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ANTI-LIPID PEROXIDATIVE ACTIVITY OF *COLOCASIA ESCULENTA* LEAF JUICE AGAINST CCL_4 AND ACETAMINOPHEN MEDIATED CELL DAMAGE

Bhagyashree R Patil and Hussein M Ageely
Faculty of Medicine, Jazan University, Jazan, Saudi Arabia

ABSTRACT

The effect of free radicals on the cell metabolism is well established. Plants are rich natural sources of antioxidants, which are most effective agents to prevent free radical associated adverse cellular interactions. The purpose of this investigation was to find out the free radical scavenging efficacy associated with *Colocasia esculenta* whole leaf juice. The free radicals were generated using the two well known hepatotoxins CCl_4 and acetaminophen. The effect of free radicals was studied on liver cells *in vitro* by using rat liver slice model. The liver slices were incubated in presence of cytotoxic concentrations of CCl_4 and acetaminophen. Co-incubation of liver slices with the hepatotoxins and *Colocasia esculenta* leaf juice was conducted to assess the potency of natural components of *Colocasia esculenta* leaf juice in scavenging the free radicals formed due to the metabolism of CCl_4 and acetaminophen. The evaluation was carried using the Thio-Barbituric Acid Reactive Substances and the total glutathione levels in the liver tissue. After the statistical treatment results revealed that the *Colocasia esculenta* whole leaf juice prevented the elicit of lipid peroxidative reactions caused due to the presence of free radicals generated by the hepatotoxins. Simultaneously marked elevations and prevention of depletion of total tissue glutathione were observed in presence of *Colocasia esculenta* whole leaf juice. From the results it is concluded that the *Colocasia esculenta* whole leaf juice contains free radical scavenging efficacy.

Key words: *Colocacia esculenta,* hepatoprotecitve *in vitro,* liver, CCl_4, Acetaminophen TBARS, Lipid peroxidation, Glutathione.

[I]INTRODUCTION

Medical treatments for acute and chronic liver diseases are often difficult to handle and have limited efficacy; hence considerable efforts are currently underway to obtain useful herbal medicines from documented medicinal plants for a wide variety of clinical conditions. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically [1]. Recently, natural plants have received much attention as sources of biologically active substances including antioxidants. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effect by scavenging reactive oxygen species, activating a buttery of detoxifying proteins or preventing the generation of reactive oxygen species Numerous studies have been carried out on plants, vegetables and fruits because they are rich sources of antioxidants, such as vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds and flavonoids which prevent free radical damage, reducing risk of chronic diseases. [2].

Colocasia esculenta (L) Schott is a member of family Araceae. It is an herbaceous perennial plant cultivated as annuals. The starchy, tuberous root is the main edible part of the crop; however the leaves are also used as a leafy vegetable. *Colocasia esculenta* leaves have been reported to be rich in nutrients including minerals and vitamins such as calcium, phosphorours, iron,

vitamin C, thiamine riboflavin and niacin [3]. Its edible corms and leaves are traditionally used for hepatic ailments [4]. Leaf juice of this plant is applied over scorpion sting or in snake bite as well as it is used in food poisoning of plant origin. Ayurveda identified ailments *viz. vata* and *pitta* are supposed to be pacified by the leaf juice and so also the constipation, stomatitis, alopecia, hemorrhoids as well as general weakness [5]. *Colocasia antiquorum* is reported to possess hepatoprotective activity against experimentally induced liver injury in rats [4]. It possess hypoglycemic efficacy due to the presence of cyanoglucoside [6]. Hypolipidemic and antihyperlipidemic activity has been reported due to the presence of arabinogalactan [7] and mono and digalactocyl diacylglycerols [8]. It also possesses antifungal activity due to presence of cystatin [9]. Antibacterial activity of *Colocasia esculenta* has been mentioned by Ravikumar and co-workers [10].

Acetaminophen overdose 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 increased level of protein adducts formation, mitochondrial dysfunction and oxidative stress [11]. In cases of acetaminophen overdose and related toxicities, N-acetylcysteine (NAC) is used as an antidote, however its efficacy is still in question in treatment of acute acetaminophen poisoning [12]. It is found that acetaminophen attenuates mast cell and peripheral blood mononucleocyte cell histamine release induced by this antidote [12]. Hence there is a necessity to find a safer and more effective antioxidant and hepatoprotective agent against acetaminophen as well as a general anti-hepatotoxic.

