Antineoplastic Approach of *Semecarpus anacardium* Leaves against N-Nitroso Diethylamine Initiated Hepatocellular Carcinoma

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**ABSTRACT**

**Objective:** To evaluate the effect of MESA on NDEA induced hepatocellular carcinoma on Sprague Dawley rats. **Methodology:** *Semecarpus anacardium* commonly known as ‘marking nut tree’ is a rich antioxidant and in Ayurveda it has been used for treatment of various forms of cancer. Methanolic extract of leaves of *Semecarpus anacardium* (MESA) was studied for its potential antioxidant property both *in vitro* and *in vivo*. NDEA 200 mg/kg, single *i.p* was the hepatocarcinogen while 200 mg/kg and 400 mg/kg of MESA was administered daily orally for 12 weeks. Protective effect of MESA was evaluated by measuring biochemical parameters like SGOT, SGPT, ALP and LDH. Alpha fetoprotein test was performed and histopathology changes of livers were assessed. **Results:** Preliminary phytochemical study revealed the presence of flavonoids, tannins, steroids, alkaloids, glycosides and vitamin C. MESA restored the level of antioxidants near to normal and reduced the elevated serum level of SGOT, SGPT, AST, LDH and AFP. The histopathology changes i.e. necrosis, widened sinusoids, elevated inflammatory cell infiltrate were partly or fully prevented in animals treated with the extract. **Conclusion:** The result of the present study indicates MESA to be rich in antioxidants and its active constituents mediated the free radical scavenging activity. 400 mg/kg extract showed promising effect in management of primary liver cancer.

**Key words:** *Semecarpus anacardium*, Hepatocellular carcinoma, N-nitroso diethylamine, Alpha fetoprotein, Liver marker enzymes.

**INTRODUCTION**

Cancer is an abnormal, uncoordinated, autonomous and rapid growth of cells due to several changes in the gene expression that invades the tissues and metastases to the distant sites leading to death of the host.¹ Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver accounting for about 90% of all liver cancer and it represents more than 4% of all cancer cases worldwide and is the fourth most common cause of cancer mortality.² It is a major health problem especially in developing countries where the incidence rates are two to three times higher compared to developed countries. Some of the available treatment strategies are liver transplantation, surgical resection, systemic chemotherapy, transcatheter arterial chemoembolization, hormonal therapy and proton therapy. However, a recent approach for the control of liver cancer widely used these days is chemoprevention. Chemopreventive agents have ability to treat cancer with less toxicity and are one of the alternative sources of drugs for treatment of cancer of liver, lungs, breast and epithelial tissues.³ Traditional medicines are widely used by about 60% of world’s population not only in the rural areas of developing countries but in developed countries as well.⁴ Medicinal
plants have been an important source of several useful anticancer agents such as vincristine, vinblastine, paclitaxel, etoposide and topotecan.  

*Semecarpus anacardium*, family- Anacardiaceae is one of the ancient traditional medicinal plants widely used in Ayurveda and Siddha system of medicine for treatment of various ailments such as tumours, haemoptysis, skin diseases, deficient lactation and constipation. It is a medium sized tree of around 20-15 m height found in Himalayas, Indian subcontinent, Africa, western peninsula and N. Australia. It contains rich amount of flavonoids and biflavonoids such as A, C, A1, A2, semecarpuflanvanone, galluflavanone, polyphenols such as bhilawanos, semecarpol and anacardol which are responsible for exhibiting several pharmacological effect due to its high free radical scavenging power. Nut, bark and fruit part of the plant has been reported to possess anti-inflammatory, antiarthritic, cardioprotective, anti-diabetic, spermicidal and antineoplastic activities. However, complete pharmacological utilization of the leaves of *Semecarpus anacardium* has not yet been established hence present study will help to establish the chemotherapeutic and preventive effect of the leaves part of the plant in the management of HCC.

**MATERIALS AND METHODS**

**Chemicals, reagents and diagnostic kits**

N- Nitroso diethyl amine (NDEA) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Aldrich Chemicals Co., St. Louis, USA, Phenobarbitone (Abbott healthcare Pvt. Ltd.), Diagnostic kits for Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamate Pyruvate Transferase (SGPT), Lactate Dehydrogenase (LDH) and alkaline phosphatase (ALP) were procured from Erba Diagnostics. All the chemicals used in the study were of analytical grade.

