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Antioxidant and Hepatoprotective Potential of Traditional Siddha Formulation Seenakara Parpam against D-Galactosamine Induced Oxidative Stress in Rats

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ABSTRACT

In recent days oxidative stress associates disease attains considerable importance in the developing countries like India due to constant change in life style and food habits. Free radical offers tireless quenching on the healthy tissue which in turn affects the entire physiology of the biological system. Siddha system of traditional medicine has wide range of formulations with the prospective anti-oxidant property and also having potential of curing several dreadful diseases. One such noble formulation is Seenakara parpam (SKP), Formulation like parpam presently used for clinical management of dysuria, blood stained dysentery, menorrhagia and other eye disorders. Still now there is no proper literature evidence claiming the antioxidant and hepatoprotective activity of the formulation SKP, hence this prompted us to pursue the preclinical investigation on exploring the efficacy of the drug SKP in selective animal model. The main aim of the present investigation is to evaluate the anti-oxidant and hepatoprotective activity of the formulation SKP against D-galactosamine induced oxidative stress in rats. Experimental rats were treated with test drug SKP at the dose of 200 and 400 mg/kg for the period of 21 days followed by this single I.P injection of D-galactosamine at the dose of 400 mg/kg to all the rats except control group. At the end of the study serum samples of collected and were analyzed for biochemical investigations including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), Total Protein (TP) and lactate dehydrogenase (LDH) level. Antioxidant profiling was carried out in liver tissue homogenate for the estimation of Lipid peroxidation (LPO), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) level. Results of the study clearly indicates that there was a significant increase in ($P<0.01$) serum AST, ALT, ALP, TB , LDH and LPO level further there was a significant decrease in ($P<0.01$) SOD, CAT, GPX and TP levels were observed in animals treated with D-galactosamine 400 mg/kg (Group II) as compared to normal control group (Group I). Pretreatment with SKP at the dose of 200 mg and 400 mg/kg to group IV and V and silymarin at the dose of 25 mg/kg to group III shown significant decrease in the levels of above indices like AST ,ALT, ALP, TB, LDH, LPO and increased level of SOD, CAT, GPX, TP in treated group. From the result of the present study it was concluded that the formulation SKP has promising antioxidant and hepatoprotective activity and it may be considered as drug of choice for the treatment of oxidative stress induced liver disease in future.

Keywords: Antioxidant; Hepatoprotective; Seenakara parpam; D-galactosamine; Silymarin; Biochemical investigation

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INTRODUCTION

Liver being the major organ of metabolic events often exposed to toxic metabolites as an outcome of oxidative stress, metabolism pertains to drugs, hazardous chemical agents, toxicants in food and beverages. Oxygen free radicals, such as superoxide, hydroxyl radicals, and peroxyl radicals, with the addition of non-radicals, such as hydrogen peroxide, hypochlorous acid and ozone, are known as reactive oxygen species (ROS), which are generated during the metabolism process of oxygen. Reactive nitrogen species (RNS), including nitrogen based radicals and non-radicals, such as nitrogen dioxide, nitric oxide radicals and peroxynitrite, are derived from nitric oxide and superoxide via inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, respectively [1,2]. Due to their special chemical characteristics, ROS/RNS can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminately oxidize virtually all molecules in biological membranes and tissues, resulting in injury. Anti-oxidant enzymes plays vital role in quenching and alleviation of generated ROS. The SODs convert superoxide radicals into hydrogen peroxide and molecular oxygen (O_2), while catalase and GPx convert hydrogen peroxide into water, and in the case of catalase, to oxygen and water.

