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Hepatoprotective effect of methanolic extract of *Syzygium aromaticum* against hydralazine induced toxicity: An *In vitro* study

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Abstract
The main objective of present study was to investigate protective effect of methanolic extract of *Syzygium aromaticum* against hydralazine induced hepatotoxicity *in vitro*. Experimental study involved administration of hypertension drug hydralazine to goat liver homogenate *in vitro* in order to induce hepatotoxicity. Experimental protocol involved four experimental groups each containing 1ml goat liver homogenate. Group-I represented untreated control group. Group-II involved administration of *Syzygium aromaticum* extract (0.18mg/ml) to liver homogenate. Group-III represented toxin exposed group and involved administration of hydralazine (0.27mg/ml) to liver homogenate. Group-IV involved co-administration of hydralazine (0.27mg/ml) and *Syzygium aromaticum* extract (0.18mg/ml) to liver homogenate. Homogenate cultures were incubated in BOD incubator containing 5% CO_2 at 37ºC for 1-hour duration. After incubation period, experimental samples were subjected to biochemical analysis for evaluation of hydralazine induced hepatic injury and ameliorative potential of clove extract. Results revealed statistically significant decline in protein levels and activities of liver biomarkers like alkaline phosphatase, acid phosphatase, alanine aminotransferase and aspartate aminotransferase while significant elevation was observed in lipid peroxidation in hydralazine exposed group as compared to control group. Co-administration of *Syzygium aromaticum* extract to hydralazine exposed liver homogenate cultures maintained all studied biochemical parameters nearest to control group. In conclusion, methanolic extract of *Syzygium aromaticum* remarkably exhibited hepatoprotective activity and therapeutic potential against hydralazine induced toxicity.

Keywords: hydralazine toxicity, hypertension drug, hepatotoxicity, *Syzygium aromaticum*

Introduction
Spices are dried seeds, fruits, roots, bark or vegetative parts of plants used nutritionally in substantial amount as food supplement for the reason of flavoring due to their taste and aroma [1-2]. Spices constitute a significant group of agricultural commodities. They are widely used in medicine, alcoholic beverages, cosmetics, religious rituals, perfumery, preservatives and colorants [3]. Ayurvedic extracts of spices are used in the variety of forms including infusions, decoctions, macerations, tinctures, fluid extracts, tea, juices, syrups, oils, ointments and powders. Spices are rich sources of calcium, iron, vitamin-B, vitamin-C, carotene and other antioxidants [1-2]. They have therapeutic potential to fight against multiple metabolic disorders like diabetes, obesity, altered lipid profile and hypertension.

Syzygium aromaticum (Clove) is one of the earliest known spices in trade. Clove is the aromatic dried flower bud of a tree belonging to Myrtaceae family. Clove grows best on tropical mountain slopes at lower elevations as part of a mixed forest. Clove is a natural antiviral, antimicrobial, antiseptic and antifungal agent. It also holds aphrodisiac and circulation-stimulating capacities. Clove oil has been used in a variety of health conditions including indigestion, generalized stress, parasitic infections, cough, toothache, headache and blood impurities. Metabolism, in the long run, increases free radical production and alters lipid profile along with decreasing the antioxidant levels in the liver. Clove extract is helpful in counteracting deleterious effect of free radicals due to its hepatoprotective properties which may be attributed to presence of high amount of antioxidative phytochemicals. *Syzygium aromaticum* extract denotes one of the major vegetal sources of phenolic compounds especially flavonoids, hydroxybenzoic acids, hydroxycinnamic acids and hydroxiphenylpropens. Eugenol is the main bioactive compound of *Syzygium aromaticum* [4]. Hydrolysable tannins of ellagic acid and gallic acid are the other major compounds found in higher concentration in clove extract [5]. Other phenolic acids found in clove are

caffeic acid, ferulic acid and salicylic acid. Flavonoids like kaempferol, quercetin and its derivatives (glycosylated) are also found in clove in lower concentrations. Clove flower buds contain approximately 18% of essential oil [6].

