



Original Research Article

ANTIOXIDANT EVALUATION AND CYTOTOXIC ACTIVITY OF *ASCLEPIAS CURASSAVICA* LINN.Soma Das^{*1}, K Chandrashekar Naidu² and Y Venkateswara Rao¹¹Department of Botany, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India²Vemu Institute of Technology, P. Kothakota, Chittoor Dist, Andhra Pradesh, India

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Abstract: *Asclepias curassavica* L. (Aselepiadaceae) has been known since ancient times for its curative properties and has been utilized for the treatment of various ailments such as tumor, asthma, fever, homeostasis, inflammation, diarrhea, and warts. In recent times a great number of chemical and pharmacological studies have been done on *Asclepias curassavica* L. In the present study hexane, chloroform and methanol extracts of *Asclepias curassavica* L. were evaluated for their antioxidant and cytotoxic activities. The *in vitro* antioxidant activity was conducted by determining the total phenolic content by using Folin-ciocalteu reagent and DPPH free radical scavenging activity. In all the three solvent highest total phenolic content and highest DPPH free radical scavenging activity percentage was shown by chloroform extract of *Asclepias curassavica* L. i.e. 105.6GAE_{mg}/gram dry material and 93.8% respectively which are nearly equal to Ascorbic acid and Gallic acid standard. The cytotoxic activities of *Asclepias curassavica* L. was also studied *in vitro* by using two assay i.e. ATP chemiluminescent and flow cytometry on two cell lines i.e. HeLa and MDA-MB-231. *Asclepias curassavica* L. exhibited potential cytotoxic activity. Thus results indicate that this plant might be used as a potential source of natural antioxidant and as anti-cancer agent.

Key words: *Asclepias curassavica*, Antioxidant activity, ATP- chemiluminescent, Flow cytometry, HeLa and MDA-MB-231 cell line.

INTRODUCTION

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past decades. Antioxidants are defined as compounds that can delay inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and Nitrogen species (ROS/RNS, e.g. superoxide, hydroxyl radical, peroxyxynitrite) overcome endogenous antioxidant capacity, leading to oxidation of varieties of bio macromolecules, such as enzymes, proteins, DNA and lipid. Oxidative stress plays a vital role in the development of chronic degenerative disease including coronary heart disease, cancer and aging (1). Recently, Phenols have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than vitamin C and E (2, 3). It has been proposed that the antioxidant properties of phenol compounds can be mediated by the following mechanism.

1. Scavenging radical species such as ROS/RNS
2. Suppressing ROS/RNS formation by inhibiting some enzymes (or) chelating trace metals involved in free radical production.
3. Up regulating or protecting antioxidant defense (4).

Asclepias curassavica is a traditional medicinal plant used by tribal people in Eastern Ghats, India, to treat piles, Gonorrhoea, roundworm infestation and abdominal tumors. To better understand its efficiency it is necessary to further investigate the biological

activities at *Asclepias curassavica*. The objectives of this work were to investigate the antioxidant and cytotoxic activities. With this series of experiment, we hope to help clinician to better understand its clinical usage.

MATERIALS AND METHODS

Plant Materials

Asclepias curassavica was collected from Eastern Ghats in Visakhapatnam. It was identified with the help of Gamble, "Flora of the Presidency of Madras" and later verified by comparison with the authentic specimens available in the herbariums of BSI, Hyderabad and the Department of Botany, Andhra University, Visakhapatnam.

Preparation of Plant Extracts

The collected plant material was chopped into small pieces, shade dried and coarsely powdered in Willy mill. The coarsely powdered material weighed and extracted with hexane, chloroform and methanol in sequential order of polarity using a soxhlet extractor for five to six hours at temperature not exceeding the boiling point of the solvents. For each gram of dry material 2ml of solvent was used. The extracted solvents were filtered through Whatman no-1 filter paper and subsequently concentrated under reduced pressure (in vacuo at 40°C) using a rotary evaporator. The residue obtained were designated as crude extracts and stored in a freezer at -20°C until assayed.

