ORIGINAL ARTICLE

In vitro anti-proliferative, anti-bacterial potential and induction of DNA strand break of partially purified *Cuscuta reflexa* Roxb.

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Cuscuta reflexa is an important medicinal plant, mentioned in Ayurveda, an ancient Indian system of medicine. The plant is selected to evaluate the possibility for novel pharmaceuticals for anticancer and antibiotics drugs. Since most of these drugs had developed resisitance against currently used chemotherapeutics. This study describes the *in vitro* anti-proliferative, anti-bacterial and single stand DNA break of the holoprasitic plant *Cuscuta reflexa*. Bioassay-guided fractionation and partial purification of the plant were done and evaluated for antiproliferative activity against human cancer cell lines by SRB assay and single strand DNA break by comet assay. Further antibacterial activity was also performed by agar well diffusion assay. The alcoholic extract, chloroform fraction and partially purified ethylacetate–methanol (1:1) sub-fraction of *C. reflexa* showed anti-proliferative potential against IMR-32 and 502713 human cancer cell lines. Alcoholic extract exhibited anti-proliferative activity of 74% and 72%, chloroform fraction demonstrated 91% and 95% against neuroblastoma (IMR-32) and colon (502713) cancer cell lines at 100 µg/ml. Single strand DNA break of the chloroform fraction was also demonstrated using comet assay, indicating that possible mode of cell death may be apoptosis. Anti-microbial properties were evaluated against eight species of pathogenic and non-pathogenic microorganisms and maximum zone of inhibition for anti-bacterial activity was found against *Staphylococcus aureus* (22 mm) by alcoholic extract, 21 mm by chloroform fraction and 12 mm by ethylacetate–methanol (1:1) sub-fraction. Minimum inhibitory concentration (MIC) of the chloroform fraction was 1500 µg/ml for *S. aureus*. The plant was found to be equally effective against gram-positive and negative bacteria. Studies are well underway to isolate and identify active compounds from chloroform fraction and ethyl acetate:methanol (1:1) sub-fraction, which can be used as effective drug for various diseases.

Key words: Anti-bacterial, anti-proliferation, bioassay-guided fractions, comet assay, *Cuscuta reflexa*, minimum inhibitory concentration

INTRODUCTION

Natural products and related drugs are used to treat 87% of all categorised human diseases including cancer, bacterial infection and immunological disorders.[1] Approximately 15 million people die each year due to infectious diseases – nearly all live in developing countries.[2] Chemotherapy is one of the most widely used approaches for the treatment of many cancers, but the long-term use of chemotherapy can lead to drug resistance via several different mechanisms, such as gene mutation, DNA methylation and histone modification. Overuse of antibiotics has become the major factor in the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms. Patients are gradually developing resistance to widely used and standard chemotherapeutic agents, such as 5-fluorouracil, taxol, doxorubicin, cisplatin, campothecin, paclitaxel and topotecan. Due to this resistance to cancer drugs, it is important to find new anti-cancer and antibiotic agents in order that they can be developed into novel drugs that can circumvent the existing resistance mechanisms.[3] Since plants possess bioactive substances, which are safer to use without any side effects, there is need to screen out medicinal plants for potent anti-cancer and anti-microbial activities.[4]

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.94353

Cuscuta reflexa Roxb. (Family: Convolvulaceae, Amervel in Hindi). It is a common parasite found in several part of India and also well known as medicinal plant reported in Indian system of medicine and used for various ailments.[5,6] The chemical compounds isolated from the plants are mainly flavonoids.[7,8] Various studies conducted on the plant reported for anti-bacterial activity,[9] anti-fertility activity[10] and anti-oxidant activity.[11] Whereas petroleum ether extract and methanolic extract from stem part had psychopharmacological effect[12] and

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Received: 12-10-2011; **Accepted:** 02-01-2012

anti-steroidogenic effect on mice.[13] The present work was undertaken to evaluate the anti-proliferative and anti-bacterial properties of *C. reflexa* and to identify the cause of cell death.

