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Chemopreventive Effect of *Gliricidia sepium* Plant Extracts in MNU Induced Mammary Tumor Model

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To investigate various facets of breast cancer, a number of animal models have been developed. Chemically induced rodent models, like the MNU model, are suitable for studying malignant progression because rat mammary tumors induced by MNU are predominantly ductal, similar to human breast cancer is strong proof that N-methylnitrosourea (MNU) animal models closely resemble human breast cancer. As a result, it has been extensively utilized to assess his therapeutic and preventative medications for human breast cancer. The progression of malignant tumors can also be studied using chemically produced animal models. Chemopreventive activity of Gliricidia sepium plant extract was studied by MNU induced mammary tumors and animals were evaluated by various parameters like tumor parameter, hematological parameter and histopathological parameters. In current investigation along with chemopreventive effect Serum biochemical parameters, histological results of breast tissue, tumor volume, tumor incidence, tumor burden, and weakly changing body weight have all been measured and it was found that in all parameters Gliricidia sepium plant extract treated animal shows significant effect. Gliricidia sepium could be one of the effective naturally obtained anticancer remedy for treatment of mammary cancer. The objective for future research is to isolate and characterize each of Gliricidia sepium bioactive components.

Keywords: Antioxidant; Breast Tumor; Ductal Carcinoma; Gliricidia sepium; MNU.

Breast cancer is the result of uncontrolled breast cell proliferation that develops into tumors. These tumors are typically palpable as lumps. The types of breast cancer and how they develop affect how they are treated. For the diagnosis, assessment, and treatment of breast cancer, a variety of innovative methods and surgical procedures are now accessible, which has improved the disease's prognosis.[1,] In certain situations, a single breast tumor may combine elements of both invasive and in situ cancer types. Human breast cancer risk factors can be divided into two categories: risk factors that can be modified (things that can be changed, such as alcohol consumption) and fixed risk factors (things that have never shown symptoms of change, such as age and sex). Ionizing radiation, hormone replacement therapy for menopause, early age from the first month to the next, older age range, and hereditary factor.[2] Due to the mammary tissues of rodents and humans having a significant level of similarity, it is typically able to predict the course of mammary carcinogenesis in humans as well in experimental studies of breast cancer.[3] N-methyl N-nitrosourea (MNU) is a typical carcinogen that is utilized in rodents to cause breast cancer. Cribriform and papillary

carcinomas were the most significantly caused lesions.[4] finding new sources of substances with therapeutic value depends on screening medicinal plants for phytochemicals. In addition to these expensive medicines, phytotherapy is currently one of accessible option to treat cancer. Major goal of our current research is to ascertain the extracts of *Gliricidia sepium* (GS) anticancer potential in order to develop a possible chemopreventive drug for human cancers, more research is required in order to identify and characterize its active principle and mechanism of action.[5, 6]

MATERIALS AND METHODS

Collection of plant and authentication

Gliricidia sepium plant specimens were obtained from the districts of Sangli and Kolhapur, and they were further verified by the Botanical Survey of India (BSI) in Pune, with certificate reference number NO. BSI/WRC/100-1/ Tech./2020/128 dated 28/01/2021.

Preparation of plant extract

The collected aerial parts were washed and then dried under shade. The plant material was subjected to crushing, resulting in the production of coarsely powdered particles. The coarse particles were subsequently subjected to sequential extraction using chloroform, and ethanol solvents by using Soxhlet apparatus. [7, 8]

Animal Experimentation

Female Wistar rats were used for all the experimental procedure and were obtained from animal house of Appasaheb Birnale college of pharmacy, sangli (Approved from CCSEA of Appasaheb Birnale college of pharmacy, sangli IAEC no: IAEC/ABCP/18/2019-20) at 45th days of age weighing 150 – 200 gm. The animals were kept in a 12-hour light/dark cycle with typical laboratory settings. They were given food and water ad libitum. 42 animals were divided randomly into seven groups (N=6) N-methyl-N- nitrosourea (50 mg/kg) dissolved in isotonic saline solution. The first dose of NMU injected by IP route on 45th day postnatal day, second one on the 55th day and 70th day postnatal day drug while standard drug tamoxifen, chloroform extract of *Gliricidia sepium* at dose of 200mg/kg (CHGS 200) and ethanol extract of *Gliricidia sepium* at dose of 200mg/kg (ETGS 200) were used as test compound and is administered from 1st day of model after end of study model animal will be sacrificed for biochemical and Histopathological studies [9,10,11]

