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IN VITRO CYTOTOXICITY ASSAY, ANTIOXIDANT SCREENING AND HEPATOPROTECTIVE ACTIVITY OF SUCCESSIVE EXTRACTS OF *ALYSICARPUS VAGINALIS* VAR. *NUMMULARIFOLIUS* (DC.) MIQ. (FAMILY: FABACEAE) IN HEPG2 CELL LINES

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ABSTRACT

The present study aims to screen the in vitro cytotoxicity, antioxidant potential and hepatoprotective activity of hexane (AVH), ethyl acetate (AVE) and methanol (AVM) extracts of *Alysicarpus vaginalis* var. *nummularifolius*, which is a traditionally used plant for jaundice. The cytotoxicity screening of different extracts was determined by MTT cell viability assay using L929 normal fibroblast cell lines. Antioxidant potential of plant extracts was studied by ABTS and hydroxyl radical scavenging activity. Total antioxidant capacity was evaluated by phosphomolybdenum method. *In vitro* hepatoprotective activity of the extracts was evaluated against CCl_4 induced toxicity in HepG2cell lines. *Invitro* cytotoxicity study report the IC_{50} values of hexane, ethyl acetate and methanol extracts of *A.vaginalis* as 55.26µg/ml,41.51µg/ml and 112.6µg/ml respectively. Significant dose dependent increase in percentage viability of HepG2 cells at the dose 6.25, 12.5,25,50 and 100µg/ml concentration of different extracts compared to CCl_4 (0.1%) exposed cells. AVM showed high hepatoprotective activity than AVH. AVE

showed dose dependent action, lower concentrations give hepatoprotective activity while at higher concentration an inhibitory effect is noticed. Thus the present study shows that methanol extract is non-toxic in nature while hexane and ethyl acetate extract of *A.vaginalis* are moderately toxic. High antioxidant activity is exhibited by ethyl acetate and methanol extract than the hexane extract. A good hepatoprotective effect is exhibited by the methanol extract than the other two extracts.

Keywords: *Alysicarpus vaginalis* var. *nummularifolius*, *in vitro* cytotoxicity, hepatoprotective activity, antioxidant

INTRODUCTION

Plant kingdom is the source of phytochemicals that are having broad spectrum of pharmacological activities **[1]**. Herbal drugs assumed to be safe for treatments in traditional system of medicine are very often identified toxic to vital organs **[1]**. Hence plants used in traditional medicines should be analyzed for its pharmacological potentiality and possibility of toxicity effects. Free radicals cause various ailments like liver cirrhosis, atherosclerosis, diabetes, cancer etc. **[2]**. Reactive oxygen species inactivates the enzymes and damage the cellular integrity resulting in injury **[3]**. Many herbal plants contain antioxidant which can protect the body against these free radicals **[4]**.

Liver diseases create a major problem around the world. The major risk factors of liver diseases are hepatitis, viral infection, toxic chemicals, alcohol etc. Herbal medicines are viable alternative to synthetic drugs **[5]**.

Alysicarpus vaginalis which is commonly known as Alyce clover or one leaf clover, coming under the family Fabacea. Traditionally it is used with goat milk for the treatment of jaundice. It is used in Ayurvedic medicine (Indian traditional system of medicine) and in folk medicine for the treatment of coughs, pulmonary and renal disorders. It is also used for the treatment of diarrhea, Ascariasis and constipation **[6]**.

On the basis of its diversified pharmacological properties and its use in liver disease treatment formulation, the main objective of the present study was to screen the hepatoprotective activity of the various extracts of *A.vaginalis* against CCl_4 toxicity in HepG2 cell lines.

MATERIALS AND METHODS

Collection of Plant Materials

Alysicarpus vaginalis var. *nummularifolius* (DC.) Miq. was collected from Nalanchira, Thiruvananthapuram, Kerala, India. It is a

common creeping herb found in open ground and waste land and is widely distributed in India, Sri Lanka, Pakistan, Africa and Australia. The leaves are obovate –oblong, obtuse at both ends and having a diameter of 1-2.5 cm. Its flowers are pink in dense recemes. They flower and fruit during the month of September to January. The plant specimen was authenticated by Dr. G. Valsaladevi, Curator, Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala and the voucher specimen has been deposited in the Herbarium of Department of Botany, University of Kerala with voucher no KUBH -5928 for future reference.