Lack of defensive mechanism between antioxidant versus oxidant being the significant factor contributes to the condition of oxidative stress. During homeostasis, SOD sufficiently inactivates superoxides. However, during pathological states such as oxidative stress and diseases, increased levels of superoxides are not inactivated by SOD in the cells and can result in ROS-induced damage. There are three SOD enzymes. MnSOD is localized in the mitochondria, Cu/ZnSOD is located in the cytoplasm and nucleus, and ECSOD is expressed extracellularly in some tissues. Other antioxidant enzymes include catalase, which is found in peroxisomes and cytoplasm, and GPx, which can be found in many sub-cellular components including the mitochondria and nucleus [3-5].

Liver is a major organ attacked by ROS [6]. Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. The mitochondrion, microsomes and peroxisomes in parenchymal cells can produce ROS, regulating on PPARα, which is mainly related to the liver fatty acid oxidation gene expression. Moreover, *Kupffer* cells, hepatic stellate cells and endothelial cells are potentially more exposed or sensitive to oxidative stress-related molecules. A variety of cytokines like TNF-α can be produced in *Kupffer* cells induced by oxidative stress, which might increase inflammation and apoptosis. With regard to hepatic stellate cells, the proliferation and collagen synthesis of hepatic stellate cells is triggered by lipid peroxidation caused by oxidative stress [7-9].

Most of the siddha formulation are considered as excellent anti-oxidants this property of the formulation synergies its potency of reducing the active radicals. For many decades siddha formulations have been used to prevent or treat various diseases. The medicine Seenakara parpam (SKP) derived from purified Seenakaaram (Alum), Vediuppu (Potassium nitrate/salt petre) and Karchunna Neer (Quick lime solution). SKP clinically used for the treatment of urolithiasis and other kidney stone related disorders [10].

The main aim of the present investigation is to evaluate the anti-oxidant and hepatoprotective activity of the formulation Seenakara parpam SKP against D-galactosamine induced oxidative stress in rats.

MATERIALS AND METHODS

Raw Materials

Seenakaaram, Vediuppu, Karchunnam.

The above materials are identified and collected from different sources probably. These are authenticated by Geologist by analysing the following parameters: Macroscopic features, Optical properties, qualitative and quantitative analyses.

Purification of Raw Materials [11,12]

Purification of seenakaaram:

Required amount of seenakaaram is dissolved in water and filtered. Then it is boiled until it attains semisolid form (kuzhambu). It is then cooled to get the purified form.

Purification of vediyuppu:

Potassium nitrate (1 part), water (2 parts) are taken. Potassium nitrate is finely powdered and dissolved in water. The clear fluid is poured in white colored iron pot and heated till a semisolid consistency is obtained. This is then poured in a copper pot and placed in a cool place, now the salt will form. The process is repeated for five times.

Preparation of Seenakara Parpam

1. Purified Seenakaaram (Alum/Alumen)
2. Purified Vediuppu (Potassium nitrate/salt petre)

} Equal quantity

3. Karchunna Neer (Quick Lime solution) - required quantity

Method of Preparation

All the materials used in preparation of parpam are subjected to the process of purification as mentioned in Siddha literature. The purified ingredients will be ground with Karchunna Neer (Quick Lime solution) and made into tablets and subjected to calcination with 10 cow dung cakes. The calcined tablets will be ground to fine powder and stored in an air tight glass container.

Animals

Healthy adult albino wistar male rats weighing between 200-220 g were used for the study. The animals were accommodated in poly propylene cages and were kept in well ventilated with 100% fresh air by air conditioning. A 12 hr dark/light visual cycle was maintained. Room temperature was maintained between 22 ± 2°C and humidity level of about 40-65%. Animals were provided with standard laboratory rodent food and water *ad libitum*. All the animals were acclimatized to the laboratory about 7 days prior to that of the experimentation. The study protocol was reviewed and approved by Institutional Animal Ethical Committee (IAEC).