Hydralazine is one of the most frequently prescribed drugs for the treatment of moderate to severe hypertension especially during pregnancy. Hydralazine ($C_8H_8N_4$-PubChem CID-3637) belongs to the hydrazine phthalazine class of drugs and used in combination with a suitable β-blocking drug to treat hypertension [7]. In addition, it is being used increasingly for the treatment of congestive heart failure when most of the traditional approaches fail. Hydralazine is not only a peripheral vasodilator but also a potent and irreversible inhibitor of Semi-carbazide Sensitive Amine Oxidase (SSAO). Hydralazine was one of the first oral antihypertensive medications introduced into clinical medicine and first used in the late 1950s. Hydralazine was officially approved by the U.S. Food and Drug Administration (USFDA) in 1984. Hydralazine is available in generic forms and sold under the brand name of Apresoline in tablet forms of 10, 25, 50 and 100 mg as well as in parenteral forms. Most common side effects of hydralazine include dizziness, nausea, headache, orthostatic hypotension, tachycardia, flushing and gastrointestinal upset [8]. Hydralazine has been linked to several forms of acute liver injury as well as a lupus-like syndrome [9]. Antihypertensive drug hydralazine has also been reported to induce severe hepatic damage in patients during clinical treatment [10-11]. Hydralazine-induced hepatotoxicity may manifest as acute hepatitis, hypersensitivity-type injury, cholestatic jaundice, mixed hepatocellular injury or centrilobular necrosis [12].

Keeping this view, the major aim of the present *in vitro* study was to analyze the possible ameliorative potential of medicinal plant *Syzygium aromaticum* as a novel hepatoprotective agent against hydralazine induced toxicity in mammalian tissue.

2. Materials and Methods

Experimental design

In the present study, experimental protocol involved exposure of goat liver homogenate cultures to hepatotoxic drug hydralazine and herbal antidote *Syzygium aromaticum* in order to evaluate alterations in biochemical parameters of Protein content, enzymatic activities of Alkaline phosphatase, Acid phosphatase, Alanine aminotransferase, Aspartate aminotransferase and Lipid peroxidation level *in vitro*. The dosage selection for hydralazine [12] and *Syzygium aromaticum* [13] was based on reported literature. *In vitro* study doses for toxin and antidote were determined through extrapolation of available data from literature through consideration of organ weight and body weight of mammalian tissues. The dosage selection was also based on standardization carried out in laboratory to get effective concentrations for *in vitro* analysis.

2.1 Chemicals

Hydralazine drug (Apresol tablets, Ordain Health Care Global Pvt. Ltd.) was purchased from registered pharmacy store of Ahmedabad, India. All the other chemicals used in present study were analytical grade reagents (AR) and purchased from Hi-Media, Sigma and Merck Laboratory Pvt. Ltd., India.

2.2 Plant material collection and preparation of *Syzygium aromaticum* extract

The plant extract was prepared from the *Syzygium aromaticum* (Clove bud) powder obtained from recognized Ayurvedic store L.V.G., Ahmedabad, India. 5 gm of dried powder of *Syzygium aromaticum* was mixed in 50 ml methanol and kept in orbital shaker incubator for 48-hours at the speed of 110 rpm at 30°C. After that the content was filtered through Whatman filter paper no. 1. The filtrate was collected and concentrated by evaporating methanol till dryness. After complete evaporation of methanol, resultant semi-solid extract was stored in a sterile brown bottle under the refrigerated condition until further use [14].

2.3 Preparation of reagents

0.0072 gm of hydralazine tablet was dissolved in 10 ml of double distilled water to prepare the toxin stock solution. A definite volume of this stock solution was used in a final volume of the reaction mixture, so as to get the required concentration of 0.27mg/ml for hydralazine dosage. 5 mg/ml of stock solution of *Syzygium aromaticum* extract was prepared by dissolving it in double distilled water for qualitative and quantitative estimation during phytochemical screening while 0.0108 gm of extract was dissolved in 10 ml of double distilled water to prepare antidote stock solution for hepatoprotective analysis in the present investigation.

2.4 Collection of tissue

In present *in vitro* study, fresh liver sample of healthy adult goat (*Capra hircus*) was obtained from approved local slaughter house and brought to laboratory under frozen condition. Liver tissue was washed in normal saline, blotted dry by pressing between 2-3 folds of filter paper and subjected to homogenization process.

2.5 Preparation of tissue homogenates

Goat liver tissue was subjected to process of homogenization with constant speed under suitable condition of 4°C in chilled glass mortar pestles. 10% tissue homogenate of liver was prepared in chilled double distilled water for estimation of protein content and enzyme activities, while 10% tissue homogenate was prepared in ice cold 0.1M phosphate buffer (pH=7.4) for estimation of lipid peroxidation. The tissue homogenate was divided into different experimental groups for *in vitro* study.

