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ISOLATION AND IDENTIFICATION OF STIGMASTEROL FROM *ASCLEPIAS CURASSAVICA* AND EVALUATION OF ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Asclepias curassavica is a milk weed plant known for its medicinal properties.The present investigation was carried out to evaluate the anti-inflammatory potential of chloroform fraction of *A. curassavica* in modulating the inflammatory effect in carrageenan-induced mice. The effect of sub-fraction 2 obtained from chloroform extract was tested for the above model with varying dose. The reduction in the paw edema was measured using plethysmometer for the duration of 1-4 h. The MTT analysis was carried out to study the toxicity using SH-SY5Y cells and cell viability was checked. The anti-inflammatory activity was expressed as the mean increase in paw volume in terms of mL and percentage inhibition in paw volume. The sub-fraction 2 of chloroform extract of *A. curassavica* at 45 and 180 mg/kg.b.w dose significantly reduced ($p \leq 0.01$) paw edema when compared with control. Further, characterization of sub-fraction 2 using LCMS, NMR and IR revealed the presence of Stigmasterol, which is known to possess anti-inflammatory and other beneficial effects. In conclusion, the plant *A. curassavica* may be useful in treating inflammatory responses associated with different disorders.

KEYWORDS: **Anti-inflammatory; Column chromatography; *Asclepias curassavica*; Stigmasterol**

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INTRODUCTION

Inflammation is a complex biological response of vascular tissues to harmful stimuli, pathogens, irritants and damaged cells characterized by redness, swelling, warmth and pain[1]. Despite the progress made in medical research, the current treatment involves the use of NSAIDs to reduce inflammation. However, long term usage of these drugs can cause adverse side effects and leads to damage of liver, gastrointestinal tract, etc. and in some cases, cardiovascular and renal failure[2]. Chronic inflammatory diseases still remain one of the world's major health problems[3] . Therefore, at present there is a great demand for a more benign anti-inflammatory drug. Phytomedicine plays a major role in many developed and developing countries as a means of primary healthcare and according to WHO, 80% of the worldwide population rely on plant based medicine for the treatment of different diseases and ailments. *Asclepias curassavica* (Apocynaceae) is one such plant, which has very high medicinal property. It is a species of an evergreen perennial plant in the milkweed family, commonly called as milk weed. It is used in the treatment of swelling, bruises, wounds, skin ulcers and chronic cough. It has a specific action on the lungs, making it a valuable medicinal herb in all chest complaints and in the treatment of many lung diseases. Internally, it is used to treat diarrhea, dysentery and chronic rheumatism[4]. Therefore, the present study was taken up to check the potent anti-inflammatory effect by using various models.

MATERIALS AND METHODS

Collection and identification of plant material

The plant, *Asclepias curassavica* was collected from in and around Mysore during October 2013. The specimen sample was identified and authenticated by Central National Herbarium, BSI , Howrah. (Voucher specimen number: CNH/31/2014/Tech.11/58).

Preparation of crude extract

The collected plant material was shade dried and powdered using a commercial blender. The powdered plant material was sequentially extracted in a soxhlet apparatus using petroleum ether, chloroform and ethanol with increasing polarities, starting with the least polar solvent. The solvent extracts were concentrated in a rotary flash evaporator, lyophilized and stored at -20°C until use.

Column Chromatography

Separation of compounds by column chromatography is one of the most widely used techniques in biochemical work. Silica gel was chosen as the stationary phase and solvents are taken as mobile phase. The column was packed with the activated silica gel (120-200 mesh size) using hexane. The chloroform extract was transferred to the bed of silica gel by means of wet packing. Varying combinations of hexane and ethyl acetate in the ratio of 9:1, 8:2, 7:3, 6:4 and 5:5 were used in order to obtain 6 different fractions. Based on the Rf values, the fractions were pooled and subjected for second column chromatography step. The fraction obtained by 6:4 hexane and ethyl acetate combination was further orderly eluted and the sub-fraction 1 and 2 was pooled, evaporated under reduced pressure, which produced brownish amorphous powder. The structure was elucidated using NMR, LCMS and IR data.