Preparation of the plant Extracts

The entire fresh plant materials were collected washed and shade dried. The dried plant materials were grinded to fine powder using an electric grinder. The dried and powdered plant material was successively extracted with hexane (AVH), ethyl acetate (AVE) and methanol (AVM) in the order of their increasing polarity in soxhlet apparatus until it became colourless according to the standard methods **[7]**. Each extract was concentrated by using rotary vacuum evaporator (Buchi Rotavapor) and stored in refrigerator for further analysis.

***Invitro* cytotoxicity study on L929 cell lines**

Cell lines –Its culture

L929 (normal mouse fibroblast) cells were obtained from NCCS, Pune, India and maintained in Dulbecos modified Eagles medium. The cell lines were cultured with DMEM supplemented with 10% Foetal Bovine Serum (FBS) and was kept at 37ºC in a humidified 5% CO_2 incubator (New Brunswick Scientific, Eppendorf, Germany).

MTT Assay

L929 cells were seeded in triplicate in 96-well microplates and kept at 37˚C in 5% CO_2 incubator. After 24h, the cells were treated with different concentrations of plant extracts (6.25, 12.5, 25, 50 & 100µg/ml). Cells without plant extracts were used as negative control.

The viability of cells were evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay.

The percentage of growth inhibition was calculated using the formula:

$$\text{\% of viability} = \frac{\text{Mean OD Samples x 100}}{\text{Mean OD of control group}}$$

Antioxidant activity

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al*., (1987) **[8]**. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM),

Ascorbic Acid (1 mM), H_2O_2 (1mM) and Deoxyribose (10mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of the extract of different concentration (125, 250, 500 & 1000 µg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM , pH 7.9), 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37ºC for 1 hour. 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025 % BHA) to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation:

$$\textbf{\% inhibition} = \frac{control-test}{control} X\mathbf{100}$$

ABTS Radical Scavenging activity

ABTS radical scavenging activity was measured using an improved assay (Re *et al*., 1999) **[9]**. The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants.20mM ABTS and 17mM potassium persulphate stock solutions were prepared. 0.3ml of potassium persulphate was added 50ml of ABTS and was left to stand overnight in dark before use. 1ml of distilled water 0.2ml of various concentration of the samples and 0.16ml of ABTS solution were mixed and made up to the final volume 1.36ml. Absorbance was measured after 20min at 734nm and the percentage inhibition was calculated using the equation.

$$\textbf{\% inhibition} = \frac{control-test}{control} X\mathbf{100}$$

Total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al*., (1999) **[10]**. 0.3 ml of extract was added to 3 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min then, the absorbance of the solution was measured at 695nm using a UV-VIS spectrophotometer against blank after cooling down to room temperature. Methanol (0.3ml) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid with methanol.

Hepatoprotective effect of plant extracts in HepG2 cell line

The screening of hepatoprotective activity of *A.vaginalis* was based on the protection of human liver-derived HepG2 cell lines against CCl_4 –induced damage. HepG2 cells were obtained from National Centre for Cell Science, Pune, India. They are considered a good model to study toxicity *in vitro*to the liver since it retains many of the specialized functions which shows the characteristics of normal human hepatocytes **[11]** and retain many morphological characters of liver cells **[12]**. The cell line was cultured in DMEM supplemented with 10% FBS, -L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100µg/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml) and kept at 37ºC in a humidified 5% CO_2 incubator.

Confluent monolayer of cells were trypsinized and 100µl cell suspension ($5x10^4$cell/well) was seeded in 96 well tissue culture plate and incubated at 37˚C in a humidified 5% CO_2 incubator. The cells after attaining sufficient growth were then exposed toCCl_4 (0.1%) and incubated for one hour;plant extracts in 5%DMEM with different concentration (100µg, 50µg, 25µg, 12.5µg & 6.25µg) were added and incubated at 37ºC in a humidified 5% CO_2 incubator. No plant extract was added to the negative control. Cytotoxicity was assayed by estimating the viability of the HepG2 cells by the MTT reduction assay [13]. Supernatant was removed and 3µl of reconstituted MTT solution was added to all and then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. The absorbance values were measured by using microplate reader at a wavelength of 570 nm **[14]**.

The percentage of growth inhibition was calculated using the formula:

$$\%\ \boldsymbol{of\ Viability} = \frac{\boldsymbol{Mean\ OD\ of\ Sample}}{\boldsymbol{Mean\ OD\ of\ Control}} \times \mathbf{100}$$

Statistical analysis

All data were represented as mean ± SE of triplicates. Linear regression analysis in MS Excel 2013were carried out for *in vitro* cytotoxicity study. The IC_{50} value of free radical scavenging activity was calculated using probit analysis method using SPSS (version 19).

