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Research Article

Curative Effect of the Butanol Fraction of Root Bark of *Gardenia gummifera* L.f against N-nitrosodiethylamine-induced Hepatocellular Carcinoma in Experimental Rats

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ABSTRACT

Gardenia gummifera Linn. f. belongs to the family *Rubiaceae*, which is found in rocky hilltops, widely used in traditional medicine. As the plant is rich in active phytoconstituents, there is immense scope for future researches that targeted the studies on experimental animals against various ailments that have been entertained. Curative efficacy of butanol fraction of ethanol extract of root bark of *G. gummifera* L.f was evaluated on N'Nitrosodiethylamine induced hepatocellular carcinoma in experimental rats. The curative efficacy of two doses of G. gummifera butanol fractions (100 mg/kg bw and 200 mg/kg bw) was evaluated against NDEA induced liver cancer in male Wistar rats. At the end of the experiment, animals were sacrificed and the percentage of nodule incidence and all biochemical parameters, including liver cancer markers were analyzed along with histopathological investigation in the experimental animals.

The rats treated with butanol fraction of ethanol extract of *Gardenia gummifera* L.f root bark (BUGG) remarkably repressed the NDEA-induced increase of hepatic nodule incidence, nodule multiplicity, serum biochemical indices. They improved the normal hepatocellular architecture in the toxic group. Also, the biochemical analysis of treated groups revealed that BUGG neutralizes NDEA-induced oxidative stress by restoring antioxidant enzymes. In NDEA administered rats the decreased concentration of proliferative marker, alpha fetoprotein (AFP) was observed upon the supplementation of BUGG. Notably, 200 mg/kg bw BUGG supplementation showed better results than the standard drug-treated group. These results might be linked with the improvement of antioxidant activity and inhibition of hepatic cell proliferation. The conclusions of the present investigation highlight the curative effect of BUGG against the chemical carcinogenesis model and the study suggests that BUGG can be a promising source of chemopreventive, HCC inhibiting agent.

Introduction

Hepatocellular carcinoma (HCC) represents more than 5% of cancers worldwide and is the most common liver malignancy.[1] Recent data stated that the major risk factors for HCC are hepatitis B, hepatitis C infections, and autoimmune hepatitis. In developing countries, the survival rate in all stages of liver cancer is very low compared to developed countries and it is extremely difficult to cure completely. Surgical resection, chemotherapy, radiotherapy, and immunotherapy are considered promising methods of treatment.[2] So, the most effective way to prevent HCC is to evade the development of liver diseases and their development to cirrhosis. In the past decade, HCC has gone from being an almost universal death sentence to cancer that can be prevented, detected early, and effectively treated with appropriate actions.[3]

NDEA is a commonly used xenobiotic agent in *in-vivo* experimental animal studies. NDEA primarily induces the formation of liver tumors in rodents and has been used as a typical hepatic carcinogen in experimental

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studies of carcinogenesis and chemoprevention.[4] N-nitrosodiethylamine (NDEA) is metabolically converted into its active ethyl radical metabolite (CH^3CH^{2+}), which covalently bonds with nucleophilic residues in DNA. The alkylation of DNA bases is mutagenic and stimulates carcinogenesis. Also, this biotransformation of NDEA creates ROS, thus prompting oxidative stress.[5] They initiate the development of chronic inflammation, oxidative stress, and cellular propagation in response to tissue injury, which finally leads to hepatic carcinoma.[6]

Plant-based polyphenolic antioxidants are important in ameliorating most chronic diseases.[7] Hence the cellular antioxidant action is strengthened by the presence of dietary antioxidants.[8] Polyphenols, a broad group of phytocompounds in our daily diet, particularly in fruits and vegetables, with antioxidant and anticancer properties play a critical role against NDEA or CCl_4 intoxication by scavenging active oxygen and free radicals and neutralizing lipid peroxides.[9]

