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Hepatoprotective Activity of Leaf and Leaf Callus Extracts of *Orthosiphon aristatus* (Blume) Miq.

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Authors' contributions

This work was carried out in collaboration between all authors. Author NAR designed and executed the experimental work, managed the statistical analysis and drafted the manuscript. Author SMS supervised the study and approved the final draft of the manuscript. Author GVH managed the analyses of study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The study was carried out to evaluate the *in vitro* hepatoprotective activity of leaf and leaf callus extracts of *Orthosiphon aristatus* against alcohol induced toxicity using HepG2 cell line. **Materials and Methods:** Leaf segments were cultured on Murashige and Skoog solid medium fortified with different auxins alone and in combination. Prior to the determination of hepatoprotective property leaf and leaf callus extracts were subjected to the toxic dose study. The degree of hepatoprotection of extracts was determined by measuring cell viability percentage by MTT assay. Leaf and leaf callus extracts were subjected to the preliminary phytochemical analysis. **Results:** Maximum percentage of callus formation (94%) was obtained in MS medium augmented with 2 mg/L of 2,4-D. HepG2 cells were pretreated with the different concentrations (below toxic dose) of leaf and leaf callus extracts for 72 hrs. followed by alcohol intoxication. Results revealed that aqueous leaf extract pretreated HepG2 cells show 90% cell viability compared to the standard silymarin pretreated HepG2 cells which showed 81% cell viability. Leaf callus extracts also showed significant hepatoprotective activity where ethanolic callus extract pretreated HepG2 cells showed

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82% viability after intoxication with alcohol. Results revealed that HepG2 cell viability percentage is dose dependent. Phytochemical studies revealed the presence of different secondary metabolites in leaf and leaf callus extracts.

Conclusion: The bio-efficacy study confirms the presence of secondary metabolites of hepatoprotective nature. Callus mediated tissues show hepatoprotection which paves a way for the mass production of desired biologically active principles.

Keywords: Leaf callus; leaf; ethanol; hepatoprotection; HepG2 cell line; MTT assay.

ABBREVIATIONS

MS: Murashige and Skoog; 2,4-D: 2,4-Dichlorophenoxyaceticacid; NAA: Naphthalene acetic acid; IAA: Indole-3-acetic acid; MTT: 3-(4,5-dimethylthiazole-2 yl)-2,5 diphenyl tetrazolium bromide; DMEM: Dulbecco's Modified Eagle's Medium; OD: Optical density; DMSO: Dimethyl Sulfoxide; ROS: Reactive Oxygen Species.

1. INTRODUCTION

Use of plants as medicine is as old as mankind. From last few decades, there has been a surge in public interest for the use of herbal medicine due to the ill effects associated with synthetic drugs. The popular observation and scientific validation of medicinal uses of traditionally used medicinal plants has gained a global attention with the aim to isolate and characterise new natural compounds. It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families possess hepatoprotective activity [1].

Alcohol Liver Diseases (ALD) which is one of the most serious consequences of the chronic alcohol abuse and is the second leading cause of death among all liver diseases has necessitated a search for the new hepatoprotective drugs [2]. Alcohol induces a number of adverse metabolic changes in liver. Excessive consumption of alcohol for a long time leads to the steatosis, alcoholic hepatitis and cirrhosis resulting in weight and volume change of liver [3]. Studies on human HepG2 cell lines have shown in earlier studies that ethanol is cytotoxic and is apoptotic in nature predominantly in liver [4]. There are no satisfactory remedies available for liver diseases; hence search for effective hepatoprotective drugs from natural products is continued [5].

Plants have been source of diverse medicinal therapeutic agents. Only a small number of traditionally used medicinal plants have been scientifically evaluated for the hepatoprotective property [6]. Screening of callus extracts for biological activities paves a way for the mass production of biologically viable secondary metabolites and thus provides an alternative for the *in vivo* plant material thereby minimising any possible threat to the natural habitat of medicinal plants.