2.6 Experimental groups

Study protocol includes four experimental groups:

Group-I: Control Group

Group-II: Herbal Antidote (*Syzygium aromaticum* extract-0.18mg/ml) Exposed Group

Group-III: Hydralazine Toxin (0.27mg/ml) Exposed Group

Group-IV: Hydralazine (0.27mg/ml) and *Syzygium aromaticum* extract (0.18mg/ml) Exposed Group

2.7 *In vitro* study protocol

The tissue homogenate (1 ml) was exposed to aqueous solutions of hydralazine (0.27mg/ml) and plant extract of *Syzygium aromaticum* (0.18 mg/ml) as per experimental group design. The unexposed control and exposed liver homogenates were maintained in 5% CO_2 containing BOD incubator at 37°C for 1-hour time duration and then subjected to centrifugation at 2000 rpm for 15 minutes to settle down clumps. The supernatant from each centrifuge tube was collected and pellet was discarded. Supernatant was subjected to analysis of various biochemical indices for investigating ameliorative effect of the antidote against hydralazine induced

hepatotoxicity.

2.8 Biochemical analysis

At the end of the hydralazine and *Syzygium aromaticum* treatment; protein content, liver biomarker enzyme activities and lipid peroxidation levels were measured from unexposed and exposed goat liver homogenate cultures using standard protocols. To study the impact of hydralazine on protein content, levels of soluble proteins were determined in goat liver homogenates by the standard method of Lowery *et al*. [15]. The estimation of alkaline phosphatase (ALP) and acid phosphatase (ACP) was done by the method of Bessey *et al*. [16]. Activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by method of Reitman and Frankel [17]. The measurement of lipid peroxidation (LPO) level in the liver homogenates of control and exposed groups was carried out by the method of Ohkawa *et al*. [18].

2.9 Statistical analysis

Student's 't-test' was used for the statistical analysis of the data. For each parameter (n=5), the data were expressed as Mean ± SEM after subjecting to Student's 't-test' using GraphPad software for interpretation of results. The significance difference was statistically considered at level of $p<0.05$.

3. Results

Effect of Hydralazine and *Syzygium aromaticum* on Protein Levels

Hydralazine exposure to goat liver homogenate for 1-hour duration caused statistically extremely significant ($p<0.001$) decline in soluble protein levels *in vitro* as compared to control group. However, co-supplementation of hydralazine and methanolic extract of *Syzygium aromaticum* in goat liver homogenate protected against reduction in protein levels and resulted in maintenance of soluble protein levels ($p<0.01$) as compared to control group (Table-1).

Effect of hydralazine and *Syzygium aromaticum* on alkaline phosphatase activity

Hydralazine exposure to goat liver homogenate resulted in significant alteration in alkaline phosphatase activity. Results revealed that alkaline phosphatase activity was statistically significantly ($p<0.001$) declined at 0.27mg/ml hydralazine exposure as compared to control group. Co-administration of hydralazine and methanolic extract of *Syzygium aromaticum* in goat liver homogenate provided significant protection against reduction in alkaline phosphatase activity as represented by $p<0.05$ (Table-1).

Effect of hydralazine and *Syzygium aromaticum* on acid phosphatase activity

Results revealed that acid phosphatase activity significantly ($p<0.001$) decreased in hydralazine exposed goat liver homogenate as compared to control group (Table-1). Administration of methanolic extract of *Syzygium aromaticum* to hydralazine exposed goat liver homogenate almost completely ameliorated acid phosphatase activity and maintained it nearest to control group which suggests therapeutic efficiency of herbal antidote against hydralazine toxicity.

Effect of hydralazine and *Syzygium aromaticum* on alanine aminotransferase activity

Statistically extremely significant ($p<0.001$) decline was observed as compared to control group in the alanine aminotransferase activity in goat liver homogenate exposed to hydralazine. Co-supplementation of hydralazine and *Syzygium aromaticum* extract in goat liver homogenate revealed amelioration in enzyme activity as represented by $p<0.01$ (Table-1).

Effect of hydralazine and *Syzygium aromaticum* on aspartate aminotransferase activity

Results revealed that aspartate aminotransferase activity was significantly ($p<0.001$) decreased in hydralazine exposed goat liver homogenate as compared to control group. Addition of hydralazine and *Syzygium aromaticum* extract simultaneously in goat liver homogenate imparted protection against enzyme activity alteration as compared to control group as represented by $p<0.01$ (Table-1).