G. gummifera Linn. f. belongs to the family Rubiaceae and is considered one of the rare plant species of India.[10] Thousands of phytoconstituents have been isolated from the plants and many of them have powerful antioxidant properties and can be used to treat many life-threatening diseases.[11] Several medicinal properties have been attributed to *G. gummifera*, including anthelmintic, diaphoretic, expectorant, cardiotonic, antispasmodic, carminative, antioxidant, antiepileptic, peripheral and central analgesic, and antihyperlipidemic.[12] The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites found in *G. gummifera*. Antiaging, anticarcinogenesis, antiinflammation, antiatherosclerosis, cardiovascular protection, inhibition of angiogenesis, and cell proliferation activities are some pharmacological properties attributed to phenolic compounds.[13]

The current study was designed to evaluate the curative efficacy of butanol fraction of ethanol extract of root bark of *G. gummifera* L.f on N-nitrosodiethylamine (NDEA)-induced liver carcinogenesis in male Albino Wistar rats. So far, the mitigative efficacy of butanol fraction of *G. gummifera* against N-nitrosodiethylamine induced neoplasia is not accessible in the animal model and its evaluation of activity can bring more light to the pharmacological efficacy of the plant.

Materials and Methods

Preparation of Plant Fraction and Identification of Active Components

The fresh parts of the root bark of the *G. gummifera* were collected from Kanyakumari district, Tamilnadu, identified and authenticated. A voucher specimen (SBSBRL29) is kept in the School of Biosciences, Mahatma Gandhi University, Kottayam.

The fresh parts of the root bark of the *G. gummifera* were dried under shade and powdered. For extraction, 50 g of dried powder was extracted with 400 mL of ethanol for 24 h at 60°C. The ethanolic extract (ETGG) was concentrated under reduced pressure using a rotary evaporator and the concentrated extract was kept under refrigeration and used for further studies. The most active ethanol extract of the root bark of *G. gummifera* was subjected to liquid-liquid partitioning in a separating funnel. The layers were carefully collected and the process was continued with different immiscible solvents to obtain separate layers. The resultant most active butanol fraction was used for the present study. The dried fraction was dissolved in normal saline to acquire the desired concentration.

Experimental Animals, Carcinogens, and Chemicals

Male Wistar albino rats (weighing 130 ± 10 g) were purchased from a small animal breeding station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala, and used for this study. They were maintained under standard conditions and provided with adequate water ad libitum and fed with a standard balanced diet, allowed to acclimatize for one week before starting the experiment. All the experiments were performed according to CPCSEA guidelines approved by the Institutional Animal Ethics Committee at the School of Biosciences, Mahatma Gandhi University, Kottayam, (IAEC NO. 23092019-1), according to the Government of India accepted principles for laboratory animals' use and care. *In vivo* anticancer efficacy of BUGG was assessed in NDEA induced HCC in male Wistar rats according to Sundaresan and Subramanian, and Sarkar et al., with suitable alterations.[14],[15] NDEA and all other reagents used were of analytical grade. All assays were analyzed by commercial kits obtained from Span Diagnostic Limited, India and the absorbance was read in a UV-vis spectrometer.

Experimental Design

Assessment of NDEA-induced Liver Carcinogenesis

The details of experimental groups and their corresponding treatments are as follows: -

Group I (Normal): Normal control (Untreated animals)
Group II (NDEA): Toxic control (Treated with NDEA alone)
Group III (Vehicle): NDEA + 0.9% NaCl (Vehicle control)
Group IV (BUGG -100): NDEA + BUGG 100 mg/kg body weight
Group V (BUGG -200): NDEA + BUGG 200 mg/kg body weight
Group VI (Silymarin): NDEA + Silymarin 100 mg/kg body weight

