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IN VITRO PROTECTION OF HEPATOCYTES BY *ALOCASIA MACRORRHIZA* LEAF JUICE AGAINST CCL_4 AND TYLENOL MEDIATED HEPATIC INJURY

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ABSTRACT
The present study was carried out to investigate the antioxidative and hepatoprotective efficacy associated with *Alocasia macrorrhiza* leaf juice in the quest of finding new potent antioxidant and hepatoprotective molecules from nature using *in vitro* technology. To test the antioxidant and hepatoprotective efficacy *in vitro* liver slice model was used where in the hepatic damage was induced by the hepatotoxins CCl_4 and Tylenol. The free radicals generated by CCl_4 and Tylenol cause oxidative stress and result in injury to the hepatocytes. The extent of damage caused by these free radicals as well as evaluation of hepatoprotective and antioxidative efficacy associated with the *Alocasia macrorrhiza* leaf juice was measured in terms of leakage of marker enzymes of liver function *viz* AST, ALT and ALP in the surrounding incubation medium from the liver slices. Additionally the TBARS and Glutathione contents were measured in the liver slices. In presence of CCL_4 as well as Tylenol there was increase in the levels of marker enzymes and TBARS coupled with depletion of total glutathione contents (GSH). However the leaf juice of *Alocacisa macrorrhiza* remarkably decreased the leakage of AST, ALT and ALP in the medium. The results obtained from TBARS and Glutathione assays were also supportive to conclude that the *Alocasia macrorrhiza* leaf juice as a whole possesses hepatoprotective and antioxidative efficacy when tested *in vitro* using rat liver slice model.

Key words: *Alocasia macrorrhiza*, hepatoprotecitve *in vitro*, liver, CCl_4, Paracetamol, Acetaminophen, Tylenol, antioxidant
Running Title : In vitro studies of *Alocasia macrorrhiza*

[I]INTRODUCTION

Cell culture models exhibit limitations with missing cell-to-cell interactions and the complexity of isolated perfused organs. In view of these limitations, methods using liver slices were established which can retain tissue organization and cell-to-cell matrix interactions as in perfused organs [1]. Liver-slice models have been used in investigational pathology to assess a number of hepatotoxic effects and a variety of substances have been studied including CCl_4 and paracetamol. The main advantages are represented by the preservation of lobular structures in contrast to cell cultures and the possible application of biochemical and molecular biological methods in contrast to organ perfusions [1]. The *in vitro* liver slice model is widely used to test toxicity or protectivity associated with many other herbal preparations [2].

Plants as a source of medicines: Plants form the backbone of traditional system of medicine all over the world. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds. Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design.

Medicinal plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs [3].

Alocasia macrorrhiza: *A. macrorrhiza* is recorded among cultivated medicinal as well as vegetable plants by the folklore of south Asia [4]. The leaves of this plant are used to prevent iron deficiency, to enhance eye sight and as a good source of protein. Also the whole plant is used for jaundice or constipation. It is also utilized as a functional food which is specially included in diet as a preventive measure [4]. Efforts are continued to identify the potential medicinal uses of the *Alocasia macrorrhiza*, especially on liver [5]. An anti-fungal protein designated alocasin is isolated from the plant which can reduce the activity of HIV-1 reverse transcriptase [6]. One of the species of Alocacia, *A. indica* has been worked out for its efficacy to protect the liver and found to possess significant potential as hepatoprotective agent [7]. The leaves of *Alocasia macrorrhiza* are nutritionally found to contain varying amounts of proteins, Ash, crude fibre, carbohydrate, starch, Ascorbic acid, oxalates, proteases, Nitrate, and Tannin. It is a cultivated edible aroid in India [8].

Tylenol and CCl_4: Tylenol is available as over the counter medicine and over dosage of tylenol leads to the saturation of conjugation pathway leading to glutathione depletion and increase in the formation of toxic reactive metabolites. A high level of reactive metabolites increases the level of hepatotoxicity, with increases level of protein adducts formation, mitochondrial dysfunction and oxidative stress [9]. Carbon tereachloride (CCl_4) is a well known hepatotoxin causing hepatocyte injury by formation of the free radicals and is used extensively in toxicity models to induce hepatic injury. The present study reveals the hepatoprotective activity and antioxidant efficacy of *Alocasia macrorrhiza* leaf juice against Tylenol and CCl_4.

[II] Materials and Methods

2.1 Plant material: *Alocacisa macrorrhiza* plants were collected from Kolhapur, MS India. The species identification was confirmed from Dr A. R. Jadhav, of Department of Botany, Yashavantarao Chavan College, Warananagar, Kolhapur, MS India. The leaves were removed carefully, washed thoroughly using distilled water and blotted briefly. The juice of whole leaves was prepared and filtered through Whatman filter paper. The filterate was collected in sterilized and aseptic conditions and was refrigerated till further use.

