Evaluation of Chemo-Preventive Effect of Methanolic Fruit Extract of *Cucumis melo* var. *agrestis* on DEN-Induced HCC in Sprague Dawley Rats

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ABSTRACT

**Aim:** To investigate chemo-preventive effect of methanolic fruit extract of *Cucumis melo* var. *agrestis* on DEN (diethyl-nitrosamine) induced hepatocellular carcinoma in Sprague Dawley rats.

**Materials and Methods:** Five groups of rats randomly selected comprising of 6 animals on each group, all groups, except Group-1 were administered single dose of DEN (200 mg/kg, i.p. dissolved in PBS) for the induction of HCC and promoted by phenobarbital (0.05%) for 16 weeks. Group-1 was administered only normal saline, however Group-3, 4 and were intervened by the standard (5-FU) and Test-1 and 2 by MECM. At the end of 16th week, overnight fasted rats were sacrificed under anaesthesia then blood samples and liver were collected to analyse morphological liver, to evaluate serum antioxidant, liver enzymatic, non-enzymatic, haematological parameters as well as to evaluate histopathology to assess the effect of standard (5-FU) as well as test (MECM) on DEN induced HCC in rats.

**Results:** As in the case of Test-1 and 2 groups treated by low and high dose (200 mg/kg and 400 mg/kg) of MECM has showed only mild to moderate dose dependant effect on serum anti-oxidant as well as liver enzymatic and non-enzymatic activity. However, other parameter such as in body weight, liver morphology, tumour incidence, haematological as well as histopathology results with respect to standard drug (5-FU) was very poor.

**Conclusion:** This study has demonstrated that MECM clearly lack its ability to act as a chemo-preventive agent, however the moderate antioxidant effect might place it as an effective adjuvant on the treatment of HCC.

**Keywords:** Diethyl-nitrosamine, Hepatocellular carcinoma, Methanolic fruit extract of *Cucumis melo* var. *agrestis*, 5-Fluro-uracil and Phosphate buffer solution.

INTRODUCTION

Hepatocellular Carcinoma (HCC) seems to be one of the leading type of cancer affecting worldwide with high mortality. Evidence suggest HCG is promoted by risk factors such as alcohol consumption, hormone exposure, haemochromatosis, aflatoxin B₁, Hepatitis B and C, and DEN, among factors DEN was found to be the potent environmental hazards agent as it is directly and indirectly consumed through chemical, tobacco, chemical, fried foods, cosmetics as well as by pharmaceutical substance and drugs. In human consumption of DEN leads to conversion of free radicals by CYP450 dependent oxygenase forms alkyl DNA adducts and mutates the liver resulting in formation of liver cancer. Among the HCC model, DEN-induced HCC was most widely accepted model for the screening of chemo-preventive and chemotherapeutic potential of test drugs. As chemo-protective effect is evidenced in phytochemicals such as, vincristine, paclitaxel, etoposide, teniposide, irinotecan and topotecan, it is also further evident as literature review suggests the daily diets of flavonoids (triterpenoids) reduce the cancer occurrence substantially. Hence identification, isolation and pharmacological exploration of various medicinal plant might be a promising step towards effective pharmacotherapy against HCC.

