

***In-vivo* anti colorectal cancer activity of *Coldenia procumbens* on DMH induced colon cancer in wistar rats**

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ABSTRACT

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. Polyps which are the small growths in the colon are most often benign, even though some have the potentiality to become cancerous. Up to two-thirds of the colorectal polyps are pre-malignant and they are linked with a risk of colorectal cancer. About 60% of currently used anti-cancer agents are derived from a natural source (i.e. plants). In our study anti-cancer potential of the extract of *Coldenia procumbens* was studied and the synthesis of the anti-cancer moiety has been done. With the findings, it can be concluded that the plant *Coldenia procumbens* Linn and possess anti colorectal cancer activity. Before the clinical usage of extract, through 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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INTRODUCTION

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 [1]. Polyps which are the small growths in the colon are most often benign, even though some have the potential-

ity to become cancerous. Up to two-thirds of the colorectal polyps are pre-malignant and they are linked with a risk of colorectal cancer [2]. As there will be no initial signs and symptoms, by the time he was diagnosed, cancer may spread to other parts of the body [3]. 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. It plays an essential role in the discovery of lead compound for the development of conventional drugs. About 60% of currently used anti-cancer agents are derived from a natural source (i.e.

plants).

In our study anti-cancer potential of the extract of *Coldenia procumbens* was studied and the synthesis of the anti-cancer moiety has been done. It is a herb lying flat on the ground, common on dry rice grounds. The stem reaches to 10-50 cm long and it is shaggy with white hairs. The stem produces numerous branches. It is most commonly seen in South India especially in tropical and subtropical zones of South India. Leaves are alternate, short, sessile, crisped and rounded at the apex. The basal leave is a rosette with veins of 4-6 pairs on each side. The leaves are most often used as poultice to mature abscess and also applied to rheumatoid arthritis patients for reducing swellings. Oil is most commonly used as a liniment to knees and joints which are swollen.

MATERIALS AND METHODS

Plant material

Coldenia procumbens, the whole plant was collected in Tirunelveli district of Tamilnadu in India in April 2012. The plant was confirmed by botanical identity and also authenticated by a Taxonomist Dr V. Cheladurai (Research officer, Botany, C. C. R. A. S) Government of India. The plant was wholly dried under controlled temperature. After that, it was powdered and allowed to pass through sieve 40. 150 grams of plant powder was packed in Soxhlet apparatus and it is refluxed with Dichloroethane until it gets a clear solution. Then the extract was taken and dried and weighed. Amount of the dried DCP was suspended in Distilled water was used for our present study.

Selection and acclimatization of animals

Male Wistar rats were bought from National Institute of Nutrition in Hyderabad. The age group of rats we used was five weeks and the bodyweight of the rats was 150 grams. Room temperature of $22 \pm 2^{\circ}$ C and humidity of 60-70% were maintained in the animal house. Modified pellet diet and water ad libitum freely throughout the study were done, including one week of acclimatization.

The proper care and use of laboratory animals and all other animal procedures have been performed following CPCSEA. The IAEC approved the study proposal of RVS College of Pharmaceutical Sciences, Coimbatore. (IAEC NO: IAEC/ 1012/ C/ 06/ CPCSEA which was approved on 24-12-2018).

In-vivo anti-cancer activity

Preparation of DMH solution

DMH 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 dissolved in 1mM EDTA just before 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 bodyweight for 15 weeks [4].

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 of by incineration in compliance with institutions biosafety guidelines.

Drug samples preparation

200 mg/kg and 400 mg/kg of the two plant extracts were weighed and dissolved in distilled water to get a clear solution. The resulting solution was administered to the animals via the oral route.

Treatment schedule

The animals were grouped into three groups of six animals in each upon the administration of DMH. The control group received normal saline only and the two groups received 200 & 400 mg/kg dose of DCP for 30 weeks. The bodyweight of the individual animal was recorded weekly during the study (Table 1). The percentage difference in the weights has been calculated.

Table 1: Animal Experimental Design

Animal Experimental Design	
Group 1	Normal saline
Group 2	DMH only (weekly once)
Group 3	DMH (weekly once) + DCP extract (200mg/kg dose), PO daily
Group 4	DMH (weekly once) + DCP extract (400mg/kg dose). PO daily

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 haemoglobin and evaluation of various biochemical parameters. The estimation of haemoglobin [5], WBC [6] and RBC [7] was carried out using standard procedures.