2.2 Chemicals: All the chemicals used were highly pure and of analytical grade. Tylenol (Acetaminophen) and CCl_4 were obtained from S D fine chemicals, Mumbai while the culture media M199 was procured from Himedia. Pathological diagnostic kits were purchased from AGAPPE Diagnostics, India.

2.3 Experimental Animals: Albino male rats of Wistar strain weighing 175 to 225 gm, bred and reared under standard housing conditions were obtained from the registered animal house of Tatyasaheb Kore College of Pharmacy, Waranangar, Dist. Kolhapur, Maharashtra, India. Animals were sacrificed giving deep ether anesthesia.

2.4 Experimental Procedure: Surgical procedures were carried out on fed rats under deep ether anesthesia to obtain whole liver. The liver slices (LS) were prepared from the whole liver without distinction of lobes. LS were incubated in presence of incubation medium M199 in a shaking water bath (100 cycles/min)

at 37 °C to randomize and avoid any variability between slices that may come from localization in liver lobes or size. After 1 hour of preincubation, allowing fresh slices to recover, the slices were transferred to other vials ready with different combinations of hepatotoxins (Tylenol and CCl_4) and hepatoprotectants (Sylimarin as reference hepatoprotective drug, *Alocasia macrorrhiza* leaf juice). These vials contained fresh medium M199 and supplemented with CCl_4 or Tylenol concentration of 1.0×10^{-3} M, with or without *Alocasia macrorrhiza* leaf juice. *Alocasia macrorrhiza* leaf juice concentrations used in the present study were 5µl /ml (AM1) and 10 µl/ml (AM2) of medium. After transferring the LS to these vials containing different concentrations of Tylenol/CCl_4 and AM1/AM2 the vials were incubated for 2 hours in standard incubation conditions. Sylimarin concentration of 0.15% of the medium was used as standard hepatoprotective agent. Unsupplemented slices were used as control/s.

2.5 Assessment by Biochemical parameters: At the end of two hours of incubation, liver slices were removed from the vials, homogenized and utilized for assessment of TBA Reactive Substances. This was carried by the method suggested by Buege and Aust (1978) [10]. Glutathione contents were assayed by the method suggested by Grunert and Phillips (1951) [11] in the homogenate prepared from the LS. The medium used for the incubation of the respective liver slices was used to test the amount of leakage of ALT/alanine transaminase [12], AST/aspartate transaminase [12] and ALP/alkaline phosphatase [13].

2.6 Statistical Analysis of the data: Statistical analysis of the results obtained from the experiments was carried out using ANOVA. The values with their respective units are expressed as mean of 5 sets ± S.

RESULTS:

Results of the present study are represented in Table 1.

	Medium			LS	
In vitro test	AST (U/dl ± SE)	ALT (U/dl ± SE)	ALP (U/dl ± SE)	TBARS (µ mols /gm wet wt of tissue ± SE)	Total Glutathione (µg / gm wet wt of liver ± SE)
Control	25.75±1.91	43.20±2.11	364.45±10.77	54.40±3.42	39.65±3.13
CCl_4	52.50±2.13^c	75.10±3.19^c	543.60±12.24^c	98.70±5.97^c	20.22±1.87^c
Tyl	55.35±3.45^c	70.35±4.77^c	501.15±13.21^c	95.60±6.19^c	22.94±2.07^c
CCl_4+SM	26.25±1.32	42.70±2.09	388.30±14.87	56.30±4.95	38.45±2.41
Tyl+SM	25.50±1.64	43.45±3.02	368.25±14.08	53.90±5.43	40.04±3.12
CCl_4+AM1	27.00±1.21	44.00±3.24	361.75±13.98	55.90±4.86	39.01±3.08
CCl_4+AM2	26.50±1.43	42.90±3.02	369.15±15.96	54.20±4.71	38.68±3.39
Tyl+AM1	26.75±2.31	44.50±3.81	366.45±14.78	56.80±4.96	41.21±3.78
Tyl+AM2	24.75±1.33	43.80±4.12	363.95±25.91	55.70±4.01	38.46±3.54
AM1	25.25±1.98	45.60±2.98	359.25±11.65	56.30±5.08	40.88±3.26
AM2	26.00±2.57	42.80±2.66	367.55±16.29	54.50±4.76	41.09±3.72

Values are mean ± SE of 5 sets *p – values : a < 0.05, b < 0.01, c < 0.001 vs. control*

Table 1: Alterations in biochemical parameters during *in vitro* protection of hepatocytes by *Alocasia macrorrhiza* leaf juice against CCl_4 and Tylenol mediated hepatic injury