The fruit of *Cucumis melo* var. *agrestis* popularly known as Wild Melon also known as Muskmelon, which is an annual creeper widely consumed vegetable distributed most parts of India. The methanolic extract of seed and fruit contains rich phytochemicals such as triterpenes flavonoids, phenolic compounds, steroids, alkaloids, essential oil, and tannins. Muskmelon is one of the valuable medicinal plant against pain, immunity and inflammatory management. In addition to that it is also possess anti-cancer, anti-oxidant, anti-atherosclerosis.
Table 1: Experimental design.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample size</th>
<th>Group specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1 (Control)</td>
<td>6</td>
<td>Received only Normal saline for 16 weeks.</td>
</tr>
<tr>
<td>Group-2 (Negative Control)</td>
<td>6</td>
<td>Received single dose of DEN (200 mg/kg) by i.p. after two weeks, Phenobarbital (0.05%) dissolved in drinking water for 14 weeks.</td>
</tr>
<tr>
<td>Group-3 (Positive Control)</td>
<td>6</td>
<td>DEN with Standard (5-Flourouracil 20 mg/kg) by i.p. weekly dose for 6 weeks, after 10th week of DEN treatment.</td>
</tr>
<tr>
<td>Group-4 (Test–Low dose)</td>
<td>6</td>
<td>DEN with low dose of MECM (200mg/kg) by p.o. for 16 weeks.</td>
</tr>
<tr>
<td>Group-5 (Test–High dose)</td>
<td>6</td>
<td>DEN with high dose of MECM (400mg/kg) by p.o. for 16 weeks.</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Collection of Plant and Authentication

The fresh fruit of the plant *Cucumis melo var. agrestis* was collected from Thiyagai village, Kallakurichi (Dt), Tamil Nadu, India on December 2019 and authenticated from the Government Siddha Medical College, Chennai, (Voucher specimen No. GSMC/MB-87/18).

Extraction of the Plant Material

In a cold maceration technique 300 g of coarse powdered fruit of *Cucumis melo var. agrestis* was defatted with 1L of petroleum ether for 72 hr, and the obtained marc was further extracted with 1L of 70% methanol (700mL of methanol: 300ml of water) in a conical flask. The mixture was stirred thoroughly with a glass rod. The conical flask was kept for 72 hr with intermittent shaking, filtered by Whatman No.1 filter paper and concentrated to dry using heating mantle at 40°C, and the resultant residue was freeze dried and kept in a refrigerator (−20°C) till further use.

Animal

For the experimental study, Sprague Dawley male rats (160 - 180g) were used, housed standard condition of relative temperature (25 ± 2°C) and relative humidity (45-55%) at the animal house of KMCH College of Pharmacy with 12/12 hr light/dark cycle, fed with commercially available diet with water *ad libitum*. The experiment procedure was approved by the Institutional Animal Ethical Committee (IAEC) and experiment was conducted at KMCH College of Pharmacy Animal Facility, Coimbatore, Tamil Nadu, India (IAEC/SHIATS/PA16III/SDSAV08).

Induction of Hepatocellular Carcinoma

Thirty Sprague Dawley male rats divided into 5 groups, comprising 6 in each group. All groups, except Group-1 were administered single dose of DEN (200mg/kg, i.p. dissolved in PBS for the induction of HCC, after 2nd week of HCC induction, the carcinogenicity was accelerated by administration of phenobarbital (0.05%) dissolved in drinking water for continuously for 16 weeks. After 16 weeks the treatment was started such that the normal group (Group-1) was treated with saline. However, all Groups, except Group-3, 4 and 5 were intervened by the standard and test drug as described (Table 1). At the end of 16th week, overnight fasted rats were sacrificed under 10% chloral hydrate (0.3 mL/100 g, i.p.) anaesthesia. Blood samples were collected via cardiac puncture and serum samples were stored at (−30°C) until biochemical analysis, liver is excised immediately, weighed and homogenised for the study for biochemical parameters.

Qualitative Phytochemical Analysis MECM

In order to verify the presence of specified phytochemicals as coated on the literature review the MECM was subjected qualitative phytochemical analysis as per the standard protocol.

Oral Toxicity Study

As Acute Oral Toxicity Study was essential in order to find out LD₅₀ value and to fix the Test doses of MECM, it was conducted as per OECD-423 guidelines, by randomly selected and overnight fasted with free access to water, four groups of 3 animals of each were administered MECM of (5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg, p.o.) to the respective groups as single dose. The animals are closely monitored periodically for 30 min during 1st 24 hr and specific attention given during first 4 hr daily for a total period of 14 days for mortality, toxic symptoms through direct and indirect behavioural changes affecting skin, eyes, mucus membrane, respiratory, circulatory autonomic and CNS and somato-motor activates.