RESULT AND DISCUSSION

***In vitro* cytotoxicity study**

In the present study, the effect of the three extracts of *A. vaginalis* on the percentage viability of L929 was evaluated **(Figure 1)**. Of the three extracts, the high percentage viability is shown in the methanol extract when compared with the AVH and AVE at the highest concentration (100µg/ml). From the linear regression analysis the IC_{50} values

of the AVH, AVE & AVM were calculated as 55.26, 41.51 and 112.61µg/ml, respectively **(Table 1)**. Higher the IC_{50} values, lesser is the toxicity of the material **[15]**. IC_{50} value lesser than 100µg/ml can be considered as moderately toxic. AVM shows higher IC_{50} value which can be considered as non-toxic. A similar report was given by the brine shrimp lethality assay. The toxicity of a compound can influence the normal activity of the cell **[16]**.

***In vitro* antioxidant activity**

Antioxidant activity in the present study were assessed by ABTS and hydroxyl radical scavenging assay. The ABTS and HO radical scavenging activities for all the extracts showed concentration dependent patterns, which is exhibited in **Figure 2 and Figure 3**. IC_{50} is the concentration which is required to scavenge 50% free radical **(Table 1)**. A low IC_{50} value shows a high antioxidant activity. AVE shows a high antioxidant activity with lowest IC_{50} value as 401.02± 0.08 and229.85±0.07 for ABTS and HO respectively, which is followed by AVM and AVH. Total antioxidant capacity of the AVH, AVE and AVM were found to be 145.1 ±1.9, 153.97 ± 0.89 and 127.19 ± 4.34 mg Ascorbic Acid equivalent/g plant extract respectively which shows that AVE with highest antioxidant activity **[2, 4]**.

Hepatoprotective effect in HepG2 cell lines

Liver damage induced by CCl_4 is conventionally used model for the screening of hepatoprotective drugs **[17]**. CCl_4 undergoes metabolic activation by a cytochrome P-450 dependent step to free radical products which can initiate lipid peroxidation. Many previous reports confirm the decrease of both toxicity and lipid peroxidation induced by CCl_4 by many natural antioxidants **[17, 19]**. HepG2 cell lines has increasingly been used as a model to study the hepatoprotective action of natural antioxidants **[17, 19, 20, 21]**. The hepatoprotective effect of the extracts of *A.vaginalis* was evaluated in HepG2 cell lines. HepG2 cell lines exposed to CCl_4 showed a percentage viability of 29.6%. These CCl_4 exposed cells when treated with different concentrations of plant extract of *A.vaginalis* showed a dose – dependent increase in percentage viability in the case of AVM and AVH, while for AVE, the lower concentration showed a hepatoprotective activity which at higher concentrations produced an inhibitory effect. The highest hepatoprotective activity was seen in methanolic extract (56.1µg/ml) than the hexane extract (53.86µg/ml). High phenolic and flavonoid compounds present in the methanol extract was reported **[16]**. A high

correlation between total phenolic content and the hepatoprotective activity was reported **[20]**. The high phenolic content of AVM gives the high hepatoprotective activity than the other two extracts. AVE showed high cytotoxic effect in L929 cell lines with low IC_{50} value. The compound of ethyl acetate extract that gives cytotoxic effects produce an inhibitory effect to the HepG2 cell lines under higher concentrations. Considering the different extracts of this plant, the methanolic fraction can be identified as the less toxic and it displayed good antioxidant activity with higher hepatoprotective activity. The antioxidative compound might be contributing the hepatoprotective effect against CCl_4 induced toxicity. Therefore AVE under lower concentration and AVM (100µg/ml) can be considered for further pharmacological evaluation **(Figure 4)**.