Histopathology

Thin tissue slices (3–5 mm) were extracted from both normal and pathologically altered tissues. The samples were fixed in 10% formalin at room temperature for 24 to 48 hours. After fixation, the tissues were deparaffinized using xylene for 5 to 10 minutes, followed by dehydration with 100% alcohol. The sections were then stained with hematoxylin for 3 to 4 minutes and counterstained with 0.5% eosin for 15 to 30 seconds to achieve a pale pink appearance. After blotting, the slides were cleaned with xylene for 15 to 30 seconds, mounted with DPX, and dried to remove any air bubbles.[12]

Determination of Antioxidant parameters

Antioxidants play a crucial role in cancer research and can be assessed through various parameters to understand their impact on cancer development, progression, and treatment. Antioxidants are compounds that protect cells from oxidative stress by neutralizing harmful free radicals, which can damage DNA and lead to cancer.[13] Here are some key antioxidant parameters commonly studied in the context of cancer:

Superoxide Dismutase (SOD)

One important enzyme in the body's defense against oxidative stress is superoxide dismutase (SOD). It assists in transforming superoxide radicals into less dangerous compounds. With a final concentration of 0.1 M phosphate buffer (pH 6.5), 1 nM CDNB (1-chloro-2,4-dinitrobenzene) in 95% ethanol, and 1 mM GSH, the reaction volume (3 ml) was incubated for 5 minutes at 37 °C. The reaction was initiated by the addition of the enzyme sample, and for five minutes, the enzyme activity was monitored at 340 nm. [14]

Catalase (CAT)

This antioxidant enzyme aids in the reduction of oxidative damage by dissolving hydrogen peroxide into oxygen and water. To start the reaction, 0.5 mL of hydrogen peroxide (H, O,) solution was added to 200 ìL of diluted homogenate, 1.0 mL of phosphate buffer, and 0.4 mL of distilled water. In control test tubes, the hydrogen peroxide (H, O,) solution was not present. To halt the process, 2 mL of potassium

dichromate acetic acid was added after it had been incubated for 1 minute at 37°C. After 15 minutes in a boiling water bath, the samples were cooled, and the absorbance at 570 nm was measured in comparison to the control.[15]

Glutathione (GSH)

Glutathione is an essential intracellular antioxidant that participates in detoxification processes and helps protect cells from oxidative damage.

Analytical statistics

All of the data were compared using a one-way ANOVA and Dunnett's posttest. Each outcome was reported as a rise or fall relative to the values of the control results. For all statistical studies, Graph Pad Prism version 8 was utilized. A variance was deemed statistically significant if $p < 0.05$.

RESULTS

Effect of plant extract on body weight

As shown in table no 1 N-methyl-N-nitrosourea (NMU) was administered by intraperitoneal route, the body weight of the animals in the NMU control group significantly decreased (91.51±0.45) from their starting weights. Simultaneous treatment of Tamoxifen (3.3 mg/kg) and plant extracts of *Gliricidia sepium* orally resulted in a considerable rise Standard group (122.3±0.66), TEST 1 CHGS 200 mg/kg (116.3±1.64) and TEST 2 ETGS 200mg/kg (119.5±1.37) in body weight when compared to the initial weight of the animals.

Effect of plant extract on Hematological parameter

It was observed that NMU induced control group and Tamoxifen treated standard group shows significantly decrease in haematological parameters. Simultaneous treatment of and plant extracts of *Gliricidia sepium* orally resulted in restoration and maintain normal Haematological Parameter as shown in table no 2

Tumourological Parameters

The effect of *Gliricidia sepium* plant extract on tumor parameters observation in figure no.: 5 and result obtained. It was seen that tumor parameters such as tumor incidence, tumor volume, tumor weight, and tumor burden in the MNU induced control group are increased as compared to normal animals. The tumor indicators listed above significantly decreased.