Orthosiphon aristatus (Blume) Miq. also called as cat's whiskers, is an ethnomedicinal perennial herb which belongs to the family Lamiaceae and is a native of Indonesia. The plant is one of the popular traditional folk medicines extensively used in Southeast Asia for the treatment of wide range of ailments. In Indonesia leaves are used to treat rheumatism, diabetes mellitus, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhoea, syphilis, renal calculi, gallstone, acute and chronic nephritis, gout arthritis, urinary tract, renal diseases and fever [7-10]. It is also traditionally used to treat edema, eruptive fever, influenza, hepatitis, jaundice and biliary lithiasis [11,12]. Traditionally it is also called Kidney Tea Plant as it is used to treat all types of kidney ailments like nephrocirrhosis, phosphaturia, hepatorenal syndrome, renal ischaemia [13,14]. Owing to the multipurpose ethnomedicinal use of *O. aristatus*, present study was taken up to screen its leaf and leaf callus extracts for hepatoprotective activity against ethanol induced toxicity using HepG2 cell line.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material and Callus Induction

O. aristatus plants were collected from the Nilgiri Hills, Western Ghats of Tamil Nadu, India and are being maintained in the medicinal plant garden of Department of Studies in Botany, University of Mysore, Mysuru. Healthy leaf explants were collected from the mother plant

and were washed under running tap water for ten minutes to remove the soil and dust particles followed by treatment with fungicide bavistin (1%, w/v) for five minutes. Explants were then washed thrice with sterile double distilled water and were treated with mercuric chloride (0.01%, w/v) for five minutes followed by washing thrice with sterile double distilled water. Inside laminar airflow, leaf explants were cut into small pieces (1 sq.cm) and were inoculated onto the MS medium containing 3% sucrose, 0.08% agar and fortified with different concentrations of auxins. Prior to autoclaving at 121℃ for 15 min, pH of the medium was adjusted to 5.8 using 1 N NaOH and 1 N HCl. The culture flasks were maintained in the incubation chamber under a 16 hrs photoperiod at light intensity of 25 $\mu mol/s^2/m^2$ for four weeks. Each experiment was performed with five replicate and repeated thrice. The callus cultures were maintained for the period of five months and were periodically subcultured with 2-3 weeks of interval onto the fresh MS medium for callus proliferation. Consequently, the callus was harvested at the transfer age of 3 weeks, kept in hot air oven at 60℃ for 24 hrs. till a constant dry weight was obtained and the callus was then further exploited for extraction and hepatoprotective evaluation.

Dried leaves and the *in vitro* leaf derived callus was coarse powdered using electric homogenizer. Aqueous extraction was carried out by mixing leaf and leaf callus coarse powder separately with deionised water in the ratio of 1:5 (w/v) in conical flask and allowed to settle in an oven at 50℃ for 72 hrs. with occasional shaking. After 72 hours the extracts were filtered using Whatman filter paper [15]. The filtrate was lyophilised to dryness and stored in vials at 5℃ for further use. Solvent extraction was carried out by taking 25 grams of both dry leaf and callus coarse powder and filled in a thimble separately and extracted sequentially with 200 mL of petroleum ether, chloroform, ethyl acetate, ethanol and methanol in Soxhlet extractor for 48 hours. The solvent extracts were concentrated under reduced pressure and were stored at 5℃ in vials for further use. All the leaf and leaf callus extracts were dissolved in 1% of DMSO for the bio-efficacy evaluation [16]. HepG2 (Normal Human Liver Cell Line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of HepG2 cell line were subcultured as monolayers in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum) and penicillin (100 µg/mL) [17]. All the leaf and leaf callus extracts were dissolved in 1% of DMSO and were used for further studies. Prior to the screening of hepatoprotective activity, toxic dose studies of extracts (both leaf and leaf callus) and silymarin were carried out by MTT assay (3-(4,5-dimethylthiazole-2 yl)-2,5 diphenyl tetrazolium bromide).

IC_{50} value of ethanol was also calculated by MTT assay [1].