MATERIALS AND METHODS

Plant Collection

Whole plant of *C. reflexa* Roxb., Family: Convolvulaceae, was collected from Nagrotra region of Jammu (India) in the month of December and was authenticated at source by taxonomist of the institute. A voucher specimen has been deposited at the herbarium of the Institute vide IIIM collection No.17148, Acc. No.17719. The authenticated and freshly collected whole plant was chopped and dried under shade.

Extraction

Three extracts of the plant material were made with 95% alcohol, alcohol–water (1:1) and water by using repeated solvent extraction procedure. Dried powdered plant material (1 kg) was percolated in 95% ethanol (5 L) at ambient temperature for 16 h. The solvent was decanted and the process was repeated four times. The pooled solvent was evaporated under reduced pressure to yield alcoholic extract 160 g. Similarly, hydro-alcoholic extract was prepared. The dried plant material (200 g) was soaked in alcohol–water (1:1, 1 L) and the extract obtained was 72 g. The dried powdered plant material (200 g) was heated with distilled water (1.5 L) on steam bath for 2 h for the preparation of aqueous extract, the supernatant was decanted and filtered through celite powder and the process was repeated four times, pooled extract was concentrated on rotavapour and dried in a lyophilizer, 40 g extract was obtained.

Partial Purification of the Crude Alcoholic Extract of *C. Reflexa* by Solvent Partitioning with Different Polarity Solvents

The alcoholic extract was fractionated sequentially with *n*-hexane, chloroform, *n*-butanol and water. The dried alcoholic extract (20 g) was macerated with *n*-hexane (4×500 ml). The combined solvent portion was evaporated under reduced pressure to yield hexane fraction (1.5 g). The residue was further macerated with chloroform (4×500 ml). The combined organic layer was evaporated under reduced pressure to yield chloroform fraction (2.25 g). The residue obtained was dissolved in distilled water (1 L) and partitioned between *n*-butanol and water. The process was repeated four times (4×500 ml); the organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield *n*-butanol fraction (8.55 g). The aqueous part was concentrated under reduced pressure to give aqueous fraction (6.4 g).

Chromatography

Flash chromatography

The chloroform fraction was sub-fractionated on flash chromatography using silica gel 230–400 (mesh size). Elution was carried out using petroleum ether with increasing concentration of ethyl alcohol and methanol. Eight fractions were prepared CR-1 (petroleum ether), CR-2 (petroleum ether: ethyl acetate 3:1), CR-3 (petroleum ether: ethyl acetate 1:1), CR-4 (petroleum ether: ethyl acetate 1:3), CR-5 (ethyl acetate 100%), CR-6 (ethyl acetate: methanol 3:1), CR-7 (ethyl acetate: methanol 1:1) and CR-8 (methanol 100%) were collected.

Thin layer chromatography

One-dimensional thin layer chromatography (TLC) was used in order to group the obtained components and to determine the purity of fractions. A silica gel plate (10 cm in height) was used as the stationary phase, and the respective extracts or fractions were spotted at the starting line at 0.5–1 cm intervals. The mobile phase solvents used (one per TLC plate) were 1:0, 3:1 and 1:1 (v/v) ratio of CH_2Cl_2: hexane. When the mobile phase had almost reached the top of the plate, the samples were visualised under UV light (254 nm). Alternatively, the gel plate was sprayed by a 5% (v/v) H_2SO_4/0.03% (w/v) α-naphtal methanolic solution, dried in an oven or hot plate and visualised under UV light (350 nm).

Bioassay-guided isolation

Each of the three crude extracts (alcoholic, 50% alcoholic and aqueous) of *C. reflexa*, obtained as detailed above, were evaluated for their *in vitro* anti-proliferative activity against two selected cancer cell lines, i.e. neuroblastoma (IMR-32) and colon (502713) using the SRB assay as detailed below. The extract which provided the best selective anti-proliferative activity, which is the highest activity on the cancer cell lines, was selected for further partial purification by flash chromatography. In the same way, each fraction obtained from the flash chromatography was likewise assayed for selective anti-proliferative activity on the cell lines.