Serum Biochemical Parameters Estimation

The estimation of serum biochemical parameters indicates the pathogenesis of HCC and the impact of standard and test drug, hence the following hepatic parameters such as Aspartate Amino Transferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) and Lactate Dehydrogenase (LDH) and were performed at the end of 16th week of DEN treatment in all groups.
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Enzymatic and Non-Enzymatic Antioxidant Parameters Determination

As the pathology of HCC effect is directly reflected on the liver damage by free radical damage, the test and standard drug might provide clear picture of the anti-cancer activity, therefore the liver homogenates were collected to study the various enzymatic antioxidant parameters, which includes such as Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and non-enzymatic antioxidant parameters such as Glutathione (GSH) and Glutathione Reductase (GR).

Haematological Parameters Estimation

Haematological parameters mainly RBC, WBC, and Haemoglobin estimation might demonstrate the disease progress due to the suppression of bone marrow and also distinguish the effectiveness of the standard and test drug against the disease control group, hence RBC, WBC count and Haemoglobin estimations were done as per the standard procedure.

Histopathological Examination of Liver

The liver excised from each group were embedded in paraffin, deparaffinised, rehydrated and further stained with an eosin and hematoxylin to distinguish the damage and the improvement on the liver tissue between disease control, standard and test group were done by visualising about 5 μm area under light microscope.

Statistical Analysis

All the experimental data were analysed by Graph Pad Prism by One-Way Analysis of Variance (ANOVA) followed by Dunnett’s Multiple Comparisons Test. All statistical values are expressed as mean ± SEM (n=6 rats/group).

**RESULTS**

**Qualitative Phytochemical Analysis of MECM**

The qualitative Phytochemical screening of MECM found to possess flavonoids, phenolic compounds, steroids, tannins, terpenoids, alkaloids, proteins, amino acids and carbohydrates.
Oral toxicity study

Although the MECM showed mild sign of drowsiness, sedation and lethargy, no lethal or toxic effects were observed even with the highest dose of 2 g/kg body weight till the end of the study. Hence, MECM 200 mg/kg and MECM 400 mg/kg were chosen as low and high dose respectively for in vivo chemo-preventive activity.

Effect of 5-FU and MECM on body weight

MECM low dose (200 mg/kg) and high dose (400 mg/kg) once daily for 16 weeks for Test-1, Test-2 and Standard (5-Flurouracil 20 mg/kg) by weekly dose for 6 weeks, after 10th week of DEN treatment were used for studying the body weight changes in HCC rats, showed the body weights were slightly (10.95%) decreased in DEN treated group. However, the standard treatment (5-FU) showed significant protection in body weight (12.74%) as compared to DEN control group, whereas MECM Test-1 and Test-2 has mild to moderate increase in body weight (2.82%) and (6.51%) was observed. It indicates that the extract is less and dose dependent effect than standard (5-FU) as represented (Table 2 and Figure 1).

Impact of 5-FU and MECM on liver morphology and Tumour Incidence

The study demonstrates that the liver weight and no. of nodules and tumour incidence were significantly increased in DEN (38.38%, 89 and 100%) than control group, indicates the development of HCC whereas, the Standard treatment (5-FU) showed significant protection by means decreased liver weight, no of nodules and tumour incidence (24.89%, 23% and 25.84%). However, MECM Test-1 and 2 has shown no significant difference was observed as compare to DEN control group in terms increased liver weight, no. of nodules and tumour incidence (36.43, 79 and 88.7) and (32.76, 72, and 80.90) percentage respectively, indicate no chemo-preventive effect of Test drug on DEN induced HCC, as depicted in (Table 3 and Figure 1).

