

EVALUATION OF ANTICANCER ACTIVITY OF *T. DIVARICATA* EXTRACT FOR SKIN CANCER INDUCED BY DMBA IN EXPERIMENTAL ANIMALS

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ABSTRACT

Through DMBA/croton oil-induced carcinogenesis, the current work sought to ascertain the anticancer potential of *T. divaricata* leaf extracts in Swiss albino mice. The DPPH radical scavenging experiment was used to assess the extracts' antioxidant capacity. The animals were randomly assigned to seven groups: Group I was the Normal Control; Group II was the Disease Control group, which received DMBA and croton oil; Group IV, V, VI, and VII received 200 and 400 mg/kg dosages of methanol and aqueous extract; and Group III received methotrexate in addition to Group II treatment. Tumour morphological and biochemical parameters were established at the conclusion of the investigation. Methanol and *T. divaricata* aqueous extract demonstrated antioxidant activity in the DPPH free radical experiment, with IC₅₀ values of 7.65±0.74 and 13.76±1.13 µg/mL, respectively. In comparison to the disease control group, the results indicated a decrease in the percentage of tumour occurrences, burden, and yield in the treatment groups. When compared to mice in the disease control group, serum biochemical markers such as total protein, LPO, SOD, catalase, GSH, SGOT, and SGPT were reported to improve towards the normal range in the therapy groups. *T. divaricata* extracts show strong antioxidant and chemopreventive properties against DMBA/croton oil-induced carcinogenesis, according to the study's findings. It is still necessary to identify particular phytoconstituents and chemoprevention mechanisms.

INTRODUCTION

Approximately 100 plant-based novel medications, such as reserpine, deserpidine, vinblastine, and vincristine, were launched to the US market between 1950 and 1970. Medicinal plants have made a significant contribution to modern therapeutics. They were all descended from higher plants. New plant-based medications, including ginkgolides, ectoposide, eguggulsterone, and artemisinin, were developed worldwide between 1971 and 1990. Two percent of medications, such as paclitaxel, topotecan, gormetecan, irinotecan, and others, were introduced between 1991 and 1995 (1). However, as researchers began to favour the use of synthetic chemicals for illness treatment in the middle of the 20th century, the usage of medicinal plants was cut in half. The current tendency is about to shift, and people will now prefer medical plants since they include natural ingredients that are less harmful than synthetic chemicals, effective, and chemically balanced (2). Anticancer drugs can be effectively obtained from plants, and more than 60% of anticancer agents come from natural resources such as microbes, marine organisms, and plants (3). There are now some plants that show anticancer promise but have not yet been well studied, even though many anticancer drugs are originated from medicinal plants. Approximately 114,000 extracts have been tested for anticancer activity by the National Cancer Institute (4).

Skin cancer is the most common and deadly public health issue that is steadily increasing globally, with white people being particularly at risk (5). In general, there are two types of skin cancer: melanoma skin cancer (MSC) and nonmelanoma skin cancer (NMSC). Despite low reporting and diagnosis rates, epidemiologic studies have shown an increase in melanoma and NMSC occurrences over the previous few decades. The risk of skin cancer is increased because the skin, being the outermost layer, is constantly exposed to many environmental carcinogens, UV radiation, and microbes (6). The need for new and varied treatment options arose as a result of the rising prevalence of skin cancer and the dearth of effective treatments. (7) Michael Sporn coined the term "chemoprevention" in 1976, and it currently describes the application of synthetic or natural chemicals to stop the development of tumours or to stop their growth. Since then, research on chemoprevention has persisted, particularly in the area of cancer prevention. People have used plants and herbal items for medicinal purposes throughout history. (8, 9) In addition to their nutritional importance, plants also contain a range of phytoconstituents. Because of their biological activity, these phytoconstituents may be able to predict a number of diseases through a variety of processes, including the suppression of angiogenesis and cell proliferation or the modification of cell signalling. Several medications used today are derived from plants, and 80% of the world's population still receives their medical care from herbal remedies or vegetable extracts, according to WHO reports. Ten of the 55 new medications that the U.S. Food and Drug Administration (FDA) approved in 2023 came from natural sources (10).

