Abstract: Aim of the study is to investigate the Nephroprotective activity of ethanolic extract taken from Melia azadirachta leaves, against APAP (Acetaminophen) induced nephrotoxicity. The phytochemical screening was carried out on the leaf extracts of Melia azadirachta, revealed the presence of active ingredients such as Phytol, Squalene, Oleic Acid, 2-Piperidinone, N-[4-bromo-n-butyl]. Leaves of Melia Azadirachta (MA) were successively extracted with ethanol and dried in powder form. The present study was conducted on group of male albino Wister rats. On the rats with APAP induced Nephrotoxicity, different concentration of MA powder extract such as, 250, 500, 750 mg/kg of body weight of rats administered orally. Significant changes were noticed in biochemical parameters (increases in serum urea, creatinine except uric acid) in APAP induced male albino Wister rats, which were restored towards normalization in Melia Azadirachta treated animals. Thus the present study ascertains that the leaf extract of Melia azadirachta possesses significant Nephroprotective activity.

Keywords: Melia Azadirachta, Nephroprotective activity, APAP, ethanol.

I. Introduction

The kidney plays a crucial role in all animals, which is necessary for good health, overall development and growth. The main function of kidney is to maintain total body fluids and composition of acid base balance. Some drugs can influence these functions [1,2,3], one such drug is Acetaminophen (APAP). Acetaminophen (APAP), discovered in 1889, has been a widely used analgesic and antipyretic agent. Cause of hepatic and renal failure is due to the overdose of APAP [4]. These serious disorders are required to be treated to overcome the APAP overdose.

Most widely used method for monitoring renal function is creatinine concentration. This creatinine level in blood is used to compute creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR) [5]. A simple blood test will reveal the presence of nephrotoxicity. Renal failure is diagnosed by a decreased creatinine clearance, whose normal level should be in the range of 80 - 120 µmol/L.
Beneficial effects of several medicinal plants especially from India have been vastly studied in our literature [6, 7, 8, 9] to protect against nephrosis. *Melia Azadirachta* is one such medicinal plant, which has been used in this paper. *Melia Azadirachta*, family Meliaceae is from west Asia [10]. It is widely distributed in Himalayan region between the attitudes of 700 to 1000 m. It is a moderate-sized deciduous tree 9-12m in height with a cylindrical bole with dark grey bark having shallow longitudinal furrows. The leaves are bi- or trip innate, pinnate opposite or alternate, ovate or lanceolate, serrate, acuminate, glabrous on both surfaces, slightly oblique at the base. Its uses are enormous. They are,

1. It has been used for various medicinal purposes.

2. The leaf juice is used as an anthelmintic [11]. It is also used to cure strangury, amenorrhea, bronchitis, leprosy, eczema, asthma and as an antipyretic.

3. Phytochemical studies reveal the presence of Alkaloids, flavanoids, steroids, tannins and lycopena etc.

Nephroprotective effects of *Zingiber zerumbet* for Paracetamol (PCM) induced nephrotoxicity on fifty male Sprague Dawley rats have been discussed in [4]. This paper claims that amount of antioxidants present in Z. zerumbet contribute to the protection of kidney from nephrotoxicity. Reduced water intake, food consumption and reduced body weight are the criteria used to study the effects of Z. zerumbet . Nidhal AK[12] studied the aqueous effect of *Punica granatum* against the Gentamicin-Induced Nephrotoxicity in Rats. And also has investigated the changes in serum levels of urea, creatinine, uric acid, sodium, potassium and chloride in Male Albino rats.

Olagunju, J.A.et al [13], and Subal Debnath et al,[14] have studied the effect of Carica papaya and Pumpkin Fruit against the carbon tetrachloride and Cisplatin induced Nephrotoxicity respectively. S. Palani et al[15] , Shelke TT et al[16] have evaluated the nephroprotective effects of Pimpinella Tirupatiensis and Pedalium Murex Linn against acetaminophen and cisplatin induced nephrotoxicity respectively.