Cell lines and culture

The human cancer cell lines were obtained either from National Center for Cell Science, Pune, India or National Cancer Institute, Fredrick, USA. The colon (502713) cancer cell line was grown and maintained in RPMI-1640 medium, pH 7.4, whereas DMEM was used for neuroblastoma (IMR-32) cell line. The media were supplemented with FCS (10%), penicillin (100 units/ml), streptomycin (100 µg/ml) and glutamine (2 mM).

Anti-proliferative activity against human tumour lines

The anti-proliferative activity of extracts, fractions and

partially purified fractions were determined using sulforhodamine-B (SRB) as described previously.[14] The cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity in a CO_2 incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in the growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. 100 µl of cell suspension (10^5 to 2×10^5 cells/ml) was seeded in 96-well tissue culture plate and incubated for 24 h; further test materials (100 µl) were added to the wells and again incubated for another 48 h. The cell growth was stopped by adding 50 µl of 50% trichloroacetic acid and again incubated at 4°C for an hour. The plates were washed with distilled water and air-dried. Sulforhadamine B (100 µl, 0.4% in 1% acetic acid) was added to each well and plates were incubated at room temperature for 30 min and washed with 1% acetic acid. Plates were air dried, tris-HCL buffer (100 µl, 0.01 M, pH 10.4) was added to all the wells and plates were gently stirred for 5 min on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm. Suitable blanks and positive controls were also included. Each test was done in triplicate. The value reported here in are mean of two experiments.

COMET assay (Single cell gel electrophoresis)

Drug-induced DNA damage was analyzed using the comet assay with modifications.[15] Cell pellets (treated with 0, 10, 30 and 100 µg/ml of chloroform fraction for 72 h) were collected by centrifugation and re-suspended with 200 µl PBS and 800 µl of 1% low melting point (LMP) agarose. The mixture was then pipetted onto a frosted glass microscope slide pre-coated with a layer of 1.0% normal melting point agarose, prepared in PBS, covered with cover slips and incubated at 4°C for 10 min. After the LMP agarose solidified, the cover slips were gently removed, then 0.8% LMP agarose pre-coated cover slips were added and the slides were allowed to solidify at 4°C for 10 min. After 10 min, the cover slips were removed and the cells were lysed in high salt solution (2.5 M NaCl, 10 mM tris–HCl, 100 mM EDTA, pH 10, with 1% Triton and 10% dimethyl sulfoxide added fresh) for 1 h. The slides were then placed in a horizontal electrophoresis unit containing fresh buffer (1 mM EDTA, 300 mM NaOH, pH 13) and incubated for 20 min to allow unwinding of DNA. Electrophoresis was then conducted in freshly prepared electrophoresis buffer (pH 13) for 20 min at 25 V and 300 mA (0.8 V/cm) at 4°C. Subsequently, the slides were gently washed with neutralization solution (0.4 M tris–HCl, pH 7.5) for 20 min and stained with 20 µl ethidium bromide (15 µg/ml). Stained nucleoids were scored visually using a fluorescence microscope equipped with a digital camera. 100 comets on two slides were acquired using the IM50 software image analysis system. Tail length was calculated and expressed in mean±S.E.M.

Microbial Cultures

Eight pathogenic bacteria, *Staphylococcus aureus* (MTCC 1144), *Escherichia coli* (MTCC 1089), *Bacillus subtilis* (MTCC 7164), *Bacillus licheniformis* (MTCC 7425), *Staphylococcus epidermidis* (MTCC 3615), *Bacillus brevis* (MTCC 7404), *Vibrio cholerae* (MTCC 3904) and *Pseudomonas aeruginosa* (MTCC 1034) used in the study, were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. Organisms were maintained on nutrient agar (Hi-Media, India) slopes at 4°C and sub-cultured before use. Active cultures for experiments were prepared by transferring a loopful of cells from stock cultures to test tubes of Muller-Hinton broth (MHB) that were incubated without agitation for 24 h at 37°C.

