

Article

In-vitro cytotoxicity and antioxidant property evaluation from methanolic extract of *Cuscuta Reflexa* flowers

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Abstract: This investigation is made upon the plant *Cuscuta reflexa*, the flowers of it, to find out its Cytotoxicity property. The anti-oxidant property of this plant part was investigated using methanol extraction. Methanolic extract of *Cuscuta reflexa*. Was used to evaluate its cytotoxicity in Brine shrimp lethality bioassay where vincristine sulphate was used as standard drug. In Brine shrimp lethality bioassay, LC₅₀ value of the extract was 36.72µg/ml and vincristine sulphate served as the positive control showed LC₅₀ value 10.51µg/ml. So, compared to vincristine sulphate, it is evident that the methanol extract of flowers of *Cuscuta reflexa* was cytotoxic. In case of anti-oxidant the scavenging power (IC₅₀) of DPPH radical was 29.26, 17.07, 18.29, 19.55 and 54.87µg/ml respectively.

Keywords: *Cuscuta reflexa*; cytotoxicity; scavenging; reducing power; anti-oxidant

1. Introduction

The traditional medicine has been the focus for wider coverage of primary health care delivery all over the world. World Health Organization (WHO, 1978) defined traditional medicine as the sum total of knowledge or practices whether explicable or inexplicable used in diagnosing, preventing or eliminating a physical, mental or social disease, which may rely exclusively on past experience or observation handed down from generation to generation, verbally or in writing. It comprises therapeutic practices in existence for hundreds of years before the development of modern scientific medicine and is still in use today without much documented evidence of adverse effects. This traditional medicine comprised the use of plant, animal or mineral materials for healing (WHO, 1978) but the focus here is on phytomedicine (plant medicine) type.

Cytotoxicity is the standard of being toxic to cells. Cells exposed to a cytotoxic compound can respond in a lot of ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly caused by cell lysis; they can stop expanding and dividing; or they can stimulate a genetic program of controlled mobile or portable death, termed apoptosis. A cell undergoing necrosis normally exhibit rapid swelling, loses membrane honesty, shut down metabolism, and releases their contents in the environment upon lysis. Apoptosis is seen as well-defined cytological and molecular events, including a difference in the refractive index of your cell, cytoplasmic shrinkage, nuclear condensation, along with cleavage of DNA. Cytotoxicity assays are widely-used widely in drug discovery research to help you predict which lead compounds might get safety concerns in humans before significant time and expense are incurred of their development. Other researchers study mechanisms of cytotoxicity so that you can gain a better understanding of the traditional and abnormal biological processes that command cell growth, division, and death.

Human begins need oxygen for their living and also need anti-oxidative agent for controlling oxidation. It is to be said that anti-oxidants are used to protect human begins from different ill effects due to increased production of reactive oxygen species (ROS) as a result of exposure to pollutants. The body has its natural mechanism to interact with oxidative species (ROS), but sometime it's not capable to fight strongly when they need anti-oxidant, most of time supplied through food (exogenous anti-oxidant). During the process of oxygen utilization about 5% of oxygen converts to its univalent derivative free radicals like superoxide, peroxide etc. All these are known as reactive oxidative species (ROS), which attack cells of the body and each cell have face more than 1000 species of ROS per second. Free radicals are always involved in different kind of degenerative diseases like diabetes, liver damage, nephrotoxicity, cancer, inflammation and in the process of aging. Plants that have enriched phytochemistry along with anti-oxidant significance are needed to cure disease like cancer.

2. Materials and Methods

2.1. Collection of plant materials

The flowers of *Cuscuta reflexa* were collected from Chittagong local forest area; the leaves of *Cuscuta reflexa* were collected at their fully mature form. After cleaning, the leaves were taken and splitting the peel, then air dried for 8 days, and then kept in an oven at 45°C at 72 hours. 250 gm of dried powder was cold extracted with Methanol. Dried powder soaked with methanol for 7 days. Then filtered to take the concentrated extract, extract containing beaker was placed on the water bath (at 40°C-45°C) to evaporate the solvent from the extract.

