Phytochemistry and Hepatoprotective Activity of Chloroform Extract of NKC Ingredient in Santalum album Against D-Galactosamine Induced Hepatotoxicity in Rats

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ABSTRACT
Objective: Nilavembu Kudineer Chooranam is the combination of nine plant materials. The nine components are Nilavempu (Andrographis paniculata), Vettiver (Vetiveria zizanioides), Vilamicamver (plectranthus vettiveroides), Santanam (Santalum album), Peyputtal (Trichosanthes dioica), Koraikkilanku (Cyperus rotundus), Cukku (Zingeber officinale), Milaku (Piper nigrum), Parpatakam (Mollugo cerviana). All these plants are used conventionally in the treatment of fever, inflammation, arthralgia, arthritis, gastric ulcer, jaundice, and general weakness conditions.

Methods: About 500gm of dried fine powder of Santalum album were soaked in the extractor and macerated for 30 hrs with petroleum ether. On the 22nd day after overnight fast the blood was collected from retro-orbital plexus. After the separation of serum from the blood assay of ALT, AST, ALP, γGT and bilirubin were done using standard methods and enzyme assay tests.

Results: The preliminary phytochemical analysis of the Chloroform extract of Santalum album reveals the presence of alkaloid, flavonoid, phenol, coumarin, and tannin. The pretreatment of chloroform extract of Santalum album at a dose of 200mg and 400mg/kg (group IV and V) appeared to significantly prevent the galactosamine toxicity as revealed by the hepatic cells which were preserved in cytoplasms.

Conclusion: Our findings demonstrated that chloroform extract of Santalum album at both doses possesses hepatoprotective and antioxidant activity, which is evidenced by lowered serum hepatic marker enzyme activities. Among the two dosages tested, 400 mg/kg/body weight showed more promising hepatoprotective and antioxidant activity, and is comparable to the standard drug Silymarin.

Keywords: Nilavembu Kudineer Chooranam, Chloroform extract of Santalum album, preliminary phytochemical analysis, Hepatoprotective activity, D-Galactosamine induced hepatotoxicity in rats.

INTRODUCTION:
Siddha, Ayurveda, and Unani are the three medicinal systems of India. That is practiced from ancient time to this modern era. Among this Siddha which originated in the Southern part of India is the best conventional medicinal system in the world as it treats together with the body, mind and the soul. In this era, it has been proved that Nilavembu Kudineer Chooranam (NKC) is bestowed with many therapeutic potentials and is considered as one of the best medicines in Siddha. It is regarded as a perfect health drink to cure many diseases. Nilavembu Kudineer Chooranam is the combination of nine plant materials. The nine components are Nilavempu (Andrographis paniculata), Vettiver (Vetiveria zizanioides), Vilamicamver (plectranthus vettiveroides), Santanam (Santalum album), Peyputtal (Trichosanthes dioica), Koraikkilanku (Cyperus rotundus), Cukku (Zingeber officinale), Milaku (Piper nigrum), Parpatakam (Mollugo cerviana). Equal Proportion of the above nine ingredients are used in NKC. All these plants are used conventionally in the treatment of fever, inflammation, arthralgia, arthritis, gastric ulcer, jaundice, and general

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weakness conditions 2-4. Among the nine components, the Santalum album plays a vital role contributing largely to the medicinal values of NKC. It is learned from the ancient Indian the plant has played Medicinal System that the plant Santalum album (L) has played a significant role as it has been used as an astringent, antipyretic, blood purifier, disinfectant in bronchial and genitourinary tract infections, diuretic, expectorant, memory enhancer, and sedative, the tonic for heart, liver and stomach 5. The present study aims at the evaluation of the hepatoprotective potential of Santalum album (stem), against paracetamol-induced hepatotoxicity. According to the literature of Siddha and Ayurveda NKC improves liver functions. The hepatoprotective activity of NKC warrants a scientific pharmacological study. The preliminary phytochemical analysis of the Chloroform extract of Santalum album reveals the presence of alkaloid, flavonoid, phenol, coumarin, and tannin. Hepatoprotective screening of Santalum album renders a better method for the evaluation of liver protecting the role of NKC. The model system of liver damage produced by D-galactosamine (GalN) is comparable with viral hepatitis in humans morphologically and functionally 6. As a result, this model is now accepted as one of the most authentic test systems of liver damage in experimental animals. GalN has great liver specificity because the hepatocytes have high levels of galactokinase and galactose-1-P uridylyltransferase.