Anti-bacterial Assay

In vitro anti-bacterial activities of all extracts, fractions and sub-fractions of *C. reflexa* were determined by standard agar well diffusion assay.[16] Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar (Merck) seeded with 100 µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6 CFU/ml). Media were allowed to solidify and then individual petri dishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilised cup-borer. 100 µl of each extract was poured in the respective wells and the plates were incubated at 37°C for overnight. DMSO and sterilised distilled water were used as negative control, while kanamycin antibiotic (1 U strength) was used as positive control. The experiment was performed in triplicate under strict aseptic conditions and the anti-bacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by the respective extract at the end of incubation period.

MIC for the Bacteria

The anti-bacterial activity of the chloroform fraction was examined by determining the minimum inhibitory concentration (MIC) in accordance with Clinical and Laboratory Standard Institute (CLSI) methodology.[17] All tests were performed in Mueller–Hinton broth supplemented with DMSO at a final concentration of 10% (v/v) to enhance their solubility. The chloroform fraction was dissolved in MHB. Test strains were suspended in MHB to give a final density of 5×10^5 CFU/ml and these were confirmed by viable counts. Dilutions ranging from 100 to 2000 mg/ml of the fraction was prepared in tubes, including one growth control, MHB+DMSO 10% (v/v) and one sterility control MHB+DMSO 10% (v/v+test extracts). The MIC values initially recorded were from visual examinations as being

the lowest concentration of the chloroform fraction with no bacterial growth. Plates were incubated under normal atmospheric conditions at 37°C for 24 h for bacteria.

RESULTS

In this research, the therapeutic potential of *C. reflexa* was evaluated against cancer and also for various infective diseases caused by bacteria. Anti-proliferative activity of the plant was demonstrated on cell lines derived from neurobalstoma (IMR-32) and colon (502713) cancer, whereas anti-bacterial potential was confirmed against eight pathogenic and non-pathogenic strains of the bacteria. Additional studies using the comet assay permitted determination of the role in which apoptosis may play the role in the anti-cancer activity. Bioassay-guided fractionation of *C. reflexa* was done and active fraction was further partially purified using flash choromatography. The anti-proliferative potential at 10, 30 and 100 µg/ml showed growth inhibition in a dose-dependent manner against both the cell lines by all the three extracts [Figure 1]. Among them the aqueous extract was found to be least effective against both the cell lines, whereas alcoholic extract showed more pronounced cell growth inhibition against neuroblastoma cancer cell line (24, 53 and 74 at 10, 30 and 100 µg/ml, respectively) and also at colon cancer cell line (18, 64 and 72 at 10, 30 and 100 µg/ml, respectively), whereas hydro-alcoholic and aqueous extract had showed activity only at 100 µg/ml.

In case of fractions, it was observed that all the four fractions of the alcoholic extract had demonstrated strong growth inhibition in dose-dependent manner in all the cell lines at 10, 30 and 100 µg/ml [Figures 2 and 3] and among all the four fractions the chloroform fractions was most active than rest of the fractions (11, 52 and 91 at 10, 30 and 100 µg/ml, respectively) and aqueous fraction was least effective for neuroblastoma cancer cell line [Figure 3]. Similarly, chloroform fraction (14, 92 and 95 at 10, 30 and 100 µg/ml, respectively) was also most active for colon cancer cell line, followed by *n*-butanol and *n*-hexane fractions, whereas aqueous extract was least active [Figure 2].

Further partially purified chloroform fraction was also evaluated for anti-proliferative potential, it was observed that ethylacetate and methanol (1:1) sub-fraction (CR -7) was found to be more potential for both the cancer cell lines [Figure 4]. Petroleum ether: ethyl acetate (1:3) (CR-4) and ethyl acetate 100% (CR-5) sub-fractions had shown 50 and 54% growth inhibition at 100 µg/ml for neuroblastoma cell lines and rest of the sub-fractions had shown less than 50% growth inhibition. For colon cancer cell lines rest of the sub-fractions had shown more than 52% growth inhibition except ethyl acetate: methanol (3:1) (CR-6), which has shown least growth inhibition.