Aim of this study was to evaluate the effect of ethanolic extract of *Melia Azadirachta*, against APAP-induced nephritic damage in male albino Wister rats. This has been observed through the biochemical parameters such as creatinine, urea and uric acid.

II. Materials and Methods

Collection of plant material:

Fresh Matured leaves of *Melia Azadirachta* were collected from Thirumalai hills, Andrapradesh, India in the month of January, 2011. The plant material was taxonomically identified and authenticated by our Botany department of SIVET College, Gowrivakkam, Chennai -73. Botanical Information of the plant is as follows:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Sapindales
Family: Meliaceae
Genus: Melia

At the time of collection, standard herbarium record sheets were completed with the name of the collector, collection number (MA001), date, locality and local name. Authenticated by Dr.V.Chelladurai (Research Officer), Botany (C.C.R.A.S), Government of India, Voucher specimen (SIVET MA-276/2011-2012).
Extraction of plant material:

Fresh matured Melia Azadirachta leaves were extracted and air-dried at room temperature for two weeks. Dried leaves were ground to coarse powder and passed through a 30 mesh sieve. Leaf extract of Melia Azadirachta was separated by using soxhlet apparatus along with 95% C_2H_5OH and finally dried in a vacuum desiccator. The residue was dissolved in distilled water and filtered. The filtrate was evaporated to dryness. The dried mass was diluted with normal saline and used for our experimental work.

Animals:

Male albino Wister rats strain of (100-150g; 3-4 weeks old) were maintained under controlled conditions of light (12h/24h) and temperature (23 ± 1°C). Food pellets (Hindustan Lever Ltd., India) and tap water was provided ad libitum. GC-MS analysis of ethanol extract of Melia Azadirachta for identification of chemical composition was performed using a gas chromatography-mass spectograph (GC-MS). And (Agilant6890 / Hewlett- Packard 5975) fitted with electron impact (EI) mode. 2.0 µL of the ethanol extract of Melia Azadirachta was injected with Hamilton syringe to the GC-MS manually for total ion chromatographic analysis in split mode. In quantitative analysis, selected ion monitoring (SIM) mode was employed during the GC/MS analysis. SIM plot of the ion current resulting from very small mass range with only compounds of the selected mass were detected and plotted.

Experimental treatments:

Male albino wistor rats were divided into five groups of six animals each. Group I was treated with Physiological saline and kept as control. Group II was treated with a single dose of acetaminophen (APAP) of 750mg/kg body weight, was kept as toxin induced. Group III and IV were treated with ethanol extract of Melia Azadirachta at two different doses of 250 and 500 mg/kg body weight and APAP 750mg/kg of body weight of each group respectively. Group V was treated with 500 mg of extract alone. The extract was administered by oral gavages 1 hr before APAP administration [17].

Invivo study:

After 48 hours, the animals were sacrificed by using chloroform. Blood samples were collected by cardiac puncture using 21 gauge (21 G) needles mounted on a 5ml syringe (Hindustan syringes and medical devices Ltd, Faridabad, India). These were kept in ethylene diamine tetra-acetic acid (EDTA) – coated sample bottles. And were used for analyzing biochemical parameters like urea, uric acid, creatinine.

Biochemical analysis:

Creatinine level was determined according to Jaffe’s reaction, based on creatinine reaction with picric acid in alkaline solution, to form a red solution measured colorimetrically at 520 nm. The level of creatinine was extrapolated from the standard graph and the values were then stated as milligram per deciliter (mg/dl). Urea was estimated by DAM – TSC method using colorimeter and uric acid was estimated using the same technique.

Histology study:

Regular histology techniques were followed for the examination of renal tissues. After the animal was sacrificed, renal tissues were dissected, rinsed in normal saline, and sectioned into small pieces. The sectioned tissue was then fixed in 10% formalin, dehydrated in stepwise with increasing concentration of ethanol solution (50% to 100%), and embedded in paraffin. Using microtome, tissue sections of 4 µm thickness were produced, fixed overnight on the slide, subsequently stained with Hematoxylin and Eosin (H&E) and were then observed under a light microscope.