HepG2 cells were subcultured, after 72 hrs. medium supernatant was flicked off, cell monolayer was trypsinized and the cell count was adjusted to the 1.0×10^5 cells/mL. To each well of the 96 well plate 200 µL of diluted cell suspension ($1x10^5$ cells approximately) was added and the plates were incubated for 72 hours at 37℃ and 5% of CO_2 for 24 hrs. After 72 hrs. when monolayer of cells was formed, medium supernatant was flicked off and the 200 µL of DMEM containing leaf and leaf callus extracts in concentrations below toxic dose was added to each well containing cell monolayer. The plates were then incubated for 72 hrs. at 37℃ and 5% CO_2. This boosting of cells with the different extracts is also known as pre-treatment protocol. After 72 hrs., the medium containing extracts was flicked off and DMEM containing ethanol at 150 mM concentration was added to the each well containing extract and silymarin pretreated cell monolayers. The plates were again incubated for 72 hours at 37℃ and 5 % of CO_2. After 72 hrs. of incubation, medium containing ethanol was flicked off and 100 µL of 5% MTT reagent in DMEM was added to each well and the plates were again incubated at 37℃ for 3 hours. After 3 hrs., the supernatant was discarded and 100 µL of solubilisation solution (1% of DMSO) was added to each well and the plates were gently shaken in gyratory shaker to solubilise the formed formazan. The absorbance was then measured by using microplate reader at 630 nm and the percentage of growth inhibition was calculated using formula given as under:

Percentage growth inhibition =

$$\left\{\frac{\text{Mean OD of the individual test group}}{\text{Mean OD of control group}}\right\} \times 100$$

All the assays were carried out in triplicates and the data analysis was carried out by using SPSS, DMRT. Preliminary phytochemical analysis of leaf and leaf callus extracts were carried out by the method described by Rajan et al. [18].

3. RESULTS

Callus induction was observed along the cut edges of leaf segments after two weeks of inoculation on MS medium supplemented with auxins. Callus induction percentage varied with respect to the concentration of the growth regulator used and the age of the explant. Young explants cultured on MS medium augmented with 2,4-D (1-2 mg/L) showed high percentage of callus induction and proliferation. IBA and NAA supplemented medium induced hard and compact callus with low percentage of proliferation (Table 1).

Cytotoxic study of leaf and leaf derived callus of *O. aristatus* was carried out to standardise extract concentrations and evaluate their hepatoprotective activity. Results revealed that *O. aristatus* (both leaf and leaf callus) above 225 µg/mL concentration is toxic to the HepG2 cells where the cell viability percentage was reduced to 40%. MTT assay for cytotoxicity of standard drug silymarin revealed that concentration above 75 µg/mL is toxic to the cells and hence 75 µg/mL was used as the test concentration for the subsequent study. IC_{50} value of ethanol was reported to be 150 mM (0.69% ethanol). Cytotoxic effect of different concentrations of leaf, leaf callus (extracts) and silymarin is presented in Fig. 1.

Change in cell viability percentage on ethanol induced toxicity in HepG2 cells pretreated with different leaf and leaf callus extracts was carried out by MTT assay. Ethanol (150 mM) intoxicated HepG2 cells showed a percentage viability of 50% after 72 hrs. *O. aristatus* leaf and leaf callus extracts were tested at 100 µg/mL and 200 µg/mL concentration for their hepatoprotective activity. Out of the different extracts of leaf and leaf callus, polar solvent extracts like aqueous, ethanol and methanol pretreated cells showed significant viability percentage after intoxication with ethanol. HepG2 cells pretreated with leaf and leaf callus extracts showed a dose dependent increase in percentage viability. The cell viability ranged from 58% to 90% in leaf extracts and 60% to 82% in leaf callus extract pretreated cells. The maximum cell viability percentage (90%) was reported in aqueous leaf extract pretreated HepG2 cells at 200 µg/mL. Out of the callus extracts, 82% cell viability was reported in ethanolic callus extract pretreated HepG2 cells. Silymarin pretreated HepG2 cells showed maximum 81% cell viability. The cell viability percentage of HepG2 cells pretreated with leaf and leaf callus extracts is presented in Tables 2 and 3.

Preliminary phytochemical analysis of leaf and leaf callus extracts revealed the presence of Alkaloids, flavonoids, saponins, phytosterols, phenols, terpenoids, triterpenoids, cardiac glycosides and anthraquinones.