The experiment comprised different treatment groups and lasted for 12 weeks. Male albino rats of the Wistar strain were separated into six groups with 6 rats in

each group. The hepatocellular carcinoma was initiated by 2 intraperitoneal injections of NDEA (200 mg/kg body weight) every fortnight convoyed by the weekly subcutaneous injections of CCl_4 for 8 weeks. The group I kept as the control for the experiment without receiving any treatment. All the treatments (group III, group IV, group V, and Group VI) were started subsequently the first dose of NDEA administration and continued up to the end of the experiment. All drug treatments were given orally as aqueous suspension once a day. Group III was treated with 0.9% NaCl. Group IV and Group V received BUGG at the doses of 100 mg/kg body weight and 200 mg/kg body weight, respectively. Group VI received Silymarin, the known hepatoprotective and anti-hepatocellular carcinoma compound at a dose of 100 mg/kg, serving as the experiment's positive control. At the end of the experiment, overnight fasted animals were anesthetized and sacrificed 48 hours after the last dose of the drug. Blood was collected by retro-orbital puncture. Ice cold saline was used to wash the liver tissue, then blotted, dried, observed for the presence of nodules, and then weighed. A small portion of the cleaned liver tissue was fixed in formalin for histological examination.[16-18]

Parameters Analyzed

Change in Body Weight and Relative Liver Weight

For the entire period of study, the bodyweight of each animal was checked daily and the difference in body weight per day was calculated and recorded. At the end of the experimental period the final bodyweight and post-dissection liver weights were also taken. The relative liver weight was calculated as per the formula: -

Relative liver weight (RLW) = (Liver weight of the animal/Bodyweight of the animal) × 100

Morphometric Evaluation

After the end of the 3 months study, the liver tissue was excised and the dissected livers were observed for any morphological changes in the nodule formation. The neoplastic liver nodules formed in each rat were counted and the percentage of nodule incidence was calculated as follows:

Percentage of nodule incidence in a group = (number of rats with nodules/Total no. of rats in the group) × 100

The nodule multiplicity was calculated as per the formula:-

The nodule multiplicity in a group = (Total no. of nodules/Total no of rats in the group) × 100

Level of Alpha-fetoprotein (AFP)

The AFP tumor marker is a plasma protein used to detect primary hepatocellular carcinoma. The level of AFP in serum was determined after following the instructions from the manufacturer, using a diagnostic kit (Span Diagnostic Limited, India) and the absorbance was read in a UV-VIS spectrometer.

Assays of Liver Function and Kidney Functional Markers

The collected blood was allowed to clot and serum was used to analyze the liver function parameters such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase enzyme (LDH) gamma glutamate transaminase (GGT), alkaline phosphatase (ALP), albumin, globulin, A/G ratio, and bilirubin. The renal functional markers such as total protein, urea, uric acid, and creatinine were also analyzed in the study. All assays were done using the commercially available kit (Span Diagnostic Limited, India) and the absorbance was read in a UV-VIS spectrometer.

Assay of Oxidative Stress Markers

The biomarkers of oxidative stress including superoxide dismutase (SOD),[19] catalase,[20] and reduced glutathione (GSH),[21] level of malondialdehyde (MDA),[19] glutathione peroxidase,[22] glutathione reductase[23] and the level of tissue protein were analyzed by Khan *et al.*[24] The level of reactive oxygen species was determined according to the method described by Tohamy *et al.*[25]

Histopathology

Histopathology of the liver tissues was carried out to evaluate the extent of tissue damage. The rats were painlessly killed under mild euthanasia and the organ, the liver was harvested for histopathological examination. The organs were then fixed in 10% formalin. The fixed tissues were embedded and cut into 5 μm thick sections. The hematoxylin and eosin-stained sections were observed under the light microscope and examined the photograph for signs of toxicity.

Statistical Analysis

All the results were stated as mean ± standard deviation (SD). The statistical evaluation among different groups was shown by one-way ANOVA followed by Tukey's post-doc analysis (Dunnett's multiple comparison test) with the help of Graph pad prism software version 5.0. P values <0.05 were measured as statistically significant.

Results

Bodyweight Gain Pattern and Change in Bodyweight

BUGG-treated rats showed an entirely different bodyweight pattern than the normal control group (Fig. 1). In the early weeks of the study, the NDEA administered rats exhibited a below-normal weight, and later at the end of the study, it showed remarkable weight loss. But, the BUGG administered rats, displayed substantial progress in the body weight gain pattern, and during the entire study period, those rats prevented bodyweight drop. BUGG administered group showed a similar body weight pattern as of Silymarin group.