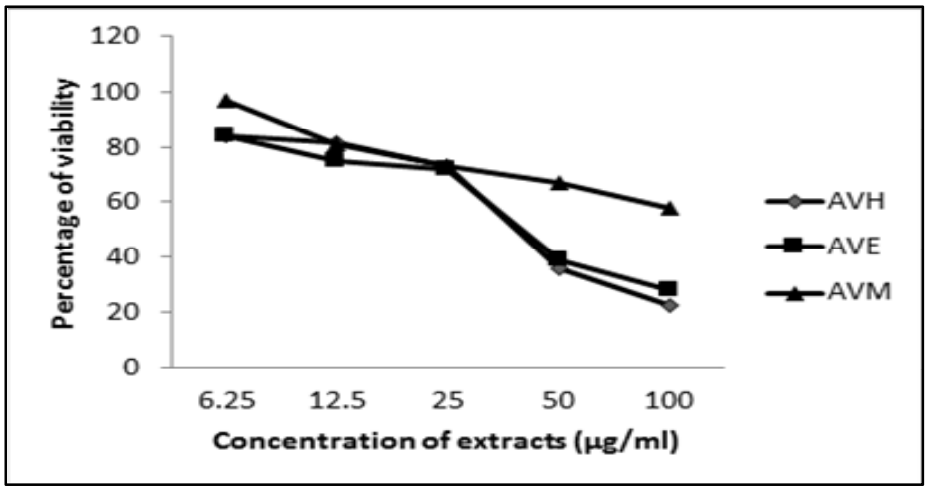

Figure 1: ***In vitro*** **cytotoxicity effects of different extracts of** ***A.vaginalis*** **on L929 fibroblast cell lines**

Table 1: ***In vitro*** **cytotoxicity, Antioxidant activity and hepatoprotective effects of different extracts of** ***A.vaginalis*** **against CCl_4 induced toxicity**

Compound	*Invitro* cytotoxicity (IC_{50} µg/ml)	Hydroxyl radical (IC_{50} µg/ml)	ABTS (IC_{50} µg/ml)	Total Antioxidant (mg AAE/g plant extract)	HepG2 Cells (EC_{50} µg/ml)
AVH	55.26	582 ±0.08	877 ±0.08	145.1 ±1.9	78.44
AVE	41.51	229.85 ±0.07	401.02 ±0.08	153.97 ±0.89	12.32
AVM	112.61	248.28± 0.078	431.02 ±0.08	127.19 ±4.34	53.38
L-Ascorbic Acid		314.87± 0.07	411.82 ±0.08		

Values are mean ±SE of three determinations. Hexane (AVH), Ethyl acetate (AVE) and Methanol (AVM) extract of ***A.vaginalis*****. AAE-ascorbic acid equivalents**

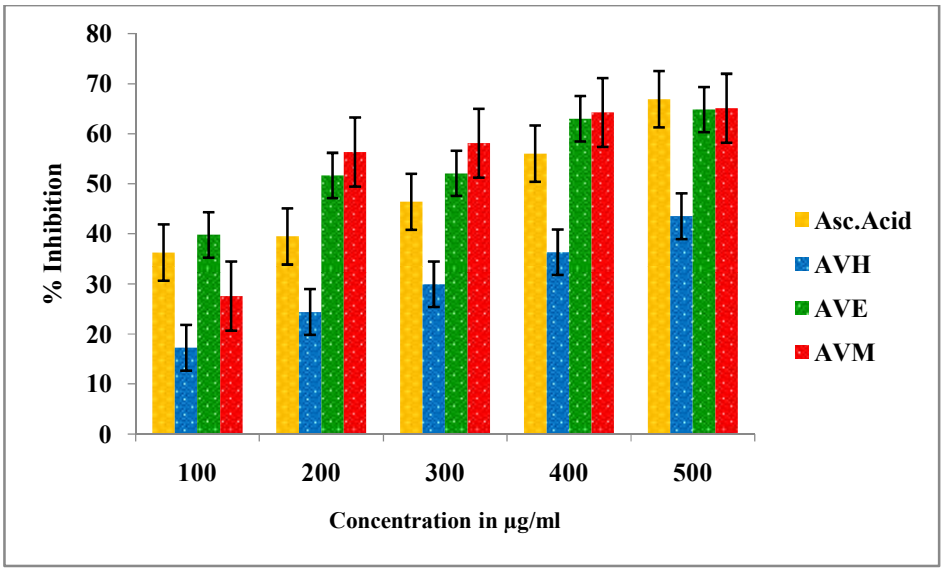

Figure 2: Hydroxyl radical scavenging activity of different extracts of *Alysicarpus vaginalis*
Values are represented as mean ± SEM of three replicates