Statistical analysis:

Statistical analysis was done using Statistical Package, the Prism 6 software. Data were analyzed using Shapiro-Wilk normality test and one way analysis of variance (ANOVA) was used for comparison between groups followed by post-hoc Tukey test. Data were expressed as means ± standard deviation (SD) and P-value less than 0.01 and 0.001 showed statistically significance.
III. Results and Discussion

The GC device is generally a reliable analytical instrument. The GC instrument is efficient in separating compounds into their various constituent components. The MS instrument provides specific results, but produces uncertain qualitative results. When an analyst uses the GC instrument to separate compounds before analysis with an MS instrument, a complementary relationship exists. The technician has to access both the retention analysis as a tool for conclusive proof of identity.

In our work, phytoconstituents like Glycerin, propane, 1,1,3-triethoxy-3,7,11,15-Tetramethyl-2-hexadecen-1-ol 3-Heptanol, 3,6-dimethyl-Phytol 1-Octanol, 2,7-dimethyl 1,2-Benzenedicarboxylic acid, diisooctyl ester 2-piperidone, N-[4-bromo-n-butyl]-Squalene 1,1-Biphenyl, 4-iodo- Dodecanoic acid, 1,3-propanetriyl ester Oleic acid- were identified from ethanol extract of *Melia Azadirachta* by using a gas chromatograph-mass spectrograph (GC-MS) [18]. This identification was done by comparison of their mass spectra on both columns with phytochemical and ethnobotanical databases libraries or with mass spectra [19][20] and home-made library. Several pharmacological studies have been reported in literature about positive and valuable effects of certain Indian Medicinal plants to protect from kidney and renal damages. It has been observed that the ethanolic extract has extensively protected the kidneys from injuries. Most of the phytochemical compounds belong to the group of antioxidant agents [21]. Presence of antimicrobial activity of n-hexadecanoicacid was reported by [22],[23], [24],[25]. With respect to the literature data, it was understood that all the above stated compounds could efficiently contribute to the biological activities of *Melia Azadirachta*.

Overdose of Acetaminophen is normally associated with several metabolic disorders including serum electrolyte, urea and creatinine rearrangements. To investigate drug induced nephrotoxicity in animals and human [26], increased concentration of serum urea and creatinine are considered. CYP-mediated conversion of acetaminophen to a highly reactive quinone imine, N-acetyl-pbenzoquinoneimine (NAPQI) is the cause of acetaminophen toxicology. The primary role of NAPQI in the toxicity of acetaminophen has been reported by several papers in literature such as [27, 28], [29, 30], [31, 32].

Blood urea nitrogen is found in the liver protein get usually excreted in the urine. Source of blood urea nitrogen is may be through diet or tissues. Renal disease occurs due to the serum urea accumulation, which is owing to the rate of serum urea production exceeding the rate of clearance [33]. Increase in urea and creatinine levels in the serum has been considered as the index of nephrotoxicity [34, 35, 36]. Creatinine is often obtained from endogenous sources by tissue creatinine breakdown [33]. And hence serum urea concentration is mostly treated as a more reliable renal function predictor than serum creatinine.

Various peaks in the Graph (Figure 1) represent phytochemical compounds present in ethanolic extract of *MA* and also tabulated in Table 1.

**Fig 1. GC-MS Analysis: Ethanolic extract of MA**
### Table 1: Activity of phyto Components identified in the plant *Melia Azadirachta* ethanolic extract [GC MS]

