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Preliminary screening of *Cuscuta reflexa* stems for Anti inflammatory and cytotoxic activity

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ABSTRACT

Objective: Evaluation of preliminary antiinflammatory and cytotoxic activities of a parasitic plant *Cuscuta reflexa*. **Methods:** Human red blood stabilizing activity was used for the evaluation of in vitro antiinflammatory activity and Brine shrimp lethality assay was used to assess the cytotoxic potential of extract of stems of Cuscuta reflexa. **Results:** Methanolic extract and Ethyl acetate fraction of methanolic extract of *Cuscuta reflexa* (MECR & EAMECR resp.) were found to have significant antiinflammatory and cytotoxic activity with inhibitory concentration IC50% values 277.83 μg/ml & 214.94 μg/ml in HRBC stabitlity assay[table 01], and lethal concentration LC50% 257.73 μg/ml 184.86 μg/ml in BSLA respectively[table 02]. **Conclusions:** Amongst various extracts evaluated for cytotoxicity and antiinflammatory activities, methanolic extract of *Cuscuta reflexa* (MECR) and its ethyl acetate soluble fraction (EAMECR) show significant cytotoxic as well as antiinflammatory activities which may be due to the presence of phenols, polyphenols and flavonoids.

1. Introduction

According to the recent research, various stages of pathogenesis of the cancer like initiation, promotion, malignant conversion, invasion, and metastasis involves a decisive role of inflammatory reactions [1]. There are well documented evidences that progression of neoplasm involves the interplay of various inflammatory reactions and mediators [2]. Genesis of the cancerous cell and its metastasis has been found to involve chronic inflammation and this is a hallmark of cancer [2, 3]. And hence Compounds that can block or alter inflammatory reactions can have potential in the management, prevention and treatment of cancer [1, 4]. It can be assumed that drugs which inhibit inflammation may induce apoptosis in some cancerous cells and can be useful as a preventive measure as well as therapy [1].

Cuscuta reflexa (Dodder, family- Convolvulaceae) is well known as Amarwel in Ayurveda. It is a parasitic climber found commonly throughout India. The plant has no root under the ground but only grows as a parasite twiner on other plants and hence called as 'Akaswel' (Sky twiner) or Amarwel (Immortal twiner), because it grows during the rains and every year the growth is fresh on the same plant [5].

Cuscuta reflexa has been used from ancient times, for various purposes viz. as apurgative,in the treatment of liver disorders, cough and itching, and for its carminative and anthelmintic actions [6] . The parasite is known to contain several antibacterial, antiviral and antiproliferative substances [7-9].The plant is known to contain compounds like phenolics and flavonoids [10, 11], and since flavonoids exhibit antiinflammatory and anticancer activities, we decided to test the possible antiinflammatory and cytotoxic effects of a extracts of *Cuscuta reflexa* by means of HRBC membrane stabilizing method and brine shrimp cytotoxicity assay.

2. Materials and methods

2.1. Plant material

Stems of *Cuscuta reflexa* growing on the plants of Ziziphus were collected from local region of Igatpuri, District Nasik in the month of February. Plant material was identified with the help of local community and was authenticated by

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Dr.P.G. Diwakar from Botanical Survey of India, Pune (Ref no. BSI/WC/Tech/2010/374).

2.2. Preparation of extract

Plant material was separated from the host plant, cleaned, and dried under shade followed by pulverization. The coarse plant material was subjected to successive extraction method with solvent sequence petroleum ether, chloroform, and methanol. Ethylacetate fraction of Methanolic extract was obtained by partitioning.

2.3 In vitro antiinflammatory activity Human red blood cell (HRBC) membrane stabilizing activity [12, 13]

This method evaluates the membrane stabilizing activity of various agents on Red blood cells against the osmotic pressure exerted by Alsever solution. Alsever solution is prepared by dissolving 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% of sodium chloride in distilled water followed by sterilization. Blood was collected from Arpan Blood Bank, Nashik, M.S. The collected blood was mixed equal volumes of Alsevers solution. The blood was centrifuged at 3000 rpm and the packed cells were washed with isosaline and 10% (v/v) suspension was made. The drug samples ranging from a concentration of 50 μg/ml –250 μg/ml were prepared by suspending the residue in hot water. The assay mixture contained the drug, 1 ml phosphate buffer, 2 ml hyposaline (0.25% w/v), 0.5 ml HRBC suspension. Diclofenac sodium was used as the reference drug and 2 ml of distilled water as control. All the assay mixtures were incubated at 37℃for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 560nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %. Percentage of protection was calculated using the following equation

2.4. Brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay is considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and cytotoxicity testing of drugs. It can also be extrapolated for cell-line toxicity and anti tumor activity [14, 15].The in-vivo lethality in a simple zoological organism such as the brine shrimp, developed by Meyer et al, was used to evaluate cytotoxic activity. Brine shrimp eggs were collected from Department of Fisheries, Government of Maharashtra, India. Brine shrimp eggs were placed in artificial sea water (3.8% w/v NaCl in distilled water) and incubated at 24–28 ℃. Eggs were hatched for 48 hours providing large number of larvae (nauplii). Ten nauplii were placed in 5 mL of sea water and different concentrations were prepared and placed in vials. Alive nauplii were counted after 24 hours and lethal concentration (LC50) was calculated.