2.2. Preparation of extraction

The extract is prepared by cold extraction process. In this process the coarse powder was submerged in ethanol (95%) since ethanol is the most common solvent for extracting most of the constituents present in herbal materials. Amber glass bottle were used for this purpose, which were kept at room temperature and allowed to stand for 7 days with occasional shaking and stirring. When the solvent became concentrated the contents were first decanted by using cotton and then filtered through Whatmann No.1 filter paper. The filtrate so obtained was then concentrated to dryness through the evaporation of solvent using rotary evaporator. Finally we got the concentrated semi-solid extract. The concentrated were then used as crude extract of respective test experiments. In our present investigation, we used methanol extract for cytotoxic and antioxidant activity.

2.3. In-vitro cytotoxic study

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The dried cyst of the brine shrimp were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) with strong aeration for 48 hours day/dark cycles to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method.

2.4. Materials

Artemia salina Leach (brine shrimp eggs), Sea salt non ionized NaCl, Small tank with perforated dividing dam to hatch the shrimp, Lamp to attract the nauplii, Pipette (1 ml and 5 ml), Micropipette (1-10 micro liter), Glass vials (5ml), Magnifying glass, Test sample for experimental plants.

2.5. Hatching of brine shrimp eggs

Artemia salina Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5gm/L) were added to one side of the tank and this side was covered. The shrimps were allowed to one side of tank and this side was covered. The shrimp were allowed for two days to hatch and mature as nauplii (larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay.

2.6. Preparation of the simulated sea water

38 grams sea salt was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The p^H of the sea water was maintained between 8-8.5 using 1N NaOH solution.

2.7. Preparation of sample solution

At first take 19ml distilled water in beaker add 1ml DMSO (dimethyl sulfoxide) thus prepares stock solution. Clean test tubes were taken. These test tubes were used for different concentration (one test tube for each concentration) of test samples. 4 mg methanolic extracts of *cuscuta reflexa* were accurately weighed and dissolved in 4ml stock solution. Thus a concentration of 1000 µg/ml was obtained which used as an extract solution. Then taking 1ml extract solution from beaker & add 9ml stock solution In vials thus prepared final extract solution. From this extract solution 10 µg/ml, 30µg/ml, 50µg/ml, 60µg/ml, 70µg/ml, and 80µg/ml were taken in six test tubes respectively and adjusted volume 5 ml sea water. Finally 10 nauplii are then applied in each test tubes.

2.8. Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used-i) Positive control ii) Negative control.

2.9. Preparation of positive control group

Positive control in cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, vincristine sulphate was used as the positive control. 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml. This was used as stock solution of vincristine sulphate. With the help of a micropipette 80, 70, 50, 30 and 10 µl of the stock solution were transferred in 6 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 80µg/ml, 70µg/ml, 60µg/ml, 50µg/ml and 10µg/ml respectively. The experiment was repeated three times.

2.10. Preparation of negative control

100 µl of distilled water, DMSO and ethanol was added to each of the three remarked glass vials containing 5 ml of simulated sea water and 15 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the samples.

2.11. Application of brine shrimp nauplii

With the help of the Pasteur pipette 15 living nauplii were added to each of the vials containing 5 ml of simulated sea water. A magnifying glass was used for convenient count of nauplii. If the counting of the 15 nauplii was not possible accurately.

2.12. Counting of the nauplii

After 24 hours, the vials are observed using a magnifying glass and the number of survival nauplii in each vial were counted and recorded. From this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC₅₀) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

2.13. Assay for scavenging power

DPPH (1, 1-Diphenyl-2-picrylhydrazyl radical) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The radical scavenging potential of the sample was determined by measuring the decrease in absorbance due to DPPH at 517nm, representing the formation of its reduced form, 1, 1-Diphenyl-1-2-picrylhydrazine (DPPH), which was yellow in color, because of the odd electron, the purple colored methanolic solution shows a strong absorption band at 517nm.

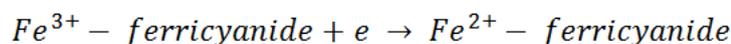
Procedure:

0.1ml of each concentration of extract (0, 5, 25, 50, 100 and 200µg/ml) was added to 3ml of 0.004% methanol solution of DPPH. The mixture was kept in dark place for proper reaction, as the reaction is light sensitive. After 30min, absorbance of the resulting solution was measured at specified wavelength. The percentage was calculated using formula following, where A₀ is absorbance of control and A₁ is absorbance of test.

$$\%SCV = \frac{A_0 - A_1}{A_0} \times 100$$

2.14. Reducing power capacity

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reluctant such as antioxidant substances in the samples causes the reduction of the Fe^{3+} -ferricyanide complex to the ferrous form by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700nm of UV spectrophotometric absorbance.