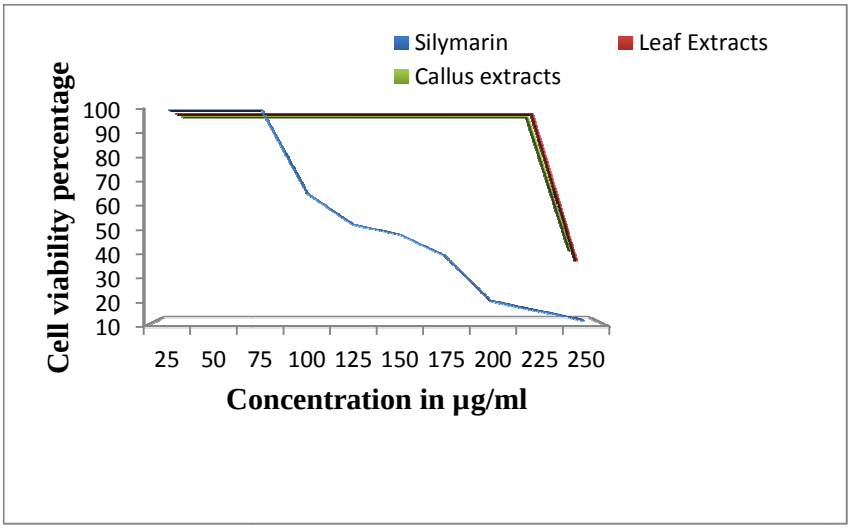

Fig. 1. Cytotoxic effect of silymarin, leaf extracts and leaf callus extracts of *O. aristatus* on HepG2 cells

Table 1. Influence of auxins on callus induction from the leaf segments of *Orthosiphon aristatus*

MS medium+Plant growth regulator mg/l	Mean percentage of callus induction	MS medium+Plant growth regulator mg/l	Mean percentage of callus induction
2,4-D		**IAA**	
0.5	68	0.5	43
1	86	1	47
1.5	90	1.5	56
2	94	2	62
2.5	81	2.5	64
3	83	3	70
3.5	72	3.5	76
4	70	4	79
4.5	74	4.5	82
5	68	5	78
IBA		**NAA**	
0.5	32	0.5	30
1	42	1	36
1.5	58	1.5	42
2	58	2	46
2.5	62	2.5	53
3	68	3	58
3.5	72	3.5	60
4	65	4	63
4.5	60	4.5	68
5	62	5	68

**All treatments with 5 replicates and were repeated thrice. Callusing percentage: (Number of explants callused/Total number of explants inoculated) x 100*

Table 2. The cell viability percentage of the HepG2 cells pretreated with leaf extracts of *O. aristatus*

Leaf extracts	Concentration (µg/mL)	% cell viability
Aqueous	100	86±0.32
	200	90±0.26
Ethanol	100	84±0.51
	200	87±0.56
Methanol	100	82±0.57
	200	84±0.38
Ethyl	100	75±0.20
acetate	200	78±0.35
Petroleum	100	62±0.31
ether	200	60±0.32
Chloroform	100	56±0.38
	200	58±0.42
Silymarin	75	81±0.53
Control	Only cells	100

**All the MTT assays were repeated thrice. Each value represents Mean±S.D. Statistical analysis done by SPSS.*

Table 3. The cell viability percentage of the HepG2 cells pretreated with leaf callus extracts of *O. aristatus*

Callus extracts	Concentration (µg/mL)	% cell viability
Aqueous	100	68±0.67
	200	73±0.31
Ethanol	100	77±0.20
	200	82±0.43
Methanol	100	78±0.43
	200	80±0.21
Ethyl acetate	100	66±0.42
	200	69±0.32
Petroleum	100	63±0.45
ether	200	65±0.40
Chloroform	100	60±0.23
	200	60±0.45
Silymarin	75	81±0.53
Control	Only cells	100