The enzyme activity of AST and ALT noted in the medium of control group were 25.75±1.91 and 43.20±2.11 U/dl respectively. The AST levels were increased markedly in presence of CCl_4 and Tylenol by 2 folds each. Similarly ALT levels were also increased significantly in presence of CCl_4 and Tylenol. These increases were attenuated when the LS were simultaneouly incubated with *Alocasia macrorrhiza* leaf juice concentrations of 5µl /ml (AM1) and 10 µl/ml (AM2) of medium. Similar to the aminotransferase activities, alkaline phosphatase enzyme levels were increased in the medium when the LS were incubated in the presence of CCl_4 and Tylenol however these levels were decreased in presence of *Alocasia macrorrhiza* leaf juice of both concentrations i.e. 5µl /ml (AM1) and 10 µl/ml (AM2) of medium. Also the TBARS levels increased significantly in presence of CCl_4 and Tylenol and were attenuated by both the concentrations of *Alocasia macrorrhiza* leaf juice. In contrast, the hepatic glutathione content in CCl_4 and Tylenol treated liver slices decreased significantly when compared with the control value and this decrease was attenuated by *Alocasia macrorrhiza* leaf juice treatment of 5µl /ml (AM1) as well as 10 µl/ml (AM2) of medium. The results obtained in presence of Alocasia macrorrhiza were almost similar to sylimarin.

DISCUSSION

In the present study, the hepatoprotective effect of *Alocasia macrorrhiza* was investigated by *in vitro* liver slice method against hepatotoxins *viz.* CCl_4 and Tylenol mediated oxidative stress caused due to the free radical generation. Free radicals and other reactive oxygen species are also derived from normal metabolic process in animals as well as from external sources such as exposure to X-rays, ozone, cigarette, smoking, air pollutants and industrial chemicals [14]. The inhibition or quenching of free radicals can serve as facile model for evaluating the activity of hepatoprotective agents [15]. The aminotransferases and alkaline phosphatase levels were increased significantly in presence of CCl_4 and Tylenol indicating severe hepatocellular damage. In contrast, incubation in presence of *Alocasia macrorrhiza* leaf juice either 5µl /ml (AM1) or 10 µl/ml (AM2) concentration of medium attenuated the release of aminotransferases and alkaline phosphate in the medium indicating the activity of the leaf juice to prevent the leakage of the enzymes through the membrane into the medium. This indirectly indicates the stabilization of the membrane by some unknown mechanism.

CCl_4 is metabolized to trichloromethyl CCl_3^* radical due to the catalytic activity of CYP450, $2E_1$ enzyme, which is further converted to trichloromethyl peroxy radical by superoxide anions. This trichloro methyl peroxy radical is the main culprit in causing hepatotoxicity. This particular radical forms a covalent bonding of free radicals with cellular macromolecules initiating the cascade of reactions leading to lipid peroxidation [16]. This causes membrane injury and the leaf juice exhibited some membrane stabilization effect against the free radical mediated injury by the results of liver function enzymes discussed above.

Tylenol is converted to N-acetyl-p-benzoquinone-imine (NAPQI) which is a highly toxic metabolite and normally conjugates with glutathione causing glutathione depletion [17] (Chandan *et al* 2007). GSH constitutes the first line of defense against free radicals and is a critical determinant of tissue susceptibility to oxidative damage. In this study *Alocasia macrorrhiza* leaf juice exhibited protective effects by impairing CCl_4 and Tylenol mediated oxidative stress through decreased production of

free radical derivations, as evidenced by the decreased levels of TBARS. Furthermore the *Alocasia macrorrhiza* leaf juice attenuated hepatic glutathione depletion. The increase in the hepatic glutathione level in *Alocasia macrorrhiza* leaf juice treated LS could be either to its effect on the *de novo* synthesis of glutathione, its regulation or both. These results suggest that the antioxidant properties may be one mechanism by which *Alocacia maccrorrhiza* leaf juice protects against CCl_4 and Tylenol induced hepatocyte injury. As it is known that the superoxide anions are generated from hepatotoxin mediated injuries [2] the antioxidant efficacy of the *Alocacia macrorrhiza* leaf juice may be due to the possible superoxide anion scavenging by some of the component of the juice. Additionally the protective effect may be due to trapping of the electron released for the generation of superoxide anion or by reducing superoxide anion to a non radical. The possibility of removal of oxygen from the reaction mixture also exists. However the present studies has limitations to assign the activity to any one of the component present in *Alocacia macrorrhiza* leaf juice or any single mechanism of protective action and antioxidant efficacy.

CONCLUSION:

From the results it can be concluded that leaf juice of *Alocasia macrorrhiza* possesses the potency to protect rat liver slices from the free radical mediated injury caused during the oxidative stress exerted by Tylenol and CCl_4 for the concentrations used in this present studies. Results of the concentrations studied in this project suggest no concentration dependent efficacy. This being preliminary study, further toxicity studies are encouraged. *In lieu* of the present poor understanding of the components present in the leaf juice of *A. Macrorrhiza* and their specific action and role in possible protection of hepatocytes, it is difficult to assign the potency to any single component. However it is highly beneficial to screen the leaf contents for the bioactive components involved in this protective activity. Further *in vivo* work is necessary to understand the synergic action of the juice on the body as a whole.

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