The extract of testing was treated with 2.5ml potassium buffer (0.2M), 2.5ml potassium ferricyanide (1%) then heated for 20min at 50°C. After that it was mixed with 2.5ml trichloro acetic acid (10%), solution was centrifuged for 10min to obtain supernatant from which 2.5ml was withdrawn and mixed with equal amount water followed by 0.5ml ferric chloride solution

3. Results and Discussion

3.1. Brine shrimp lethality bioassay

Brine shrimp lethality results of the fraction of *Cuscuta reflexa* flowers are shown in Figure 1 and LC_{50} calculated value is recorded in Table 1. The fraction showed potential cytotoxic activity with LC_{50} value of 36.72µg/ml. Vincristin sulphate served as the positive control for this brine shrimp lethality assay and its LC_{50} value was 10.51µg/ml.

Table 1. Cytotoxic activity of *Cuscuta reflexa*.

Con ^c	Nauplii	Live nauplii	Log C	% of Mortality	LC ₅₀ µg/ml
10	15	10	1	33.33	
30	15	8	1.698	46.67	
50	15	5	2	66.67	
60	15	6	2.301	86.67	36.72
70	15	5	2.477	46.66	
80	15	2	2.699	86.67	

Table 2. The table below represents the 1, 1-diphenyl-2-picrylhydrazyl radical scavenging capacity of methanolic extract of *C.reflexa* flowers.

Concentration	C.reflexa	Ascorbic acid
0	0.000	0.000
5	0.106	0.286
25	0.096	0.375
50	0.097	0.423
100	0.098	0.885
200	0.127	1.425

Table 3. Result of antioxidant activity of methanolic extracts of *C.reflexa* flowers.

Conc.	C.cuscuta	Ascorbic acid
0	0.000	0.000
5	1.806	0.286
25	1.267	0.375
50	1.266	0.423
100	1.253	0.885
200	1.250	1.425

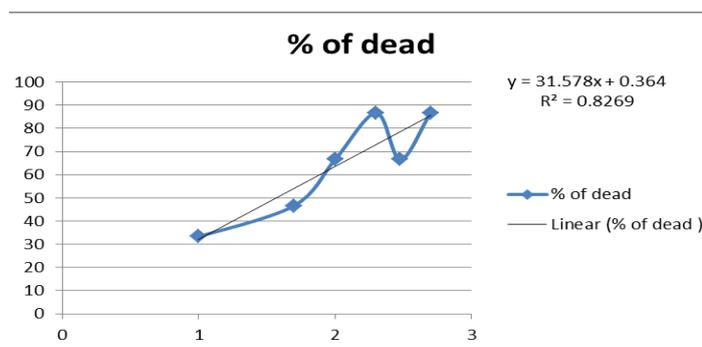


Figure 1. Determination of LC₅₀ value for extract of *Cuscuta reflexa* flowers from linear correlation between log of concentrations (Log C) versus percent (%) of mortality.

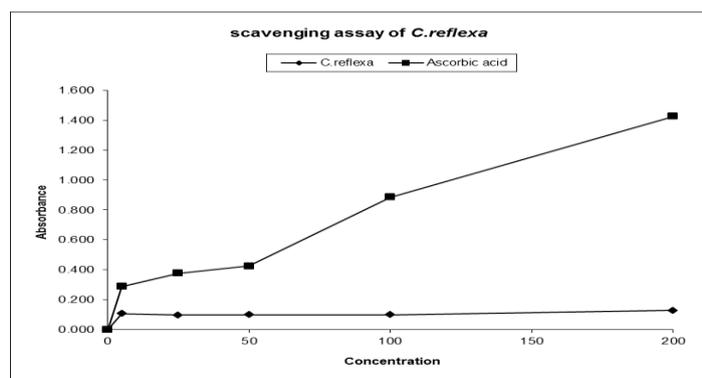


Figure 2. Scavenging activity of different fraction of *Cuscuta reflexa* flowers on DPPH radical along with comparable standard (ascorbic acid).