Impact of 5-FU and MECM on Serum Hepatic Markers

It was evident from the Table 4 and Figure 2 that DEN control group revealed significant increased level of all hepatic parameters i.e., AST (46.1%), ALT (55.2%), ALP (51.9%), and LDH (31.8%) with respect to control group at the end of 16th week. Whereas the standard group (5-FU) treated group showed significant reduction on percentage elevation as compare to DEN control group and more ever the values are very near to control group i.e. AST (12.9%), ALT (18.5%), ALP (7.6%), and LDH (7.4%). However only mild to moderate and dose dependent reduction in percentage elevation were observed for MECM treated Test 1 and 2 group i.e. [AST (38%), ALT (47.5%), ALP (44.5%), and LDH (28.1%)] and [AST (29.4%), ALT (35.7%), ALP (33.9%), and LDH (13.4%)] respectively. The results clearly demonstrate the
induction of HCC in DEN group as well as significant reduction on HCC for standard (5-FU) group, however MECM treated Test-1 and 2 group has showed only mild and moderate dose dependant anti-oxidant effect with respect to standard (5-FU) group.

**Impact of 5-FU and MECM on Antioxidant Parameters**

Table 5 and Figure 3 demonstrated that DEN control group has significant reductions on the level of almost all antioxidant parameters i.e., SOD (32.16%), CAT (34.31%), GSH (69.06%), GPx (41.17%) and GR (37.73%) with respect to control group at the end of 16th week. However, the standard group (5-FU) treated group showed significant elevation on as compare to DEN control group and more ever the values are very near to control group i.e., SOD (5.74%), CAT (5.95%), GSH (10.03%), GPx (5.29%) and GR (1.57%). However only mild to moderate and dose dependent reduction in percentage elevation were observed for MECM treated Test-1 and 2 group i.e. [SOD (17.39%), CAT (24.34%), GSH (60.15%), GPx (25.87%) and GR (23.85%)] and [SOD (13.7%), CAT (29.08%), GSH (49.2%), GPx (15.12%) and GR (15.82%)] respectively. The results prove the induction of HCC in DEN group as well as significant reduction on HCC for standard (5-FU) group, however MECM treated Test-1 and 2 group shows only mild and moderate dose dependant anti-oxidant effect with respect to standard (5-FU) group.

**Impact of 5-FU and MECM on Haematological Factors**

DEN group treated rats illustrate the clear-cut increased levels of WBC (47.75%) and significant decreased level of RBC (54.24%) and Haemoglobin (37.28%). However, there is a significant restoration of haematological parameters were observed by means of less percentage elevation and reduction on WBC (11.56%) and RBC (17.74%) as well as Haemoglobin (10.13%) in 5-FU treated rats. Whereas, only mild to moderate and dose dependent reduction in percentage elevation and reduction of haematological parameters were observed for MECM treated Test-1 and 2 group i.e. [WBC (35.41%) RBC (45.89%) and Haemoglobin (29.18%)] and [WBC (25.11%) RBC (30.98%) and Haemoglobin (16.81%)] respectively. The results indicate development of cancer in DEN treated group and significant restoration for standard (5-FU) group, however only mild to moderate dose dependent restoration on MECM treated Test-1 and 2 groups (Table 6 and Figure 4) was observed.

**Impact of 5-FU and MECM on Histopathological Examination**

Histopathological liver section of control group depicted normal lobular architecture with normal sinusoids, hepatocytes consists of small uniform nuclei scattered in cytoplasm with normal portal triad was observed, as in the case of DEN induced cancer...
rats loss of architecture with interface hepatitis, individual hepatocytes appeared as binucleations and parenchymal necrosis evidence of focal proliferation was observed reveals the evidence of development of cancer. Whereas DEN treated with standard (5-FU) group showed significant improvement in histological features by means of mild altered architecture with improved cytoplasm and interface hepatitis. Portal tract appears to be normal with dilated sinusoidal was noted. However, DEN treated with chemo-preventive MECM group of Test-1 group appeared to be severely altered lobular architecture with interface hepatitis. Individual hepatocytes showed cytoplasmic vacuolations and degenerative changes with no evidence of recovery than DEN control group. MECM treated group (Test-2) showed as mild restoration of lobular architecture with interface hepatitis with congested central vein, substantiate mild restoration of hepatocyte with reference to DEN control (Figure 5).