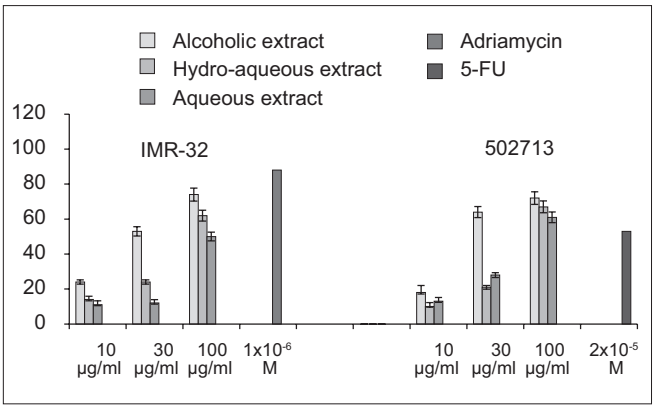

Figure 1: *In vitro* cytotoxicity of the extracts of *Cuscuta reflexa* against neuroblastoma and colon human cancer cell lines

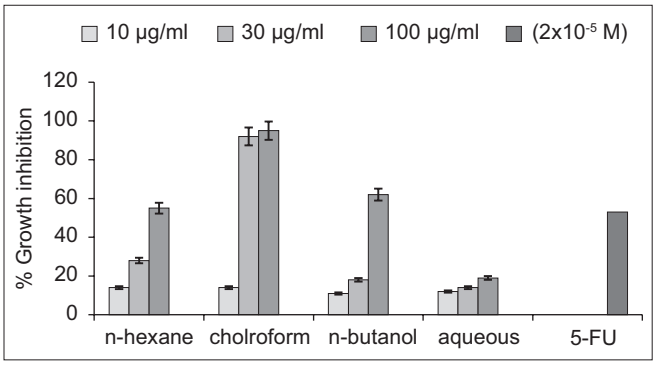

Figure 2: *In vitro* cytotoxicity of the fractions of *Cuscuta reflexa* against colon (502713) human cancer cell lines

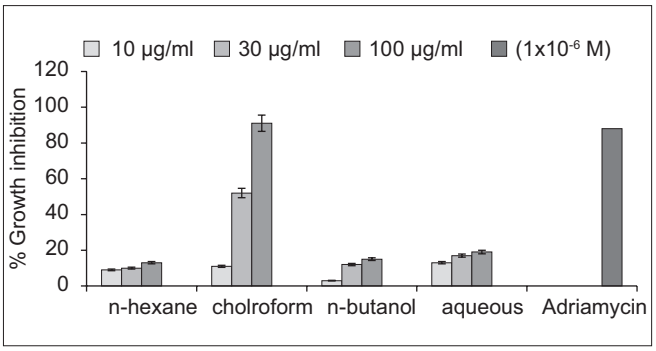

Figure 3: *In vitro* cytotoxicity of the fractions of *Cuscuta reflexa* against neuroblastoma (IMR-32) human cancer cell lines

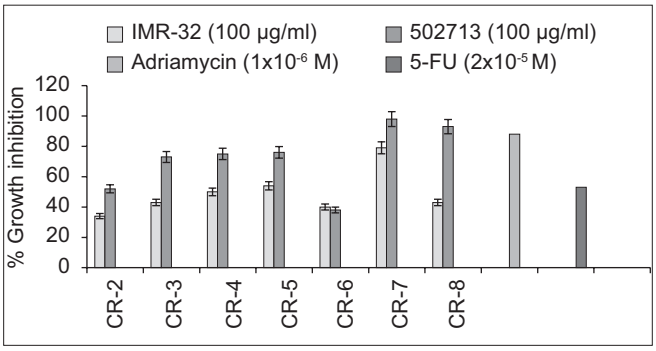

Figure 4: *In vitro* cytotoxicity of the sub-fractions of *Cuscuta reflexa* against neuroblastoma (IMR-32) and colon (502713) human cancer cell lines