Animal Grouping

The acclimatized animals were divided into 5 groups of each 6 animals and the grouping details are as follows.
Group I: Served as control administered with 1% CMC, p.o for 21 days.
Group II: Disease control received D-galactosamine (400 mg/kg), i.p on 21st day.
Group III: Received 25 mg/kg of silymarin (25 mg/kg) p.o. for 21 days.
Group IV: Served as a treatment group administered SKP 200 mg/kg in 1% CMC, p.o for 21 days.
Group IV: Served as a treatment group administered SKP 400 mg/kg in 1% CMC, p.o for 21 days.

Treatment Protocol

Animal belongs to group I received vehicle 1% CMC for the period of 21 days and served as normal control group. Rat belongs to group II has no treatment and received single i.p injection of D-galactosamine (400 mg/kg) on 21st day and served as disease control group. Animal belongs to group III received silymarin (25 mg/kg) p.o. for 21 days, whereas animals belongs to group IV and V received test formulation SKP at the dose of 200 and 400 mg/kg. Single i.p injection of D-galactosamine (400 mg/kg) was given to all the animals belongs to group II to IV on 21st day. On day 22nd day after 24 hr of Galactosamine injection all the animals were humanely sacrificed using high dose of thiopentone sodium from which the blood samples were collected by standard technique. Liver was collected and the liver homogenate were prepared.

Biochemical Parameters

Serum separated from the collected blood samples were used for evaluation of different biochemical parameters like ALT, AST [13,14], ALP [15]. Total proteins [16], bilirubin, LDH respectively were estimated in serum. TBARS, GPx [17], SOD [18], CAT respectively were estimated in homogenized liver tissue.

Histopathological Analysis

Immediately after collecting the blood samples, vascular perfusion will be performed for tissue fixation using isotonic saline (250 ml) followed by 10% buffered formalin solution (250 ml). Liver will be removed and weighed immediately on an electronic balance for subsequent analysis. Liver tissue were embedded in paraffin and subjected to hematoxillin-eosin staining [19]. The pathological observation of tissues was performed on gross and microscopic bases. Histological slides of the preserved tissues will be encrypted for analysis by a veterinary pathologist.

Statistical Analysis

The Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Newmann Keul's multiple range tests. The values are represented as Mean ± SEM. Probability value of $P < 0.01$ was determined to be statistically significant.

RESULTS

Effect of Seenakara Parpam and Silymarin Pre-treatment on Biochemical Parameters of D-Galactosamine Intoxicated Rats

There was a significant increase in (P<0.01) Serum Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline phosphatase (ALP), Total bilirubin (TB) and Lactate dehydrogenase (LDH) level and decrease in total protein level was observed in rat belongs to group II treated with D-galactosamine 400 mg/kg when compared to that of the normal control group (Group I). Pretreatment with SKP at a dose 200 mg (group IV) and 400 mg/kg (group V), orally for 21 days dose dependently decreased the levels of AST, ALT, ALP, TB, LDH, and increased levels of TP (P<0.01) in group VI when compare to that of the disease control group. Similar pattern of response was observed in group III rats pretreated with standard drug silymarin at the dose of 25 mg/kg when compare to that of the group II rats (Table 1).

Effect of Siddha Formulation Seenakara parpam and Silymarin Pre-treatment on Liver Anti-oxidant Enzyme Profile in D-Galactosamine Induced Oxidative Stress

In liver homogenate, there was significant decrease in SOD, CAT and GPx levels and increase in LPO levels were observed in animals treated with galactosamine 400 mg/kg (group II) as compared to normal control group (group I). Pretreatment with SKP at a dose of 200 mg/kg (group IV) and 400 mg/kg (group V) orally for 21 days increased the levels of SOD, CAT and GPx levels and decreased the level of LPO significantly (P<0.01) when compare to that of the group II rats (Table 2).