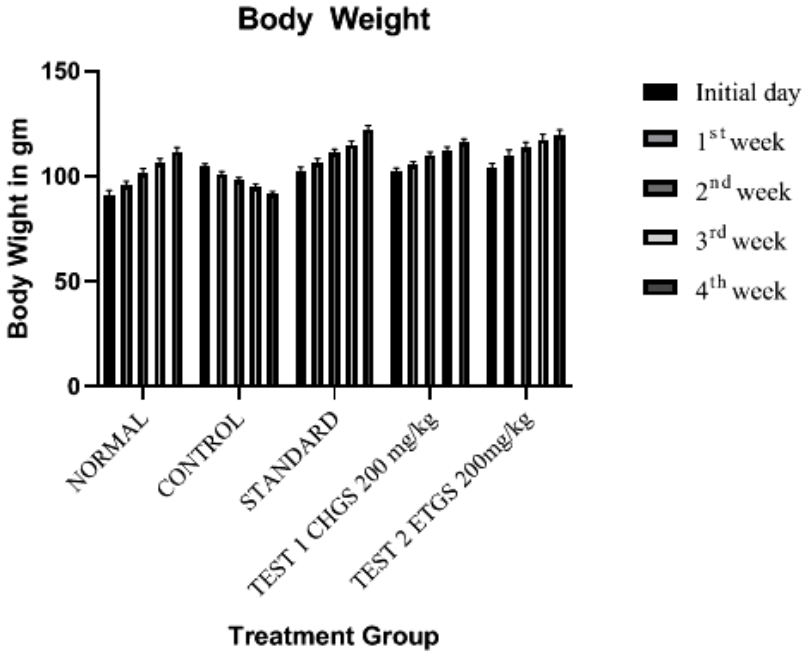


Fig. 1. Graph of animal body weight

Among treatment groups of the tamoxifen and the *Gliricidia sepium* extract treated group. In test groups, chloroform and ethanol extracts were used, but among them it was found that Ethanol extract showed more significant results in comparison with chloroform extract treated group.

Histopathology

As shown in figure no.: 4 a breast section from normal rats (N1) showed no evidence of malignancy (D-Duct). MNU induced rats (C1) showed hyperplasia of the acinar epithelium and atypical ductal hyperplasia. The MNU-induced tamoxifen-treated group (S1 and S2) and the plant extract-treated group show reduced proliferative activity in the mammary gland. Specifically, it inhibited the incidence of ductal carcinoma in situ (DCIS) or intraductal papilloma (IDP).

In vivo antioxidant activity

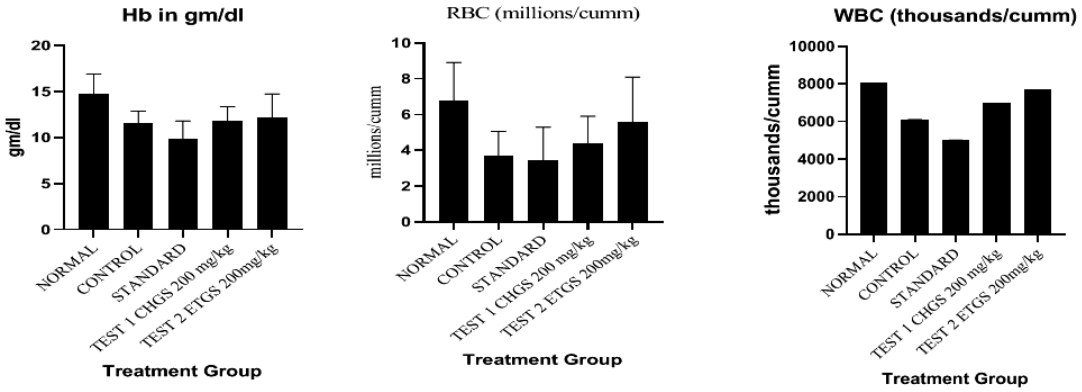
Oxidative stress is associated with cancer, antioxidant parameters were conducted on mammary carcinoma in female rats. The liver tissues of every test rat had the following antioxidant enzymes measured. As results shown in table no. 4.