Morphometric Evaluation

Table 1 demonstrated the data which showed the total number of neoplastic liver nodules formed in each rat under different treatment groups along with the percentage of nodule incidence and nodule multiplicity. 100% nodule incidence with 15.26 multiplicities was observed in the NDEA intoxicated group. BUGG's ability to prevent nodule formation was evident from the BUGG-treated rats.

Measurement of Liver Weight and Relative Liver Weight (RLW)

When measured at the end of the experiment, the group of rats administered with NDEA alone showed abnormal weight gain in the liver tissue, whereas BUGG and Silymarin treated rats showed considerably reduced weight gain in a dose-dependent manner. According to the results shown in the Table 2, a great difference was observed in the relative liver weight of the rats. The liver weight and relative liver weight of the toxic control group (NDEA alone) were (12.02 ± 0.008g and 8.92 ± 0.015) significantly varied from the values of normal rats (7.81 ± 0.014g and 2.38 ± 0.00. But the BUGG-200 treated group exhibited a significant reduction from the elevated levels of liver weight and relative liver weight into a normal value of 7.41 ± 0.008 and 2.597 ± 0.008, respectively, and these values were parallel with the Silymarin group (7.51 ± 0.044g and 2.585 ± 0.015).

Effect of BUGG on Alpha-fetoprotein Level

The level of tumor marker, the alpha-fetoprotein was found to significantly increased during the NDEA administration and its level was found to be declined after the treatment with BUGG as illustrated in Fig. 2. It was observed that

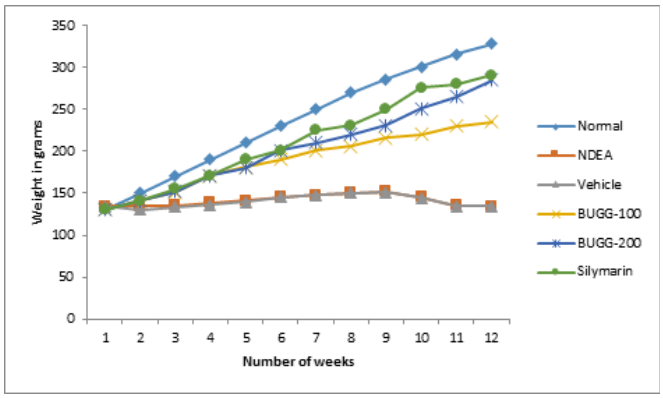

Fig. 1: Effects of BUGG on bodyweight patterns of rats in NDEA-induced HCC
The values are depicted as mean ± SD, n =6.

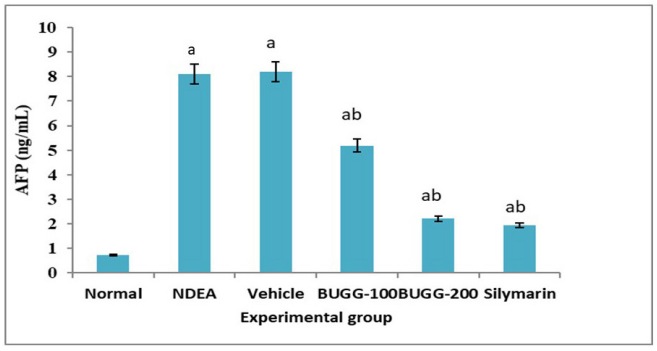

Fig. 2: Change in the level of alpha-fetoprotein in NDEA-induced HCC
The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

Table 1: Incidence of liver nodules and nodule multiplicity

Groups	*Rats with nodules*	*Nodule incidence (%)*	*Total number of nodules*	*Nodule multiplicity*
Normal	0	0	0	0
NDEA	6^{a}	100^{a}	91.58 ± 1.26^{a}	15.26^{a}
Vehicle	6^{a}	100^{a}	93.86 ± 0.93^{a}	15.64^{a}
BUGG -100	4^{ab}	66.66^{ab}	23.02 ± 0.88^{ab}	5.75^{ab}
BUGG -200	1^{b}	16.66^{b}	2.54 ± 0.16^{b}	2.54^{b}
Silymarin	1^{b}	16.66^{b}	1.49 ± 0.04^{b}	1.49^{b}

The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

Table 2: Effect of BUGG (Dose in g/kg body weight) on relative organ weight in NDEA induced study.

