the natural compounds strictly under platform of a traditional system of medicine. Moreover, the studies focused towards proper standardisation and formulation of a suitable dosage form in due consideration of their therapeutic toxicity ratio will, however, be necessary with controlled clinical trials to prove their efficacy. Plants affects different facts of life such as cultural, economical, medical and spiritual [1]

The history of herbs is as long as the history of mankind. People have used these plants for medicinal purposes since the earliest time, and the knowledge of herbs has been handed down generation to generation for thousands of years. The term "herb" are plants, some parts of which contain essential oils useful in food, medicine, and/or cosmetics and which usually grow in temperate regions, both in the wild and under cultivation. They do not develop persistent woody tissue [2-5]

The phytochemical studies include extraction and isolating compounds from the origin plant, followed by defining their structure and therapeutic potential in laboratory model systems, such as cell cultures, in vitro experiments, or in vivo studies using laboratory animals. Challenges in that field include isolating specific compounds and determining their structures, which are often complex, and identifying what specific phytochemical is primarily responsible for any given biological activity [6-8].

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Antimicrobial activity has been demonstrated *in vitro* for several herb and spice extracts and essential oils from thyme, oregano, parsley, cilantro, and cinnamon. Growth of several bacteria strains has been shown to be inhibited by various concentrations of these culinary herb and spice extracts in the culture medium. Antimicrobial phytochemicals fall into several categories: phenolics/polyphenols, terpenoids and essential oils, alkaloids, and lectins and polypeptides. Phenolic and polyphenols possess multiple antimicrobial mechanisms of action when compared to other phytochemical categories [9]. They are capable of forming an irreversible complex with nucleophilic amino acids in protein, leading to their inactivation and therefore loss of function in bacteria. Phenolic and polyphenols have been reported to disrupt microbial membranes, inactivate microbial enzymes, and activate immune response by stimulating macrophages [10,11].

MATERIALS & METHODS

SUCCESSIVE SOLVENT EXTRACTION

Successive solvent extraction of the dried powder of aerial parts of *Cyperus iria* was carried out by using solvents of increasing polarity viz. petroleum ether, chloroform, acetone, methanol and water. Around 18.6 g of dried powder was weighed, moistened with the respective solvent and packed in the soxhlet extractor and was then extracted with 500 ml each of the petroleum ether, chloroform, acetone, methanol and water. After each extraction, the same dried marc was used for the subsequent extraction. Each extract was then filtered, the solvent distilled off and finally the dried extract was obtained. The percentage yield of each extract was calculated. These extracts were subjected to preliminary phytochemical screening [12].

Thin Layer Chromatographic Analysis

TLC was introduced by Izmailov and Schriber in 1938. Separation of individual components on TLC plate is due to adsorption/partition chromatography or a combination of these two processes. TLC is a very sensitive technique and requires very little amount of substance for analysis. Selection of solvent for TLC is based upon its polarity. The developing period is relatively short, 15 to 60 seconds. The TLC plate can be heated to higher temperature and aggressive reagents can be sprayed on the plate for identification [13, 14]. The movement of chemical compounds relative to solvent front in a chromatographic system is constant. The relative movement is called the retardation factor represented as Rf value, and it is calculated as follows.

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

Sample preparation

The plant extracts dissolved in suitable solvent, filtered.

TLC plates

For this study TLC plates were prepared with silica gel and activated in hot air oven for 30 minutes at the temperature of 105˚C.

Spotting of sample

The sample was spotted using a capillary tube on TLC plates 2 cm above from the bottom of the plate.

Selection of a mobile phase

The selection of a mobile phase and detecting agents depends upon nature of active principles in each extract.

Thin layer chromatography study was tried on the chloroform extract of *C.iria* using different mobile phases. The stationary phase used was silica gel G and the detection by iodine chamber method and vanilline sulphuric acid reagent.