**Phytochemical screening**

Methanolic extract of *Semecarpus anacardium* leaves (MESA) were subjected to phytochemical screening for the presence of phytoconstituents such as alkaloids, flavonoids, tannins, steroids, phenols and terpenoids using standard conventional protocols.

**Estimation of in vitro antioxidants**

**Reducing power assay**

Reducing power assay was carried out by the method of Oyaizu. Substances having reducing potential reacts with potassium ferricyanide (Fe$^{3+}$) to form potassium ferrocyanide (Fe$^{2+}$), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Increase in the absorbance of reaction mixture indicates the reducing power of the samples.

$$\text{Percentage increase in Reducing Power} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{blank}}} \times 100$$

Where  
- $A_{\text{test}}$ - absorbance of test sample  
- $A_{\text{blank}}$ - absorbance of control reaction

**DPPH radical scavenging assay**

The antioxidant potential of the methanolic extract and standard compound (ascorbic acid) were assessed on the basis of the free radical scavenging effect of the DPPH free radical. The amount of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. 1 ml of 0.1 mM DPPH solution was mixed with 3.0 ml of extract solution / standard solution in methanol at different concentrations and the absorbance was measured at 517 nm after 30 min. The capability of scavenging the DPPH radical was calculated using the following equation.

$$\text{DPPH Scavenged} (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$

Where  
- $A_{\text{control}}$ - absorbance of control reaction  
- $A_{\text{test}}$ - absorbance of test sample

**Animals**

Male Sprague Dawley rats (200-240 g) obtained from the institutional animal house facility of Krupanidhi College of Pharmacy, Chikkabellandur, Carmelaram post, Bengaluru, India were used for the study. The rats were housed in groups in polypropylene cages under controlled condition of temperature 25°C, 65% relative humidity and 12 h light and dark cycle and provided with the standard balanced diet and water ad libitum. The protocol was approved by the Institutional Animal
Ethics Committee (IAEC) as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA); Government of India and the approval no is 2016/PCOL/06/KCP/IAEC.

**Induction of hepatocellular carcinoma**

Single carcinogenic dose of NDEA; 200 mg/kg, i.p was used as the inducer of hepatocellular carcinoma. Phenobarbitone (PB) (0.05%) was given as a promoter from the 14th day of NDEA administration till end of the experiment via drinking water.13

**Experimental design**

The rats were acclimatized for a week and body weight of each rat was noted. They were randomly divided into four groups (n = 6) for a 14 week study.

**Group I** - Negative control provided normal water and food ad libitum.

**Group II** - Positive control; Hepatocellular carcinoma induced by single dose of NDEA (200 mg/kg, i.p), followed by PB (0.05 %) after two weeks of NDEA administration till 14th week.

**Group III** – HCC induced animals administered 200 mg/kg MESA, p.o, daily after 14 days of NDEA administration along with PB till 14th week.

**Group IV** – HCC induced animals administered 400 mg/kg MESA, p.o, daily after 14 days of NDEA administration along with PB till 14th week.

The low and high dose (200 & 400 mg/kg) of MESA was selected from the oral acute and sub-acute toxicity studies performed on rats.14

**Sample preparation**

At the end of the experimental period, the animals were subjected to ketamine anaesthesia, followed by cervical dislocation. Blood was collected from retro orbital plexus without anticoagulant and was allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min and kept at 2-4°C for further use.

**Liver tissue preparation**

Liver was excised from sacrificed animal and rinsed in ice-cold normal saline solution, blotted dried in filter paper and weighed in analytical balance. Tissue was centrifuged at 3000 rpm for 20 min at 4 °C and 10% of tissue homogenate was prepared in 0.1 M Tris HCl buffer (pH-7.4).

**Estimation of biochemical parameters**

Serum samples were analysed for the estimation of SGOT, SGPT, LDH, ALP by semi autoanalyzer using suitable diagnostic kits and serum alpha fetoprotein was estimated using ELISA reader working on the principle of Chemiluminescent microparticle immunoassay. Lipid peroxidation was determined in the liver homogenate measured as MDA according to the method of Devasagayam,15 SOD level were determined using liver homogenate using the method of Kono Y.16

**Statistical analysis**

The results were expressed as mean and Standard Error of Mean (SEM). Comparisons among the groups were analysed with ANOVA followed by Dunnett’s test using GraphPad Prism version 5 and p<0.05 was considered to be statistically significant.