Experimental groups	*Initial body weight (g)*	*Final body weight (g)*	*Liver weight (g)*	*Relative liver weight*
Normal	130.6 ± 0.35	327.6 ± 0.23	7.81 ± 0.014	2.38 ± 0.006
NDEA	134.3 ± 0.54	134.6 ± 0.23	12.02 ± 0.008	8.92 ± 0.015^{a}
Vehicle	134.2 ± 0.63	134.6 ± 0.24	11.92 ± 0.075	8.847 ± 0.059^{a}
BUGG -100	130.5 ± 0.42	235.4 ± 0.50	8.23 ± 0.040	3.493 ± 0.015^{ab}
BUGG -200	130.8 ± 0.75	285.1 ± 0.48	7.41 ± 0.008	2.597 ± 0.008^{b}
Silymarin	130.8 ± 0.49	290.4 ± 0.24	7.51 ± 0.044	2.585 ± 0.015^{b}

The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

the AFP value of the BUGG-200 group (2.212 ± 0.11) was analogous with silymarin (1.93 ± 0.039) treated animals.

Effect of BUGG on Serum Levels of Liver Enzymes

The elevated serum levels of liver enzymes including ALT, AST, GGT, ALP, and LDH were significantly reduced by the treatment with BUGG. The decreased liver enzyme levels of BUGG treated groups showed the fraction's ability to improve the liver condition to normal. The results obtained were significantly compared with the level of the silymarin-treated group (Table 3).

Effect of BUGG on Biochemical Parameters of Liver Function

Fig. 3 displayed the levels of liver function parameters that established the effect of BUGG treatment during NDEA-induced carcinogenesis. NDEA administered control rats were showed higher values of both direct and indirect bilirubin, whereas these levels were found significantly returned after the treatment with BUGG. A similar protective effect was found in the Silymarin group.

The BUGG treated NDEA groups were also showed a normal range of albumin, globulin, total protein, and A/G ratio levels after a drastic reduction in their respective values before treatment (Fig. 4). Silymarin also formed a significant defensive effect in the biochemical parameters.

Effect of BUGG on the Liver Antioxidant Status

Table 4 shows that the levels of all the antioxidant enzymes were declined from their normal level when NDEA was administered. But after BUGG treatment, the groups gradually rose to the normal level. Tissue antioxidant enzymes and reduced glutathione were ominously

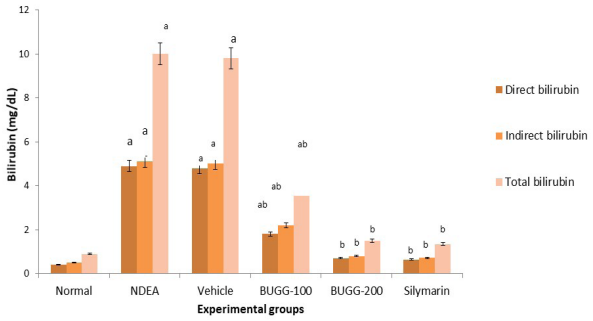

Fig. 3: Effect of BUGG on the level of bilirubin
The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

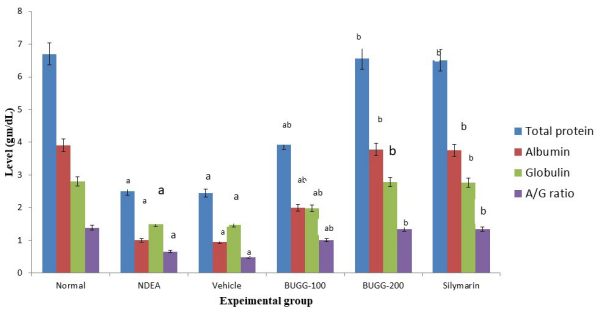

Fig. 4: Effect of BUGG on the level of total protein, albumin, globulin, A/G ratio
The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