The different solvent phases tried include:

- Ethyl acetate: methanol (9:1)
- Ethyl acetate: methanol: water (8:1.5:0.5)
- Chloroform: glacial acetic acid: methanol: water (5:4:1:2)
- Chloroform: Methanol: acetone (8:1:1)
- Ethyl acetate: chloroform (7.5:2.5)

Antimicrobial Activity

Agar disc diffusion method

Antimicrobial activity was determined by agar disc diffusion method using Ciprofloxacin as standard. Activity of petroleum ether, chloroform, acetone, methanol and water extract was tested against *Staphylococus aureus, Enterococcus, Klebsiella and, Escherichia coli*

MEDIA: Muller-hinton agar {Hi media)

Ingredients

Beef infusion	-300gm/ litre
Casein acid hydrolysate	- 17.50
Starch	- 1.50
Agar	- 17.06

Preparation

The ingredients were dissolved in distilled water with the aid of heat and pH was adjusted to 7.2-7.6 using alkali or dilute acid [15, 16].

Sterlization

A solution of 10- 15ml of Muller – Hington agar (Hi media) was transferred to tubes and sealed with non-absorbent cotton. It was then autoclaved at a temperature of 121˚C and a pressure of 15 psi for not less than 20 min.

- Drug used: various extracts of *Cyperus iria* at a concentration of 200 and 400 µg/ml
- Standard: Ciprofloxacin (50 µg/ml)
- Vehicle: DMSO (dimethyl sulfoxide)
- Culture used: *Staphylococus aureus* NCIM 5201, *Enterococcus* NCIM 5318, *Klebsiella* NCIM 2957 *and, Escherichia coli* NCIM 2027 were collected from the national chemistry laboratory, Pune and stored in our pharmaceutical biotechnological laboratory

Maintenance of culture

The selected strains were confirmed for their purity and identity by Gram staining method and by their characteristic biochemical reaction. The selected strains were preserved by sub culturing them periodically on nutrient agar slant and storing them under frozen condition. For the study fresh 24-hour broth cultures were used after standardisation of culture.

Standardization of bacterial cultures

The organisms were grown overnight (24 hours) at 37˚C on nutrient agar and harvested during the stationary growth phase. For preparing fresh cultures a loopful of cells from the stock cultures were added to the nutrient broth and incubated for 24 hours at 37˚C. Inoculum was standardized by matching the turbidity of culture to 0.5 McFarland standards. This was produced by mixing 0.5ml of 0.048 M $BaCl_2$ (1.175% w/v Barium Chloride dehydrates) with 99.5 ml of 0.36N H_2SO_4. This produced an inoculating suspension of approximately 2.0×10^6 (CFU/ml) for bacteria [17].

Method

Muller – Hington agar plates were prepared aseptically to get a thickness of 5-6 mm, the plates were allowed to solidify and inverted to prevent condensate falling on the agar surface. The test organism was spreaded on Muller – Hington agar plates by Streak plate method. Now, disc of various concentration of sample (200µg/ml and 400µg/ml) and standard (50 µg/ml) were placed on agar plates. The plates were incubated for 18 – 24 hours [18]. Observations were made for zone of inhibition of various extracts of *C.iria* against *Staphylococus aureus & Enterococcus* (Gram positive) and *Klebsiella & Escherichia coli* (Gram negative) was determined and tabulated in table no: 5.14

***Ex Vivo* Cytotoxicity Studies**

The test samples were studied for short term *ex vivo* cytotoxicity using Dalton's lymphoma ascites (DLA) cell.