Acute toxicity test

MTT Analysis of Cell Viability

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The SH-SY5Y cells were seeded in 96-well plates at a density of 1 9 104 cells/well and incubated for 24 h before experimental treatments. The cells were then subjected to the treatments of interest. After 24 h incubation, MTT (0.5 mg/mL) was added to each well. Following additional 2 h incubation at 37°C, 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 540 nm using a VERSA max Hidex plate chameleon TM V (Finland). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as percentage of control.

In vivo Anti-inflammatory activity

Hind paw edema method

The animals used in this study were male and female mice weighing between 25-30gms. Animals were maintained at 22 ± 2°C with 12 h light and dark cycle, fed on standard rodent chow with free access to water. All animal studies conducted were approved by the Institutional Animal Ethics Committee, DFRL Mysore, as stated by prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Protocol

Carrageenan induced paw edema is a classical model for determination of acute phase inflammation. The mice paw edema was provoked by sub planter injection 0.1 ml of 1% w/v of carrageenan in 0.9% saline in right hind paw. The hind paw volume was measured by dipping the foot in digital plethysmometer up to the lateral malleolus[5] . The displacement of sodium chloride solution was measured by the plethysmometer. The initial paw volume considered as 0 h reading was measured and recorded. The drug or test substances like 1% DMSO (vehicle control), Diclofenac Sodium, and various extract doses were administered orally 60 min before administration of carrageenan. The hind Paw volume was measured at 1 h interval up to 4th h of experiment. The difference between paw volumes at various time intervals indicated the edema volume due to inflammation. The percentage inhibition produced by the drug and extracts were calculated by following formula

Percentage inhibition of paw edema (%) = (Control - Treated) / Control X 100

Carrageenan induced paw edema model

Group I - Vehicle control treated with 1% of DMSO.

Group II - Animals treated with 1% of carrageenan.

Group III – Carrageenan induced animals treated with diclofenac sodium (dose 50 mg/kg.b.w).

Group IV - Carrageenan induced animals treated with sub-fraction 2 (dose 45 mg/kg.b.w).

GroupV - Carrageenan induced animals treated with sub-fraction 2 (dose 90 mg/kg.b.w).

Group VI - Carrageenan induced animals treated with sub-fraction 2 (dose 180 mg/kg.b.w).

RESULTS AND DISCUSSIONS

The HPLC chromatogram and the mass spectrum of isolated compound showed retention time of 17.7 to 18.5 (fig. 1) and molecular ion peaks at m/z 413.4 (M +1) with that corresponds to Stigmasterol (fig. 2). One major compound in sub-fraction 2 is a flavonoid glycoside with mass of 412. This is represented by the m/z ion at 413 (M+1). The mass spectral fragments suggest the presence of a flavonoid moiety. Based on the mass spectral fragmentation pattern and the NMR, the structure is proposed. The identified compound Stigmasterol[6] (Fig:3), also known as stigmasta-5,22-dien-3-ol ($C_{29}H_{48}O$) is reported in plant *Calotropis procera*. In the present study, we have isolated the same compound from *Asclepias curassavica,* both the plants belonging to the family Apocynaceae. Similar derivaties have been isolated viz., Stigmasta - 7,22- dien-3 β-ol (Spinasterol) from the plant extract of *Impatiens balsamina*[7] and in the dichloromethane extract of the flowers of *Adenocalymmer imperatoris - maximilianii*. Earlier reports by many investigators have proved the biological effects of stigmasterol to have anti-inflammatory, inhibiting tumor promotion, anti HIV reverse transcriptase[8] and it is known to possess anti-tumor effect against breast, ovarian and skin cancer cells[9]. Carrageenan induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation[10,11]. Development of edema induced by carrageenan is commonly correlated with early exudative stage of inflammation[12] and it is a bi-phasic phenomenon. The early phase of edema is attributed to the release of histamine, serotonin and similar substances. The later phase results mainly from the potentiating effects of