**RESULTS**

Percentage yield of methanolic extract of *Semecarpus anacardium* (MESA) was found to be 3.6%

**Preliminary Phytochemical screening**

The preliminary phytochemical screening showed the presence of various phytoconstituents in the extract. The extract had given positive test for alkaloids, carbohydrates, flavonoids, tannins, saponins, vitamin C, steroids and terpenoids.

**In vitro antioxidant assay**

The free radical scavenging activity of MESA was evaluated by the DPPH free radical and reducing power assay. The antioxidant quality of an extract is determined by the IC_{50} value. The result of the reducing power and DPPH scavenging activity of MESA is shown in Table 1.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Samples</th>
<th>IC_{50} Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing power assay</td>
<td>MESA</td>
<td>82.76 μg/ml</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>15.25 μg/ml</td>
</tr>
<tr>
<td>DPPH assay</td>
<td>MESA</td>
<td>78.89 μg/ml</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>17.72 μg/ml</td>
</tr>
</tbody>
</table>
Effect of MESA on body weight and organ weight at end of the experiment

Decrease in the animal body weight, increase in the liver organ weight and increase in the relative liver to body weight ratio indicates signs of hepatocarcinogenesis in the experimental rat receiving NDEA with PB. However decrease in the liver weight of the respective treatment groups implies curative effect on the enlarged liver. Effect of low and high dose of extract on the liver weight and relative organ to body weight is shown in Table 2.

Morphological examination of liver tissue on exposure to NDEA with respective treatment

Figure 1: A Liver tissue of negative control group with smooth surface and no sign of liver damage; B. NDEA/PB administered positive control rat liver tissue with appearance of multiple white nodules; C. Low dose 200 mg/kg MESA treated rat liver with smaller white nodule with rough surface bearing min depressions; D. High dose 400 mg/kg MESA treated liver with lesser sign of liver damage indicating preventive effect of the extract on hepatocarcinogenesis. MESA- Methanolic leaves extract of Semecarpus anacardium; NDEA- N-nitroso diethyl amine; PB- Phenobarbitone.

Effect of various treatments on liver enzymes in different groups of Sprague Dawley rats

Table 3 shows the status of SGOT, SGPT, ALP and LDH in the experimental animals. Animals administered with NDEA/PB showed increase in the level of liver function enzymes when compared to the negative control. On treatment with low and high dose of MESA, it significantly reduced the activity of these marker enzymes compared to the untreated HCC bearing rats.

Biomarker of Hepatocellular carcinoma

Alpha fetoprotein, a specific biomarker of HCC level was significantly (p < 0.05) increased in NDEA/PB administered rat (21.2 ±0.30) compared to negative control animals (4.90 ±0.35). On treatment with the extract, the level decreased significantly (p < 0.05) in a dose dependent manner (Figure 3).

In vivo antioxidant

In positive control group, the level of lipid peroxides significantly increased relative to negative control while the activity of liver superoxide dismutase was significantly decreased. On treatment with MESA significant inhibition (p < 0.05) on the altered antioxidant enzyme level was noticed; shown in Table 4.
Metastasis of Hepatocellular carcinoma on administration of NDEA/PB

**Figure 2: Metastasis of HCC to other vital organs after metabolism of NDEA by liver. NDEA- N-nitroso diethyl amine; PB- Phenobarbitone; HCC- Hepatocellular carcinoma.**

**Histopathological examination of hepatic tissues**

The study of the liver tissue by light microscopy revealed normal hepatic architecture retained in the tissue with the presence of distinct hepatic cells, unremarkable central vein and sinusoidal space with no cell swelling and presence of kupffer cell (Figure: 4. A & B) in the sections from negative control rats while positive control showed lymphocyte infiltration, presence of clear cell foci, necrosis of cell surrounding central vein, hepatocyte hypertrophy and inflammatory cell infiltrate in portal area (Figure: 4.C). Low and high dose of extract (200 & 400 mg/kg) on administration for 12 weeks show gradual repair of the hepatic architecture (Figure: 4. D & E)

**DISCUSSION**

Oxidative stress plays a key role in HCC development and progression. A low level of reactive oxygen species (ROS) is essential in various cellular processes such as proliferation, apoptosis and cell cycle arrest. However, increased level of ROS causes oxidative stress leading to DNA damage which leads to cancer progression.[17] NDEA is a potent hepatocarcinogen known to cause perturbation in the nuclear enzymes involved in DNA repair/replication.[18] It is a well-known liver carcinogen

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**Table 3: Effect of MESA on serum SGOT, SGPT, ALP and LDH of animals.**