Effect of hydralazine and *Syzygium aromaticum* on lipid peroxidation level

Hydralazine exposure to goat liver homogenate *in vitro* for 1-hour was found to enhance production of thiobarbituric acid reactive substances (TBARS) like malondialdehyde (MDA) as marked by highly significant elevation ($p<0.001$) in lipid peroxidation with respect to control group. However, co-supplementation of hydralazine and *Syzygium aromaticum* methanolic extract in goat liver homogenate provided protection against hydralazine induced hepatotoxicity as represented by $p<0.01$ (Table-1). *Syzygium aromaticum* extract showed ameliorative effect in hydralazine exposed goat liver homogenate and acted as therapeutic agent against hydralazine induced oxidative stress.

Table 1: Biochemical Parameters of Control and Exposed Liver Homogenates *In Vitro*

Biochemical Parameters	Group I Control	Group II SAE (0.18 mg/ml)	Group III HLZ (0.27 mg/ml)	Group IV HLZ (0.27 mg/ml)+ SAE (0.18 mg/ml)
Protein[a]	3.867 ± 0.006	3.870 ± 0.003^{NS}	$3.500 \pm 0.007^{***}$	$3.814 \pm 0.005^{**}$
Alkaline Phosphatase[b]	0.614 ± 0.002	0.609 ± 0.001^{NS}	$0.590 \pm 0.001^{***}$	$0.609 \pm 0.001^{*}$
Acid Phosphatase[c]	1.476 ± 0.002	1.475 ± 0.003^{NS}	$1.446 \pm 0.002^{***}$	1.473 ± 0.003^{NS}
Alanine Aminotransferase[d]	178.929 ± 1.33	175.836 ± 0.893^{NS}	$134.449 \pm 1.97^{***}$	$172.269 \pm 1.15^{**}$
Aspartate Aminotransferase[e]	278.221 ± 1.17	275.62 ± 1.066^{NS}	$186.693 \pm 2.30^{***}$	$271.291 \pm 1.306^{**}$
Lipid Peroxidation[f]	817.313 ± 26.92	816.610 ± 10.36^{NS}	$1412.155 \pm 16.94^{***}$	$921.132 \pm 17.624^{**}$

p – Values: *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control group

Values are expressed as Mean ± SEM; (n=5)

NS = Non Significant as compared to control group

SAE = *Syzygium aromaticum* extract; HLZ = Hydralazine Drug

a = mg Protein/100 mg fresh tissue weight; b, c = μ moles of p–nitrophenol released/30 minutes/mg protein

d, e = mU/mg protein; **f** = nano moles of MDA/hr/mg protein

4. Discussion

Findings of current *in vitro* study revealed statistically significant reduction in protein level in hydralazine exposed group of goat liver homogenate as compared to control group. The liver disorder is related to a decrease in the level of total protein due to inhibition of RNA, DNA and protein synthesis. Reduction in level of protein might be due to detachment of polyribosomes from endoplasmic reticulum or defect in protein biosynthesis due to hydralazine induced hepatotoxicity. *Syzygium aromaticum* extract administration protected against hepatic damage caused by hydralazine toxicity and maintained level of tissue protein nearest to control group which might be attributed to presence of numerous phenolic compounds and flavonoids present in plant extract in form of secondary metabolites [19].

Significant alteration in alkaline phosphatase and acid phosphatase activities was observed in hydralazine exposed tissue homogenate as compared to control group. Decline in alkaline phosphatase and acid phosphatase activities in hydralazine exposed tissue might be due to increase in cell membrane permeability and damage or necrosis of hepatocytes [20]. Hydralazine toxicity could damage organelles especially, plasma membrane of hepatic parenchymal cells and might interfere with their functions. While exposing the hydralazine treated tissue homogenate to *Syzygium aromaticum* extract, it significantly protected against decline in activities of alkaline and acid phosphatases which emphasized the protective effect of the plant by resuming the plasma membrane functions, maintaining structural integrity of cell and displaying the ability to heal hepatic tissue damage.