In this work, assessment of DNA damage of the cancer cells was also performed by using comet assay in neuroblastoma and colon cancer cells. The comet assay is a sensitive method used to monitor single strand (ss) DNA breaks at the single-cell level. Any DNA damage is represented as a tail length (tail migration) of the DNA strand. When cancer cells were treated with 0, 10, 30 and 100 µg/ml of the chloroform fraction for 72 h, ssDNA damage was significant as indicated by the increased tail length when compared with the controls [Figure 5]. Moreover, the DNA damage induced by the chloroform extract was also dose-dependent.

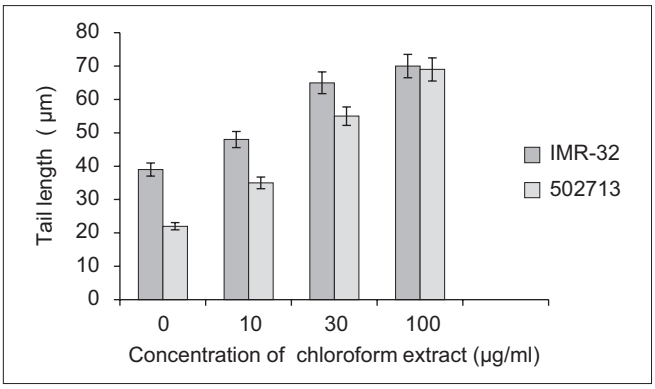

Figure 5: Evaluation of single strand DNA break of the chloroform fraction against IMR-32 and 502713 human cancer cells

Anti-infective potential of the plant was demonstrated by the plant against eight pathogenic and non-pathogenic strains. Anti-bacterial property of the alcoholic extract was better than those of hydro-alcoholic and aqueous extract. Aqueous extract was found almost ineffective against bacterial strains producing zone of inhibition ≤9 mm. Hydro-alcoholic extract showed zone of inhibition ranging from 13 to 10 mm, whereas alcoholic extract showed maximum zone of inhibition of 22–18 mm. It was found that alcoholic extract was equally inhibitory for gram-positive bacteria as well as for gram-negative bacteria. Among all the bacterial strains tested, *S. aureus* was found most susceptible with maximum inhibition by alcoholic extract producing zone of inhibition 22 mm followed by hydro-alcoholic extract and least activity was observed by aqueous extract [Table 1]. Further, chloroform fraction of the active alcoholic extract was comparatively more active producing zone of inhibition of 21–18 mm, followed by *n*-hexane fraction demonstrating zone of inhibition of 19–15 mm, whereas *n*-butanol and aqueous fraction showed least anti-bacterial activity following in range of 11–9 mm, respectively [Table 2]. Kanamycin (a positive control) showed inhibition diameters ranging from ~30 to 32 mm against all test microorganisms. Control experiments using sterile distilled water and DMSO (negative control) showed no inhibition of any bacteria.

Table 1: Results of anti-microbial screening of *Cuscuta reflexa* extracts determined by agar diffusion method

Bacterium name	Alcoholic extract	Hydro-alcoholic extract	Aqueous extract
Staphylococcus aureus	22±0.24	13±0.16	8±0.16
Escherichia coli	19±0.22	10±0.16	8±0.14
Bacillus subtilis	18±0.21	11±0.14	9±0.13
Bacillus licheniformis	21±0.24	11±0.11	9±0.16
Staphylococcus epidermidis	18±0.20	12±0.13	8±0.16
Bacillus brevis	19±0.22	10±0.13	9±0.16
Vibrio cholera	19±0.22	10±0.10	9±0.14
Pseudomonas aeruginosa	20±0.23	11±0.11	8±0.13
Kanamycin	31±1.5	32±2.2	30±2.1
DMSO	NA	NA	NA