Dalton's Lymphoma Ascites

Varying concentrations of extracts were prepared (12.5, 25, 50, 100 & 200 µg/ml). The cancer cells were aspirated from the peritoneal cavity of cancer bearing mice and were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method (cell viability should be above 98%). Different dilutions of 10^{-1}, 10^{-2}, 10^{-3} were made. The number of cells in the 10^{-3} dilution was counted using a haemocytometer and the cell number was adjusted to 1×10^7 cells/ml. The experiment was setup by incubating different concentration of the drug and standard with 1×10^{-6} cells [19]. The final volume of assay mixture was made upto 1ml using phosphate buffer saline (PBS) incubated for 3 hrs at 37^0C. Cyclophosphamide was used as a standard drug (12.5, 25, 50, 100 & 200µg/ml). Control tube contained only cell suspension. After incubation the cell suspension was mixed with 100µl of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye [20]. The number of stained and unstained cells were counted separately.

10 % cytotoxicity = (No. of dead cells/ No. of live cells + No. of dead cells) × 100

RESULTS AND DISCUSSION

TLC of Chloroform Extract of *Cyperus Iria*

TLC of chloroform extract using solvents Chloroform: Methanol: Acetone (8:1:1), the solvent system suitable as a screening system for the TLC investigation of alkaloids and flavonoid glycosides. The solvent system showed good separation and three yellowish green clear spot. The results of the TLC analysis of chloroform extract are tabulated below in table no 1 and fig no. 1

Antimicrobial Studies

Agar disc diffusion method

The antimicrobial activity of petroleum ether, chloroform, acetone, methanol and aqueous extracts of *Cyperus iria* was determined using Agar Disc Diffusion method. The antimicrobial activity was tested against two gram-positive organisms namely *Staphylococcus aureus* and *Enterococcus* and two gram-negative organisms namely *Escherichia coli* and *Klebsiella.* 200µg/ml and 400µg/ml concentrations of each sample and 50µg/ml of standard was taken for the assay. Ciprofloxacin was used as standard. The zone of inhibition of the extracts was compared with that of standard Ciprofloxacin. The result shows that all the extract shows limited inhibition of the bacterial growth against the tested organisms, in which chloroform, methanol and acetone shows moderate activity. The results are depicted in table no 2 and fig no. 2-5 respectively.

The results were interpreted as per Agar Disc Diffusion Method (17mm & above: sensitive, 13-16mm: moderate sensitive, less than 12mm: resistant).

***Ex-Vivo* Cytotoxicity Studies**

Dalton's Lymphoma Ascites Method

Cytotoxicity screening was done by using chloroform, acetone, methanol and aqueous extracts. Varying concentrations of extracts 12.5, 25, 50, 100 and 200µg/ml was taken. Cyclophosphamide was used as the standard drug in concentrations of 12.5, 25, 50, 100 and 200µg/ml. Control tube contained only cell suspension. The result of cytotoxicity screening confirms that chloroform extract shows higher activity when compared with cyclophosphamide as standard. From the research study, gives a scientific basis for the therapeutic activity of *Cyperus iria* plant for raises its medicinal value in anticancer therapy and it shown in table no 3 and figure no 6

IC_{50} value was calculated for each extract and standard from the graph. Among them significant 50% inhibition was shown by chloroform extract when compared with the concentration of standard drug. The values are shown in table no 4.

Table 1: Results of the TLC analysis of chloroform extract of Cyperus iria

Sl. no	Solvent system	No. of spots	Detection	Colour of spot	Rf value
1.	Chloroform: Methanol: Acetone	3	Iodine chamber	Yellow	0.25 (Spot 1) 0.59 (Spot 2) 0.64 (Spot 3)

Table 2: Results showing the antimicrobial activity of various extracts of C. iria using Agar Disc Diffusion Method

Sl. No	Organisms	Diameter of the zone of inhibition (mm)										
		Standard (µg/ml)	Petroleum ether (µg/ml)		Chloroform (µg/ml)		Acetone (µg/ml)		Methanol (µg/ml)		Aqueous (µg/ml)	
		50 (S)	200 (a)	400 (b)	200 (a)	400 (b)	200 (a)	400 (b)	200 (a)	400 (b)	200 (a)	400 (b)
1	*Staphylococcus aureus*	27	6	6	9	11	8	6	9	10	5	6
2	*Enterococcus*	25	6	6	12	14	10	14	12	16	12	13
3	*Escherichia coli*	20	3	3	8	10	6	10	6	6	3	3
4	*Klebsiella*	28	3	3	13	15	5	6	10	12	9	9