Separation of serum

Estimation of Serum Biochemical Parameters

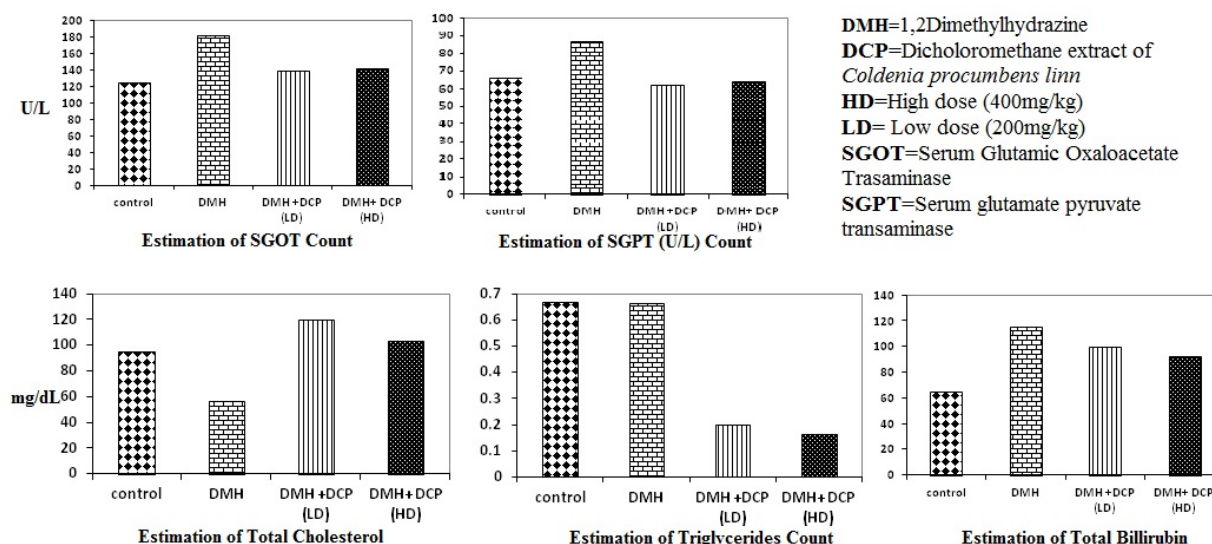


Figure 1: Estimation of serum biochemical parameters

In Vitro Cytotoxicity activity of MCP on HT-29 Cell line

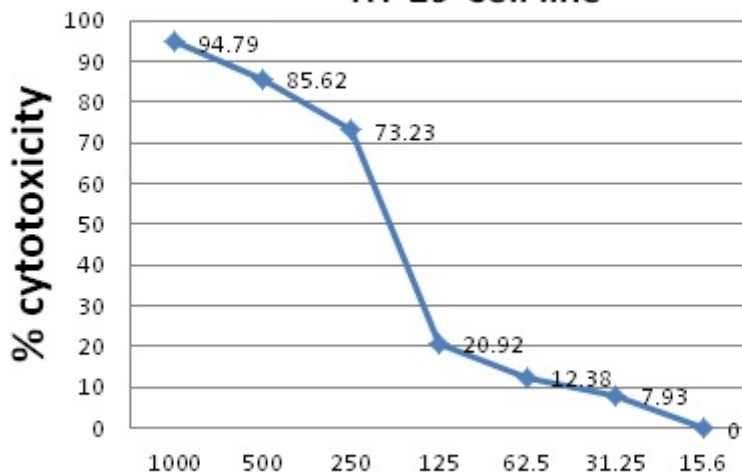


Figure 2: In vitro cytotoxicity study using HT-29 Cell line

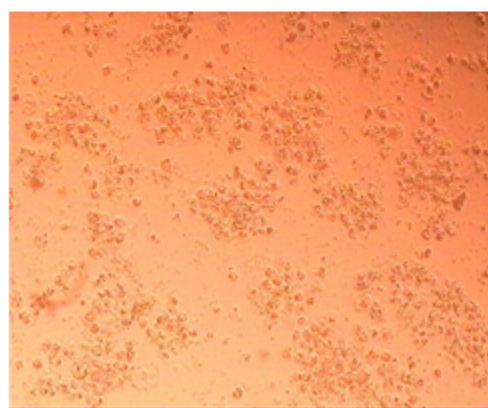
Table 2: Estimation of Hematological Parameters for DCP