The attractive, blooming, evergreen shrub *Tabernaemontana divaricata*, which belongs to the genus *Tabernaemontana*, which has 100–110 species of flowering plants, is a member of the Apocynaceae family. It typically reaches a height of 6 feet. (11) Tropical nations including Brazil, Egypt, India, Sri Lanka, Vietnam, Malaysia, and Thailand are home to this common garden plant. (12) The plant's white, sweetly fragrant flowers are one of its medicinally valuable features. Its leaves, flowers, roots, and stem have all been used historically to treat rheumatism and ulcers. Other medicinal qualities of the plant include anxiolytic, antidiabetic, and anticonvulsant effects. Evaluating *T. divaricata* leaves' anticancer potential in experimental mice was the primary goal of this study.

MATERIALS AND METHODS

Collection and Extraction of the plant material: The leaves of the plants were gathered locally after the knowledgeable botanist verified that *T. divaricata* was the species. A mechanical grinder was used to crush the leaves into a coarse powder after they had been dried

and shed. Using solvents such as petroleum ether, methanol, and water in order of increasing polarity, 100 g of the powdered material was subjected to consecutive hot extraction. A rotary vacuum evaporator was used to chill and condense the extracted materials. The extracted material was kept between 2 and 8°C until it was needed. (17)

Preliminary Phytochemical Screening: Using the techniques outlined in Trease and Evans, the extracted *T. divaricata* samples were qualitatively evaluated for the presence of various classes of phytoconstituents. (18)

Antioxidant Activity via DPPH Free Radical Scavenging Assay: The DPPH Free Radical Scavenging Assay was used to assess the antioxidant activity of *T. divaricata* extracts (19, 20). The decolorisation of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution was used to gauge the plant extractives' capacity to donate hydrogen atoms. When antioxidants are present, the violet/purple colour that DPPH imparts to methanol changes to yellow hues. Petroleum ether, methanol, and aqueous extract in methanol at different concentrations (10–50 µg/mL) were mixed with 2.4 mL of the 0.1 mM DPPH solution in methanol. After 30 minutes of incubation, the mixes' absorbance at 517 nm was measured. The reference is butylated hydroxytoluene (BHT). Plotting the concentration against the percentage of inhibition allowed for the analysis of the curve to estimate the IC₅₀. The following formula was used to calculate the percentage of DPPH free radical scavenging activity:

$$\% \text{ inhibition} = \left[\frac{A_{\text{Control}} - A_{\text{sample}}}{A_{\text{Control}}} \right] \times 100$$

Where A_{control} and A_{sample} are the absorbances of the control and extracts or standard.

Pharmacological Activity: For the investigation, 42 female Swiss albino mice weighing 25–35 g and aged 5–7 weeks were employed. The animals were housed in the Animal House with a 12/12 hour light and dark cycle at a temperature of 25±2°C. The animals were kept in clear polypropylene cages of standard size (530 cm²) and randomly assigned to seven groups, each consisting of six mice. During the week-long adaption period, there was unrestricted access to food and water. The Committee for Control and Supervision of Experiments on Animals (CCSEA) and the Institutional Animal Ethical Committee (IAEC) provided the ethical clearance. Each mouse's dorsal hairs, measuring 2 cm by 2 cm, were trimmed with an electric trimmer before to the study's commencement. The study did not include any animals that had cuts on shaved surfaces. (21, 22)

Acute Oral Toxicity Study: In accordance with Organisation for Economic Cooperation and Development (OECD) 425: up- and down-procedure, the acute oral toxicity research was conducted (23). In accordance with the OECD rules for chemical testing, an oral dose of extract at a concentration of 2000 mg/kg b.w. was selected for the limit test. The animals were monitored for any indication of toxicity for the following 14 days after the extract was given just once.