Table 3. Results showing the percentage cell death of various extracts of Cyperus iria by Dalton's Lymphoma Method

Sl No.	Drug concentration (µg/ml)	Percentage Cell Death (%)				
		Standard	Chloroform	Water	Acetone	Methanol
1	12.5	17	11	4.67	4.59	4.59
2	25	30	25	4.67	4.61	4.59
3	50	45	41	4.65	7.22	4.6
4	100	85	77	8.34	11.6	10.4
5	200	120	100	15	20.4	16.2

Table 4: Results showing IC50 value of various extracts of Cyperus iria by DLA cytotoxicity screening

Sl. No	Sample	IC_{50} (µg/ml)
1	Standard	74.59
2	Chloroform	89.30
3	Water	628
4	Acetone	461.25
5	Methanol	568.82

Figure 1: TLC of chloroform extract of Cyperus iria

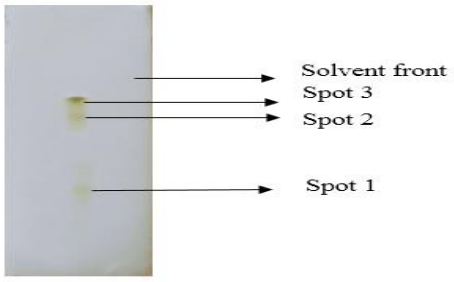
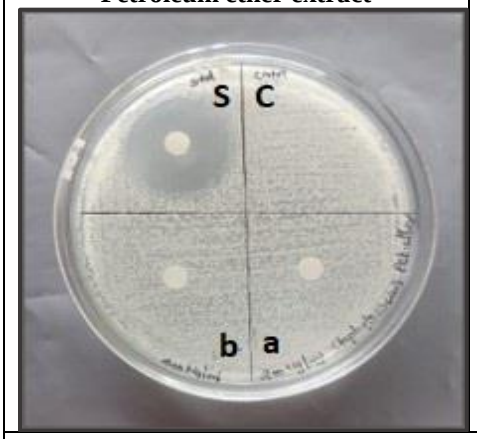
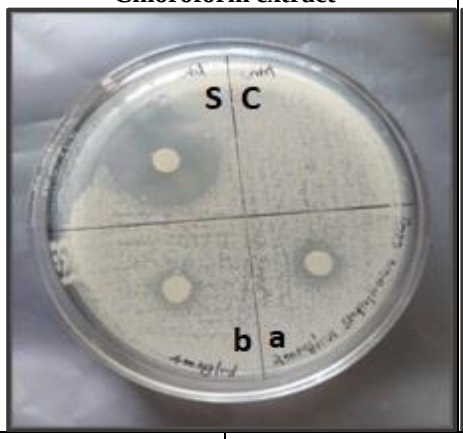


Figure 2: Antimicrobial activity of various extracts of aerial parts of Cyperus iria against gram positive Staphylococcus aureus Organism: Staphylococcus aureus