Quantitative estimation

Total Phenolic content: Phenolic compounds are secondary metabolites which get synthesized in

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plants. They possess biological properties such as antioxidant activity, anti-apoptosis, anti-ageing, anti-carcinogenic, anti-inflammatory, anti-arthrosclerosis and cardiovascular protection, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capacities (Han 2007).

Total phenol of extract can be evaluated with spectrophotometric method using Folin-ciocalteu reagent. The principle of this method is reduction ability of phenol functional group. Oxidation and reduction reaction of phenolate ion takes place at basic condition. The reduction of Phosphotungstan – Phosphomolybdenum complex (Fc reagent) by phenolate ion will change its colour to blue. The reduction of complex will increase when the extract contain more phenolic compounds. Thus the colour will be darker and the absorbance will be higher.

Procedure

1 mg/ml solution of Gallic acid was prepared and serially diluted to 5 concentrations. 10µl of 1mg/ml plant extract was prepared and 150µl of Folin-Ciocalteu reagent (dilute 1:4 in water) was added and incubated for 15 min at room temperature. Similar process was done for standard. After 15 min of incubation 50µl of 1 M sodium carbonate was added and incubated for 60 min at room temperature in dark. The absorbance was taken at 725nm. The total phenolic content is expressed as Gallic acid Equivalent (GAE) in mg per gram extract.

Qualitative estimation

DPPH radical scavenging assay: Free radical scavenging is one of the mechanisms involved in antioxidant action, a good antioxidant (AH) able to scavenge the DPPH (1,1 Di phenyl 2-picrylhydrazyl) radical and retain its own stability due to its reduction ability as shown in the equation below.



Procedure

Plant extracts was tested for the scavenging effect on DPPH radical according to the method of Al – Dabbas et al., (2006). 2 ml of extract solution of different solvent (Hexane, chloroform and methanol) was taken in different concentration (5,50,100 and 400µg) to which 2 ml of 0.4 m mol/L DPPH methanolic solution was added. Solution containing 2 ml of methanol and 2 ml of the DPPH solution was used as negative control and synthetic antioxidant ascorbic acid was used as positive control. Different concentrations were kept in the dark at room temperature for 30min. The scavenging activity of the DPPH was determined by measuring the absorbance at

517 nm until the reaction reached the steady state, using a spectrophotometer. All the determination was performed five times.

The DPPH radical scavenging activity was calculated using the following equation.

$$\% \text{ inhibition} = (1 - A_1/A_0) \times 100$$

A₁ and A₀ are the absorbance of the tested sample and control respectively.

In vitro cell based assay

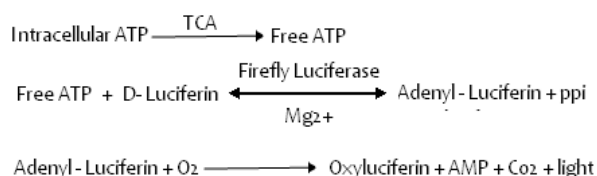
Assessment of *in vitro* anticancer activity is based on the concept of ‘basal’ cytotoxicity of the compounds/drug which affects the basic functions of cells which in turn leads to cellular damage. Change in metabolic activity such as mitochondrial activity and change in Adenosine Triphosphate (ATP) levels are the early indication of cellular damage; damage to cell membrane integrity increases the concentration of enzymes such as Lactate Dehydrogenase (LDH); and uptake of fluorescent dye after DNA fragmentation are some of the basic parameters for the estimation of cellular damage. Hence anticancer activity/cytotoxicity can be evaluated by measuring the basic parameter of cellular damage.

Assays Based on Determination of ATP

The nucleotide Adenosine 5'-triphosphate is produced only in living cells during photosynthesis and cellular respiration and it acts as the important chemical energy reservoir in cells which is used for biological synthesis, signalling, transport, and movement processes which in turn helps in performing their specialized function and keep the cells alive. Most ATP is found within living cells which associate with catabolic and anabolic processes. However, cell injuries and oxygen depletion results in a rapid decrease in cytoplasmic ATP. Hence ATP is a key indicator of cellular activity and utilized to measure the cell viability and cytotoxicity in research and drug discovery. The most successful technique for the determination of ATP is bioluminescent method, because of its sensitivity and the wide dynamic range.

