Anticancer potential of the extract of *Melothoria maderaspatana* on DMH induced colon cancer in albino rats

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**ABSTRACT**

Cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality. Out of all the cancers, colon cancer is one of the most common diseases in the world. Every year 1.2 million patients are diagnosed for colon cancer. The rate of colon cancer incidence was low in India but is presently increasing; out of 3.5 million cancer cases, 35,000 have colon cancer. As a part of chemotherapy, lots of anticancer drugs are in the market, but the main problem associated with these drugs is their side effects. Because of chemotherapy treatment side effects, the patient needs secondary palliative care treatment. Plant medicines are well known for their non-toxic side effects, so the objective of the study is to develop a drug from medicinal plant against colon cancer with non-toxic side effects. It plays an important role in the discovery of lead compound for the development of conventional drugs. About 60% of currently used anticancer agents are derived from a natural source. In the present study, *Melothoria maderaspatana* was used to study the anticancer potential of the extract and to synthesize new anticancer moiety. With the findings of the study, it can be concluded that the plant *Melothoria maderaspatana* and possess anti-colorectal cancer activity. Before the clinical usage of extract, thorough toxicological profile has to be determined on the crude extracts as well as on isolated compounds to confirm the safety of the drug.

INTRODUCTION

Cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality. Out of all the cancers, colon cancer is one of the most common diseases in the world. Every year 1.2 million patients are diagnosed for colon cancer. Colorectal cancer is the second leading cause of cancer death in the United States for both men and women. The rate of colon cancer incidence was low in India but is presently increasing; out of 3.5 million cancer cases, 35,000 have colon cancer (*Shrikhande et al.*, 2007).

The small growths (known as polyps) in the colon are often benign, although some have the potential to develop and become cancerous. It is estimated that up to two-thirds of colorectal polyps are premalignant and associated with a risk of colorectal cancer. However, there are often no initial symptoms, and cancer may already have spread to other parts of the body by the time the patient is diagnosed (*Edwards et al.*, 2010).
Worldwide, colorectal cancer is diagnosed in over 1.2 million people globally each year; it is the second most common cancer in women and the third most common cancer in men. The disease is responsible for approximately 609,000 deaths each year (8% of all cancer deaths), making it the fourth leading cause of cancer death after lung, stomach and liver cancers. Europe colorectal cancer is the most common cancer in Europe, with approximately 430,000 new cases each year; the highest incidence rate of colorectal cancer in the world. It is also the second highest cause of cancer death in Europe following lung cancer, accounting for 12% of all cancer deaths. North America there was approximately 177,000 new cases of colorectal cancer in North America in 2008, making it the second most commonly diagnosed cancer in the region. Colorectal cancer accounted for 11% of all cancer incidence and 9% of all cancer deaths in North America in the same year.

There are two pathogenetically distinct pathways for the development of colon cancer, both of which involve the stepwise accumulation of multiple mutations. However, the genes involved and the mechanisms by which the mutations accumulate are different. There are a few standard ways in which the pathogenesis of colon cancer occurs. They are APC/β-catenin pathway, Loss of the APC tumour suppressor gene, Mutation of K-RAS, Loss of 18q21 deletion, Loss of TP53, Microsatellite instability pathway.

However, there are multiple treatment ways there are four types of treatment used to treat cancers. Surgery (removing cancer in operation) is the most common treatment for all stages of colon cancer, Cryosurgery, Radiation therapy, Chemotherapy. As a part of chemotherapy, lots of anticancer drugs are in the market, but the main problem associated with these drugs is their side effects. Because of chemotherapy treatment side effects, the patient needs secondary palliative care treatment. Plant medicines are well known for their non-toxic side effects, so the objective of the study is to develop a drug from medicinal plant against colon cancer with non-toxic side effects. It plays a vital role in the discovery of lead compound for the development of conventional drugs. About 60% of currently used anticancer agents are derived from a natural source (i.e. plants). Phytochemically the plant has been investigated for cardenolides, alkaloids, triterpenes and saponins and it is found to contain a variety of triterpenes and steroidal compounds and also to find out, a newer synthetic drug, for its anti-colon cancer potential and its toxic profile. In the present study, Melothoria maderaspatana was used to study the anticancer potential of the extract and to find new anticancer moiety.