Induction of Skin Cancer in Mice: The process of causing skin tumours through initiation, development, and advancement is known as skin carcinogenesis. After the mice were split up into seven groups, depilatory cream was applied to each mouse's back to eliminate the hairs. Two days prior to the start of the trial, they were housed in typical laboratory settings. The mice's dorsal side was then treated with 100 µL DMBA (100 µg/100 µL acetone). Following two weeks of initiation, 100 µL of 1% v/v croton oil in acetone was applied topically three times a week for 56 days in order to promote the tumour. All of the animals were routinely monitored every day for the duration of the eight-week experiment, and their body weight was measured every week. All were sacrificed at the conclusion of the experiment, and the chosen parameters were assessed. (24, 25)

Experimental Protocol:

- Group-I: This group served as a normal saline control group.
- Group-II: This group served as a negative control. for two months on the dorsal surface of the skin.
- Group-III: This group served as positive control by using standard drug (5-Fluorouracil) for two months.
- Group-IV: This group served test group I by using a test drug i.e METD 200mg/kg.p.o for two months.
- Group-V: This group served test group II. by using a test drug i.e METD 400mg/kg.p.o; for two months.
- Group-VI: This group served test group III by using a test drug, i.e., AETD 200mg/kg.p.o for two months.
- Group-VII: This group served test group IV by using a test drug, i.e., AETD 400mg/kg.p.o for two months.

Determination of Morphological Profile: Every week, from the commencement to the end day of the experiment, the body weight of every mouse was recorded. On the final day of the trial, the tumour incidence was calculated and expressed as a percentage of the total number of mice carrying a single tumour. The average number of tumours per tumor-bearing mouse on the final day of the trial was used to assess the tumour burden. Tumour yield is determined on the final day of the trial and is based on the average number of tumours per mouse. The diameter of the tumours per mouse over the last days of the study was used to calculate the tumour diameter. (26)

Blood biochemical parameters: Using BD Ultra-Fine TM syringes (needle 25 X 5 mm), blood samples were taken from each animal group at the conclusion of the trial and placed in heparinised tubes. Centrifugation was used to separate the serum for five minutes at 2500 rpm. The Erba Chem 5X semi-automated analyser and associated kits were then used to measure the serum sample's levels of total protein, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), in accordance with the manufacturer's instructions. However, the methods outlined by Okhawa et al. (27), McCord and Fridovich (28), Aebi (29), and Moron et al. (30) were used to evaluate lipid peroxidase (LPO), glutathione reductase (GSH), and superoxide dismutase (SOD).

Statistical analysis: The results are shown as the mean $\pm$ standard error of the mean (SEM) and include the number of observations. (31) One-way analysis of variance (ANOVA) was used to examine the data, and Dunnet's t-test was used to determine whether the results were statistically significant. When the p-value was less than 0.05, the results were deemed significant.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening: Both methanol and aqueous extracts of *T. divaricata* showed the presence of carbohydrates tannins, alkaloids, flavonoids, flavanones, steroids, phenols, terpenoids, and isoflavones.

DPPH Free Radical Scavenging Assay: BHT is used as a standard to demonstrate the free radical scavenging activity of leaf extracts, namely methanol extract (METD) and aqueous extract (AETD). METD had the most free radical scavenging activity of the three extracts. The percentage free radical scavenging activity of METD, AETD, and BHT was found to be 94.35 ± 1.54 , 90.11 ± 0.32 , and $97.26 \pm 0.64\%$, respectively, at the maximum concentration, or 50 $\mu\text{g/mL}$. METD, AETD, and BHT were determined to have respective IC₅₀ values of 7.65 ± 0.74 , 13.76 ± 1.13 , and 6.11 ± 0.35 $\mu\text{g/mL}$. Therefore, the order of the free radical scavenging activity was BHT>METD>AETD.

Acute Oral Toxicity Study: A single dose of 2000 mg/kg b.w. was used to test the extract's acute oral toxicity in six mice, and the extract's ant toxicity was monitored for two weeks.