Zone of inhibition (in mm diameter) including the diameter of well (6 mm) in agar well diffusion assay. In each well, the sample size was 100 µl. Kanamycin (positive control) 1 U strength. DMSO (negative control) 100 µl was used NA (no activity). Data are represented in the form of mean of three tests±SEM of the standard group

Table 2: Results of anti-microbial screening of *Cuscuta reflexa* fractions determined by agar diffusion method

Bacterium name	Alcoholic extract			
	n-Hexane fraction	Chloroform fraction	*n*-Butanol fraction	Aqueous fraction
Staphylococcus aureus	19±0.20	21±0.15	11±0.16	10±0.15
Escherichia coli	17±0.19	20±0.14	10±0.14	10±0.14
Bacillus subtilis	18±0.20	18±0.11	10±0.13	11±0.13
Bacillus licheniformis	15±0.21	20±0.12	11±0.16	10±0.16
Staphylococcus epidermidis	17±0.20	20±0.13	10±0.16	ND
Bacillus brevis	18±0.23	19±0.13	10±0.16	9±0.16
Vibrio cholera	17±0.20	18±0.14	9±0.14	9±0.14
Pseudomonas aeruginosa	18±0.21	21±0.13	10±0.13	10±0.13
Kanamycin	31±1.3	30±2.2	31±2.2	30±2.0
DMSO	NA	NA	NA	NA

Zone of inhibition (in mm diameter) including the diameter of well (6 mm) in agar well diffusion assay. In each well, the sample size was 100 µl. Kanamycin (positive control) 1 U strength. DMSO (negative control) 100 µl was used. Data are represented in the form of mean of three tests±SEM of the standard group. NA - No activity; ND - Not detected

Again the anti-bacterial potential of partially purified sub-fractions of chloroform fraction was evaluated and it was observed that ethyl acetate and methanol (1:1) sub-fraction (CR-7) followed by ethyl acetate: methanol (3:1) sub-fraction was comparatively more active by producing zone of inhibition of ≤12 mm and ≤10 mm, whereas rest of the sub-fractions (CR-6) showed very low activity or were not detectable [Table 3]. Fractions and sub-fractions obtained after purification have low anti-microbial activity and the zone of inhibition was found to have decreased by extract purification. It was noted during purification that some compounds are lost, which may explain the lesser anti-microbial properties observed with purified sub-fractions than with crude extract, or may suggest the presence of more than one active compound. MIC against susceptible bacterial species

by chloroform extract was evaluated [Table 4]. MIC of chloroform fraction was 1,500 µg/ml against *S. aureus*, 1000 µg/ml against *E. coil*, 2000 µg/ml against *B. subtilis* and 1200 µg/ml against *B. licheniformis*, while it was 800 µg/ml against *B. brevis* and *S. epidermidis* and 700 µg/ml against *V. cholera* and *P. aeruginosa*. All anti-microbial activity occurred in a concentration-dependent manner as suggested by MIC determination. However, the efficacy of fraction was less than that of the standard antibiotic, Kanamycin.