GROUPS(n=6)	CON-TROLGroup-I	Only DMHGroup-II	DMH+DCP (200 mg/kg)Group-III	DMH+DCP(400 mg/kg) Group-IV
RBC (1*10 ¹² /L)	6.633±0.1978	4.500±0.5003	6.193±0.1598**	6.360±0.1852**
WBC (1*10 ¹⁹ /L)	8147±134.8	10810±325.1	8430±260.1***	7977±279.5***
Hb (g/dL)	13.83±0.3383	10.13±0.5925	12.40±0.1155**	12.57±0.5364**

Data are expressed as Mean±SEM. (n = 6, animals in each group).



Control –HT-29 cell line



DCP CTC-50 (195 µg/ml)

Figure 3: In vitro cytotoxicity study using HT-29 Cell line

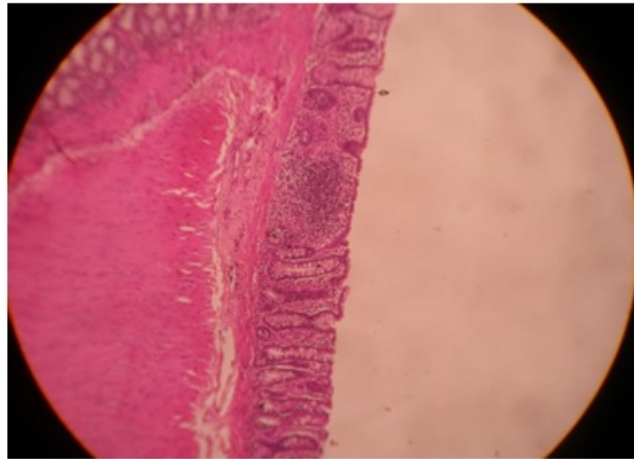
Table 3: Estimation of Serum Biochemical Parameters for DCP

GROUPS(n=6)	CONTORLGroup-I	Only DMHGroup-II	DMH+DCP (200 mg/kg)Group-III	DMH+DCP(400 mg/kg) Group-IV
SGOT(U/L)	125.0 ±4.359	181.7 ±1.453	140.0 ±8.021***	142.0 ±0.5774***
SGPT(U/L)	66.33 ±2.404	86.67 ±2.333	62.67 ±2.963**	64.67 ±4.256**
Total Cholesterol (mg/dl)	64.00 ±4.041	115.0 ±3.215	99.33 ±2.186*	92.67 ±2.333**
Triglycerides (mg/dl)	94.17 ±3.491	56.13 ±8.781	120.1 ±4.477**	103.6 ±14.47*
Total bilirubin (mg/dl)	0.6667 ±0.03333	0.6667 ±0.1202	0.2000 ±0.05774***	0.1667 ±0.03333***

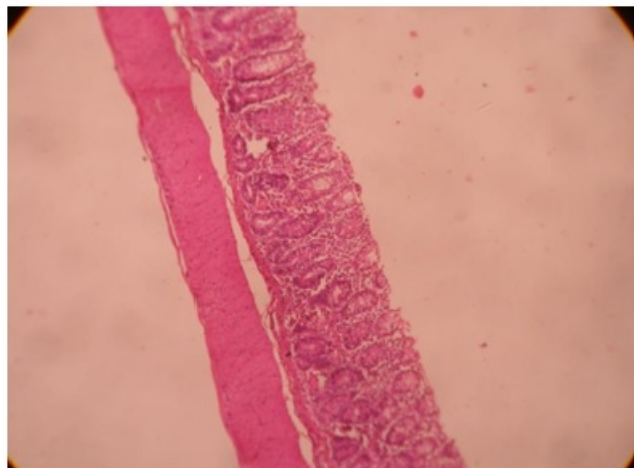
***P<0.001, **P<0.01, *P<0.05, #-Non Significant

Data are expressed as Mean±SEM. (n = 6, animals in each group).