Effect of Plant extract on superoxide dismutase (SOD)

Animals administered MNU showed a significant reduction in SOD levels (2.82±0.01) in control group and it decreases below to normal level (6.40±0.047). When rats given MNU were given treatment with extracts of *Gliricidia sepium* SOD concentrations increased significantly in ethanol extract (4.96±0.005) as comparison with chloroform (3.9±0.019) SOD level as displayed in Table no 4. According to the findings from the treated ethanolic extract aggregate, a significant increase as comparison with normal SOD was seen in the case of MNU rats administered tamoxifen.

Effect of Plant extract on catalase (CAT)

Compared to normal rats, MNU-induced animals had lower levels of catalase—26.34 ± 0.01 unit/mg. It appears that the reduction is statistically



A B C

Fig. 2. Graph of Hematological Parameter A) Hemoglobin Content B) RBC count C) WBC Count

Table 1. Animal Body Wight among different treatment groups

Treatment Groups	Weekly measurement of body weight (gm)Mean ± SEM					% Change in body weight from initial day of treatment
	Initial day	1st week	2nd week	3rd week	4th week	
Normal	91.17±0.83	95.5±1.6	101.7±1.43	106.3±1.76	111.7±1.1	22.51
Control	104.7± 1.66	101± 1.4	98.27± 1.08	95.04 ±0.51	91.51±0.45	-12.59
Standard	102.5±1.78	106.7±1.28	111±0.8	115±1.50	122.3±0.66	19.31
Test 1 CHGS 200 mg/kg	102.5±2.14	105.5±1.8	110±1.8	112.5±1.7	116.3±1.64	13.46
Test 2 ETGS 200mg/kg	103.8±1.51	110±1.22	113.7±1.53	117.5±1.23	119.5±1.37	15.12

significant. CAT levels increased in MNU-induced rats when G. sepium extract was administered. The CAT level was 17.42 ± 0.01 unit/mg when the chloroform extract was administered, and it changed when the ethanol extract was given, as shown in table no 4. Tamoxifen-treated MNU rats showed a significant increase in CAT, which is consistent with the findings from the group that received ethanol extract treatment.[16]

Effect of Plant extract on Reduced glutathione (GSH) nmol/mg

Compared to normal rats (7.3±0.043) , MNU-induced animals showed a significant drop in GSH levels (1.8 ± 0.13). GSH levels increased in MNU-induced rats when *Gliricidia sepium* extract was administered. The amount of GSH in the tissue was 3.4 ± 0.34 mg/g when chloroform extract was administered, and this changed to 3.9 ± 0.14 mg/g when ethanol extract was given. Data from the ethanol extract-treated group coincides with the considerable increase in GSH shown in the case of MNU rats treated with tamoxifen.

DISCUSSION

Carcinogenesis is an intricate and unpredictable process, often involving unexpected molecular and cellular pathways. Despite the extensive body of knowledge regarding its causes, mechanisms, and progression, cancer remains a formidable and vulnerable disease. Mortality and morbidity rates continue to be alarmingly high, even with advances in diagnostic tools and therapeutic strategies. [17, 18]

In recent years, plant-derived natural compounds have attracted considerable interest in medical research due to their diverse pharmacological properties, particularly their antioxidant and anticancer activities. Numerous studies using various cancer cell lines have demonstrated the therapeutic potential of plant extracts in combating malignancies. Furthermore, there has been a significant increase in the use of complementary therapies such as dietary supplements and phototherapeutic products especially among women with a history of breast cancer. [19,20]

Most in vitro research has centered on mechanisms such as free radical scavenging, apoptosis-induced cytotoxicity, and DNA damage. However, in vivo models are critical, as they provide histopathological evidence to distinguish between benign and malignant tumors. Traditional cancer treatments often fall short of delivering optimal results, prompting the exploration of alternative therapies. [21,22]