Meanwhile, other organs are not affected 7-8. GalN causes liver cell injury, with spotty hepatocyte necrosis and prominent portal and parenchymal inflammation 9. GalN also causes depletion of uridine diphosphate (UDP). GalN converts uridine diphosphate (UDP) into UDP-sugar derivatives. Inhibition of RNA and protein synthesis by UDP-sugar derivatives leads to deterioration of the cell membranes 10-11. Developing nations use medicinal plants for primary health care. The human body responds well when herbal drugs are administrated.

MATERIALS & METHODS:

The whole plant of Santalum album was collected from authentic herbal trader, Mylapore, Chennai, Tamilnadu, shade dried for a week in a shadow and blended to a coarse powder. Then a Taxonomist authenticated the plant sample. About 500gm of dried fine powder of Santalum album were soaked in the extractor and macerated for 3 hrs with petroleum ether. There it is refluxed successfully with ethyl acetate, after that it is extracted with chloroform by continuous hot percolation method using soxhlet apparatus for 40hrs separately. The chloroform extract was filtered and concentrated in vacuum using rotary flask evaporator under reduced pressure. After concentration chloroform extract of Santalum album gave a brownish residue that was stored in an airtight container.

Animals: In-house laboratory-bred six weeks old Wistar rats were selected for the study. Animals were maintained under the controlled temperature at 20±20 °C and relative humidity of 50-60% with an alternating 12hr light/dark cycle. The animals were acclimatized for one week before the study and had free access to standard laboratory feed and water ad libitum.

Study Design:

Rats of body weight 180-220 g were selected. The total number of 30 rats was divided into five groups of 5 animals each.

Group I: Normal Control: The animals received distilled water 5 ml/kg b.w. p.o. for 21 days.

Group II: Toxicant N-Galactosamine Group: Also received distilled water 5ml/kg b.w. p.o. for 21 days. A dose of D-Gal N in D.W 400 mg/kg b.w was given i.p. after one hour of the vehicle 12.

Group III: Standard Silymarin Group: The animals received silymarin 75 mg/kg b.w. p.o. was given for 21 days. The animals received a single dose of D-Gal N in D.W 400 mg/kg b.w.p after 1 hour of std drug on the 21st day.

Group IV Toxicant + Santalum album: 200 mg/kg was p.o. for 21 days. A dose of D-Gal N in D.W 400 mg/kg b.w. was given i.p. after 1 hour of the test drug.

Group V Toxicant + Santalum album: 400 mg/kg was p.o. for 21 days. The animals received a dose of D-GalN in D.W 400 mg/kg b.w.p after 1 hour of the test drug.

Biochemical Parameters: On the 22nd day after overnight fast, the blood was collected from retro-orbital plexus. Before centrifugation at 2500 rpm for ten minutes, blood was allowed to clot. After the separation of serum from the blood assay of alanine transaminase [ALT] 13, aspartate transaminase [AST] 14, alkaline phosphatase [ALP] 15, γ-glutamyl transferase [γGT] 16 and bilirubin were done using standard methods and enzyme assay tests. Transasia Biomedical Ltd Kit for ALT, AST, LDH and Accurex Biomedicals Ltd Kit for γ GT & ALP. The enzyme assays were performed on a semi-autoanalyzer ERBA Chem.

Evaluation of Biochemical Parameters: The livers were dissected out immediately, washed with ice-cold saline and 10% homogenates in phosphate buffer solution (pH 7.4) were prepared. The following biochemical measurements were carried out in the liver tissues. Liver homogenate was used for the assay of MOA while some fraction of homogenates were centrifuged at 2500 rpm for 10 min at 4 °C using refrigerated centrifuge, and the supernatants were used for the assay of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) 17-19 by standard methods using enzyme assay kits. The enzyme assays were performed on a semi-autoanalyzer ERBA Chem.

Histopathological Observation: The liver from each group was aseptically excused stored separately for analysis of oxidative stress-related biomarkers and in phosphate - buffered formalin (10%) for histopathological evaluation.

Statistical Analysis: The Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Newman–Keul’s multiple range tests. The values are represented as Mean ± SEM. Probability value at p < 0.01 was considered statistically significant.