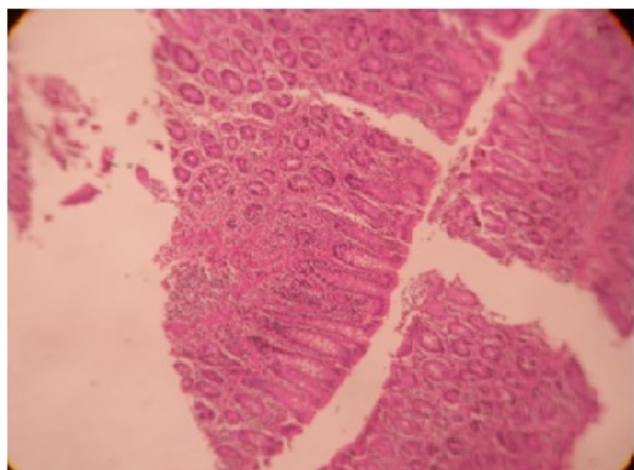
Statistical comparison: One way ANOVA, followed by Dunnet’s comparison, was performed.



Colon show no obvious abnormality in control group



DMH treated colon tissue shows the presence of tiny pedunculated polyp probably a benign tubular adenoma



colon shows no obvious abnormality in DCP (400 mg/kg) treated

Figure 4: Histopathological study of colon tissue treated with DCP

Table 4: Estimation of Alpha-Feto-Protein(AFP) for DCP

GROUPS (n=6)	CONTROL	Only DMH	DMH+DCP (200 mg/kg)	DMH+CP (400 mg/kg)
AFP (ng/dL)	0.4800 ±0.01528	0.6780 ±0.007234	0.5000 ±0.005773***	0.4633 ±0.01856***
Carcinoembryonic Antigen (ng/dL)	0.1953 ±0.002906	0.4733 ±0.007126	0.2660 ±0.006083***	0.2177 ±0.002404***
ACF & Polyps	0.0 ±0.0	13.33 ±1.856***	6.667 ±1.202**	5.000 ±0.5774**

Data is expressed as Mean±SEM. (n = 6, animals in each group).

Statistical comparison: One way ANOVA, followed by Dunnet's comparison, was performed.

For estimating the biochemical parameters such as SGOT, SGPT using Optimized UV- test according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine), [8] Total Cholesterol by CHOD-PAP: enzymatic photometric assay, Triglycerides by Colorimetric enzymatic analysis using glycerol-3-phosphate-oxidase (GPO) and Total Bilirubin [9]. 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-fetoprotein (AFP), Carcinoembryonic 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 tumour markers

Secured the desired number of coated wells in the holder. It has dispensed 50 µl of standard, specimens, and controls into appropriate wells and dispensed 100 µl of Enzyme Conjugate Reagent to each well. Thoroughly mixed for 30 seconds. It is essential to have a complete mixing in this setup. It was incubated at room temperature (18-25°C) for 60 minutes. Removed the incubation mixture by emptying plate content into a waste container. Rinsed and emptied the microtiter wells five times with distilled or deionized water. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets. It dispensed 100 µl of TMB Reagent into each well. Gently mix for 10 seconds. It was 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 essential to make sure 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 [10].

Alpha-fetoprotein (AFP)

All reagents were brought to room temperature (18-

25 °C) before use and reconstituted each lyophilized standard with 1.0 ml distilled water. Allow the reconstituted material to stand for at least 20 minutes and mix gently.[10] Reconstituted standards will be stable for up to 30 days when stored sealed at 2-8°C. Secured the desired number of coated wells in the holder.

20 µl of standard, specimens, and controls were dispensed into appropriate wells. Add 100 µl of Zero Buffer into each well and mix it thoroughly for 30 seconds. Complete mixing is essential. The mixture is incubated at room temperature of 18-25°C for 30 minutes. By flipping plate content into thrash bin, we can remove the incubation mixture. Microtiter wells were flicked and rinsed for five times with distilled or deionized water.