Effect of Seenakara Parpam and Silymarin on Liver Histology of Galactosamine Intoxicated Rats

Histopathological section of normal control rats showing normal liver lobular architecture with well projected central vein and prominent nucleus and nucleolus. Samples belongs to group II D- Galactosamine (400 mg/kg) treated rats showing severe toxicity with congested blood vessels with infiltration of inflammatory cell and swollen endothelial cell projection. Liver section of rats treated with silymarin 25 mg/kg showing only a few inflammatory cells around portal tract. Histology of rats belongs to group IV treated with SKP 200 mg/kg showing marginal inflammatory changes with occasional swollen nucleus. Sample belongs to group V treated with SKP 400 mg/kg showing slight inflammation changes with prominent hepatocyte architecture. As shown in Figure 1.

Table 1: Effect of siddha formulation seenakara parpam and silymarin pre-treatment on biochemical parameters of D-galactosamine intoxicated rats

Group	Treatment	AST (IU/mL)	ALT (IU/mL)	ALP (IU/mL)	TP (gm/dl)	TB (mg/dl)	LDH (U/L)
I	Normal control	82.32 ± 3.78	44.10 ± 2.32	66.40 ± 2.5	6.22 ± 0.75	0.52 ± 0.15	318.40 ± 6.45
II	D-galactosamine 400 mg/kg	275.28 ± 11.32*a	228.22 ± 5.35*a	218.35 ± 4.25*a	3.24 ± 0.34 *a	1.30 ± 0.32*a	415.30 ± 7.85 *a
III	Silymarin 25 mg/kg	150.22 ± 5.25 *b	155.25 ± 4.30*b	120.50 ± 3.14*b	5.65 ± 0.48*b	0.58 ± 0.18*b	340.12 ± 6.60*b
IV	SKP 200 mg/kg	208.30 ± 7.25*b	210.58 ± 5.05*b	150.20 ± 4.30*b	4.62 ± 0.32*b	0.76 ± 0.28*b	380.18 ± 7.15*b
V	SKP 400 mg/kg	187.35 ± 6.40*b	190.28 ± 4.68*b	132.40 ± 3.88*b	4.86 ± 0.40*b	0.68 ± 0.22*b	362.32 ± 6.75*b

- Values were expressed as Mean ± SEM.
- Values were found out by using one way ANOVA followed by Newmann Keul's multiple range tests.
- *a – values were significantly different from Normal control at P<0.01.
- *b – values were significantly different from Toxic control (G2) at P<0.01.

Table 2: Effect of siddha formulation seenakara parpam and silymarin pre-treatment on liver Anti-oxidant enzyme profile in D-galactosamine induced oxidative stress

Group	Treatment	SOD (U/mg) Protein	CAT (U/mg) Protein	GPx (U/mg) Protein	Lipid Peroxidation (nmoles of MDA/g)
I	Normal control	9.14 ± 0.9	0.195 ± 0.08	10.6 ± 0.92	108.85 ± 4.18
II	D-galactosamine 400 mg/kg	2.72 ± 0.10*a	0.041 ± 0.06*a	2.65 ± 0.22*a	182.88 ± 7.95*a
III	Silymarin 25 mg/kg	7.84 ± 0.60*b	0.166 ± 0.04*b	7.80 ± 0.60*b	114.80 ± 5.20*b
IV	SKP 200 mg/kg	6.26 ± 0.48*b	0.094 ± 0.03*b	6.20 ± 0.50*b	144.30 ± 6.58*b
V	SKP 400 mg/kg	6.80 ± 0.52*b	0.121 ± 0.02*b	6.48 ± 0.54*b	126.32 ± 6.32*b

- Values were expressed as Mean ± SEM.
- Values were found out by using one way ANOVA followed by Newmann Keul's multiple range tests.

- *a – values were significantly different from Normal control at P<0.01.
- *b – values were significantly different from Toxic control (G2) at P<0.01.