Carbon tetrachloride is also a widely used hepatotoxin that is known to induce toxic liver injuries in laboratory animals. The hepatic necrosis caused by CCl_4 involves bioactivation by cytochrome P450 2E1 (CYP2E1) resulting in the formation of trichloromethyl free radicals and reactive oxygen species (ROS), which initiate lipid peroxidation and protein oxidation and damage the hepatocellular membranes [13]

The development of new drugs consists of a variety of single steps leading from the discovery of pharmacological effects in cell and animal models to the assessment of toxicity and finally to the demonstration of efficacy and safety in humans. In the field of investigational toxicology, different models have been established to assess the toxicity of a compound/newly developing drug in an early stage. [14]. Cell lines [12], isolated primary hepatocytes [15, 16, 17] and liver slices [18, 19, 20, 21] are widely used in assessing the early efficacy evaluation and toxicity associated with the new drugs.

Hepatic drug metabolism occurs in the hepatocytes that represent with 80% of the total volume and 60% of the total cell number the predominant cell type found in the liver [14]. In lieu of this our laboratory has preferred to work with liver slices over the isolated hepatocytes [18, 19, 20]

[II] Materials and Methods

2.1 Plant material: *Colocacia esculenta* plants were collected locally, from Kolhapur, MS India. The plant identification was done by an expert in Botany, Dr A. R. Jadhav, from Department of Botany, Yashavantarao Chavan College, Warananagar, Kolhapur, MS India. Leaves were removed carefully and washed thoroughly using distilled water. Also the leaves were blotted briefly prior to the preparation of crude juice. The juice of whole leaves was prepared and filtered through Whatman filter paper. The filtrate was collected in sterilized and aseptic conditions and was refrigerated till further use.

2.2 Chemicals: Highly pure and of analytical grade chemicals were utilized for the present studies. Acetaminophen / paracetamol and CCl_4 were purchased from S D fine chemicals, Mumbai while the culture media was obtained from Himedia (M199).

2.3 Experimental Animals: Healthy Wistar strain Albino male rats 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. The animals were kept in standard plastic animal cages with a 12 hours light and dark cycle and fed on standard rat chow and provided pure water *ad libitum*. The experiments were carried out according to guidelines of 'Committee for Prevention and Control of Scientific Experimentation on Animals' (CPCSEA) New Delhi. 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 as described earlier [18] The slices were transferred to experimental vials with combinations of hepatotoxins i.e. acetaminophen and/or CCl_4 with/without hepatoprotectant *Colocacia esculenta* leaf juice as described below. Unsupplemented slices were used as control/s. Following text explains in brief the experimental Design for testing of antihepatotoxic effects of *Colocacia esculenta* leaf juice against acetaminophen and CCl_4. CCl_4 concentration is cytotoxic in rat hepatocytes from 0.5 X 10^{-3} M to 2.5 X 10^{-3} M hence cytotoxic concentrations, 1 X 10^{-3} M of CCl4 was used in the present project. Similarly acetaminophen concentration is cytotoxic for rat hepatocytes from 0.6 X 10^{-3} M and above. Hence 1 X 10^{-3} M concentration of acetaminophen which is cytotoxic was used in this experiment. Based upon preliminary studies the concentration of leaf juice used in the present project was 5 and 10 µl /ml of the medium.

Test 1: Control: LS in these vials contained only the medium M199. It was not supplemented with hepatotoxin or hepatoprotectant.

Test 2: CCl_4 Control: These vials contained 1.0×10^{-3} M CCl_4 in the medium

Test 3: CE1 Control: These vials contained medium supplemented with 5µl /ml of *Colocasia esculenta* fresh leaf juice

Test 4: CE2 Control: LS in these vials were incubated in presence of 10µl /ml concentration of *Colocasia esculenta* fresh leaf juice

Test 5: CCl_4+CE1 : The medium in this vial was modified with CCl_4 and 5µl /ml of *Colocasia esculenta* fresh leaf juice

Test 6: CCl_4+CE2 : These vials contained CCl_4 and 10µl /ml of *Colocasia esculenta* fresh leaf juice

Test 7: PA Control: The LS in these vials were incubated in presence of acetaminophen of 1.0×10^{-3} M concentration in the medium

Test 8: PA + CE1: The medium in this vial contained acetaminophen and 5µl /ml of *Colocasia esculenta* fresh leaf juice

Test 9: PA +CE2: The medium was modified by addition of acetaminophen and 10µl /ml of *Colocasia esculenta* fresh leaf juice

As stated above these vials contained fresh medium M199 and supplemented with CCl_4 or acetaminophen (PA) concentration of 1.0×10^{-3} M, with or without *Colocacia esculenta* leaf juice either 5µl /ml or 10µl /ml of the medium. Based upon preliminary studies carried in our laboratory, *Colocacia esculenta* leaf juice concentrations decided to be used in the present study were 5µl /ml (CE1) and 10 µl/ml (CE2) of medium. After transferring the LS to these vials containing different concentrations of acetaminophen/CCl_4 and CE1/CE2 the vials were incubated for 1, 2 and 4 hours in standard incubation conditions.