Principle of ATP chemiluminescent assay

Anticancer drug /cytotoxic compounds damage the cancerous cells which results in depletion of ATP level in the cells which are extracted using Trichloroacetic acid (TCA). The freed ATP is allowed to react with luciferin in presence of Mg²⁺ ions and enzyme luciferase (catalyst) to form Adenyl-luciferin, which in turn react with oxygen to give oxyluciferin and short half-life luminescent signal (green light) which are measured at 562 nm.



Protocol for the cell viability assay

180µl of HeLa and MDA-MB-231 cells were seeded at a density of 5000 cells per well in a white opaque plate and incubated for 24 hrs at 37°C, 5% CO₂ incubator. 24 hrs post cell seeding, 20µl of 10x crude extract were treated such that the final concentration of the compound ranged from 40 to 0.0097µg/ml. As reference, Puromycin was treated simultaneously to both cells and incubated for 72 hrs at 37°C, 5% CO₂ incubator. After 72hrs, 100µl per well cell titre luminescent reagent was added to the plates and incubated at room temperature for 30min. Luminescence signal was captured using En vision Multilabel reader. Puromycin was diluted serially (half log) from 10µM through 0.000114 µM.

Flow cytometry assays for analysis of cell viability using propidium iodide

Flow cytometry provides a rapid and reliable method to quantify viable cells in a cell suspension. Determination of cell viability is critical when evaluating the response to cytotoxic drugs or other environmental factors. In addition, it is often necessary to detect dead cells in a cell suspension in order to exclude them from the analysis. Dead cells can generate artifacts as a result of non-specific antibody binding or through unwanted uptake of fluorescent probes. One method to assess cell viability is through the use of dye exclusion. Normal cells have intact membranes that exclude a variety of dyes that easily penetrate the damaged, permeable membranes of non-viable cells.

Propidium iodide (PI) is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between base pairs. PI is excited at 488nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617nm. Because of these spectral characteristics, PI can be used in analyzing cell viability. PI is a suspected carcinogen and should be handled with care. The dye must be disposed of safely.

Methodology of propidium iodide cell viability assay

Cells were harvested by centrifugation at 1200rpm at 4°C for 5 minutes by adding PBS/EDTA/trypsin solution. Before putting the cells in to the tube it was counted with the help of haemocytometer and then washed one time by putting 1×10⁶ cells per tube by adding 1ml PBS and centrifuged at 1200rpm at 4°C. Re-suspended pelleted cells in 0.3ml

of PBS buffer. To fix the cells, cold 70% ethanol was added drop wise to the tube containing 0.3ml of cell suspension in PBS while vortexing gently. The tube was left for 1 hour at 4°C. Cells were centrifuged as above, washed 1 time with cold PBS and re-centrifuged. Then the cells were treated with ribonuclease by adding 50µl of a 100µg/ml stock of RNase. This will ensure only DNA, not RNA in stained. In the last 200µl PI (from 50µg/ml stock solution) was added. The tube was kept in dark at 4°C until analyzed. For analyzing cell viability reading was taken by cytometer at 488nm. The results are shown based on a forward scatter (FSC) depicting the morphology of the cells and side scatter (SSC) depicting the granularity of the cell.

RESULTS

The result clearly shows that the plant *Asclepias curassavica* have potent total phenolic content and free radical scavenging capacity. Of all the three solvent extracts (Hexane, Chloroform and methanol) tested, the potent total phenolic content was shown by chloroform and methanolic extract of *Asclepias curassavica* i.e. 105.6 and 122.6 GAE mg/gram dry material respectively. Similarly the height DPPH-free radical scavenging activity percentage was shown by chloroform and methanol extract i.e. 93.8% and 84.6% respectively as shown in table no.1.