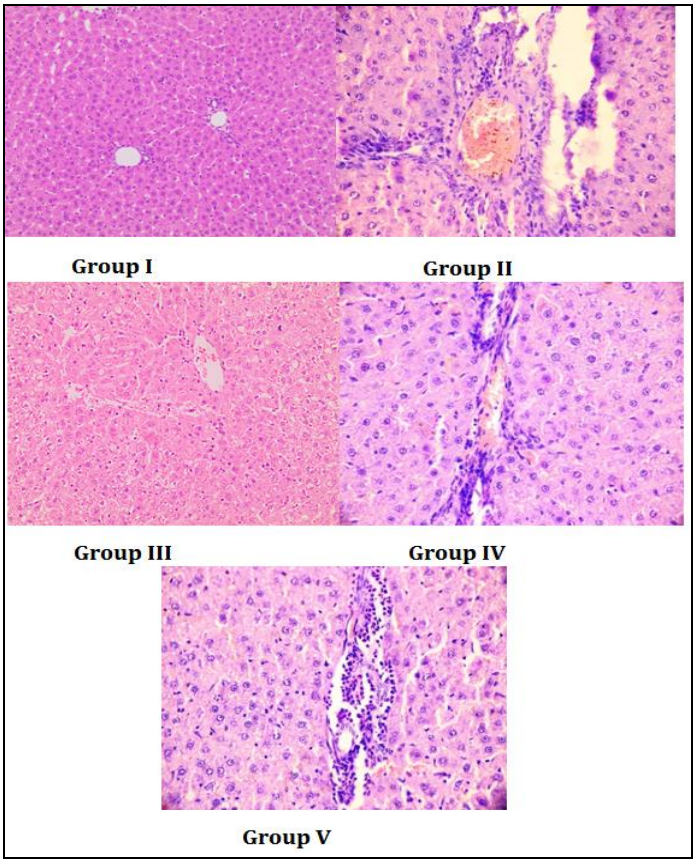

Figure 1: Effect of seenakara parpam and silymarin on liver histology of D-galactosamine intoxicated rats

DISCUSSION

One of the important pathology lies behind the oxidative stress is damage occurred to the several cellular macromolecules due to imbalance between the generation of ROS and the rate of scavenging. ROS have direct and indirect relationships with oxidation of cellular biomolecules resulting in many health disorders such as neurodegenerative disease, hypertension, inflammation, diabetes, cancer and aging [20]. ROS play an important role in the pathogenesis of various serious diseases, such as livery disease, neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation.

Although multiple free radical species, atoms or molecules with an unpaired electron in the outer shell are present in the body, the most common radicals are derived from the reduction of molecular oxygen to water during oxidative phosphorylation and as a group are termed reactive oxygen species (ROS) [21]. Normally, a balance is maintained between ROS generation and antioxidant protection mediated through antioxidant enzymes including copper/zinc superoxide dismutase, manganese superoxide dismutase, glutathione peroxidase and catalase among others and small antioxidant molecules such as glutathione, vitamin E, and vitamin C.

Imbalance between free radical generation and antioxidant capacities is disturbed and free radical generation is no longer (or less effectively) countered by such defense mechanisms, oxidative stress occurs leading to oxidative damage to lipids, proteins, RNA, and DNA. Oxidative damage to these biomolecules can contribute to loss of function leading to exacerbation of damage. The brain is particularly susceptible to oxidative damage due to its high oxygen consumption rate [22]. The mechanism of inflammation injury partially involves the release of ROS from activated neutrophils and macrophages. ROS propagate inflammation by stimulating the release of cytokines such as interleukin-1, tumor necrosis factor-α, and interferon-α, which stimulate recruitment of additional neutrophils and macrophages. Free radicals are important mediators that provoke or sustain inflammatory processes, and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation [23,24].