Table 2. Hematological parameter

Treatment Groups	Hb	RBC (millions/cumm)	WBC (thousands/cumm)
Normal	14.75±0.36	6.783±0.36	8063±247.2
Control	11.52±0.47	3.733±0.25	6109±206.75
Standard	9.9±0.12	3.417±0.20	5007±210.6
Test 1 CHGS 200 mg/kg	11.83±0.4364	4.383±0.2286	7014±119.5
Test 2 ETGS 200mg/kg	12.18±0.22	5.567±0.28	7739±109.6

Table 3. Tumor Parameter

Treatment Groups	Tumor mass (g)	Tumor size (mm)	Tumor Incidence
Normal	0	0	0
Control	23.35±4.8	30.41±2.9	87.63
Standard	10±2.56	15±6.16	34
Test 1 CHGS 200 mg/kg	13±3.78	18±2.35	46
Test 2 ETGS 200mg/kg	11±4.34	16.34±3.12	41

One such alternative is *Gliricidia sepium*, whose antitumor effects have been evaluated in rodent models bearing induced tumors. In the present study, body weight was monitored throughout the experimental period. The weight loss observed in the MNU-treated (control) group was likely due to increased metabolic demand and systemic cancer-related effects. In contrast, the treatment group exhibited a comparatively higher percentage gain in body weight, indicating a potential protective role of the plant extract in countering cancer-induced weight loss. [23,24]

Histological analysis provided further evidence of tumor regression in the treated group, revealing a significant reduction in proliferative activity within the mammary gland tissue. Notably, the treatment inhibited the development of ductal carcinoma in situ (DCIS) and intraductal papilloma (IDP), both of which are precancerous lesions.[25, 26]

Reduced glutathione (GSH) is vital for maintaining cellular viability, acting as a major intracellular antioxidant that protects cells against oxidative damage by neutralizing reactive oxygen species (ROS). Previous studies have shown that GSH depletion in mammary cancer models is associated with increased lipid peroxidation, a process that damages cellular membranes and contributes to carcinogenesis. Moreover, tumor cells often exhibit heightened GSH consumption due to their rapid proliferation and increased metabolic demand, indicating its complex role in tumor biology. In this study, treatment with Gliricidia sepium led to significantly lower malondialdehyde (MDA) levelsa biomarker of lipid peroxidationand elevated serum GSH levels, suggesting a reduction in oxidative stress and an enhancement of the endogenous antioxidant defense system. This biochemical shift indicates that G. sepium may mitigate the oxidative environment typically favorable for cancer progression. The role of antioxidants in cancer therapy is multifaceted. On one hand, they can scavenge harmful free radicals and inhibit oxidative DNA damage, thus preventing the initiation and promotion stages of carcinogenesis. On the other hand, enhancing antioxidant capacity in normal tissues may help reduce the side effects of conventional therapies such as chemotherapy and radiotherapy, which often generate excessive ROS. In the context of G. sepium treatment, the observed increase in GSH

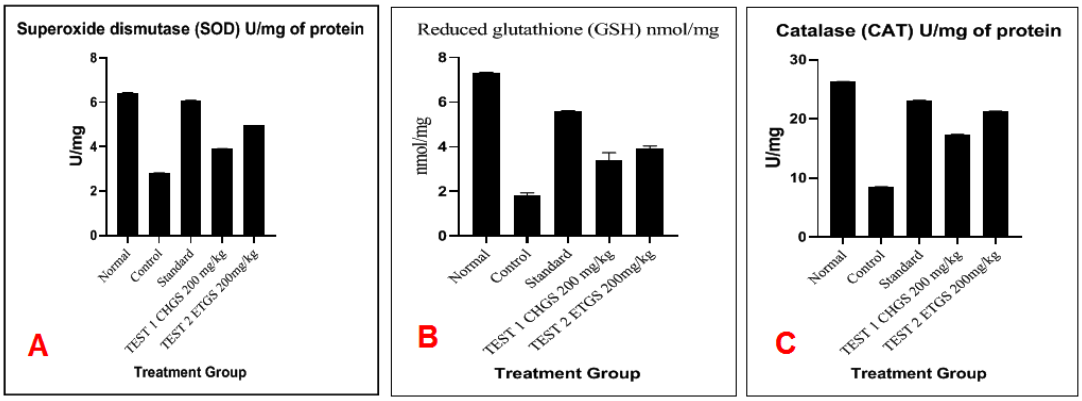


Fig. 3. Graph of Anti-oxidant parameter A: SOD, B: GSH, C: CAT.