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular Formula</th>
<th>MW</th>
<th>Peak Area %</th>
<th>Nature of Compound</th>
<th>Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.23</td>
<td>Glycerin</td>
<td>C₃H₈O₃</td>
<td>92</td>
<td>23.48</td>
<td>Alcohol compound</td>
<td>Antimicrobial, Preservative</td>
</tr>
<tr>
<td>2.</td>
<td>2.84</td>
<td>Propane, 1,1,3-triethoxy-</td>
<td>C₉H₂₀O₃</td>
<td>176</td>
<td>7.88</td>
<td>Ether compound</td>
<td>No activity reported</td>
</tr>
<tr>
<td>3.</td>
<td>11.68</td>
<td>3,7,11,15-Tetramethyl-2-hexadecen-1-ol</td>
<td>C₂₀H₄₀O</td>
<td>296</td>
<td>0.63</td>
<td>Terpene alcohol</td>
<td>Antimicrobial, Anti-inflammatory</td>
</tr>
<tr>
<td>4.</td>
<td>13.67</td>
<td>3-Heptanol, 3,6-dimethyl-</td>
<td>C₉H₂₀O</td>
<td>144</td>
<td>0.58</td>
<td>Alcoholic compound</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>5.</td>
<td>15.04</td>
<td>Phytol</td>
<td>C₂₀H₄₀O</td>
<td>296</td>
<td>3.09</td>
<td>Diterpene</td>
<td>Antimicrobial, Anti-inflammatory, Anticancer, Diuretic</td>
</tr>
<tr>
<td>6.</td>
<td>17.40</td>
<td>1-Octanol, 2,7-dimethyl-</td>
<td>C₁₀H₂₂O</td>
<td>158</td>
<td>0.12</td>
<td>Alcoholic compound</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>7.</td>
<td>20.94</td>
<td>1,2-Benzenedicarboxylic acid, diisonoic acid, diisoctyl ester</td>
<td>C₂₄H₃₈O₄</td>
<td>390</td>
<td>2.74</td>
<td>Plasticizer compound</td>
<td>Antimicrobial, Antifouling</td>
</tr>
<tr>
<td>8.</td>
<td>23.26</td>
<td>2-Piperidinone, N-[4-bromo-n-butyl]-</td>
<td>C₉H₁₆BrNO</td>
<td>233</td>
<td>0.74</td>
<td>Alkaloid compound</td>
<td>Antimicrobial, Antioxidant, Anti-inflammatory</td>
</tr>
<tr>
<td>9.</td>
<td>24.81</td>
<td>Squalene</td>
<td>C₃₀H₅₀</td>
<td>410</td>
<td>9.60</td>
<td>Triterpene</td>
<td>Anticancer, Antimicrobial, Antioxidant, Chemo preventive, Pesticide, Anti-tumor, Sunscreen</td>
</tr>
<tr>
<td>10.</td>
<td>28.91</td>
<td>1,1'-Biphenyl, 4-iodo-</td>
<td>C₁₂H₉I</td>
<td>280</td>
<td>4.84</td>
<td>Biphenyl Iodo compound</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>11.</td>
<td>30.60</td>
<td>Dodecanoic acid, 1,2,3-propanetriyl ester</td>
<td>C₃₉H₇₄O₆</td>
<td>638</td>
<td>36.04</td>
<td>Lauric acid ester</td>
<td>Antioxidant, Antibacterial, COX-1 &amp; COX-2 inhibitor, Antiviral, Hypocholesterolemic, Candidicide</td>
</tr>
<tr>
<td>12.</td>
<td>32.29</td>
<td>Hexadecenoic acid, Z-11-</td>
<td>C₁₆H₃₀O₂</td>
<td>254</td>
<td>8.63</td>
<td>Palmitoleic acid</td>
<td>No activity reported</td>
</tr>
<tr>
<td>13.</td>
<td>33.60</td>
<td>Oleic Acid</td>
<td>C₁₈H₃₄O₂</td>
<td>282</td>
<td>1.63</td>
<td>Oleic acid</td>
<td>Anti-inflammatory, Antiandrogenic, Cancer preventive, Dermatitisigenic, Hypocholesterolemic, 5-Alpha reductase inhibitor, Anemiagenic, Insectifuge, Flavor</td>
</tr>
</tbody>
</table>

**Source: Dr.Duke’s: Phytochemical and Ethnobotanical Databases [37]**
In the present study, administration of APAP to rats resulted in nephrotoxicity and development of oxidative stress damage in renal tissues. Biochemical parameters such as urea, uric acid and creatinine levels obtained through this study is tabulated in Table 2. In this study, APAP induced nephrotoxicity demonstrates a significant (p<0.01) increase in the serum urea and creatinine concentrations in Group II (APAP induced) rats when compared to the normal group (Group I). In addition, oral administration of ethanol extract of Melia Azadirachta has significantly (p<0.01) decreased the concentration of urea and creatinine in Group III, IV and V respectively, when compared with the p values of Group II. However the level of uric acid is significantly decreased (P<0.01) in the Group II rats when compared to Group I. Oral administration of plant extract significantly (P<0.01) increases the uric acid level in Group III, IV and V respectively, when compared to the APAP induced rats of Group II. The above discussion can also be seen from the pictorial representations of the three phytochemical parameters, Urea, Uric acid and Creatinine in figures 2, 3 and 4.