Table: 1 Phytochemical analysis

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Chloroform extract</th>
</tr>
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<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
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Table: 2 Effect of santalum album and silymarin pre-treatment on biochemical parameters of the rats intoxicated with d-galactosamine.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>TREATMENT</th>
<th>DOSE (mg/Kg)</th>
<th>AST (IU/mL)</th>
<th>ALT (IU/mL)</th>
<th>ALP (IU/mL)</th>
<th>TP (gm/dl)</th>
<th>TB (mg/dl)</th>
<th>GGTP (mg/dl)</th>
<th>Total Albumin(g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>10ml/kg normal saline</td>
<td>45.35±1.48</td>
<td>30.20±1.02</td>
<td>22.70±1.28</td>
<td>5.16±0.32</td>
<td>0.11±0.02</td>
<td>2.64±0.18</td>
<td>3.78±0.10</td>
</tr>
<tr>
<td>II</td>
<td>Toxic control</td>
<td>400mg/kg D-galactosamine</td>
<td>108.65±3.45</td>
<td>93.40±3.32</td>
<td>141.20±5.25</td>
<td>3.18±0.14</td>
<td>4.34±0.22</td>
<td>172.30±5.90</td>
<td>2.14±0.11</td>
</tr>
<tr>
<td>III</td>
<td>Standard control</td>
<td>Silymarin 75mg/kg</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
</tr>
<tr>
<td>IV</td>
<td>Treatment control</td>
<td>Santalum album 200mg/kg</td>
<td>65.80±2.14</td>
<td>51.60±2.72</td>
<td>67.45±2.80</td>
<td>4.48±0.28</td>
<td>5.04±0.20</td>
<td>137.20±3.62</td>
<td>2.52±0.16</td>
</tr>
<tr>
<td>V</td>
<td>Treatment control</td>
<td>Santalum album 400mg/kg</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
</tr>
</tbody>
</table>

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

Table: 3 Effect Santalum album and Silymarin pre-treatment on biochemical liver parameter in d-galactosamine induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>TREATMENT</th>
<th>DOSE (mg/Kg)</th>
<th>SOD (U/mg)</th>
<th>CATALASE (U/mg)</th>
<th>GPX (U/mg)</th>
<th>MOA (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>10ml/kg normal saline</td>
<td>132.25±2.38</td>
<td>290.35±4.38</td>
<td>1.20±0.12</td>
<td>3.95±0.24</td>
</tr>
<tr>
<td>II</td>
<td>Toxic control</td>
<td>400mg/kg D-galactosamine</td>
<td>*a</td>
<td>*a</td>
<td>*a</td>
<td>*a</td>
</tr>
<tr>
<td>III</td>
<td>Standard control</td>
<td>Silymarin 75mg/kg</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
</tr>
<tr>
<td>IV</td>
<td>Treatment control</td>
<td>Santalum album 200mg/kg</td>
<td>67.18±1.26</td>
<td>191.75±2.80</td>
<td>0.36±0.03</td>
<td>7.42±0.45</td>
</tr>
<tr>
<td>V</td>
<td>Treatment control</td>
<td>Santalum album 400mg/kg</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
<td>*b</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM. Values are found out by using one way ANOVA followed by Newmann-Keul's multiple range tests. *a – values are significantly different from Normal control at P< 0.01. *b – values are significantly different from Toxic control (G2) at p< 0.01.
Table: 4 Effect of *santalum album* on the levels of non enzymatic antioxidants in the liver tissue of d-galactosamine hepatotoxic and control rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>GLUTATHIONE MG/100G TISSUE</th>
<th>VITAMIN-C MG/100G TISSUE</th>
<th>VITAMIN-E MG/100G TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control 10ml/kg normal saline</td>
<td>132.65±3.64</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>II</td>
<td>Toxic control 400mg/kg D-galactosamine</td>
<td>73.18±1.78*a</td>
<td>0.32±0.04*a</td>
</tr>
<tr>
<td>III</td>
<td>Standard control Silymarin 75mg/kg</td>
<td>112.30±2.75*b</td>
<td>0.72±0.08*b</td>
</tr>
<tr>
<td>IV</td>
<td>Treatment control <em>Santalum album</em> 200mg/kg</td>
<td>97.28±2.20*b</td>
<td>0.60±0.05*b</td>
</tr>
<tr>
<td>V</td>
<td>Treatment control <em>Santalum album</em> 400mg/kg</td>
<td>91.65±1.94*b</td>
<td>0.67±0.08*b</td>
</tr>
</tbody>
</table>

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

**RESULT:**

**Phytochemical screening**

The extract of Nilavembu Kudineer Chooranam was subjected to qualitative chemical examination for the presence of alkaloid, carbohydrates, flavonoids, tannin, and phenolic compound are reported in table - 1.