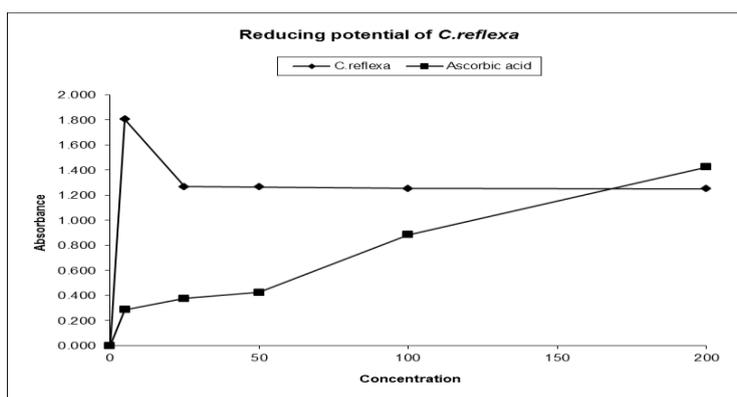


Figure 3. Reducing power capacity of *Cuscuta reflexa* in treatment with ferric chloride radical, absorbance against concentration showing the inhibition of radicals with the increase of concentration.

3.2. DPPH free radical scavenging activity

The scavenging power was estimated through DPPH testing method it is most common and considerable easy as well as time relaxing method. Result obtained by this method relatively good. 1,1-Diphenyl-2-picrylhydrazyl radical is scavenged by anti-oxidant compound. Every anti-oxidant compound doesn't have likely scavenging power, as they vary their power can be measured through this testing at 517nm UV-spectrophotometric absorbance, therefore among other use of 1,1-Diphenyl-2-picrylhydrazyl one is in anti-oxidant assay.

3.3. Reducing power capacity

Reducing power of the crude extract of *cuscuta reflexa* was monitored and concluded by the transformation of Fe^{3+} to Fe^{2+} . The reducing power of tested sample was found concentration dependent and presented graphically in following figure (Figure3).

4. Discussion

Plant-derived medicines have a long history of use for the prevention and treatment of human diseases. Today, many pharmaceuticals currently approved by the Food and Drug Administration (FDA) have origins to plant sources. A major role for plant-derived compounds based on the reported immune modulatory effects has emerged in recent times and has led to the rigorous scientific examination to determine efficacy and safety. Toxicity of plant materials is a major concern to scientists and medical practitioners and therefore cytotoxic assay was conducted in this study to determine the toxicity profile of the plant extracts through the Brine Shrimp Lethality (LC_{50} , 24 h) test. Lagarto demonstrated a good correlation ($r^2 = 0.91$) between the LC_{50} of the brine shrimp lethality test and the acute oral toxicity assay in mice. Based on that correlation, brine shrimp lethality $\text{LC}_{50} < 10 \mu\text{g/ml}$ (LD_{50} between 100 and 1000 mg/kg) is considered as the cut off value of cytotoxicity. DPPH is an established and well known free radical available commercially. Any compound or candidates prove thyself in DPPH scavenging assay would provide a promising result in in-vivo study. It was reported by Prasad *et al.* 2005 and Zhao *et al.* 2006 that phenolic and flavonoid reduces DPPH, enhancing scavenging power through their hydrogen donating ability. The result obtained by the investigation (table-2 & fig-2) revealed that the scavenging power of *cuscuta reflexa* has very good action and attributor is may be hydrogen donating ability.

Reducing power is generally associated with the presence of reductions that break down free radical chain by donating hydrogen atom, thus show anti-oxidant property. In this assay ferricyanide having Fe^{3+} reduced to Fe^{2+} , by visual measurement it has seen navy blue color and final conformation is obtained by measuring at 700nm spectrophotometric absorbance. The reducing power of *cuscuta reflexa* may be due to its di or monohydroxyl substitute in the aromatic ring system, which probably present in it.

5. Conclusions

From the above study it can be concluded that the methanolic extract of *cuscuta reflexa* may be a potential candidate for future cytotoxic agent. Furthermore study and isolation is needed to obtain site specific and more potent agent that causing this effect. The cytotoxic result obtained $36.72 \mu\text{g/ml}$ (LC_{50}) it so good, but proper isolation can make it more potent and useful. So, it could be suggested to modify for site specific use. From the experimental literature these compounds have record of broad spectrum action as anti-oxidant and they are originating for such reason from long period of time. Anti-oxidant activities of methanolics are caused by their donation of hydrogen atoms to free radical scavenging action. They possess an ideal structure for scavenging property.

Conflict of interest

None to declare.

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