**All the MTT assays were repeated thrice. Each value represents Mean±S.D. Statistical analysis done by SPSS*

4. DISCUSSION

The study was aimed to evaluate the hepatoprotective property of leaf and leaf callus extracts of *O. aristatus.* In the present study, 2,4-D supplemented MS medium was found to induce maximum callusing in leaf segments. Effect of auxins on callus induction has been reported earlier by many tissue culturists [19-21]. Our results are directly in concurrence with the earlier findings reported by Rout et al. [21] and Manickam et al. [22] in *Plumbago zeylanica* and *Withania somnifera* respectively. Drug, whether synthetic or natural, if consumed in excess dose damages the liver cells. This is the reason; toxic dose studies of drugs are carried out. In our study, cytotoxic study of *O. aristatus* leaf and leaf callus extracts was carried out by MTT assay before the extracts were subjected to the hepatoprotective activity. The principle involved is the cleavage of tetrazolium salt into its blue coloured derivative formazan; the process takes place in the inner mitochondrial membrane where an enzyme succinate dehydrogenase cleaves the tetrazolium salt. Only the living cell mitochondria reduces the MTT to coloured formazan, therefore the concentration of the dye is directly proportional to the number of metabolically active cells. The MTT assay revealed that concentration of *O. aristatus* leaf and leaf callus above 225 µg/mL kill 60% of the cells. Toxic dose studies of the extracts lay the foundation for the formulation of any type of herbal remedy [23].

In the present investigation ethanol was used as hepatotoxicant. Ethanol induced liver injury is encompassed by wide spectrum of lesions, the most characteristic being alcoholic steatosis (fatty liver), alcoholic hepatitis, alcoholic fibrosis and cirrhosis [24]. The present investigation revealed that ethanol treated groups of HepG2 cells showed a drastic decrease in the cell viability when compared to the HepG2 cells pretreated with the leaf and leaf callus extracts. Increase in the percentage of cell viability in extract pretreated HepG2 cells indicates that the cells get boosted up upon treatment with extract and does not allow oxidation to take place upon intoxication with ethanol. In the present study, HepG2 cells pretreated with leaf and leaf callus extracts showed more percentage viability than that of positive control silymarin**.** The cell viability relies on the structure of membrane and any damage to cell membrane causes leakage of the cellular enzymes and consequently a cell death [2]. Results revealed that HepG2 cells pretreated with aqueous, ethanol and methanol leaf and leaf callus extracts of *O. aristatus* showed a dose dependent increase in the cell viability. The dose dependent cytoprotection has been reported earlier in plants like *Cassia roxburghii* [25], *Polygonum multiflorum* [26]*, Andrographis paniculata* [27] and *Rumex vesicarius* [28]. Kanchana and Jayapriya [29], described in their study that the increase in the cell viability percentage may be a consequence of membrane stabilisation boosted by phytochemicals and they further demonstrated that the plant extracts elevate the tissue antioxidant defence enzymes and thus tackle oxidative stress.

Callus cultures have proved to be of great importance in alternative medicine in recent times as it paves a way for the establishment of callus cell lines for the mass production of the pharmacologically and biologically active secondary metabolites. Present investigations revealed that leaf callus extracts of *O. aristatus* possess hepatoprotective activity against ethanol induced toxicity in HepG2 cells. The chemical constituents of leaf and leaf callus extracts may have interrupted the reaction of ROS with cell proteins and nucleic acids and thus preventing the formation of adducts by acting as scavengers and thereby stabilising the cell membrane resulting in increased cell viability. The inference of the present investigation is in concurrence with the earlier studies carried out by Santhosh et al. [30]*,* Kamel et al. [31], and Bhagyashree et al. [32].

5. CONCLUSION

Ethanol induces production of reactive oxygen species (ROS), leading to huge oxidative stress which damages the liver cells. *O. aristatus* leaf and leaf callus extracts have shown the dose dependant hepatoprotection against ethanol. The results suggest the presence of active phytoconstituents in leaf and leaf callus extracts which strengths antioxidant defence in cells and thus minimising the chances of production of free radicals. The current study further confirms the hepatoprotective property of leaf callus, which adds the extra feather to our study as the callus cultures could be used for the mass production of desired secondary metabolites and may also be used as replacement for the *in vivo* plant material and thus minimising the chances of threat to natural population of medicinal plants. Furthermore phytochemical analysis needs to be carried out to isolate and characterise the bioactive compounds from leaf and leaf callus

extracts with hepatoprotective activity and for further authentication pharmacological studies also need to be carried out.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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