prostaglandins on mediator- release[13] .Soon after carrageenan injection, there is a sudden elevation of tissue volume, correlating with the action of inflammatory mediators on vascular permeability. Since several polysaccharides sharing the same back bone and branches posses the ability to inhibit the increase in vascular permeability, which is a typical model of first stage inflammatory reaction[14,15,16].The anti-inflammatory effect of sub-fraction 2 of *Asclepias curassavica* at different dose is shown in Table 1. The control group (Group I) showed no signs of inflammation or swelling during the experimental period. The group treated with carrageenan (Group II) showed elevated inflammation with respect to time interval. Significant increase in the development of edema was observed from 81.11 ± 2.62 to 94.26 ± 2.59 during 1 to 3 h. Treatment with *Asclepias curassavica* at 45 mg/kg (Group-IV) in carrageenan-inflamed mice resulted in significant ($p \leq 0.01$) decline in edema with a percentage of inhibition ranging from and initial value of 31.63 ± 1.25 to 20.8 ± 1.47 after 3 h. The inflamed mice treated with *A.curassavica* at 90 mg/kg (Group-V) showed moderate ($p \leq 0.05$) inhibition in paw edema (44.46 ± 1.37). However, a high inhibition rate was observed in mice treated with *A. curassavica* at 180 mg/kg (Group-VI) which showed a significant ($p \leq 0.01$) decline in paw edema with values ranging from 50.98 ± 1.77 at 1 h to 26.03± 1.09 after 3 h which was further declined to 17.59 ± 1.22 at 4th h. The mice treated with standard anti-inflammatory drug diclofenac sodium at 50 mg/kg.b.w (Group III) showed significant increase in inhibiting paw edema (22.18 ± 1.56 to 9.21 ± 1.48 at 1 h to 3 h respectively). The mice treated with *A. curassavica* at 45 mg/kg.b.w and 180 mg/kg.b.w were compared to mice treated with standard diclofenac sodium and from the results obtained it seems the effect of these drugs are almost similar. The anti-inflammatory activity was expressed as mean increase in paw volume in terms of mL and percentage inhibition in paw volume by different doses of the extracts. The result of the present study revealed that sub-fraction 2 of chloroform extract of *A. curassavica* present a potent anti-inflammatory effect in 'mean increase in paw volume' induced by carrageenan injection in the sub-plantar region of mice's paw. The sub-fraction of chloroform extract was tested for cytotoxic effects against SH-SY5Y cells (neuroblastoma) by MTT assay. Cell viability was evaluated by using various concentrations as shown in fig 4. The results clearly indicates that viability of cells were in a dose dependent manner. At 7.5 mg concentration, a two fold increase (from an initial value of 9.1% at 2mg to 21.99%) in the percentage of cell viability was observed. there was no change in the cell viability from 10 to 50 mg and a slight increase was observed at 75 and 100 mg. But, at 200 mg, a fourfold increase was observed when compared to initial concentration. Therefore, it is clear from the above results that the sub-fraction of chloroform extract of *A. curassavica* does not possess any toxic effects. However, reports concerning the cytotoxic constituents of *A. curassavica* is limited, but, traditionally, the plant is used in the treatment of cancer.