The plant is an annual climber with hairy shoots. Leaves simple, alternative, deltoid or sometimes ovate, 3-5 lobed, with scrubby hairs all around. Flowers small yellow, unisexual, fruits globose, greenish with streaks when unripe and bright red when ripe, with small, numerous compressed ovoid seeds. The parts of the plant are used as vermifuge and febrifuge. It is also used as animal fodder. Leafy shoots of the plant are used as laxatives.

MATERIALS AND METHODS

Plant material

Whole plant parts of Melothoria maderaspatana were collected from local region and District of Tirunelveli, Tamilnadu, India in April 2012. The botanical identity was confirmed and authenticated by a Taxonomist Dr. V.Chelladurai (Research Officer, Botany, (C.C.R.A.S) Government of India. The plant was dried under controlled temperature, powdered and passed through a 40-mesh sieve Tables 1, 2, 3 and 4.

150 g of powdered plant material was packed in Soxhlet apparatus and refluxed with Dichloroethane until to get a clear solution. The extract was dried, and weighed amount of the dried DMP was suspended in Distilled water and was used for the present study.

Selection and acclimatization of animals

Male Wistar rats were purchased from the National Institute of Nutrition, Hyderabad. Rats having the age of 5 weeks and 150 gm body weight were used for the study. All the rats were kept at room temperature of 22±2°C under 12 hr dark-light cycles, the humidity was maintained at 60-70% in the animal house. Rats were fed with a modified pellet diet, and water ad libitum freely throughout the study (including 1 week for acclimatization). All animal procedures were performed in accordance with the recommendations for CPCSEA the proper care and use of laboratory animals. The proposal of the present study was approved by IAEC of RVS College of Pharmaceutical Sciences, Coimbatore. (IAEC NO: IAEC/1012/C/06/CPCSEA approved on 24.12.2010)

In-vivo anticancer activity

Preparation of DMH solution

DMH (1,2-Dimethylhydrazine) was purchased from Sigma Chemicals, Mumbai, India. After receiving, DMH was stored in a cool and dry place to prevent decomposition and contamination. DMH was dis-
solved in 1mM EDTA just prior to use, and the pH is adjusted to 6.5 with 1mM sodium bicarbonate to ensure the stability of the chemical. Animals were given a weekly once subcutaneous (S.C.) injection of DMH in the groin region at a dose of 20 mg/kg body weight for 15 weeks (Nalini et al., 2004).

Clean up following injection

After DMH induction the excess amount of DMH and the prepared area was cleaned and chemically inactivated by using a dilute solution of Sodium carbonate was used in general & other materials used during carcinogen administration can be disposed by incineration in compliance with institutions bio safety guidelines.

Preparation of drug samples

The two plant extracts were weighed at a dose of 200mg/kg and 400mg/kg and dissolved in distilled water to provide a clear solution, which was administered to the animals through oral route.

Treatment schedule

After the administration of DMH, the animals were grouped into three groups of six animals in each. One group of animals were treated as control received normal saline only, out of three, two groups received 200 & 400mg/kg dose of DMP for 30 weeks. During the course of the study, individual animal body weight was recorded, weekly. And % difference in the weights between the groups was calculated.

| Group | Normal saline |
|-------|---------------|
| 1     | DMH only (weekly once) |
| 2     | DMH (weekly once) + DMP extract (200mg/kg dose), PO daily |
| 3     | DMH (weekly once) + DMP extract (400mg/kg dose). PO daily |

Table 1: Animal Experimental Design

Blood collection

After the end of the treatment period, the animals were anaesthetized with Ketamine 2mg/kg (i.p route), blood was collected by Retro orbital puncture, with EDTA and without EDTA for the enumeration of blood cell (i.e. RBC, WBC,), estimation of Hemoglobin and estimation of various biochemical parameters. The estimation of haemoglobin (Shiau and Chang, 1983), WBC and RBC (Natt and Herrick, 1952) were carried out using standard procedures.