Table 3: Effect of BUGG on liver function parameters

Experimental groups	*ALT (IU/L)*	*AST (IU/L)*	*ALP (IU/L)*	*LDH (IU/L)*	*GGT (IU/L)*
Normal	85.99 ± 0.67	225.8 ± 1.54	683.7 ± 0.85	354.6 ± 1.32	11.4 ± 0.42
NDEA	913.2 ± 0.49^{a}	516.4 ± 0.78^{a}	1024 ± 0.69^{a}	715.3 ± 0.89^{a}	26.61 ± 0.24^{a}
Vehicle	910.9 ± 0.65^{a}	515.4 ± 0.67^{a}	1023 ± 0.74^{a}	714.1 ± 0.62^{a}	25.63 ± 0.18^{a}
BUGG -100	373.5 ± 0.54^{ab}	386.0 ± 1.23^{ab}	874.9 ± 0.76^{ab}	483.7 ± 1.91^{ab}	18.58 ± 0.17^{ab}
BUGG -200	134.7 ± 0.46 ^{ab}	236 ± 0.54^{ab}	714.1 ± 0.49 ^{ab}	371.2 ± 2.37^{ab}	13.51 ± 0.20^{ab}
Silymarin	124.1 ± 1.09 ^{b}	232.2 ± 1.49^{b}	694 ± 1.11^{b}	360.6 ± 0.16^{b}	13.1 ± 0.64^{b}

The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

Table 4: Effect of BUGG on tissue antioxidants in the liver

Groups	*SOD (U/mg protein)*	*CAT(U/mg protein)*	*GPx(U/mg protein)*	*GR(U/mg protein)*	*GSH(µM/mg protein)*
Normal	20.96 ± 0.031	10.22 ± 0.063	23.27 ± 0.47	28.36 ± 0.579	12.93 ± 0.523
NDEA	5.55 ± 0.221^{a}	4.76 ± 0.471^{a}	7.62 ± 0.185^{a}	9.78 ± 0.117^{a}	6.55 ± 0.070^{a}
Vehicle	5.79 ± 0.079^{a}	5.17 ± 0.525^{a}	12.23 ± 5.55^{a}	9.46 ± 0.128^{a}	6.345 ± 0.49^{a}
BUGG -100	13.65 ± 0.176^{ab}	6.73 ± 0.159^{ab}	12.84 ± 0.56^{ab}	17.78 ± 0.103^{ab}	7.67 ± 0.197^{ab}
BUGG -200	18.8 ± 0.133^{b}	8.63 ± 0.221^{ab}	19.73 ± 0.135^{ab}	24.67 ± 0.196^{ab}	9.74 ± 0.110^{ab}
Silymarin	19.17 ± 0.207^{b}	9.44 ± 0.185^{b}	20.61 ± 0.221^{b}	26.74 ± 0.112^{b}	10.76 ± 0.179^{b}

The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

depleted after NDEA administration. The level of GSH was found to decrease to 6.55 ± 0.070µM/mg protein from 12.93 ± 0.523µM/mg protein, the value of the normal rats. Lower and higher doses of BUGG treatment showed significantly improved GSH values of 7.67 ± 0.197 and 9.74 ± 0.110 µM/mg protein from their counterpart of 6.55 ± 0.070 µM/mg protein exhibited by intoxicated rats. These improved levels were found to be similar to the values of silymarin-treated groups. The dose-dependent beneficial effect of BUGG in the replenished levels of tissue antioxidant enzymes including SOD, CAT, GPx, and GR was also found in this analysis.

Effect of BUGG on Lipid Peroxidation

The lipid peroxidation was evaluated based on the level of malondialdehyde concentration and it was found elevated in the NDEA intoxicated groups. MDA level was significantly reduced in the BUGG treated groups indicate its reduced lipid peroxidation level (2.21 ± 0.011 µM/mg protein), which was comparable with silymarin treated group (1.97 ± 0.037 µM/mg protein) (Fig.5).