**Biochemical observations**

Significant increase in (*P* < 0.01) Serum Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline phosphatase (ALP), Total bilirubin (TB) and Gamma-glutamyl transpeptidase (GGTP) and significant decrease in (*P* < 0.01) Total protein (TP) and Total albumin (TA) levels were observed in animals treated with galactosamine-400mg/kg (Group II) as compared to normal control group (Group I).

Pretreatment with chloroform extract of *Santalum album* at a dose 200mg and 400mg/kg, orally for 21 days decreased the levels of above indices like AST, ALT, ALP, TB, GGTP and increased levels of TP and TA significantly (*P* <0.01) in group IV and V.

Silymarin pretreatment produced a significant increase in (*P* < 0.01) Liver homogenate enzyme such as SOD, CAT, GPx levels and decrease the levels of LPO significantly (*P*<0.01) in group III.

Table 4 shows the levels of non-enzymatic antioxidants such as reduced glutathione, Vitamin C and Vitamin E in the tissues (liver) of D-galactosamine hepatotoxic and control rats. There was a significant decrease in the levels of nonenzymatic antioxidant in D-galactosamine hepatotoxic rats. The chloroform extract of *Santalum album* at a dose 200mg and 400mg/kg administered rats showed significantly increased levels of these non-enzymic antioxidants as compared with untreated hepatotoxic rats.

**Histopathological observations**

Normal liver architecture was observed in the histology of liver sections of normal control animals (Group I). Liver section of control healthy rats showed normal hepatic lobular architecture. The hepatocytes were within normal limits and arranged in thin plates. No fibrous tissue or lymphocytes deposition were observed. Ther was the presence of cytoplasm and prominent nucleus and nucleolus (Fig no: 1). The liver sections of galactosamine treated animals (Group II) showed hepatic cells with serum toxicity characterized by inflammatory cell collection, scattered inflammation across liver parenchyma, focal necrosis and swelling up of vascular endothelial cells (Fig no:2).

Silymarin (Group-III) exhibited protection from galactosamine-induced changes in the liver (Fig no: 3).

The pretreatment of chloroform extract of *Santalum album* at a dose of 200mg and 400mg/kg (group IV and V) appeared to significantly prevent the galactosamine toxicity as revealed by the hepatic cells which were preserved in cytoplasm. The pretreatment of Chloroform extract of *Santalum album* also caused a marked decrease in inflammatory cells (Fig no:4 and 5).
DISCUSSION:

D-galactosamine is a hepatotoxicant. It injures the liver. Liver injury caused by D-galactosamine is similar to that of viral hepatitis. D-galactosamine disturbs the metabolism in the cells of the liver resulting in changes in the serum enzyme activities. The elevated serum enzymes indicate the cellular leakage and loss of functions of the hepatocyte. 

Enzymes such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, and gamma-glutamyltranspeptidase are released into the blood veins when the liver cell plasma membrane is damaged. The in-vivo measurement of the above mentioned quantitative markers indicates the quantity and quality of hepatocellular damage.

In D-galactosamine induced toxicity, increased activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, and gamma-glutamyl transpeptidase and decrease activities of complete protein and total albumin were observed in serum. The chloroform extract of Santalum album seems to preserve the structural integrity of the hepatocyte membrane resulting in the significant reduction in the activities of these enzymes. The 400mg/kg dose had a better effect than the low dose of the
chloroform extract of Santalum album (200mg/kg). The higher concentration might have resulted in the production of more byproducts that would have interfered with the activity. Treatment with chloroform extract of Santalum album significantly decreased these enzyme activities, indicating that chloroform extract of Santalum album has a hepatoprotective effect against a D-galactosamine-induced liver injury. The formation of the highly reactive hydroxyl radical (OH-) and lipid peroxidation are the harmful oxidative effects of D-galactosamine. Consequently, the cell membrane is destroyed. The evidence clearly shows that the D-galactosamine toxicity and lipid peroxidation damages the kidney. The presence of reduced antioxidants was observed in the kidney. The previous studies show that D-galactosamine-induced rats significantly increased thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes in liver and kidney. In the present study, the observation indicates an increase in the levels of thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes in the tissues of D-galactosamine-hepatotoxic rats. Increased lipid peroxidation in various tissues has long been known to cause functional degradation; thus, the deterioration of vital tissue leading to complications may be indirectly due to increased oxidative stress. Treatment with chloroform extract of Santalum album and silymarin showed a significant reduction which might be due to the antioxidant ability of these compounds and the consequent decrease in lipid peroxidation. The chloroform extract of Santalum album possesses antioxidative and free-radical scavenging effects. 