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (IU/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>50.96±2.04</td>
<td>21.66±1.45</td>
<td>78.48±7.08</td>
<td>124±2.28</td>
</tr>
<tr>
<td>Positive control</td>
<td>91.09±4.44</td>
<td>53.57±2.67</td>
<td>145.6±5.5</td>
<td>278.9±8.2</td>
</tr>
<tr>
<td>200 mg/kg MESA</td>
<td>68.58±3.18”</td>
<td>37.03±3.45**</td>
<td>109.2±7.2**</td>
<td>229±8.12**</td>
</tr>
<tr>
<td>400 mg/kg MESA</td>
<td>60.98±2.00”</td>
<td>30.68±2.56**</td>
<td>84.2±7.8**</td>
<td>216±7.78**</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± S.E.M (n=6); statistically significant at P <0.05; ANOVA, followed by Dunnetts’ test. When compared positive control vs. low dose and high dose MESA treatment groups.

**SOD** - Superoxide dismutase; **MDA** - Malonaldehyde.
reported to be found in tobacco smoke, alcoholic beverages, fried and processed food and underground water having more amount of nitrates.\textsuperscript{19} During the metabolism of NDEA, high amount of ROS are generated that leads to tissue damage along with transition of normal cell via so called initiated cell to a pre-neoplastic lesions that develop into malignant tumours and to clinical liver cancer.\textsuperscript{20}

\textit{Semecarpus anacardium}, traditional medicinal plant used in Ayurveda & Siddha system of medicine has been reported to be a good antioxidant and possess several phytoconstituents such as saponins, alkaloids, flavonoids, steroids, diterpines, mucilage and gums. Tannins are known to be useful in treatment of ulcerated and inflamed tissues and possess remarkable cancer preventive activity,\textsuperscript{21} saponins possess surface active characteristics and bear cytotoxicity, immune modulatory effects and normalization of carcinogen induced cell proliferation.\textsuperscript{22} Flavonoids have been known to play diverse biological effect in cancer prevention and treatment including free radical scavenging, antimutagenic and antiproliferative, regulation of cell signalling and cell cycle and inhibition of angiogenesis.\textsuperscript{23} Preliminary phytochemical test performed on methanolic extract of \textit{Semecarpus anacardium} leaves (MESA) showed the presence of alkaloids, flavonoids, saponins, tannins, phenolic compounds and vitamin C. \textit{Semecarpus anacardium} has also been reported to contain minerals such as calcium, iron, copper, sodium and aluminium which are responsible for its antiproliferative effect on several cancer cells.\textsuperscript{24} Vitamin C present in the plant protects cell membrane and lipoprotein particle from oxidative damage. Bhilwanol- a phenolic compound localised maximally in the cell membrane of the plant has been reported to exert its effect through changing the permeability of the membrane affecting cellular growth and this might contribute to its anticancer property.\textsuperscript{25}