Response of the living organism toward ROS is mediated through production of antioxidant enzymes as well as they possess genetically regulated adaptive mechanisms against ROS. However, once the free radicals and ROS overwhelm the regulatory ability of the body, a state of oxidative stress ensues. It is known that in case of extensive hepatic damages, enzymes, like AST, ALT and ALP leave the confinement (within liver tissue) and escape into the circulatory system [25,26]. Hence in this study the levels of liver enzyme markers in the serum of the Galactosamine intoxicated rats were compared to the normal control. There was a significant increase in ($P<0.01$) Serum AST, ALT, ALP and LDH level and decrease in total protein level was observed in rat belongs to group II treated with D-galactosamine 400 mg/kg when compared to that of the normal control group (group I). Pretreatment with SKP at a dose 200 mg (group IV) and 400 mg/kg (group V), orally for 21 days dose dependently decreased the levels of AST, ALT, ALP, LDH and increased levels of TP ($P<0.01$) in group VI when compare to that of the disease control group. Similar pattern of response was observed in group III rats pretreated with standard drug silymarin at the dose of 25 mg/kg when compare to that of the group II rats. Estimation of serum bilirubin is used for the assessment of hepatic function in order to diagnose the hepatobiliary diseases and severe disturbance of hepatocellular functions [27]. Increased level of bilirubin in this study is in agreement with previous reports showing that Galactosamine induced hepatitis is characterized by increased levels of bilirubin in serum [28]. The pretreatment of Galactosamine –intoxicated rats with Seenakara parpam produced significant reduction of increased bilirubin level suggests the ability of siddha formulation Seenakara parpam being to counteract biliary dysfunction. Interest in antioxidants of natural origin as food and health supplements has increased much because of their potential to prevent and to reduce the risk of several diseases without any toxic effect [29]. Protective actions against ROS are performed by several enzymes (e.g., superoxide dismutase (SOD), catalase and glutathione peroxidase) as well as nonenzymatic compounds (e.g., tocopherol, vitamin E, beta-carotene, ascorbate and glutathione (GSH)) [30-32]. When the capacity of this antioxidant system decreases, the level of inactivated ROS rises. Ultimately, a dangerous level of redox state is established, and the undesirable influences of oxidative agents appear. These consequences affect several amino acids, such as tyrosine, tryptophan, histidine and, particularly, cysteine. Proteins that are rich in these specific amino acids comprise the direct targets of ROS. ROS-mediated modification might alter both protein structure and function. Oxidized proteins are highly susceptible to proteolytic attack by proteasomes [33]. Siddha drugs being rich in anti-oxidants, helps control the ROS-mediated macromolecular damages [34]. The use of Indian system of traditional medicine as complementary and alternative drug is on rise due to the lesser side effects compared to synthetic drugs. At present, natural antioxidants are also used as alternative to synthetic antioxidants in the clinical management of several oxidative stress induced disease [35]. In liver homogenate, there was significant decrease in SOD, CAT, GPx levels and increase in LPO levels were observed in animals treated with galactosamine 400 mg/kg (group II) as compared to normal control group (Group I). Pretreatment with SKP at a dose of 200 mg/kg (group IV) and 400 mg/kg (group V) orally for 21 days increased the levels of SOD, CAT and GPx levels and decreased the level of LPO significantly ($P<0.01$) when compare to that of the group II rats.

CONCLUSION

From the results obtained from the present investigation it was concluded that the siddha formulation Seenakara parpam found to have significant hepato protective activity against D- galactosamine induced hepatic toxicity. The effect is almost comparable to standard drug silymarin. SKP at both the dose level of 200 and 400 mg/kg significantly reduced the levels of serum AST, ALT, ALP, LDH and Total bilirubin level. Histopathological evidence further strengthens the efficacy of the novel formulation SKP. The results of the present investigation showcase the efficacy of the novel formulation Seenakara parpam. The probable action of drug action may be due to stabilization of hepatocellular membrane, halting the progression of lipid peroxidation, neutrophil infiltration into the liver cells, synergizing activity of antioxidant enzymes but further molecular studies need to be carried out in order to derive the exact mechanism of drug action. By considering the merits of the drug Seenakara parpam it was further concluded that this drug may be used for better clinical management of oxidative stress induced disorder's in near future.

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