Separation of serum

For estimating the biochemical parameters such as SGOT, SGPT using Optimized UV- test according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine), Total Cholesterol by CHOD-PAP: enzymatic photometric test, Triglycerides by Colorimetric enzymatic test using glycerol-3-phosphate-oxidase (GPO) and Total Bilirubin. Serum was separated from blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum was collected and used for the parameter estimation.

Separation of plasma

For the estimation of tumour markers such as Alpha-feto-protein (AFP), Carcinoembroyonic antigen (CEA), the blood was collected with EDTA, and centrifuged at 10,000 rpm for 5 min. The separated plasma was used for the parameter estimation.

Estimation of tumor markers

Secured the desired number of coated wells in the holder. Dispensed 50 µl of standard, specimens, and controls into appropriate wells. (Symeonidis et al., 2004) Dispensed 100 µl of Enzyme Conjugate Reagent to each well. Thoroughly mixed for 30 seconds. It is very important to have a complete mixing in this setup. Incubated at room temperature (18-25ºC) for 60 minutes. Removed the incubation mixture by emptying plate content into a waste container. Rinsed and emptied the microtiter wells 5 times with distilled or deionized water. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets. Dispensed 100 µl of TMB Reagent into each well. Gently mix for 10 seconds. Incubated at room temperature for 20 minutes. Stopped the reaction by adding 100 µl of Stop Solution to each well. Gently mix for 30 seconds. It is important to have that all the blue colour changes to yellow colour completely. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

Alpha-fetoprotein (AFP)

(Symeonidis et al., 2004) All reagents were brought to room temperature (18-25 ºC) before use. Reconstituted each lyophilized standard with 1.0 ml distilled water. Allow the reconstituted material to stand for at least 20 minutes and mix gently. Reconstituted standards will be stable for up to 30 days when stored sealed at 2-8ºC. Secured the desired number of coated wells in the holder. Dispensed 20 µl of standard, specimens, and controls into appropriate wells. 5 Dispensed 100 µl of Zero Buffer into each well. Gently mix for 30 seconds. It is very important to have a complete mixing in this setup. Incubated at room temperature (18-25 ºC).
Table 2: Estimation of Hematological Parameters for DMP

| Groups (n=6) | Control Group-I | Only DMH Group-II | DMH+DMP (200 mg/kg) Group-III | DMH+DMP (400 mg/kg) Group-IV |
|-------------|----------------|------------------|-------------------------------|------------------------------|
| RBC (1*10¹²/L) | 6.633 ± 0.1978 | 4.500 ± 0.5003 | 7.6417 ± 0.0065** | 7.9433 ± 0.0065** |
| WBC (1*10¹⁹/L) | 8147 ± 134.8 | 10810 ± 325.1 | 8.441 ± 0.4279*** | 8.2167 ± 0.259.32** |
| Hb (g/dL) | 13.83 ± 0.3383 | 10.13 ± 0.5925 | 12.10 ± 0.2503** | 12.503 ± 0.1125** |

P<0.001,**P<0.01,*P<0.05,#-Non Significant
Data is expressed as Mean±SEM. (n=6, animals in each group).
Statistical comparison: One way ANOVA, followed by Dunnet’s comparison, was performed.

Table 3: Estimation of Alpha-Feto-Protein(AFP) for DMP

| Groups (n=6) | Control Group-I | Only DMH Group-II | DMH+DMP (200 mg/kg) | DMH+DMP (400 mg/kg) |
|-------------|----------------|------------------|---------------------|---------------------|
| AFP (ng/dL) | 0.4800 ± 0.01528 | 0.6780 ± 0.007234 | 0.4234 ± 0.00324*** | 0.3842 ± 0.01246*** |
| Carcinoembryogenic Antigen (ng/dL) | 0.1953 | 0.4733 | 0.148 | 0.18721 |
| ACF & Polyps | 0.0 ± 0.0 | 13.33 | 5.542 | 5.042 |

P<0.001,**P<0.01,*P<0.05,#-Non Significant
Data is expressed as Mean±SEM. (n=6, animals in each group).
Statistical comparison: One way ANOVA, followed by Dunnet’s comparison, was performed.

Collection of tissues

After the blood collection, the animals were sacrificed, and the body was cut open, and gross pathological changes were observed, and the organs like liver, kidney, and colon were excised immediately and washed with normal saline, and wet organ weight was determined.