There was no indication of toxicity in the animals. Consequently, two doses—a lower median dose of 200 mg/kg and a higher median dose of 400 mg/kg b.w.—were chosen for additional research.

Morphological Studies:

Body Weight: Every week, the body weight of each mouse was recorded. In the first and last days of the study, the value is displayed. The normal group's body weight was progressively increased. In contrast to the normal group, the control group's body weight decreased over the course of the study. When compared to the control group, the body weight of every treatment group recovered significantly. Table 1 displays all of the findings.

Tumor Incidence: In comparison to group I, the tumour incidence was considerably ($P<0.001$) higher in group II. In comparison to the control group, the tumour incidence was considerably ($P<0.001$) lower in all treated groups. At a dosage of 400 mg/kg, Group III and Group (test groups II & IV) demonstrated a greater decrease in tumour incidence than Group V and Group VII. Table 1 displays all of the findings.

Tumor Burden: Group II had a considerably ($P<0.001$) higher tumour burden than the normal group. The tumour load was considerably reduced in all therapy groups. In comparison to group II, the tumour burden was significantly ($P<0.001$) reduced in groups III and V (test groups II & IV). As seen in Table 1, group V and VII were found to be more successful than groups IV and VI.

Tumor Yield: Group II's tumour yield was substantially ($P<0.001$) higher than group I's. In comparison to group II, the tumour yield was considerably ($P<0.001$) lower in all treatment groups. At 400 mg/kg, the tumour production was significantly reduced in groups III and V (test groups II and IV). Although less successful, the groups V and VII decreased the tumour production. Table 1 displays all of the findings.

Tumor Diameter: Group II's tumour diameter was substantially ($P<0.001$) larger than Group I's. Nonetheless, all therapy groups showed a significant ($P<0.001$) reduction in tumour diameter. Nevertheless, groups III, V, and VII were more effective than groups IV and VI. Table 1 displays all of the findings.

Table 1: Morphological Profile

Groups	Body Weight (g)		Tumor Incidence	Tumor yield	Tumor diameter	Tumor Burden
	Initial	Final				
Group-I	30.12±1.45	35.43±1.54	00	00	00	00
Group-II	33.87±0.74	29.65±1.21	89.75±1.67***	7.57 ±0.86***	1.91±0.065***	7.9 ±0.48**
Group-III	32.65±1.43	35.21 ±2.83	32.65±1.62***	4.11±0.36***	1.11±0.041***	3.78 ±0.62*
Group-IV	29.12±0.35	31.35 ±1.43	57.54±1.21**	5.95 ±0.87***	1.55±0.12***	5.85 ±0.36*
Group-V	32.54±0.36	35.32 ±2.18	35.11±0.36***	4.03±0.36***	1.25±0.47***	4.07 ±0.11**
Group-VI	33.54±1.35	34.65 ±1.65	60.11±2.26**	5.11±0.65***	1.45±0.37***	5.11 ±0.76*
Group-VII	30.65±0.49	34.645±0.86	39.43±1.44***	4.65±0.14***	1.31±1.54***	4.62±1.36**

Determination of Biochemical Profile:

Catalase: In comparison to group I, the serum of DMBA-induced skin tumours in group II had a considerably ($P<0.001$) lower level of the catalase enzyme. Catalase enzyme levels were considerably ($P<0.001$) higher in treatment groups III, V, and VII than in the control group. Table 2 indicates that group VI ($P<0.05$) and group IV ($P<0.01$) were less significant.

Reduced Glutathione (GSH): Group II's GSH level was significantly ($P<0.001$) lower than group I's. According to Table 2, the GSH enzyme levels in groups II, V, and VII showed a considerable ($P<0.001$) recovery when compared to groups IV ($P<0.01$) and VI ($P<0.05$) when compared to group II.

Superoxide Dismutase (SOD): In comparison to group I, the superoxide dismutase level in group II decreased considerably ($P<0.001$). SOD levels in treatment groups III, V, and VII

recovered significantly ($P<0.001$). However, Table 2 indicates that Group IV ($P<0.01$) and Group VI ($P<0.001$) recovered the SOD level less significantly than Group II.