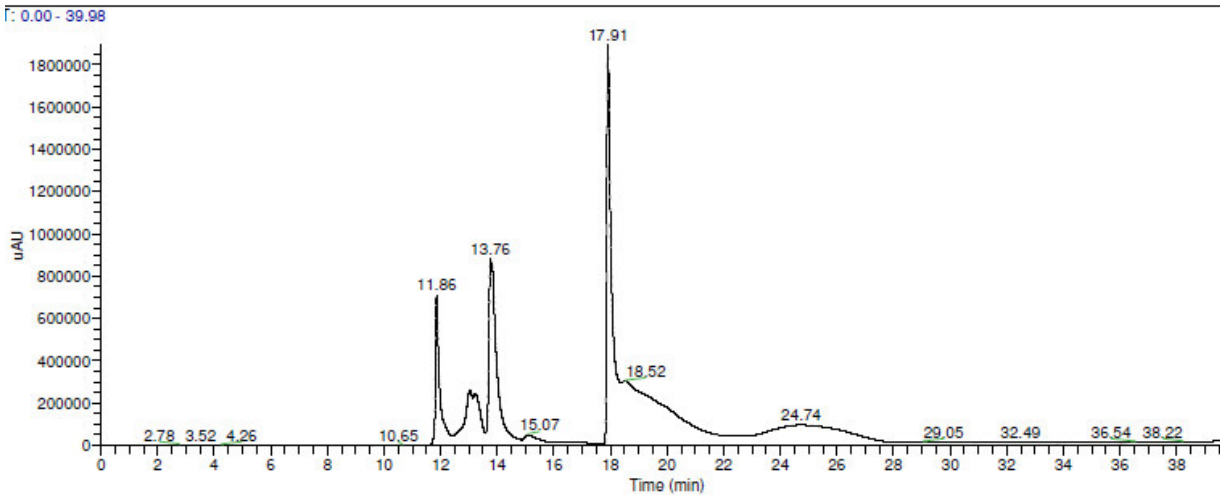

Figure 1
HPLC chromatogram of sub-fraction 2

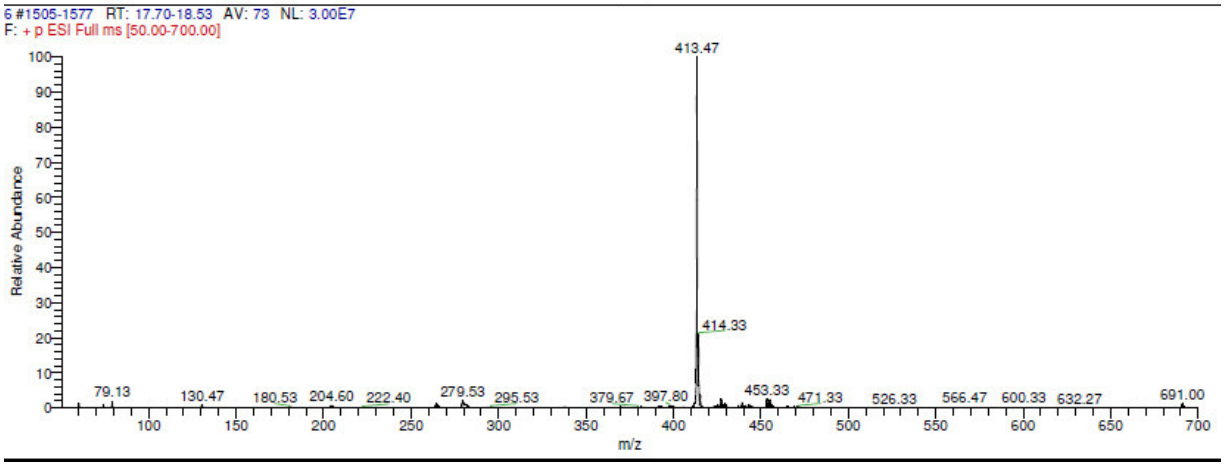

Figure 2
LCMS of sub-fraction 2 of Asclepias curassavica showing a single major peak with a molecular mass of 413 (M+1)

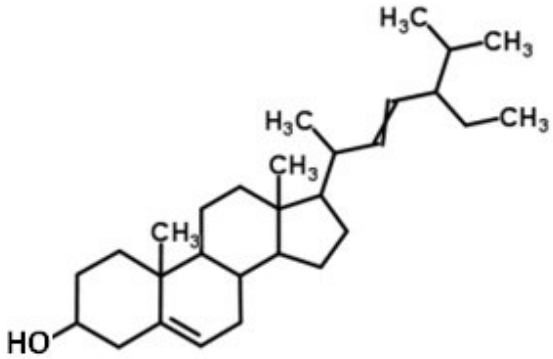

Figure 3
Stigmasta-5,22-dien-3-ol (Stigmasterol)