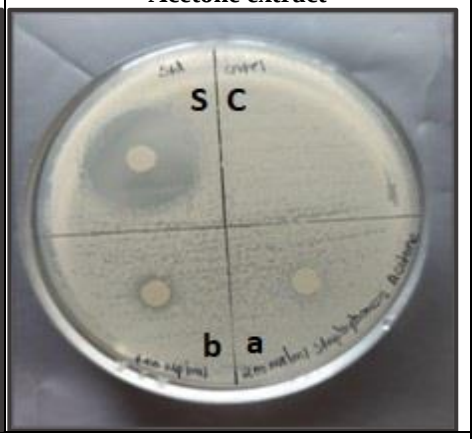
Petroleum ether extract



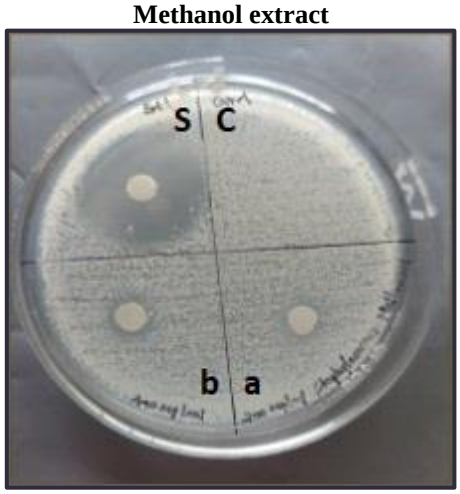
Chloroform extract



Acetone extract



Methanol extract



Aqueous extract

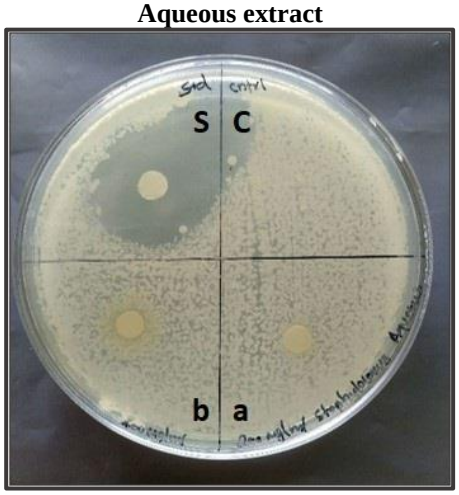
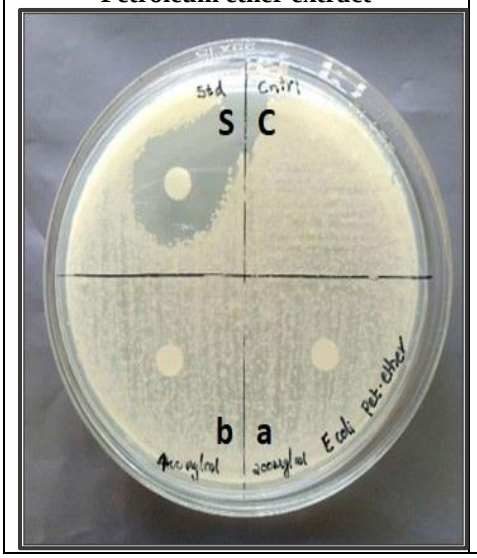
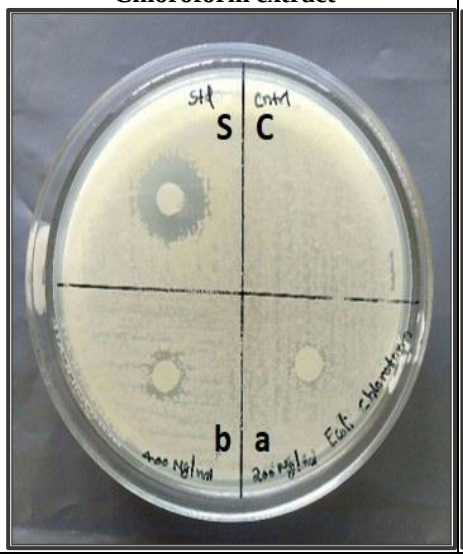


Figure 3: Antimicrobial activity of various extracts of aerial parts of Cyperus iria against gram positive Enterococcus Organism: Escherichia coli