Present *in vitro* study also revealed that alanine aminotransferase and aspartate aminotransferase activities were significantly decreased in hydralazine exposed group of liver homogenate as compared to control group. ALT and AST are normally located in the cytosol of hepatocyte as well as involved in the transfer of amino groups of aspartate and alanine to ketoglutaric acid and used as prognostic markers for identifying drug induced liver injury [21]. Alteration in ALT and AST activities might be due to cellular leakage and loss of functional integrity of cell membranes due to hydralazine toxicity [22]. In the current study, co-administration of *Syzygium aromaticum* to hydralazine exposed liver homogenate exerted protection against decline in liver biomarker enzymes which might be attributed to the presence of flavonoids which are known to possess remarkable antioxidant properties and capable of protecting normal cells from various incentive induced oxidative stress and cell death.

Alteration in ALP, ALT and AST was reported during liver failure, indicating cellular damage, obstructive damage like cholestasis or blockage of bile flow and loss of functional integrity of hepatic cell membrane [23]. However, *Syzygium aromaticum* supplementation was found to cause significant suppression of these destructive activities, indicating its inhibitory effect on hepatotoxicity.

Lipid peroxidation can be termed as oxidative degradation of lipids containing carbon-carbon double bonds, especially present in fatty acids. Lipid peroxidation results in the damaged cell membrane and altered physiological function of cells. A lipid peroxidation byproduct malondialdehyde (MDA) is used as an indicator of oxidative stress in tissues. The significant increase in lipid peroxidation as compared to control group in hydralazine exposed liver homogenate group might be due to generation of excessive free radicals. In the current study, hydralazine-depended increase in MDA levels confirmed the oxidative stress and explained the tissue damage. Co-supplementation of *Syzygium aromaticum* methanolic extract with hydralazine exposed homogenate protected against enhancement in liver tissue MDA levels as specified by a decrease in lipid peroxidation as compared to hydralazine exposed group. Ability of *Syzygium aromaticum* to encounter hydralazine induced oxidative stress might be attributed to antioxidant activity of phenolics due to their reducing potential, free radical scavenging activity and potential to decompose peroxides [24-25].

Present *in vitro* study proved that administration of *Syzygium aromaticum* extract at dose of 0.18mg/ml succeeded in maintaining lipid peroxidation as well as liver function enzymes activities of ALP, ACP, ALT and AST towards normal values of control against hydralazine induced toxicity. The beneficial effect and hepatoprotective activity of *Syzygium aromaticum* against hydralazine toxicity might be due to free radical scavenging and antioxidant activities of its phenolic components present in methanolic extract [26]. Predicted mechanism of action of various spice extracts might also be attributed to antioxidant properties of plants due to presence of flavonoids which lead to increase in reduced level of blood glutathione, enhancement in total protein levels, inhibition of lipid peroxidation, enhancement of antioxidant enzymatic activity and decrease in hepatic marker enzymes involving AST, ALT, ALP and arginase as well as total bilirubin in plasma [27]. Active principles in the clove include essential oils like acetyl eugenol, beta-caryophyllene and vanillin; crategolic acid; tannins like gallotannic acid; methyl salicylate; flavonoids like eugenin, kaempferol, rhamnetin and eugenitin; triterpenoids like oleanolic acid, stigmasterol and campesterol as well as several sesquiterpenes which are known to possess antioxidant, antiseptic, anti-inflammatory, carminative and anti-flatulent properties [28]. Hepatoprotective effects of clove essential oil and clove extracts have also been reported by several scientists which corroborates with our data [13, 19, 29-31].

5. Conclusion

From the findings of present *in vitro* study, it can be concluded that medicinal plant *Syzygium aromaticum* (clove) significantly conferred protection and acted as effective ameliorative agent against hydralazine induced hepatotoxicity. It can be inferred that methanolic extract of *Syzygium aromaticum* exerted significant therapeutic effect against hydralazine induced hepatotoxicity due to presence of numerous pharmacologically active phytochemicals in form of phenolics and flavonoids which could provide antioxidant properties to the plant for counteracting hydralazine induced oxidative stress and led to the normalization of protein content as well as activities of liver biomarker enzymes of mammalian tissue. Thus, *Syzygium aromaticum* can be employed as an effective and safe therapeutic agent in pharmaceutical research against drug induced hepatotoxicity.

6. Author's contributions

Present research work was the outcome of equal contribution from all three authors. NKJ, UDP and FCS had carried out literature review and designed the experimental protocol. UDP had performed experimental work for collection of study data. UDP had carried out statistical analysis and FCS was involved in verification. UDP and FCS had drafted the

manuscript. The final draft of the manuscript was reviewed and edited under the guidance of NKJ. All the three authors had read and approved the final manuscript.

7. Competing interests

Authors have declared that there are no conflicts of interests.

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