Lipid Peroxidation (LPO): Group II's LPO level was significantly higher than group I's ($P<0.001$). The LPO enzyme level was considerably restored in each therapy group. When compared to the control group, the LPO level in groups III, V, and VII decreased dramatically ($P<0.001$). Table 2 indicates that group IV ($P<0.01$) and group-VI ($P<0.05$) demonstrated less relevance in recovering the LPO level.

Total Protein: Group II's total protein content was considerably ($P<0.001$) lower than group I's. When compared to group I, the amount of total protein content increased significantly in all treatment groups. As indicated in Table 2, the levels of total protein in groups III, V, and VII improved significantly ($P<0.001$), while those in groups IV and VI were less significant ($P<0.01$) ($P<0.05$) than those in group II.

Table 2: Biochemical Profile

Groups	Catalase level ($\mu\text{mol H}_2\text{O}_2$ consumed/min/ mg protein)	Reduced Glutathione ($\mu\text{mol/mg tissue}$)	Superoxide Dismutase (SOD) mol/mg protein	Lipid Peroxidation (nmol/mg tissue)	Total protein (mg/g tissue)
Group-I	49.11 $\pm$ 6.65	8.62 $\pm$ 1.76	64.57 $\pm$ 7.69	5.11 $\pm$ 0.37	102.62 $\pm$ 9.29
Group-II	32.65 $\pm$ 2.32***	2.76 $\pm$ 1.76***	30.65 $\pm$ 4.92***	13.1 $\pm$ 1.62***	57.18 $\pm$ 4.28***
Group-III	48.46 $\pm$ 3.11***	6.63 $\pm$ 0.57***	56.76 $\pm$ 5.11***	7.52 $\pm$ 1.23***	105.11 $\pm$ 9.32***
Group-IV	38.32 $\pm$ 5.27**	4.64 $\pm$ 0.94**	41.43 $\pm$ 5.86**	11.32 $\pm$ 1.38**	76.64 $\pm$ 10.27**
Group-V	40.52 $\pm$ 3.22***	5.75 $\pm$ 0.54***	50.65 $\pm$ 7.82***	8.99 $\pm$ 1.28***	82.76 $\pm$ 8.27***
Group-VI	33.53 $\pm$ 4.21*	3.87 $\pm$ 0.48*	37.43 $\pm$ 3.28*	7.43 $\pm$ 1.44*	70.98 $\pm$ 8.11*
Group-VII	38.11 $\pm$ 6.21***	5.34 $\pm$ 0.54***	44.32 $\pm$ 4.54***	9.65 $\pm$ 1.29***	79.74 $\pm$ 9.76***

Discussion

T. divaricata is a popular plant that has long been used to treat a variety of illnesses. Potential phytoconstituents including alkaloids, flavonoids, tannins, steroids, phenols, terpenoids, flavanones, and isoflavones were found in the first results of the phytochemical screening. According to earlier research, tannins, flavonoids, terpenoids, and polyphenols—all of which are typically derived from common dietary sources—have anticancer effects (32). Phase I or phase II carcinogenesis enzymes are modulated by these plant secondary metabolites to exert their anticancer effects. Because of their anti-inflammatory and antioxidant properties, the majority of these plant secondary metabolites with anticancer potential cause cancer cells to undergo apoptosis. (33) The body's outermost layer, the skin, is constantly exposed to a variety of stressors, such as chemicals, radiation, pollution, and so on, which causes a rise in reactive oxygen species (ROS) and free radical production. Lack of antioxidant defence and excessive ROS generation are associated with the development of cancer. Numerous metabolic pathways and gene expression are impacted by this imbalance, which causes lipid peroxidation and DNA strand breaks (34). With an IC₅₀ value of 7.65 $\pm$ 0.74 $\mu\text{g/mL}$, the results demonstrated the strong antioxidant activity of the methanol extract of *T. divaricata* leaves.