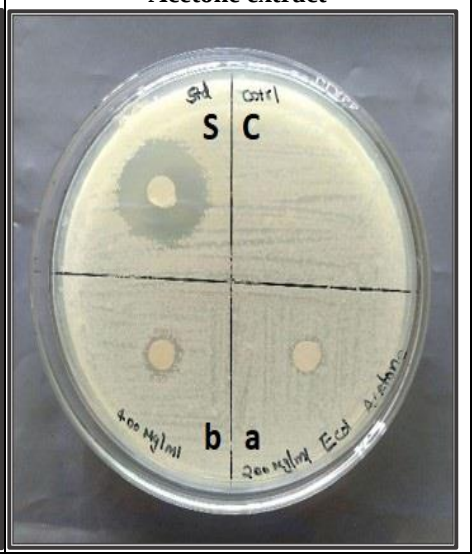
Petroleum ether extract



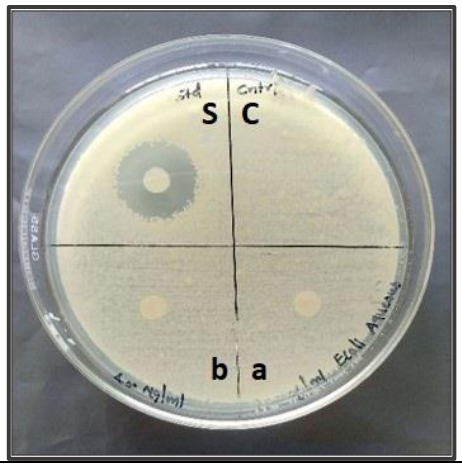
Chloroform extract



Acetone extract



Methanol extract



Aqueous extract

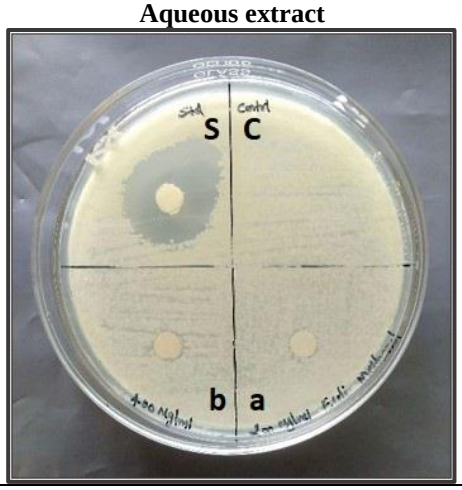
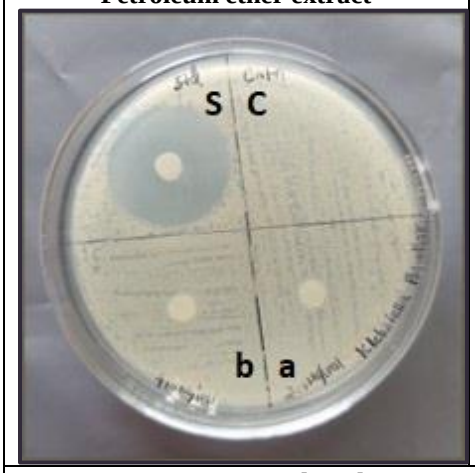
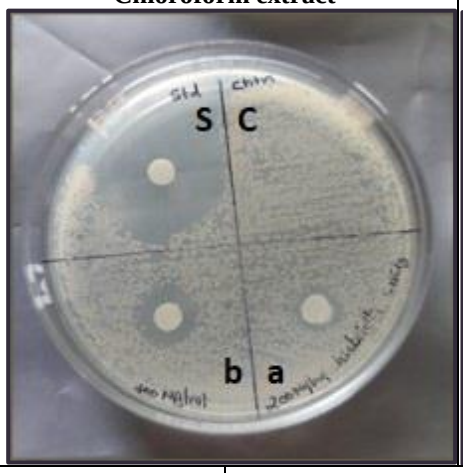


Figure 4: Antimicrobial activity of various extracts of aerial parts of Cyperus iria against gram negative Escherichia coli Organism: Klebsiella