Level of Reactive Oxygen Species (ROS)

The level of ROS was found to be higher in NDEA intoxicated with a value of 803.3 ± 2.41 µmol NBT/g tissue as compared with 350.2 ± 0.307 µmol NBT/g tissue of normal value. The BUGG and Silymarin treated groups exhibited a significantly lesser value comparable with the normal group. The noticed level of ROS for BUGG-200 and Silymarin groups were 412.6 ± 2.977 and 398.8 ± 1.89 µmol NBT/g tissue, respectively (Fig. 6).

Effect of BUGG on the Histology of Liver

The histopathological evaluations of liver sections were observed to support the above-said findings of the study. The pathological changes induced by NDEA on animals and its corresponding effects of BUGG on the different treated groups were well obtainable in the microphotographs of hematoxylin and eosin-stained liver tissue sections. The untreated rats showed the normal architecture of the liver and hepatocytes. The hepatocytes of NDEA-treated rats showed anisonucleosis with prominent nuclei and markedly dilated sinusoids. Focal inflammatory cell infiltration could be seen. Characteristic pathological changes such as the formation of hyperplastic nodules and atypical nuclei observed on the NDEA induced liver are the indicators of NDEA induced hepatocarcinogenesis. The BUGG treated group showed a marked difference compared to the toxic group as sections displayed

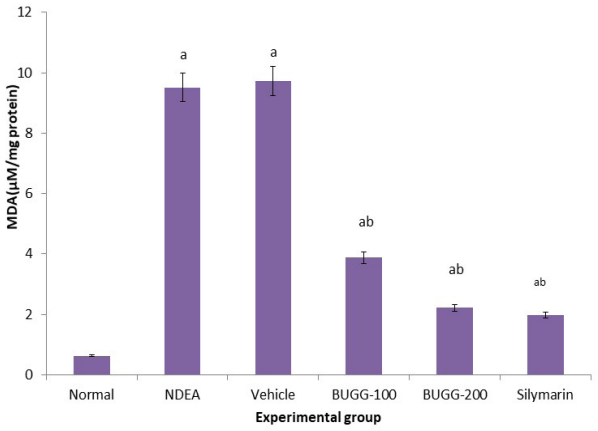

Fig. 5: Effect of BUGG on lipid peroxidation
The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

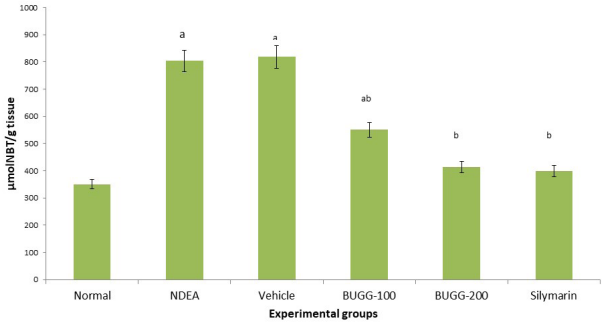

Fig. 6: Level of reactive oxygen species (ROS)
The values were depicted as mean ± S.D., n=6. 'a' represents the statistical deviation from the normal control group, 'b' indicates statistical deviation from NDEA control group and 'ab' means statistical deviation from normal control and toxic (NDEA) control group.