This work used a skin-carcinogenic mice model to show the anticancer potential of *T. divaricata* leaf extracts. In comparison to the carcinogen control group, the results demonstrated that methanol and aqueous extract treatment extended the average latency period while decreasing the tumour burden, tumour yield, cumulative number of papillomas, tumour occurrences, and tumour multiplicity. The most effective dose of *T. divaricata* methanol extract was 400 mg/kg. This impact is probably caused by the methanol extract's strong antioxidant activity, which lowers ROS chemopreventive action. It was discovered that the methanol extract of *T. divaricata* leaves included tannins, flavonoids, and polyphenols—all of which are recognised antioxidants. Alkaloids, flavonoids, steroids, saponins, terpenes, and phenolic substances were detected in TD by phytochemical screening. By either preventing the

development of cancer or preventing its progression, different tannins have in-vivo anti-cancer action against a range of animal malignancies (35, 36).

Reactive oxygen species (ROS) cause an imbalance between cellular proliferation and apoptosis, which is a major contributing factor to a number of chronic disorders, including cancer. Oxidative stress rises when antioxidant systems are overloaded or exhausted, which may harm proteins, lipids, and nucleic acids. The activation of signalling cascades and lipid peroxidation in cancer is greatly aided by high ROS levels, which are caused by enhanced generation of hydrogen peroxide (H₂O₂), hydroxyl radical (OH), and superoxide anion (O₂⁻). Levels of lipid peroxidase (LPO) signal oxidative stress in carcinogenesis and promote the development of cancer. Compared to normal mice, mice with DMBA-induced skin cancer displayed higher levels of LPO and lower levels of enzymatic antioxidants like SOD and CAT and non-enzymatic antioxidants like GSH. By consuming plant extracts, biochemical parameters were improved and returned to levels that were almost normal. (37) Free radicals produced by carcinogens damage proteins and membranes, however administering plant extract restored the concentration of total proteins, which had dropped in the carcinogenic control group. The groups treated with 400 mg/kg of methanol extract had considerably higher levels of SOD and GSH than the carcinogenic control group. Superoxide radicals are transformed into hydrogen peroxide by SOD, a metalloprotein and chain-breaking antioxidant, which stops the production of reactive oxygen species. (38)

Scavenging free radicals and serving as a vital cofactor for GSH S-transferases and GSH peroxidases in the detoxification of nonradical oxidants, reduced GSH is the most abundant cytosolic thiol and an important antioxidant. By strengthening the detoxification system, the elevated SOD and GSH levels demonstrated the substantial antioxidant and anti-cancer potential of *T. divaricata* leaves methanol extract. The activities of our body depend on the liver and kidneys. Their impairment increases the toxicity of chemotherapy medicines by disrupting their metabolism. Chemical agents have the ability to harm the liver, which metabolises xenobiotic substances. Liver and kidney damage is brought on by carcinogenic metabolites and ROS from DMBA metabolism (39). Higher SGOT, SGPT, and bilirubin levels in the DMBA-treated groups of this study indicated liver injury; these levels were lowered following treatment with extracts from *T. divaricata* leaves, demonstrating hepatoprotective properties. DMBA also has an effect on the kidneys, which are in charge of eliminating harmful metabolic waste. Increased urea and creatinine levels in DMBA-treated groups suggested kidney injury. These levels were similarly markedly lowered by *T. divaricata* extracts, indicating kidney protective properties.

CONCLUSION

To sum up, many *T. species* components have antinociceptive, cytotoxic, diuretic, wound-healing, hepatoprotective, antirheumatic, antihypertensive, anti-fisher, and skin disease properties. According to the current study, *T. divaricata* leaf extracts exhibit strong chemopreventive efficacy because they have anti-lipid peroxidative, antioxidant, phase I and II detoxification enzyme-modulating, and anti-proliferative properties in mice given DMBA/croton oil. This work urges more research to determine the phytoconstituent in charge of the activity, the exact mode of action, and the possible therapeutic application of *T. divaricata* leaf parts as a chemopreventive agent.

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