Evaluation of colon cancer by aberrant crypt foci

After the completion of 30 weeks treatment, all the animals were sacrificed and collected the colons of all the rats. Cut the Colons longitudinally, to expose the luminal surface. Flushed with potassium phosphate buffer. The opened colons were placed between the filter papers and placed in 10% formalin fixative overnight, and then placed the 2cm long segments in a petri dish and stained with 0.2% methylene blue solution. And the total number of aberrant crypt per focus was counted (Figueiredo et al., 2009).

Histopathology

The collected organ was washed with normal saline to remove the cell debris and preserved in 10% buffered neutral formalin solution, the tissues are trimmed to 2-3 mm thickness & subjected to preparation of paraffin blocks and cut into 5μ thickness & followed H&E staining, the alterations in the tissue was read and reported.
In Vitro Cytotoxicity Studies

HT-29 (Colon Carcinoma) cell culture was used to study the in-vitro cytotoxicity studies. Cell culture was procured from National Centre for Cell Sciences (NCCS), Pune. Cells were grown in Minimal essential medium supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum, Penicillin (100 μg/ml), Streptomycin (100 μg/ml) and Amphoteracin B (5 μg/ml) and The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and subculture twice a week.

Determination of Mitochondrial Synthesis by Microculture Tetrazolium (MTT) Assay

The monolayer cell culture was trypsinized using TPVG, (Nalini et al., 2004) and the cell count was adjusted to 1.0x10⁵ cells/ml using a medium containing 10% newborn calf serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once, and 100 μl of (1000 to 15.6 μg/ml) two plant extracts were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded, and 50μl of MTT (MTT: prepared in Hank’s Balanced Salt Solution without phenol red [(HBSS-PR), 2 mg/ml, Sigma Chemicals]) was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed, and 50 μl of propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a Microplate reader (ELISA Reader, Bio-rad) at a wavelength of 540nm. The percentage growth inhibition was calculated using the formula below,

\[
\% \text{ Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \right) \times 100
\]

CTC₅₀ was determined by plotting the conc Vs % growth inhibition.

RESULTS AND DISCUSSION

In vivo anticancer activity

Anticancer effect of DMP was assessed in 1, 2-dimethyl hydrazine induced colon cancer model, by administering the DMH in a dose of 20mg/kg for 15 weeks. And the efficacy of the extract was evaluated by treating the animals with two dose levels one week prior to DMH treatment and simultaneous treatment with DMH for 30 weeks by daily dosing. At the end of 31 weeks, treatment, hematological, biochemical parameters and plasma tumor markers were estimated.

In the colon cancer condition, there was alteration in the normal blood cell counts. A significant increase in the level of WBC, and a significant decrease in the level of RBC and haemoglobin when compared to control animals were observed. The extract reversed these changes towards normal values in a dose-dependent and significant manner.

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Figure 1: Estimation of biochemical parameters with DMP extract

Figure 2: In vitro cytotoxicity study using HT-29 Cell line

In the cancer condition, there will be a significant change in serum biochemical parameters. There will be a significant decrease in triglycerides and total cholesterol and a significant increase in SGOT,
Table 4: Estimation of Serum Biochemical Parameters for DMP

| Groups (n=6) | Control Group | Only DMH Group | DMH+DMP (200 mg/kg) Group | DMH+DMP (400 mg/kg) Group |
|-------------|---------------|----------------|--------------------------|--------------------------|
| SGOT(U/L)   | 125.0         | 181.7          | 142.0                    | 142.85                   |
|             | ±4.539        | ±1.453         | ±8.500***                | ±1.3165***               |
| SGPT(U/L)   | 66.33         | 86.67          | 43.4367                  | 46.0550                  |
|             | ±2.404        | ±2.333         | ±3.176**                 | ±1.744**                 |
| Total Cholesterol (mg/dl) | 64.00         | 115.0          | 108.47                   | 101.04                   |
|             | ±4.041        | ±3.215         | ±0.53**                  | ±0.61***                 |
| Triglycerides (mg/dl) | 94.17         | 56.13          | 24.52                    | 28.23                    |
|             | ±3.491        | ±8.781         | ±1.11*                   | ±0.42***                 |
| Total bilirubin (mg/dl) | 0.6667        | 0.6667         | 0.6448                   | 0.6938                   |
|             | ±0.03333      | ±0.1202        | ±0.001**                 | ±0.011**                 |

P<0.001, **P<0.01, *P<0.05, #-NonSignificant
Data is expressed as Mean±SEM. (n=6, animals in each group).
Statistical comparison: One way ANOVA, followed by Dunnet’s comparison, was performed.