DISCUSSION

Above-mentioned results have provided knowledge that a holoparasite *C. reflexa* might be potential source for producing anti-cancer and anti-infective drug. These findings may be attributed to the nature of biological active compounds and their strong solubility with appropriate solvent. It is well documented that alcohols (ethanol, methanol) are used as a solvent for preparation of plant extract for their strongly extraction power. Many researchers had already used methanol or ethanol as a solvent for cytotoxicity, phytotoxicity, anti-bacterial and anti-tumour activities in several plant species.[18-22] As there might be compounding effects due to the potential presence of catatonic agents at high bioactive concentrations mixed in with the desired bioactivity compounds, we further enriched the extracts. Solvent partitioning based upon different solvent polarities was used for further purification and the anti-proliferative [Figures 2 and 3] and anti-bacterial activities [Table 2] of active compounds and they were likely to be non-polar or low-polar chemicals in nature. Since high cytotoxicity was principally observed in the chloroform partitioned fraction, it was then further fractionated by flash chromatography with elution based upon solvents of increasing polarity to obtain seven different sub-fractions among them the ethyl acetate and methanol (1:1) sub-fraction (CR -7) Figure 4 and Table 4 demonstrated significant activity. Furthermore, considering the TLC separation pattern, more purification steps that were performed, better observed separation and migration of compounds was observed (data not shown). Anti-cancer activity of the plant primarily was elucidated by ss DNA break with help of comet assay. Here, the extent of DNA damage increased proportionately with chloroform fractions of alcoholic extract of *C. reflexa*. Overall, the bioassay-guided isolation appeared suitable in order to obtain the purified active compounds, as reported before[23] and use of non-polar solvents for the preparation of extracts provides more consistent results as compared to polar solvents. We know that plants synthesise various bioactive substances by secondary metabolism to defend themselves when attacked by bacteria, fungi, parasites, viruses or other agents.[24] These substances have also been shown to be effective in preventing malignant transformation of cells in culture and experimentally induced tumourigenesis in various animal models.[25] As reported by various workers, the presence of phytochemical like cuscutin, cuscutalin and a large quantity of flavonoids in the methanol extract of *C. reflexa*.[7,26] However, considering the results presented here, it is possible that the anti-proliferation activity and DNA damage demonstrated by the plant may be derived from a combination of compounds. Since various flavanoids have been reported to possess cytotoxic and anti-bacterial effect,[27,28] it may be considered the active fractions reported here may be due to the presence of such compounds. Further studies are in progress for the purification to homogeneity and analysis of the chemical structures of each bioactive component should be performed in order to investigate which exact compounds in the holoprasitic plant *C. reflexa* are responsible for the anti-proliferation, anti-bacterial activity and mechanistic involve in the cell death due to these compounds so that it can be further design as template for future drug design.

Table 3: Results of anti-microbial screening of *Cuscuta reflexa* sub-fractions determined by agar diffusion method

Bacterium name	CR-7 Sub-fraction	CR-6 Sub-fraction
Staphylococcus aureus	12±0.10	10±0.15
Escherichia coli	10±0.11	9±0.10
Bacillus subtilis	11±0.10	8±0.11
Bacillus licheniformis	9±0.9	8±0.9
Staphylococcus epidermidis	9±0.11	8±0.9
Bacillus brevis	8±0.10	ND
Vibrio cholera	8±0.11	8±0.14
Pseudomonas aeruginosa	9±0.10	ND
Kanamycin	31±1.3	30±2.0
DMSO	NA	NA

Zone of inhibition (in mm diameter) including the diameter of well (6 mm) in agar well diffusion assay. In each well, the sample size was 100 µl. Kanamycin (positive control) 1 U strength. DMSO (negative control) 100 µl was used. Data are represented in the form of mean of three tests±SEM of the standard group. NA no activity, ND not detected

Table 4: Results of minimum inhibitory concentration of chloroform fraction of *Cuscuta reflexa*

Bacterium name	Chloroform fraction	Positive control (Broth+TO)	Negative control (Broth+CF)
Staphylococcus aureus	1500	G	NG
Escherichia coli	1000	G	NG
Bacillus subtilis	2000	G	NG
Bacillus licheniformis	1200	G	NG
Bacillus brevis	800	G	NG
Vibrio cholera	700	G	NG
Pseudomonas aeruginosa	700	G	NG
Staphylococcus epidermidis	800	G	NG

TO - Test organisms; CF - Chloroform fraction; G - Growth; NG - No growth

ACKNOWLEDGMENT

Authors are grateful to National Centre for Cell Science, Pune

(India), and National Cancer Institute, Frederick, MD, USA, for providing human cancer cell line. We are thankful to Dr. B.K. Kapahi for their help and support.

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How to cite this article: Bhagat M, Saxena AK. *In vitro* anti-proliferative, anti-bacterial potential and induction of DNA strand break of partially purified *Cuscuta reflexa* Roxb.. Int J Green Pharm 2011;5:307-13.

Source of Support: Nil, **Conflict of Interest:** None declared.