Thus the same experimental procedure was repeated for one, two and four hours to understand time dependent toxicity, if any, associated with the xenobiotics used in this experiment.

2.5 Assessment by Biochemical parameters: At the end of one, two and four hours of incubation, the liver slices were removed from the test vials and homogenized. The homogenate was used to evaluate the Thiobarbituric Acid Reactive substances (TBARS) to assess the lipid

peroxidation by the method of Buege and Aust [22]. Also the total glutathione contents were measured by the method of Grunert and Philips [23].

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 6 sets ± SE. Value of $p < 0.05$ was considered as significant.

[III] RESULTS:

Results of the present study are represented in Fig.1 and 2, which were obtained at the end of one, two and four hours in TBARS and total glutathione contents in the liver tissue slice homogenate. Thiobarbituric Acid Reactive Substances (TBARS) measured in terms of amount of malonaldehyde in liver slice homogenate at the end of one, two and four hour was found to be 27.20± 2.06 mols, 28.56±1.60 mols and 28.72 ± 2.49 mols of MDA/gm wet wt of liver tissue. In presence of CCL_4 the same was found to be 43.35±2.81, 56.36±3.77 and 52.11±3.07 mols of MDA/gm wet wt of liver tissue respectively at the end of one, two and four hours. The liver slices incubated in presence of acetaminophen showed 34.80±1.66, 42.34±2.81 and 50.48±2.42 mols of MDA/gm wet wt of tissue. The CCl_4 treated liver slices exhibited a significant ($p < 0.05$) elevation in TBARS at the end of one, two and four hours. Similar trend was obtained with that of acetaminophen treatment. In presence of 5µl concentration of *C. esculenta* however the TBARS reported was significantly ($p < 0.05$) declined when compared with CCl_4 treated liver slices however it was not near that reported in control, although with 10 µl /ml of medium concentration of *C. esculenta* leaf juice the TBARS was normalized. Similar results were obtained with liver slices incubated in presence of acetaminophen. The liver slices incubated only in the presence of *C. esculenta* did not show any elicit of lipid peroxidation as evidenced by near control levels of TBARS. Total glutathione contents measured in the control liver slices were estimated as 19.83±1.16, 21.21±0.99 and 22.90±1.53 µg/gm wet weight of liver tissue slice. Incubation in presence of CCl_4 resulted in 17.11±0.82, 18.30±1.06 and 11.68±1.01 µg total glutathione/gm wet weight of liver tissue slice at the end of one, two and four hour respectively. In presence of acetaminophen the total glutathione in terms of µg/gm wet wt of liver slice tissue was found to be 17.47±0.66, 18.69±1.28 and 13.25±0.78 respectively after one, two and four hours. The glutathione contents observed in the liver slices treated in presence of CCl_4 and acetaminophen were significantly ($p < 0.05$) declined. The same were found near/above normancy in all the liver slices solely or co-treated with the leaf juice of *C. esculenta*.

[IV] DISCUSSION

In vivo studies on hepatotoxicity are limited by animal welfare/ethical concerns and difficulties to distinguish primary and secondary toxic effects, *in vitro* liver preparations are increasingly used as they offer different approaches on all levels of investigational toxicology [14]. Use of liver slices provides decided advantage over previous techniques because this preparation allows for maintenance of the functional acinar architecture of the liver and has displayed drug metabolism over a span of hours to days. Advantages like maintenance of the functional architecture have made liver slice method a very versatile method for the study of drug disposition *in vitro* [24]. The *in vitro* isolated hepatocyte [15, 16], cell line [12] as well as liver slice [18, 19, 20] model is earlier used to assess toxicity and reveal the underlying mechanisms. Additionally, Fraga, Leibovitz and Tappel have showed the usefulness of liver tissue slices to measure lipid peroxidation in terms of TBARS. [25] Hepatocellular injury from acetaminophen exposure is primarily initiated by CYP2E1 bioactivation to form reactive intermediates such as N-acetyl-p-benzoquinone imine that deplete glutathione and then bind to

critical cellular macromolecules. Mitochondria are thought to be primary targets in acetaminophen toxicity with particular attention on the mitochondrial permeability transition. Generation of other reactive oxygen species such as nitric oxide and superoxide anion may be important determinants in hepatocyte death. Evidence has also been accumulating for the contribution of nonparenchymal cells such as kupffer cells, natural killer cells, and neutrophils that secrete cytokines and chemokines during acetaminophen induced liver injury (26). Due to the same use of liver slices to assess the toxicity and protective mechanisms become useful as in slices the functional architecture of the tissue is retained.