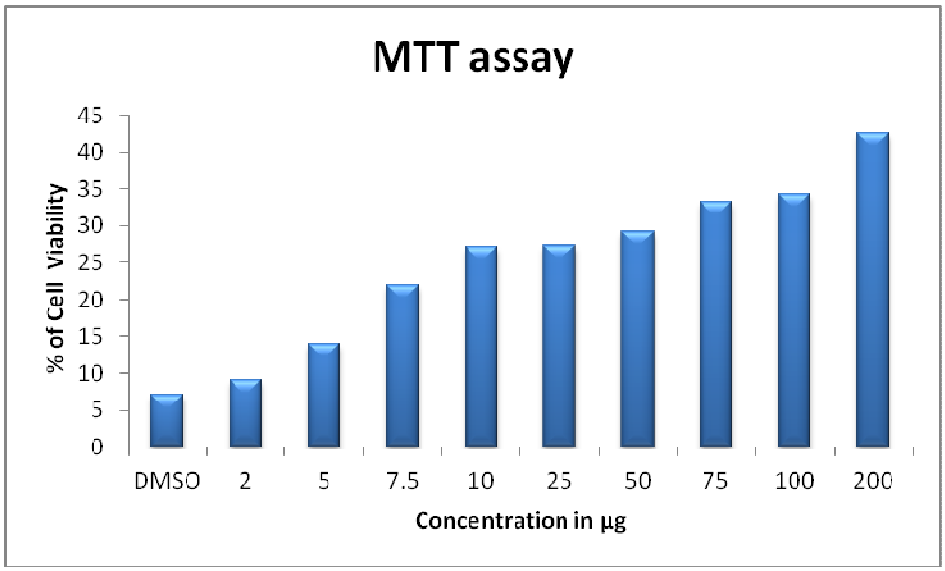

Figure 4
Effect of sub-fraction 2 of chloroform extract of A. curassavica on cell viability (SH-SY5Y cells)

Table 1
Effect of sub-fraction of chloroform extract of A. curassavica on paw edema volume in carrageenan-induced mice

Treatment Group	% inhibition of paw edema			
	1 h	2 h	3 h	4 h
Group I (Control)	0.00 (NA)	0.00 (NA)	0.00 (NA)	0.00 (NA)
Group II (Control Treated with Carrageenan at 1%)	81.11 ± 2.62	90.55 ± 1.70	94.26 ± 2.59	43.48±2.44
Group III (Carrageenan induced mice + Treated with Diclofenac sodium at 50 mg/kg)	22.18 ±1.56 ^{b}	13.06 ± 1.64^{b}	9.21 ± 1.48^{b}	4.9 ± 1.07 ^{a}
Group IV (Carrageenan induced mice + Treated with *A. curassavica* at 45 mg/kg)	31.63 ± 1.25 ^{a}	22.3 ± 1.51	20.8 ± 1.47^{b}	12.75± 0.99^{a}
Group V (Carrageenan induced mice + Treated with *A. curassavica* at 90 mg/kg)	50.51± 1.13^{a}	50.5 ± 1.75^{b}	44.46± 1.37^{a}	36.1 ± 1.7^{b}
Group VI (Carrageenan induced mice + Treated with *A. curassavica* at 180 mg/kg)	50.98 ± 1.77^{a}	35.41 ± 1.40^{b}	26.03± 1.09^{b}	17.59± 1.22^{a}

Percentage inhibition of paw edema values are presented as mean ± SD (n = 6); NA- Not Applicable.
^{a} p ≤ 0.05, ^{b} p ≤ 0.01, ^{c} p ≤ 0.001; Carrageenan v/s drug or test substance.

CONCLUSION

The results show that *A.curassavica* has promising anti-inflammatory activity against acute inflammation. The sub-fraction from chloroform extract showed significant anti-inflammatory potential, controlling the initial phase of inflammation and provoking an inhibition of edema formation similar to the positive control of diclofenac sodium. Characterization of the sub-fraction revealed the presence of stigmasterol.

Declaration of interest

The author have declared no conflict of interest. The investigator is thankful to all those who supported to carry out this work successfully. .

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