**Group-I**

Section from liver showed normal lobular architecture, individual hepatocytes has no significant pathology with normal portal triad. Central vein showed dilatation and the sinusoids appeared to be very normal, representing healthy hepatocytes.

**Group-II**

Liver section study showed altered architecture with interface hepatitis. Individual hepatocytes showed binucleations and parenchymal necrosis in addition to that Portal tract appeared to be unremarkable. The Central Sinusoidal veins were dilated affirmed the development of HCC.

**Group-III**

It demonstrates mild altered architecture with slight interface hepatitis, individual hepatocytes showed cytoplasmic vacuolations and mild parenchymal necrosis focal reactive atypia. However portal tract appears normal. Mild sinusoidal and central vein dilatation was also observed.

**Group-IV**

Section study from liver showed severely altered lobular architecture with moderate to severe interface hepatitis, individual hepatocytes with cytoplasmic vacuolations and degenerative changes. Sinusoids and central vein were depicted as dilated as well as congested.

**Group-V**

Section study showed abnormal lobular architecture with interface hepatitis. Individual hepatocytes showed binucleations. Although portal tract appeared to be normal sinusoidal showed significant pathology in terms of central vein congestion.
**DISCUSSION**

Our current study was an endeavour to evaluate whether MECM possess chemo-preventive effect against HCC, preliminary phytochemical screening confirm the presence of flavonoids, phenolic compounds, steroids, tannins, terpenoids, alkaloids, proteins, amino acids and carbohydrates to correlate the therapeutic effect of MECM on HCC, Oral Toxicity was conducted under OECD-423 guidelines in order to ensure the optimal dose for MECM such that low dose as 200 mg/kg and 400 mg/kg were selected for Test-1 and Test-2 group respectively.

DEN initiated HCC in rats was one of the renowned and more relevant model for the screening of hepatic cancer, as it clearly demonstrates the whether the test drug has the potential to act as a chemo-preventive agent, to compare level of effectiveness with relevant to standard drug (5-FU) and to weigh the therapeutic potential of MECM against HCC. There is a significant loss in body weight was observed in HCC induced rats as compared to control rats indicating the development of HCC. However as in the case of standard (5-FU) the body weights steadily increased after treatment denotes the effectiveness of treatment, whereas in case of MECM treatment on Test-1 and 2 groups has very mild effect compared with standard, indicate that MECM does not showed chemo-preventive effect it is further substantiated by the fact that Test-1 and 2 Groups doesn't reduce the tumour incidence and changes in energy metabolism.

The induction of HCC was proven by substantial increase in liver weight and relative liver weight in DEN treated control group as compare to control group, however as in the case of standard group treated by 5-FU significant reduction was observed, shows the rehabilitative capacity of the 5-FU but that is not a case with MECM affirmed that there is no chemo-preventive potency. The number and incidence of tumour nodules is the prominent future of HCC, was observed in Group-2 (treated by DEN), but not in Group-3 (treated by 5-FU), whereas in Grup-4 and 5 (Test-1 and 2) the number and incidence of tumor were almost similar to that of DEN control, clearly demonstrate the lack of chemo-preventive activity of MECM.

The elevated levels of delicate Serum biomarkers such as AST, ALT, ALP, and LDH indicated the beginning of the destruction process of liver with subsequent spilling of cytosolic enzyme into the circulatory system and these changes further reduce the liver function by means of the pathological changes such as hypofunction, decreased biosynthetic capacity of serum markers as well as altered liver cell membrane permeability, were observed
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Literature review suggests that there is decreased level of antioxidant reflected through reduced levels of enzymatic antioxidants, includes the SOD and Catalase with consequent elevation of hydrogen peroxide indicating the oxygen stress, which steadily progress from induction to the development of HCC in DEN treated Group-II. In our study 5-FU expanded the levels of hepatic SOD and Catalase enzymes with subsequent reduction in the level of Reactive Oxygen Species and almost similar trend is observed in DEN and 5-FU treated Group-2 and 3 for GSH, which is one of the vital non-enzymatic anti-oxidant parameter. In the case of MECM treated Test-1 and 2 there is dose depended elevated levels of both enzymatic and non-enzymatic anti-oxidant levels, which is close to the standard group treated by 5-FU. The elevated anti-oxidant levels indicate that MECM possess potent anti-oxidant effect.