In lieu of this, the present project was designed to study the antihepatotoxic activity of Colocasia esculenta leaf juice in terms of its anti-lipid-peroxidative efficacy measurement against the CCl_4 and paracetamol induced lipid peroxidation mediated cell damage in rat liver slices *in vitro*. In previous work from our laboratory (20) the alterations in the release of the enzymes *viz* AST, ALT and ALP in the medium were studied in presence of similar concentrations of *Colocasia esculenta* and it was found that the *Colocasia esculenta* leaf juice possesses hepatoprotective efficacy in terms of leakage of these three enzymes into the medium. The present study was carried to reveal the mechanism of protection by *Colocasia esculenta* leaf juice.

The peroxidation of membranes usually exhibited as lipid peroxidation has been implicated as one of the primary events in the oxidative damage of the cell (27). At the same time the lowered concentration of glutathione has been generally considered to be an index of increased oxidative stress (28). Hence parameters selected for the present study were lipid peroxidation measured as thiobarbituric acid reactive substance and total glutathione contents in the cells.

Acetaminophen administration selectively depletes (within 2 hr) mitochondrial glutathione, and produces local toxicity by altering membrane permeability and decreasing the efficiency of oxidative phosphorylation. This renders mitochondria more susceptible to oxidative damage [29]

A number of xenobiotics form conjugates with hepatic glutathione. Under such conditions glutathione levels are decreased and the liver cells made more susceptible to the development of lipid peroxidation (30). It is well established that isolated hepatocytes peroxidize when exposed to CCl_4 as demonstrated by an increased production of malonaldehyde. [31]

The data presented in the result section are consistent with the hypothesis that acetaminophen induced toxicity occurs by two phases, a metabolic phase and an oxidative phase [15]. A similar mechanism may be existing for CCl_4. During first two hours or maximum upto four hours the first phase i.e. metabolic phase may be occurring with glutathione depletion [15].

The hepatotoxic effects of CCl_4 are largely due to the generation of free radicals [32]. CCl_4 is biotransformed by the cytochrome P450 system to produce the trichloromethyl free radicals, which in turn covalently bind to cell membranes and organelles to elicit lipid peroxidation [33].

Colocasia esculenta leaf juice may be protecting the hepatocytes against the injurious effects of CCl_4 and acetaminophen that may result from the interference with cytochrome p450 system, resulting in the hindrance of the formation of hepatotoxic free radicals eliciting the lipid peroxidation and consequent damage to macromolecules and membrane. Earlier results (20) support these data where the leakage of enzymes AST, ALT and ALP was noted in the surrounding medium indicating the membrane damage.

The possible mechanism of action underlying the antitoxic effect of *Colocasia esculenta* leaf juice against CCl_4 may be due to interference of some component/s of it with the cytochrome p450 system involved in biotransformation of CCl_4 and responsible to produce the free radical of

trichloromethyl. However the possibility of antioxidant activity of leaf juice as a whole or some of its component appears more prominent as it may be scavenging the trichloromethyl free radicals produced by the biotransformation of CCl_4 by the cytochrome p450 systems which is evident from the available data of this study. Similarly hepatoprotection against cytotoxic concentrations of acetaminophen by *Colocasia esculenta* may also be due to its interference with the cytochrome p450 system. Additionally it may be scavenging the free radicals formed during the biotransformation of acetaminophen. Also it is possible that the leaf juice may be enhancing the synthesis of glutathione in the hepatocytes which is highly possible among all other possibilities from the data obtained during this project and hence eliminating the possibility of free radical mediated damage to the macromolecules. This is also supported by earlier studies where leakage of membrane and cytoplasmic contents in the surrounding medium is prevented in similar circumstances (20).

The crude juice of *C. esculenta* was also tested for toxicity to hepatocytes, the data of CE1 and CE2 tests suggests that the leaf juice itself did not exerts any lipid peroxidation. So also it maintains the glutathione levels unaltered in its presence. The results obtained were slightly dose dependent for the concentrations of leaf juice of *C. esculenta* tested in this *in vitro* study.