Table 4. Antioxidant Parameter

Treatment Groups	Superoxide dismutase (SOD) U/mg of protein	Reduced glutathione (GSH) nmol/mg	Catalase (CAT) U/mg of protein
Normal	6.40±0.047	7.3±0.043	26.34±0.012
Control	2.82±0.01	1.8±0.134	8.52±0.04
Standard	6.068±0.028	5.6±0.030	23.14±0.045
Test 1 CHGS 200 mg/kg	3.9±0.019	3.4±0.34	17.42±0.01
Test 2 ETGS 200mg/kg	4.96±0.005	3.9±0.14	21.34±0.023

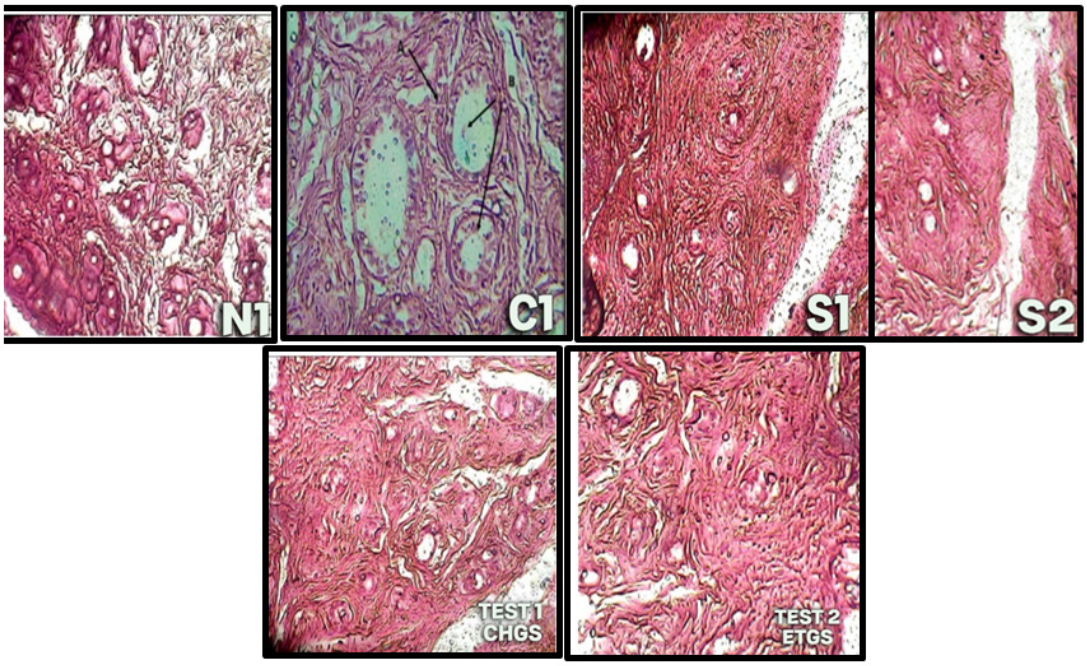


Fig. 4. H&E-stained paraffin sections N1- Normal, C1- Control shows A- Hyperplasia of acinar epithelium and B shows Atypical ductal hyperplasia, S1, S2- Standard, Test 1 CHGS- Chloroform extract of *Gliricidia sepium* and Test 2 ETGS Ethanol extract of *Gliricidia sepium*