SGPT, bilirubin levels.

Epidemiologic data related serum cholesterol levels and cancer. In a recent study, a positive association was noted between serum cholesterol levels and the risk for rectal cancer in men. A number of epidemiological studies have been published in recent years, showing an increased risk of death from cancer subjects with low plasma cholesterol levels. Although several authors proposed that hypo cholesterolemia is a predisposing factor for cancer development, no causative relation has been established so far. Current theories regarding cancer causation have generated interest in variables such as levels of serum cholesterol and triglycerides as potential associations with cancer relating to dietary factors or basic constitutional factors. Curiously enough very few studies exist concerning serum lipid profile in patients with cancer. The present study examined the lipid profile of animals with colon cancer in comparison with DMP treated cancer groups there was a significant increase in the total cholesterol level due to modification in the diet. The effects of DMP on different serum biological parameters are showing a significant reverse in altered serum biological parameters.

The carcinoembryonic antigen (CEA) test measures the amount of this protein that may appear in the blood of some people who have certain kinds of cancers, especially large intestine (colon and rectal) cancer. It may also be present in people with cancer of the pancreas, breast, ovary, or lung. CEA is normally produced during the development of a fetus. The production of CEA stops before birth, and it usually is not present in the blood of healthy adults. Alpha-feto-protein is a serum protein that is detected in elevated concentration in carcinoma conditions; it is a serum protein similar in size, structure to serum albumin. The levels of AFP will be in minute quantities in adults where there will be an elevated level in cancer condition.

In the present study, a decrease in the level of CEA and AFP was observed followed by DMP and indicates a positive prognosis the decrease levels on DMP treatment prevents the neoplastic growth and reduces the level of carcinoma, which indicates that it possesses anticarcinogenic properties.

There was a significant change in the protein levels due to impairment of glyconeolytic enzymes, groups treated with DMP shows a significant (p<0.001) reverse in the altered protein levels.

The effects of extracts on DMH induced colon cancer was evaluated by the formation of aberrant crypt foci (ACF). After the termination of the study, the no of ACF in the colon was enumerated to determine the effect of extracts on DMH induced colon cancer. From the present study, the extract treated groups shown significantly reduce in the formation of ACF.

AOM-induced ACF are characterized by an increase in the size of the crypts, the epithelial lining, and the pericryptal zone and share many morphologic and biochemical characteristics with tumors, including a comparable increase in cell proliferation. The effects of extracts on DMH induced colon cancer was evaluated by the formation of aberrant crypt foci (ACF). After the termination of the study, the no of ACF in the colon was enumerated to determine the effect of extracts on DMH induced colon cancer. From the present study, the extract treated groups shown significantly reduce in the formation of ACF.
Histopathology reports showed a DMH treated colon tissue shows the presence of tiny pednucleated polyp probably a benign tubular adenoma, and the DMP treated groups does not show any abnormality. And so it suggested that DMP have shown a good response when compared with the first group.

**In vitro cytotoxic activity**

In this phase of the study, the DMP was evaluated for the cytotoxic activity. The cytotoxic test was carried out by using the MTT method, by using different cell lines like HT-29 (colon cancer cell lines). In this study, different concentration of the DMP was treated with known quantity of cells, and the % cytotoxicity in each dose level was measured by using MTT (Micro culture Tetrazolium) method. The extract shown significant % cytotoxicity in cell lines.
CONCLUSIONS

With the above said findings, it can be concluded that the plant Melothoria maderaspatana and possess anti-colorectal cancer activity. Before the clinical usage of extract, thorough toxicological profile has to be determined on the crude extracts as well as on isolated compounds to confirm the safety of the drug.

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