Due to Oxidative stress, cell and tissue lack the antioxidant defense and the scavenging ability of reactive oxygen species. Lipid peroxidation, DNA damage, and the inactivation of many enzymes are other ill effects of oxidative stress. The enzymatic antioxidant defense system is the natural protector against lipid peroxidation that includes superoxide dismutase, catalase and glutathione peroxidase. Reduced activities of these enzymes in the tissue of D-galactosamine-hepatotoxic rats were observed in our study. Superoxide dismutase protects against the superoxide radical (O2--), which damages the membrane and its biological structure. Catalase primarily decomposes hydrogen peroxide to H2O2 at a much faster rate, sharing this function with glutathione peroxidase. Glutathione peroxidise removes lipid hydroperoxides. These enzymes remove oxygen radicals from tissues.

Due to the increase in the levels of superoxide radicals and H2O2: the liver enzymes become inactive leading to death. The administration of chloroform extract of Santalum album significantly increased the activities of these enzymes.

The non-enzymatic scavengers like glutathione, ascorbic acid, and α-tocopherol, defend the residual free radicals escaping from decomposition by the antioxidant enzymes. These scavengers play the second line defense. Moreover, enzymic antioxidants are inactivated by the excessive levels of free radicals, and hence the presence of non-enzymatic antioxidants is presumably essential for the removal of these radicals.

Glutathione is a major non-protein thiol found in a living organism. It protects important thiol group from oxidation by combining with reactive oxygen species and electrophilic metabolites. It is perceived that the glutathione behaves as a substrate for enzyme like glutathione peroxidase. The lowered glutathione in D-galactosamine induced rats represent the increased utilization of glutathione as a result of oxidative stress. Perturbation in the redox status of glutathione not only impairs cellular defense against toxic compounds but also results in enhanced oxidative stress and oxidative injury. Free-radical scavengers protect cell membrane against poisonous agents.

α-tocopherol (Vitamin E) and ascorbic acids (Vitamin C) are important free radical scavengers. They prevent oxidative damage under all types of oxidative stress. Ascorbic acid possesses excellent power to trap lipid perox radical in the aqueous phase producing dehydro dehydroascorbate. The conversion of the dehydroascorbate into ascorbate is known as thiol cycle consisting of a GSSG/GSH couple. The active form of vitamin C (ascorbic acid) is kept up in the cells by glutathione. Reduction in glutathione lowers ascorbic acid content. D-galactosamine hepatotoxic rats are found to have reduced glutathione α-tocopherol and ascorbic acid levels due to an antioxidant defense against increased ROS.

Ascorbic acid and α-tocopherol decreased the hepatotoxic levels in the rats with liver injury, which received D-galactosamine dosage. This study observes the increase in the levels of these antioxidants in chloroform extract of Santalum album and silymarin administered rats.

It is confirmed that chloroform extract of Santalum album inhibited lipid peroxidation. The antioxidant present in the extract accounts for the inhibition of lipid peroxidation. The anti-hepatotoxic activity of the chloroform extract counteracts the free radical-mediated tissue damage. Antioxidants are flavonoid s. Flavonoids contain hydroxyl groups. Hydroxyl groups present in ring B of polyphenols are essential for the free radical scavenging mechanism of the anti-oxidants. The chloroform extract of Santalum album contains flavonoids. A high dose of 400 mg/kg body weight chloroform extract of Santalum album improved the hepatoprotective activity in the rats having D-galactosamine-induced hepatitis.

CONCLUSION:

The findings demonstrated that chloroform extract of Santalum album at both doses possesses hepatoprotective and antioxidant activities. The lowered serum hepatic marker enzyme activities confirmed the antihapatotoxicity of plant extract. Among the two dosages tested, 400 mg/kg/body weight showed more promising hepatoprotective and antioxidant activities and is comparable to the standard drug Silymarin. The observed significant hepatoprotective activity of Santalum album is a solid proof for the hepatoprotective activity of NKC.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The research work was approved by the Institutional Animal Ethical Committee. (IACUC/48/PT/02/2016/PhD/KMCP/58/2019).