The wells were striped sharply onto the absorbent paper or paper towels to remove excess residual droplets of water. 150 µl of Enzyme Conjugate Reagent was dispensed into each well. Mix it gently for 10 seconds. And it is incubated at room temperature for 30 minutes. By flipping plate content into thrash bin, we can remove the incubation mixture. Microtiter wells were flicked and rinsed for five times with distilled or deionized water. Usage of tap water is strictly prohibited. The wells were striped sharply onto the absorbent paper or paper towels to remove excess residual droplets of water. 100 µl of TMB Reagent was dispensed into each well. Mix it gently for 10 seconds and incubate it at room temperature for 20 minutes. By adding 100 µl of Stop Solution to each well, the reaction will be terminated. Mix it gently for 30 seconds. Make sure that all blue colour turns to yellow completely. Within 15 minutes, optical density was read at 450 nm with a microtiter reader.

Collection of tissues

After the blood collection, the animals were sacrificed and the body was cut open. 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 of treatment, all the animals were sacrificed and collected the colons of all the rats. Cut the Colons longitudinally, to expose the luminal surface. They were flushed with a 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 [11].

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 5 μ 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 two mM L-glutamine, 10% Fetal Bovine Serum, Penicillin (100 μ g/ml), Streptomycin (100 μ g/ml) and Amphotericin B (5 μ g/ml) and The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and subculture twice a week [8].

Determination of Mitochondrial Synthesis by Micro culture Tetrazolium (MTT) assay

The monolayer cell culture was trypsinized using TPVG 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 microtiter 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 μ l of (1000 to 15.6 μ g/ml), the extracts of two plants were added to the cells of microtiter plates. After that, plates were incubated at 37°C for three days in 5% C O₂ atmosphere. Observations were recorded after microscopic evaluation after every 24 hours. After three days, the wells with drug solutions were discarded and 50 μ l of MTT (MTT solution is prepared in Hanks's Balanced salt solution without using phenol red [(HBSS-PR), 2 mg/ml, Sigma was added to each well. The plates were

shaken and it was incubated at 37C for 3 hours in 5% CO₂

Atmosphere. After removing supernatant add 50 μ l of propanol was added and stirred to solubilize the formed solution. The absorbance was measured at a wavelength of 540 nm using a Microplate reader (ELISA Reader, Bio-Rad). The growth inhibition percentage was calculated by using the below formula [4].

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

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

RESULTS

In vivo anti-cancer activity

Anti-cancer effect of DCP 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 before DMH treatment and simultaneous treatment with DMH for 30 weeks by daily dosing. At the end of 31 weeks treatment, haematological, biochemical parameters and plasma tumour markers were estimated.

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

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 considerable increase in SGOT, SGPT, bilirubin levels.

Epidemiologic data related to 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. Several epidemiological studies have been published in past years, showing an increased risk of death from cancer subjects with low plasma cholesterol levels. Although several authors proposed that hypocholesterolemia is a predisposing factor for cancer development, no causative relationship has been established so far. Committee of principal investigators. 1978. Current theories regarding can-

cer causation have generated interest in variables such as levels of serum cholesterol and triglycerides as potential associations with cancer relating to dietary factors or primary constitutional factors [12, 13]. 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 DCP treated cancer groups; there was a significant increase in the total cholesterol level due to modification in the diet. The effects of DCP on different serum biological parameters are showing 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, unusually large intestine (colon and rectal) cancer. It may also be present in people with cancer of the pancreas, breast, ovary, or lung. CEA is usually produced during the development of a fetus. The production of CEA stops before birth and it often is not present in the blood of healthy adults. Alpha-fetoprotein 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 (Figures 1, 2 and 3).

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

There was a significant change in the protein levels due to impairment of glycolytic enzymes, groups treated with DCP shows a significant ($p < 0.001$) reverse in the altered protein levels (Table 2).

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 the formation of ACF (Table 3).

AOM-induced ACF is characterized by an increase in the size of the crypts, the epithelial lining, and the pericryptal zone and shares many morphologic and biochemical characteristics with tumours, 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 the formation of ACF (Table 4).

Histopathology reports showed a DMH treated colon tissue shows the presence of tiny pedunculated polyp probably a benign tubular adenoma, and the DCP treated groups does not show any abnormality And so it suggested that DCP has shown a good response when compared with the first group (Figure 4).

Invitro cytotoxic activity:

In this phase of the study, the DCP 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 DCP was treated with a known quantity of cells and the % cytotoxicity in each dose level was measured by using MTT (Micro culture Tetrazolium) method. The extract has shown significant % cytotoxicity in cell lines.

CONCLUSION

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

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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