Antioxidants offer protection against free radical mediated diseases such as cancer and cerebro-cardiovascular disorders due to its ability to scavenge free radicals. MESA being a rich source of antioxidant has been able to combat the oxidative stress mediated by NDEA/PB and extract has been able to restore the damage incurred to the hepatocytes. In our study, \textit{in vitro} antioxidant capacity of the extract was assessed by DPPH assay and reducing power assay (Table 1). Extract was found to possess good antioxidant with ROS scavenging activities. Serum liver function tests are the indicator for liver damage. Intracellular enzymes such as SGOT, SGPT, LDH and ALP are located in the cytoplasm of hepatocytes which gets released to the circulation in case of cellular damage after excessive production of ROS\textsuperscript{26} and their elevated level indicates liver damage. In the present study, SGPT & SGOT levels were significantly increased (p < 0.05) after NDEA/PB administration signifying presence of active disease (Table 3). The abnormal variation in the marker enzymes reflects overall change in metabolism that occurs during malignancy. ALP level elevates linearly with the tumour mass and is considered to be important marker during diagnosis in early detection of cancer.\textsuperscript{27} MESA at dose of 200 mg/kg and 400 mg/kg decreased the elevated levels of these biochemical parameters in a dose dependant manner. Extract tends to prevent liver damage by maintaining integrity of plasma membrane thereby suppressing the leakage of enzyme through membranes, exhibiting hepatoprotective activity. This might be reason for restoration in activities of marker enzymes after administration of the extract. An increase in LDH in malignant liver disorder depends upon the extent of metastasis.\textsuperscript{28} In positive control group, there was a marked increase in LDH which might be due to increase in glycolysis leading to high ATP production. However in extract treatment group decrease in LDH level is significant at p<0.05 when compared to positive control and this is due to controlled glycolysis and protection to membrane integrity offered by the extract.\textsuperscript{29} Agreeing with these results, similar results were obtained by Premalatha B and team\textsuperscript{30} in HCC induced rat treated with Siddha preparation of the nut milk extract. This indicates the usefulness of this plant for anticancer properties. AFP, a serum protein is detected in elevated concentration in condition such as HCC due to its high specificity for the disease.\textsuperscript{31} In the present study, NDEA/PB treatment significantly increased AFP levels (21.2±0.30ng/ml) when compared to that of negative control (4.90±0.35ng/ml) indicating the development of carcinogenesis. Treatment with MESA at low and high doses has reduced the AFP levels (Figure 3). Our results are concurrent with the reported literature.\textsuperscript{32} MESA restored the decreased level of SOD enzyme and this indicates its free radical scavenging ability. Lipid Peroxidation plays an important role in carcinogenesis. On administration of NDEA/PB, lipid peroxidation products like Malondialdehyde and 4 hydroxy noneal reacts with other molecule to cause oxidative stress and carcinogenicity.\textsuperscript{33} Inhibition of elevated level of MDA by MESA in NDEA treated cancerous rat liver suggests its antioxidant potential. Histological examination was performed to confirm the presence of HCC and anticancerous effect of MESA. The study revealed noticeable changes in the architecture of liver in HCC bearing animals like distorted
and disorganized liver histology, tissue necrosis, highly damaged portal tracts and inflammation. This indicates the presence of neoplastic conditions following NDEA and PB administration as observed in other studies. In animals treated with low and high doses of MESA, liver displayed amelioration of hepatocellular architecture in a dose dependent manner and the damage were found to be recovered.

CONCLUSION

The present study demonstrates that MESA possesses free radical scavenging and antioxidant activity. It is able to modulate the levels of LPO and significantly increase the endogenous antioxidant defence mechanism in NDEA induced hepatocarcinogenesis. This is due to the presence of antioxidant principles such as flavonoids, vitamin C and polyphenols present in it. Further, this plant can be taken for extensive study in other animal models including mammals. Isolation of phytoconstituents is required to substantiate the antineoplastic activity of *Semecarpus anacardium*.

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ABBREVIATIONS

MESA: Methanolic leaves extract of *Semecarpus anacardium*; NDEA: N-nitroso diethyl amine; PB: Phenobarbitone; HCC: Hepatocellular carcinoma; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamate pyruvate transaminase; LDH: Lactate dehydrogenase; ALP: Alkaline Phosphatase; LPO: Lipid peroxidation; SOD: Superoxide dismutase; MDA: Malonaldehyde; ROS: Reactive oxygen species; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

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Upreti, et al.: Antineoplastic effect of *S. anacardium* against NDEA initiated HCC


**SUMMARY**

- Methanolic extract of *Semecarpus anacardium* (MESA) was prepared and was used for carrying out antioxidant and anticancer activity.
- Chemical model of HCC was developed using NDEA 200 mg/kg, *i.p.*, as an inducer and PB 0.05% in drinking water was used as promoter of HCC.
- NDEA was administered to all experimental male Sprague Dawley rats except for negative control group. 200 & 400 mg/kg, *p.o.*, daily were low and high doses of test extract treatment group.
- At end of experiment, blood was withdrawn by retro orbital plexus and serum was separated. Liver marker enzymes such as SGOT, SGPT, ALP and LDH were estimated with suitable kits using semi autoanalyzer.
- Serum alpha fetoprotein being the specific biomarker of HCC was estimated by chemiluminescent microparticle immunoassay.
- *In-vivo* antioxidant parameters were estimated using 10% liver tissue homogenate. Lipid peroxidation and superoxide dismutase level in the tissue homogenate was determined.
- Alpha fetoprotein level had increased in positive control animals suggesting development of HCC. Metastasis of cancer was observed morphologically as several other vital organs such as lungs, GIT, heart, pancreas and kidneys were severely affected.
- Histopathology study confirmed the presence of HCC in the positive control animals as the normal hepatic architecture was deranged with signs of metastasis. After treatment with Low and high dose of MESA, improvement in the damaged hepatic architecture was seen with induction of apoptosis in dose dependent manner.

**PICTORIAL ABSTRACT**

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**About Authors**

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