Table 1: Antioxidant activity of *A. curassavica*

Solvent	GAE mg/g of dry material	DPPH-Radical scavenging activity %
Hexane	54.11	52.5
chloroform	105.67	93.8
Methanol	122.67	84.6

Depending on the antioxidant and total phenolic content, the chloroform extract of *Asclepias curassavica* was evaluated for its cytotoxic activity on two cell lines i.e. HeLa and MDA-MB-231 by using ATP chemiluminescent and flow cytometry assay. The IC₅₀ value of the compound was calculated from dose response curve (Figure 1) and found to be 0.4 µg/ml with a maximum inhibition of 94% at the highest tested concentration of the extract i.e. 40 µg/ml when tested at different dilutions against MDA-MB-231 cell lines. However the compound when tested on HeLa cells showed a maximum inhibition of about 60% at the top most tested concentration of 10µg/ml and the inhibition was constant at 60% even up to 0.15 µg/ml as shown in the table 2. Based on the data the compound appears to be a partial inhibitor for HeLa cell line and a potent inhibitor for MDA-MB-231 cell lines after 72 hours as shown in the figure 2 and 3 respectively.

The IC₅₀ of reference compound Puromycin was 0.3µM and 0.5 µM against HeLa and MDA-MB-231 cell respectively. Similarly, a scatter plot analysis by flow cytometry was utilized to determine the viability

of the HeLa and MDA-MB-231 cell lines. The results are shown based on a forward scatter (FSC) depicting the cells and side scatter (SSC) depicting the granularity of the cell as shown in figure 4. Based on the data gathered the tested compound has shown potent cell death against MDA-MB-231 cell lines and partial inhibition against HeLa cell lines i.e 26.14% and 8.6% respectively at highest concentrations 40µg/ml as shown in table 3 are also supporting the results of ATP chemiluminescent assay.

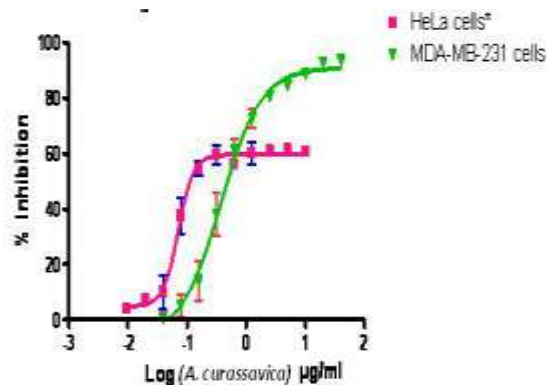
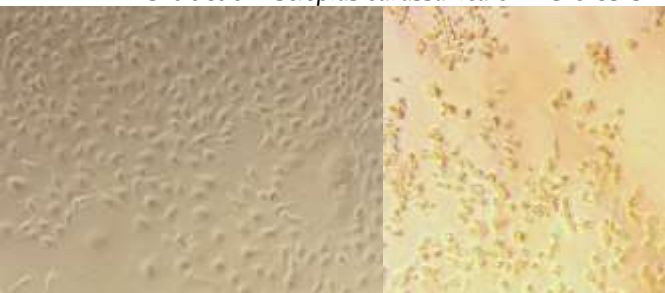


Figure 1: Dose response curve of *A. curassavica* (chloroform extract) against HeLa and MDA-MB-231 cells

Table 2: Cytotoxicity activity of *A. curassavica* (chloroform extract) against HeLa and MDA-MB-231 cells

Cell line	IC ₅₀ µg/ml	S.D	pIC ₅₀	IC ₅₀ Lower 95% confidence interval	IC ₅₀ Upper 95% confidence interval	% of Maximum Inhibition	Z'
HeLa	0.1	± 0.01	1.16	0.0614	0.08211	60	0.8
MDA-MB-231	0.4	± 0.13	0.47	0.2802	0.4665	94	0.9

Figure 2: Images of cytotoxic effects of Chloroform extract of *Asclepias curassavica* on HeLa cells



HeLa cells – Untreated cells
HeLa cells – 72hrs post treatment with *A. curassavica* extract

Figure 3: Images of cytotoxic effects of Chloroform extract of *Asclepias curassavica* on MDA-MB-231 cells