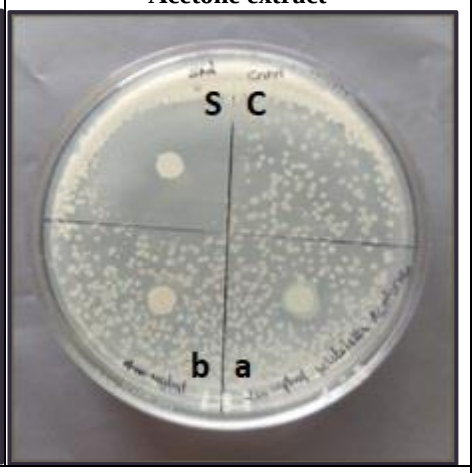
Petroleum ether extract



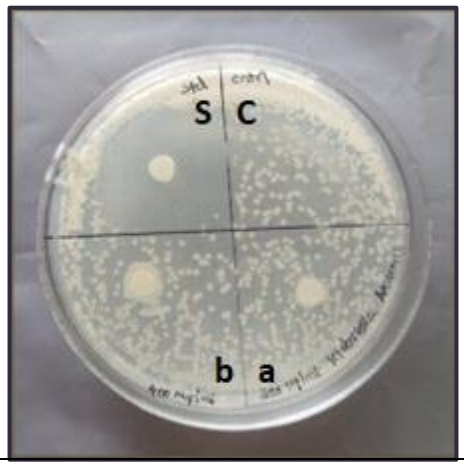
Chloroform extract



Acetone extract



Methanol extract



Aqueous extract

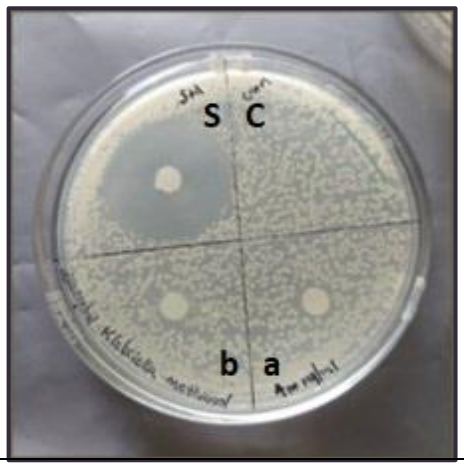
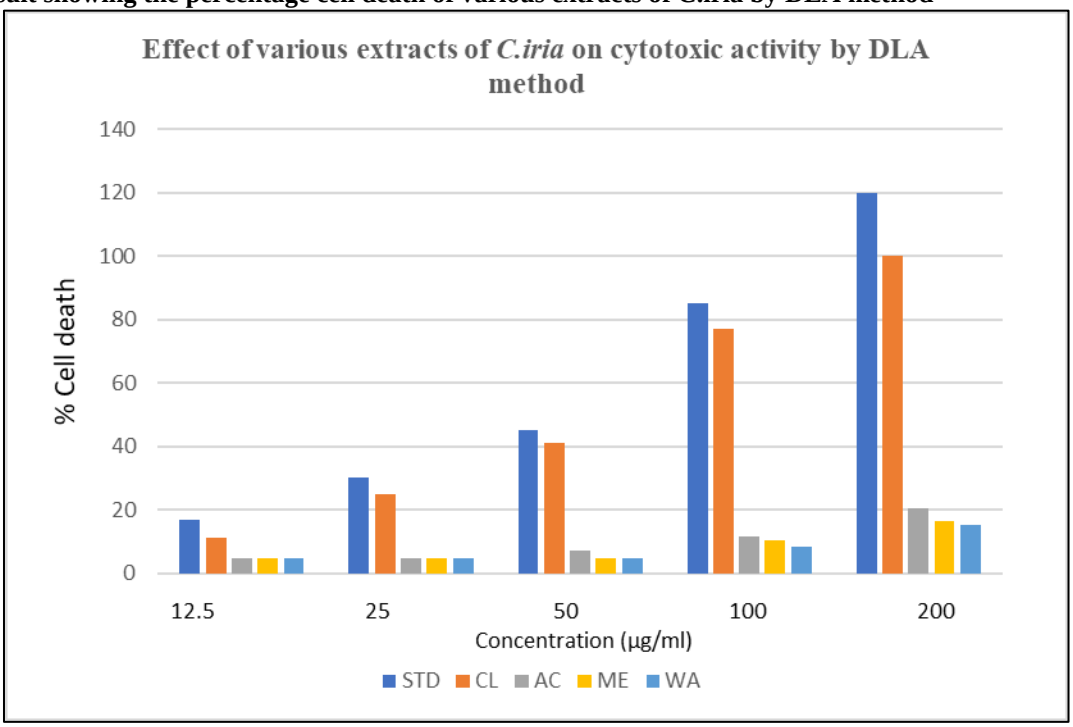