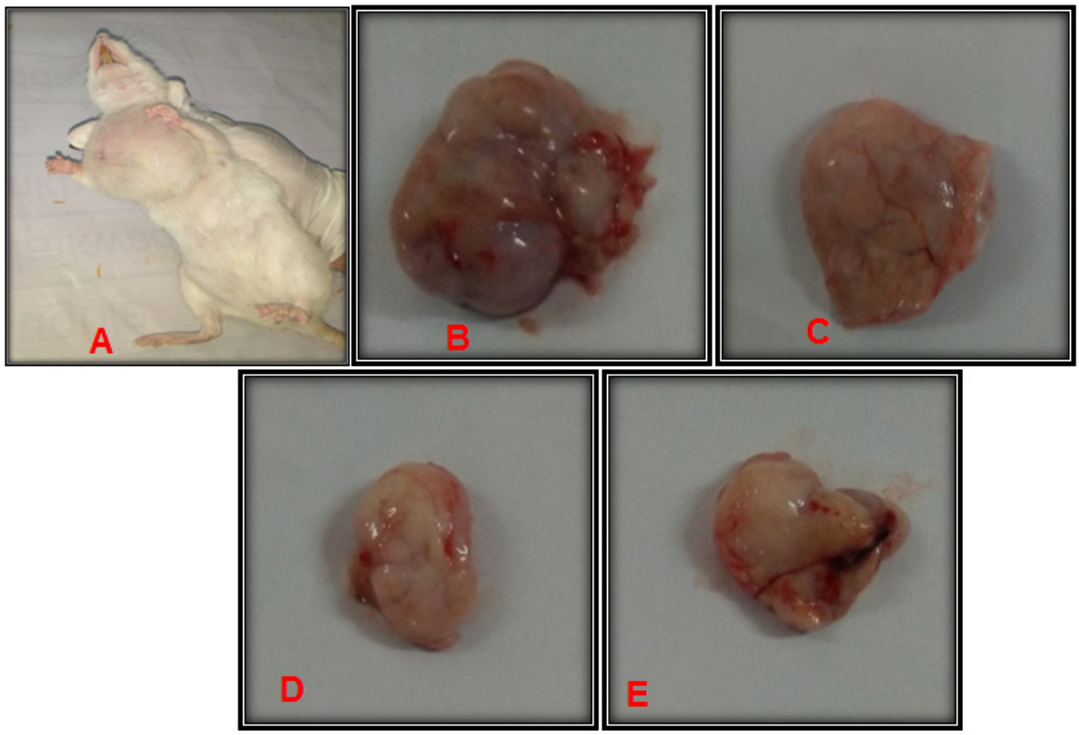


Fig. 5. Mammary tumors isolated from various treatment groups A- Control Group animal with. Mammary tumor B- Control Group C- Standard D- TEST 1 CHGS 200 mg/kg E- TEST 2 ETGS 200mg/kg

and decrease in MDA suggest a protective effect on cellular components and a potential inhibitory effect on tumor growth and proliferation.

By restoring redox balance, antioxidants may help induce apoptosis in malignant cells, modulate signaling pathways involved in cell cycle regulation, and suppress angiogenesis and metastasis. Therefore, the antioxidant properties of Gliricidia sepium could play a pivotal role in curbing breast tumor development, supporting its potential as a complementary therapeutic agent in breast cancer management.[27, 28]

CONCLUSION

Conventional cancer therapies often have limitations, leading to growing interest in plant-derived compounds for their anticancer potential. Both plant extracts of *Gliricidia sepium* has demonstrated promising antitumor activity but ethanol extract shows more promising result than chloroform by reducing tumor proliferation and enhancing antioxidant defense mechanisms in mammary cancer models. Its ability to lower lipid peroxidation and increase glutathione levels suggests a protective effect against oxidative stress in breast tissue. The findings indicate that plant-based therapies could serve as complementary treatments for cancer management. However, further clinical studies are necessary to validate these effects and explore their therapeutic applications in humans.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research involves an experiment on animals that requires ethical approval, Ethical approval has been taken from CCSEA of appasaheb birnale college of pharmacy, sangli IAEC no: IAEC/ABCP/18/2019-20

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Permission to reproduce material from other sources

Not Applicable

Authors Contributions

Sudhir Patil: Conceptualization, Methodology, Writing – review and editing, Visualization, Investigation, Formal analysis; Kiran Wadkar: Methodology, Software, Validation, Formal analysis, Resources, Supervision, Project administration

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