2.5. Statistical analysis

Each value represented the mean ±SEM of 3 consistent readings. The significance of the differences between controls and, tests were analyzed using analysis of variance followed by Dunnet multiple comparison test. Values of P<0.01are indicated by subscript 'a' and; Values of $P<0.05$ are indicated by subscript 'b' when compared with control.

3. Results

3.1. In vitro antiinflammatory activity (HRBC Stabilization)

The membrane stabilizing activity of Methanolic extract of *Cuscuta reflexa* and its Ethyl acetate fraction possess significant membrane stabilizing activity than the Pet. Ether and chloroform extracts when compared with the control group. Inhibitory concentration IC50% values ranged from 393.60 to 214.94 with values for diclofenac, MECR and EAMECR being 121.68 μg/ml, 277.83 μg/ml & 214.94 μg/ml respectively [table 01].

Table 1.
In vitro antiinflammatory activity Human red blood cell (HRBC) membrane stabilizing activity –Percent protection of membrane at various concentrations of different extracts. PECR– Petroleum ether extract of Cuscuta reflexa, CECR– Chloroform extract of Cuscuta reflexa, MECR– Methanolic extract of Cuscuta reflexa, EAMECR– Ethyl acetate fraction of methanolic extract of Cuscuta reflexa. Figures in right column indicate Effective concentration in50 % population, i.e. EC 50% μg/ml.

Sample	Conc. (μg)	O.D.Mean±SEM	% Protection	EC_{50} μg/ml
Control	––	0.912±0.012	––	
Diclofenac Sodium	50	0.593±0.006	34.98a	121.68
	100	0.496±0.007	45.62 a	
	150	0.406±0.008	55.49 a	
	200	0.305±0.005	66.56 a	
	250	0.199±0.008	78.18 a	
PECR	50	0.789±0.005	13.49 a	373.26
	100	0.722±0.005	20.84 a	
	150	0.675±0.007	25.99 a	
	200	0.627±0.004	31.25 a	
	250	0.585±0.007	35.86 a	
CHCR	50	0.770±0.008	15.58 a	393.60
	100	0.716±0.005	21.50 a	
	150	0.692±0.004	24.13 a	
	200	0.622±0.009	31.80 a	
	250	0.588±0.009	35.53 a	
MECR	50	0.729±0.004	20.07 a	277.83
	100	0.673±0.009	26.21 a	
	150	0.617±0.007	32.35 a	
	200	0.553±0.007	39.37 a	
	250	0.482±0.003	47.15 a	
EAMECR	50	0.686±0.007	24.79 a	214.93
	100	0.619±0.004	32.13 a	
	150	0.536±0.003	40.68 a	
	200	0.469±0.001	48.58 a	
	250	0.414±0.004	54.61 a	

Values expressed are mean±SEM, n=03, a– $P<0.01$and; b– $P<0.05$ with control. O.D.=Optical Density.

Table 2.
BSLA–Percent mortality at various concentrations of different extracts.
PECR– Petroleum ether extract of Cuscuta reflexa, CECR– Chloroform extract of Cuscuta reflexa, MECR– Methanolic extract of Cuscuta reflexa, EAMECR– Ethyl acetate fraction of methanolic extract of Cuscuta reflexa. Figures in right column indicate lethal concentration in50 % population, i.e. LC 50% μg/ml.

Drug/Extract	Percent mortality at various concentrations (μg/ml)						LC50 μg/ml
	12.5	25	50	100	200	400	
Colchicine	33.33±3.33 a	46.67±3.33 a	56.67±3.33 a	76.67±3.33 a	86.67±3.33 a	100±5.77 a	27.4
PECR	0.00	0.00	6.66±3.33	13.33±3.33	30.00±5.77 a	50.00±5.77 a	384.08
CECR	0.00	3.33±3.33	6.66±3.33	10.00±5.77	30.00±5.77 a	46.67±3.33 a	142.29
MECR	6.66±3.33	13.33±3.33	23.33±3.33 b	33.33±3.33 a	53.33±3.33 b	63.33±3.33 a	257.73
EAMECR	6.66±6.66	16.67±6.66	36.67±3.33a	56.67±3.33 a	66.67±3.33 a	70.00±3.33 a	184.86

Values expressed are mean±SEM, n=03, a– $P<0.01$and; b– $P<0.05$ with control.

3.2. Brine Shrimp Lethality Assay

The percentage mortality increased with an increase in concentration. LC50 values ranged from 384.08 to 184.86 μg/mL, with Ethyl acetate fraction of Methanolic extract of *Cuscuta reflexa* having the lowest value i.e. 184.86 μg/mL; this was followed by methanolic extract i.e. 257.73 μg/mL [table 02].