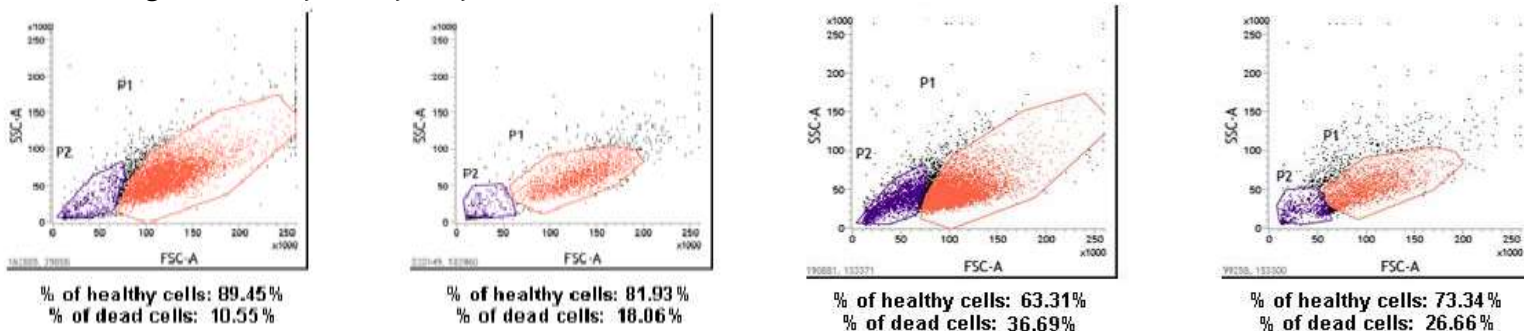


MDA-MB-231 cells – Untreated cells
MDA-MB-231 cells – 72hrs post treatment with *A. curassavica* extract

Table 3: Flow cytometry analysis

Cell lines	Cell Viability	Untreated cells	Treated cells at 40µg/ml	Treated cells at 9.7 µg/ml	Cell death at 40µg/ml	Cell death at 9.7 µg/ml
HeLa	% of Healthy cells	81.93	73.34	74.1	8.59	7.83
	% of Dead cells	18.06	26.66	25.9		
MBA-MB-231	% of Healthy cells	89.45	63.31	69.48	26.14	19.97
	% of Dead cells	10.55	36.69	30.52		

Figure 4: Flow cytometry analysis



MDA-MB-231 cells – Untreated cells
HeLa cells – Untreated cells

MDA-MB-231 cells treated with *A. curassavica* extract
HeLa cells treated with *A. curassavica* extract

DISCUSSION

The common garden plant *Asclepias curassavica* belongs to the family Asclepidaceae (Milkweeds) and is a good source of the cardenolide cardiac glycosides (9). These cardio active compounds have also been isolated from the monarch butterfly (*Danaus plexippus* L.), which feeds on the *Asclepias* genus, including *A. curassavica* (10). Butterflies and other sucking insects sequester these noxious chemicals from host plants for their protection from vertebrate predators. From an ecological point of view, this host-guest-predator relationship has been well established (11, 12). However, only limited research has been carried out concerning the cytotoxic constituents of *A. curassavica*. This plant is used as cancer treatment in traditional medical practice (13). Calotropin isolated from this plant family has been reported as potent cytotoxic agent against KB cells (14).

Recently a great emphasis has been given towards the research on complementary and alternative medicine that deals with cancer treatment. The broad aim of this study is to provide a natural drug that can prevent or suppress the two dreadful cancers that are cervical and breast cancer.

In the present study chloroform extract of *Asclepias curassavica* has shown a potent antioxidant and cytotoxic activity on MDA-MB-231 cell and partial inhibition on HeLa cell lines. The cytotoxic activity of the extract was observed to be 94% on MDA-MB cell lines which is nearly equivalent to the control drug activity i.e. 100%, where as it has shown a partial cytotoxic activity of 60% on HeLa cell line which is reported for the first time.

The flow cytometry studies also support the above finding indicating that chloroform extract of *Asclepias curassavica* is having a potent cytotoxic effect on MDA-MB-231 cell lines and a partial inhibition on HeLa cell lines.

CONCLUSION

Various scientific studies from divergent fields are investigating plants with an eye to their anticancer usefulness. Laboratories of the world have found thousands of phytochemicals which have antioxidant and anticancer capacity. From the present study it can be concluded that chloroform extract of *A. curassavica* has a potential source of cytotoxic activity on MDA-MB-231 and HeLa cells lines. Hence there is a hope for the development of new, novel and more powerful commercial drugs from plant derivatives to effectively treat cancer.

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