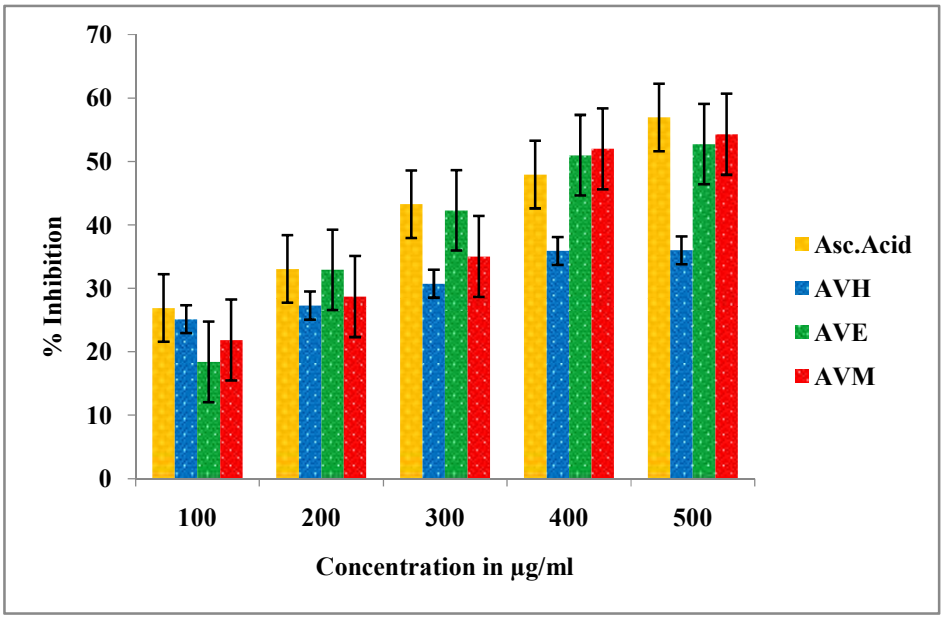

Figure 3: ABTS radical scavenging activity of different extracts of *Alysicarpus vaginalis*
Values are represented as mean ± SEM of three replicates

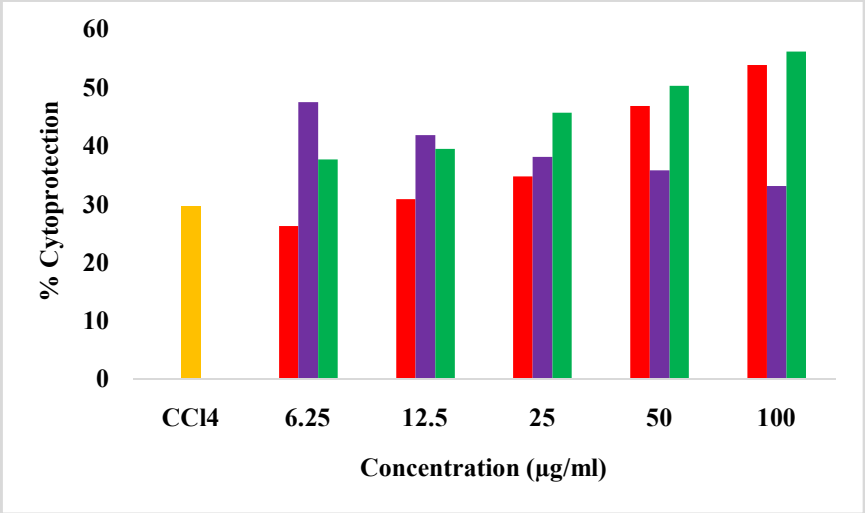

Figure 4: Hepatoprotective effect of hexane(AVH),ethyl acetate (AVE) and methanol (AVM) extract of A. vaginalis against CCl_4 induced toxicity

CONCLUSION

The present study reported the *invitro* cytotoxicity effect of the successive extracts of *A.vaginalis* on L929 cell lines. The methanol extract (AVM) showed the least cytotoxic effect. The *invitro* antioxidant activities of the plant extracts were evaluated. AVM showed a high antioxidant activity followed by AVE and AVH. Oxidative stress was introduced in HepG2 cell lines by CCl_4toxication. The cell viability was improved with the treatment of the extracts of *A.vaginalis*. AVM had the less cytotoxic effect with high antioxidant and hepatoprotective activity which is followed by AVE and AVH. The occurrence of antioxidative principles and hepatoprotective action of this plant extracts support its ethanopharmacological use for the treatment of hepatitis. Further efforts are needed for the isolation and characterization of the bioactive compound and the in vivo studies can reveal the mechanism of the action of these plant extracts.

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