4. Discussion

Lysosomes are single–membrane structures that contain digestive enzymes. When certain white blood cells engulf bacteria, the bacteria are digested and destroyed by these lysosomal enzymes. Worn–out cell parts and dead cells are also digested by these enzymes. This is a beneficial process, and is necessary before tissue repair can begin. But it does have a disadvantage in that lysosomal digestion contributes to inflammation in damaged tissues. An excess of inflammation can start a vicious cycle, actually a positive feedback mechanism that results in extensive tissue damage [16].

Various methods are employed to screen and study drugs, chemicals, herbal preparations that inhibit the inflammation. These techniques include uncoupling of oxidative phosphorylation (ATP biogenesis linked to respiration), inhibition of denaturation of protein, erythrocyte membrane stabilization, lysosomal membrane stabilization, fibrinolytic assays and platelet aggregation [17].Human red Blood Cell stabilization against hypotonicity induced lysis was selected for the assessment of antiinflammatory activity of *Cuscuta reflexa* due to its simplicity and reproducibility. HRBC membrane is similar to the lysosomal membrane, during inflammation, histamine from damaged tissues makes capillaries more permeable, and the lysosomes of damaged cells release their enzymes, which help break down damaged tissue but may also cause destruction of nearby healthy tissue [16]. Some of the NSAIDS and Glucocorticoids stabilize lysosomes in tissue cells and thereby prevent release of lysosomal enzymes into the cytoplasm of the cells, thus preventing deterioration from this source [18]. Stabilization of lysosomal membrane therefore can control inflammatory response andtherefore stabilization of human red blood cell (HRBC) from hypotonicity induced lysis can be correlated with the antiinflammatory potential of a drug. Some of the Indian medicinal plants like Punica granatum [19], Enicostemma Axillare[20],Andrographis paniculata, Crateva magna, Glariosa superb, Hydrocotyle japonica, ,Sarcostemma acidum[21], Gendarussa vulgaris [22] have been screened for their antiinflamamtory activity by various researchers so far. The assay method involves incubation of RBC′s into a hypotonic solution(less than 282 mOsm/L), so that water will diffuse into the cell, causing it to swell; water will continue to diffuse into the cell, resulting in lysis of the cell 18. In the present study various extracts of *Cuscuta reflexa* possess significant stabilizing of HRBC, the probable mechanism of protection of hypotonicity induced lysis is shrinking of the cell membrane and involves processes that prevent the migration of these intracellular components outside the cell. It has been shown that cell deformability and cell volumes of erythrocytes are closely related to their intracellular content of calcium [23]. Thus, the membrane stabilization effect by these agents may be due to alteration of the influx of calcium into the erythrocytes [23]. The precise mechanism for these effects remains to be elucidated.

BSLA has been suggested as a valid method to evaluate cytotoxic activity [24] and thus the method is commonly used as a substitute assay for the screening of cytotoxic compounds. In the present study, the BSLA of extracts of *Cuscuta reflexa* which is used in traditional medicine was determined following the modified method of Solis et al [24]. Many plants possess cytotoxic property, by virtue of the presence of antitumor compounds [25]. Cancer is the predominant threat in most parts of the world, one of the important etiological factors is oxidative stress [26, 27]. A drug from plant origin can be a useful tool to combat cancer because of the fact that most of plant contains natural antioxidants which are very effective in combating cancer. Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. Natural antioxidants are preferred in allopathic drugs to overcome the side effects. Most of the polar compounds such as phenolic and flavonoid substances are potent inhibitors of reactive oxygen species attack [28].

The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural

prototype. They include the Vinca alkaloids, vinblastine and vincristine, isolated from Catharanthus roseus, etoposide and teniposide, the semisynthetic derivatives of epipodophyllotoxin, isolated from species of the genus Podophyllum, the naturally derived taxanes isolated from species of the genus Taxus, the semisynthetic derivatives of camptothecin, irinotecan and topotecan, isolated from Camptotheca acuminata, and several others. Over 50 % of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them. Most of the research performed today focuses on the development of new drugs to treat cancer, as well as viral and microbial infections [29].

The preliminary phytochemical study of methanolic and ethylacetate fraction of *Cuscuta reflexa* revealed the presence of flavonoids, the antiinflammatory and cytotoxic effects shown by these extracts can be related to the presence of flavonoids.

The crude extracts of *Cuscuta reflexa* show preliminary cytotoxic and antiinflammatory activities. The plant significantly inhibit the hypotonicity induced erythrocyte damage, which is an indicative of potential antiinflammatory effect of the plant, the authors are assessing the mechanism of the anti-inflammatory effect of various extracts, using various in vivo models.

The plant also possesses significant cytotoxic activity, which may be attributed to the presence of phenols, polyphenols, and flavonoids in plant. As the Brine shrimp lethality assay is only a preliminary test for the evaluation of antiproliferative activity, an in vitro anticancer activity testing on human malignant cell lines using SRB assay will be used for more precise evaluation of cytotoxic effect of *Cuscuta reflexa*.

Conflict of interest statement

We declare that we have no conflict of interest.

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