Many plants of various genera and species are already tested and being studied for their efficacy in controlling the CCl_4 and acetaminophen induced liver damage as it has been established that several phytoconstituents have the ability to induce microsomal enzyms either by accelerating the excretion of toxicants or by inhibition of lipid peroxidation induced by the toxin. Phytoconstituents like flavonoids and triterpenoids are known to possess hepatoprotective activity [34, 35]. Phytochemical investigations on the *Colocasia* extracts have shown the presence of anthocyanins such as cyanidin-3-glucoside, pelargonidin-3-glucoside and cyanidin-3-rhamnoside, which have antioxidant activities as evident from previous studies [36, 37, 38]. Therefore, anthocyanins may be responsible for the hepatoprotective as well as anti-lipid peroxidative activity that was observed associated with the leaf juice of *Colocasia esculenta*. However, the results obtained in the present studies may be a synergic action of all the components present in *Colocasia esculenta* leaf juice hindering various stages of the toxicity development.

[V]CONCLUSION:

From this studies it may be concluded that the leaves of *Colocasia esculenta* possess antihepatotoxic and anti-lipid peroxidative efficacy. The exact constituent(s) responsible for this effect cannot be explained with the present data. It may be due to the antioxidant effects of the crude filtered juice of the *Colocasia esculenta* may be due to the presence of anthocyanins or some flavonoids present it. The bioactive components present in the leaf juice contributing towards the protection of hepatocyte can further be investigated as a safer and more effective antioxidant, and hepatoprotective, anti-lipid peroxidative agent against acetaminophen as well as a general anti-hepatotoxic.

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Fig 1. *Colocasia esculenta* influenced *in vitro* alterations in TBARS activities in presence of CCl_4 and acetaminophen

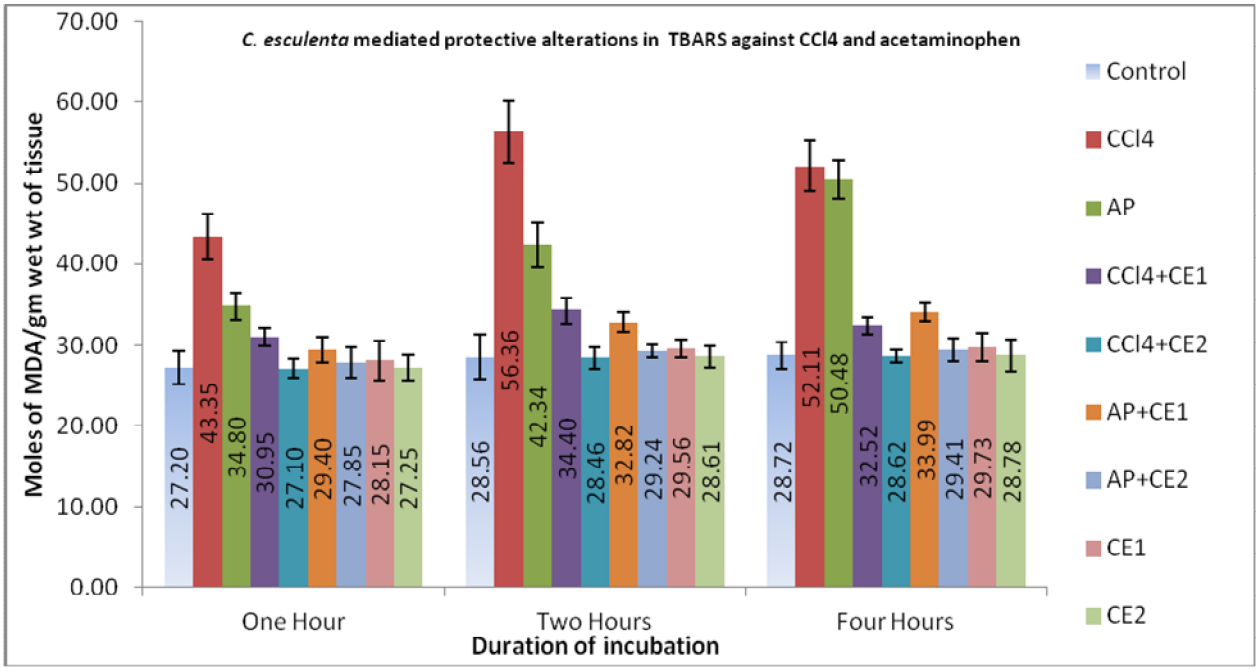

Fig 2. : *Colocasia esculenta* influenced *in vitro* alterations in total glutathione content in presence of CCl_4 and acetaminophen