Table 2: Effect of treatment with ethanol extract of MA on the serum Urea (mM/L), uric acid (µM/L) and creatinine levels (µM/L))

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Control (NaCl 0.9% w/v)</th>
<th>Group II APAP (750 mg/kg)</th>
<th>Group III MA (250mg/kg) + APAP (750mg/kg)</th>
<th>Group IV MA(500mg/kg) + APAP + 750mg/kg</th>
<th>Group V MA (500mg/kg) only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>6.62±0.44</td>
<td>12.32±0.32 a,**</td>
<td>10.28±0.22 b, *</td>
<td>8.34±0.22 b,**</td>
<td>7.43±0.14</td>
</tr>
<tr>
<td>Uric acid</td>
<td>124.64±4.22</td>
<td>113.43±6.54 a,**</td>
<td>109.74±8.02 b, *</td>
<td>123.16±8.12 b,**</td>
<td>125.62±8.22</td>
</tr>
<tr>
<td>Creatinine</td>
<td>64.52±2.12</td>
<td>94.88± 6.14 a,**</td>
<td>82.51±6.12 b, *</td>
<td>80.63± 2.52 b,**</td>
<td>65.22±2.72</td>
</tr>
</tbody>
</table>

Values are expressed mean ± S.D for six rats in each group. a - As compared with control, b - As compared with APAP, ** - represents P<0.001, * - represents P<0.01.

Fig. 2: Effect of treatment with ethanolic extract of MA on the blood Urea (mM/L)] level, in rats with APAP-induced nephrotoxicity

Data are expressed as mean ± S.D., (n = 6). a - As compared with control, b As compared with APAP, ** - represents P<0.001 , * - represents P<0.01.
Fig. 3: Effect of treatment with MA on the serum uric acid (mM/L) in rats with APAP-induced nephrotoxicity

Data are expressed as mean ± S.D., (n = 6). a - As compared with control, b As compared with APAP, ** - represents P<0.001 , * - represents P<0.01.

Fig. 4: Effect of treatment with MA on the creatinine levels (µM/L)] in rats with APAP-induced nephrotoxicity

Data are expressed as mean ± S.D., (n = 6). a - As compared with control, b As compared with APAP, ** - represents P<0.001 , * - represents P<0.01.

Figure 5a shows histological pattern of normal kidney having normal tubular brush borders and intact glomeruli and Bowman’s capsule, fig 5b, shows severe tubular necrosis and degeneration. Mild degree of necrosis and degeneration has occurred in figure 5c and with a drastic change towards cure is shown in figure 5d. The final stain in figure 5e typically similar to the control and also reveals the effect of MA over nephrotoxicity.
Fig 5. Nephroprotective effect of MA extract. Histopathological observations (kidney sections stained with Hematoxylin - Eosin, magnification-45x)

5a. Normal

5b. Acetaminophen -induced

5c. MA (250mg/kg) + APAP (750mg/kg)

5d. MA (500mg/kg) + APAP(750mg/kg)

5e. MA (500mg/kg)

IV. Conclusion

In this work, we have studied the effect of *Melia Azadirachta* ethanolic extract against APAP-induced Nephrotoxicity in albino Wister rats. These rats are divided into five groups of six rats each. Rats were administered with different concentration and combination of APAP and MA. Based on this study, we inferred that the prolonged use of extract of *Melia Azadirachta* will decrease the renal disorder. The results obtained implied, that the presence of phytoconstituents is responsible for the nephroprotective activity.
V. References


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