Figure 6: Result showing the percentage cell death of various extracts of C.iria by DLA method



CONCLUSION

The powdered drug material was then subjected to successive solvent extraction using solvents of increasing polarity viz. petroleum ether, chloroform, acetone, methanol and water. After each extraction, the same dried marc was used for the subsequent extraction. Each extract was filtered, the solvent distilled off and finally the dried extract was obtained. These extracts were used for further phytochemical screening and pharmacological studies. The phytochemical studies reveal the presence of alkaloids, carbohydrates, glycosides, flavonoids, phenolics, steroids, terpenoids and saponins.

From the TLC analysis of chloroform extract by using solvent system like Chloroform: Methanol: Acetone (8: 1: 1) showing a good separation and three clear spots with Rf value 0.25, 0.59 and 0.64 respectively.

Antimicrobial screening was carried out by disc diffusion method using various extracts with both gram-positive organisms namely *Staphylococcus aureus* and *Enterococcus* and gram-negative organism namely *Escherichia coli* and *Klebsiella.* Petroleum ether, chloroform, acetone, methanol and aqueous extracts in a concentration of 200 and 400µg/ml were used for antimicrobial screening. 50µg/ml of ciprofloxacin was taken as standard. The result shows only limited anti-bacterial activity against both gram-positive and gram-negative organism. Among the five extracts the chloroform and methanol extract shows more activity than other three extracts when compared to standard Ciprofloxacin. In total all the extracts were found to be less sensitive to both gram-positive and gram-negative organism.

Ex vivo cytotoxic screening was carried out by Dalton's Lymphoma Ascites method using four extracts viz. chloroform, acetone, methanol and water extract. The result of cytotoxicity screening shows that the chloroform extract possesses higher activity than the other three extracts when compared with standard Cyclophosphamide.

As a whole it can be concluded that the aerial parts of plant *Cyperus iria* on which not much work has been carried out, by performing in-vitro antimicrobial and ex-vivo cytotoxicity screening revealed that the drug has a significant role in cancer treatment. Hence further study is focused towards the isolation and in-vivo studies of the active principle of the plant which is responsible for the claimed use. Previous reports also confirm the said activities as some of the compounds/similar compounds present in this drug have already been seen in other herbal drugs with the above said effects.

Cyperus iria is an annual perhaps perennial plant in favorable circumstances and its sub species reported the rich content of alkaloids, flavonoids, phenolics, glycosides, steroids, terpenoids and saponins that may be responsible for antioxidant, antibacterial and cytotoxic effects. The phytochemical studies give confirmation of the above said results. The pharmacological studies give supportive results to affirm the above said activities.

therapeutical potential of *Cyperus iria* plant and raises a scientific tool for developing a novel drug development for cancer therapy. In future, further study is required for isolation of active constituents responsible for the plant's medicinal value and also done in vivo studies for novel drug therapy.

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