DEN by its basic nature as a toxic chemical, it is very much expected that it might have deleterious effect on RBC as well as
Haemoglobin and the result confirm the same. The DEN group rats revealed significant reduction RBC and Haemoglobin levels in addition to that enhanced levels of WBC also observed. The 5-FU down regulated the level of WBC and upgraded the range of RBC and Haemoglobin, whereas only mild to moderate dose dependent reduction on MECM treated Test-1 and 2 groups was observed and similar results was observed in histopathology as well.

CONCLUSION

The induction of HCC with DEN followed by phenobarbital as a tumour promoter proven to be very effective pathological development of HCC on rats, the treatment of MECM has shown mild to moderate and dose depended anti-oxidant effect on both Test-1 and 2 Groups in terms of elevating serum, enzymatic and non-enzymatic parameters as compare to the standard drug treated by 5-FU of Group-3, probably mediated by rich in flavonoids, phenolics, ascorbic acid and tannins. However, MECM doesn’t showed to be effective in reducing other parameter such as in body weight, liver morphology, tumour incidence, haematological as well as histo-pathological results with respect to standard drug (5-FU), which has proven to be a remarkable chemotherapeutic agent. Overall results conclude that the MECM was clearly lack its ability to act as a chemo-preventive agent, however the moderate antioxidant effect might place it as an effective adjuvant on the treatment of hepatocellular carcinoma.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CPCSEA: Animal Care and were in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines; DEN: Diethyl-nitrosamine; HCC: Hepatocellular carcinoma; MECM: Methanolic fruit extract of Cucumis melo var. agrestis; 5-FU: 5-Fluro-uracil; IAECS: Institutional Animal Ethical Committee; PBS: Phosphate buffer solution.

SUMMARY

The main aim of the research work was to investigate chemo-preventive effect of methanolic fruit extract of Cucumis melo var. agrestis on DEN induced hepatocellular carcinoma in Sprague Dawley rats based on the phytochemical and pharmacological activity the plant. We choose DEN induced HCC model for the induction and divided into five groups of rats randomly selected comprising of 6 animals on each group, all groups, except Group-1 were administered single dose of DEN (200 mg/kg, i.p. dissolved in PBS) for the induction of HCC and promoted by phenobarbital (0.05%) for 16 weeks. Group-1 was administered only normal saline, however Group-3, 4 and 4 were intervened by the standard (5-FU) and Test-1 and 2 by MECM. At the end of 16th week, overnight fasted rats were sacrificed under anaesthesia then blood samples and liver were collected to analyse morphological liver, to evaluate serum antioxidant, liver enzymatic, non-enzymatic, hematological parameters as well as to evaluate histopathology to assess the effect of standard (5-FU) as well as test (MECM) on DEN induced HCC in rats. The results showed in the case of Test-1 and 2 groups treated by low and high dose (200 mg/kg and 400 mg/kg) of MECM has shown only mild to moderate dose dependant effect on serum anti-oxidant as well as liver enzymatic and non-enzymatic activity. However, other parameter such as in body weight, liver morphology, tumour incidence, haematological as well as histopathology results with respect to standard drug (5-FU) was very poor. This study has demonstrated that MECM clearly lack its ability to act as a chemo-preventive agent, however the moderate antioxidant effect might place it as an effective adjuvant on the treatment of HCC.

Ethical Statement

The entire research work on animals were done by strict adherence with CPCSEA norms.

REFERENCES