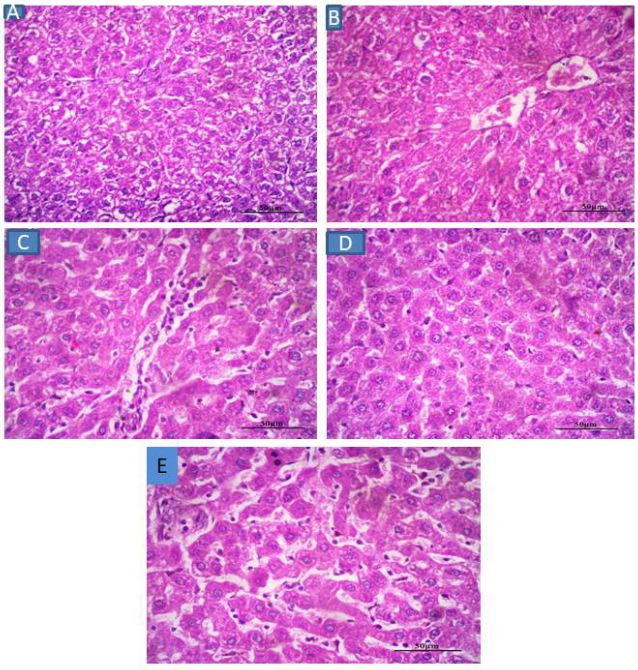

Fig. 7: Effect of BUGG on the histology of liver during the NDEA-induced HCC study (a) Normal (b) Toxic (c) Treated with 100 mg/kg of BUGG (d) Treated with 200 mg/kg of BUGG (e) Treated with 100 mg/kg of Silymarin

minimal inflammatory changes and few neoplastic cells, and apart from that, the hepatocytes maintained normal architecture. The liver sections of rats treated with 100 mg/kg of BUGG showed mild to moderate anisonucleosis and prominent nuclei. Mild dilation in sinusoids and proliferation of kupffer cells were also observed. There was no inflammation noticed in this group. Hepatocytes of rats treated with 200 mg/kg of BUGG were appeared normal with no observed nuclear or cytoplasmic atypia. There was no inflammation or necrosis noted in this group. Silymarin treated group was appeared nearly normal. The curative changes induced by the BUGG treatment in the liver tissue were comparable to that of the standard drug, Silymarin (Fig. 7).

Discussion

HCC is one of the deadly diseases which affects the liver and is a polygenic disease with a complex mechanism and signaling pathways. Herbal drugs are more widely used than allopathic drugs for hepatic ailments due to the less expensive availability of many medicinal plants as hepatoprotective agents, widely accepted by the rural people, better adaptability to the human body, and reduced side effects.[26] More than 100 phytocompounds have been identified as hepatoprotective agents from different plants.[27] Therefore, many traditional remedies originated from herbal combinations are being tested for their toxicity effect and potential hepatoprotective activity in experimental animal models and cell lines.

NDEA is an important carcinogen that produces oxidative stress through the generation of ROS and modifies the antioxidant defense system in tissues.[28] Liver injuries induced by NDEA followed by CCl_4 injection are the best-characterized system of the xenobiotic induced hepatotoxicity is a commonly used model for screening the anti-hepatotoxic/ anti-cancer potential of natural compounds.[29]

Abnormal body weight gain and loss of body weight are the implications of toxicity. Unexplained weight loss previously been reported in many cancer cases such as gastro-esophageal, pancreatic, non-Hodgkin's lymphoma, ovarian prostate, colorectal, lung, gastro-esophageal, pancreatic, non-Hodgkin's lymphoma, ovarian, myeloma, renal tract, and biliary tree, etc. Previously, weight loss was considered a symptom of the advanced stage of cancer, although reports give some conflicting opinions, that colorectal, lung, and pancreatic cancer studies have reported that even people with early-stage cancer may present with weight loss.[30] There is an alternation in body weight change pattern was observed in the NDEA intoxicated rats in the early week of the study but an insidious weight loss was observed in the final stage of the experiment. It points to the result of reduced metabolism and hepatotoxicity due to carcinogens. But the treatment with BUGG considerably improved the weight gain pattern without causing any weight loss during the entire study period, specifies the beneficial nature of the plant-derived fraction against NDEA-induced carcinogenesis. In the present investigation, absolute and relative liver weight were significantly reduced in BUGG-treated rats compared to the NDEA control group. In toxicity evaluation in animal models, relative organ weight is a more precise parameter than the absolute weight.[31]

Hepatocarcinogenesis in human chronic liver diseases is a multi-step process in which hepatic precancerous lesions grow into early hepatocellular carcinoma (HCC) and progress into HCC. Further studies showed that these nodules may be similar to hepatic precancerous lesions, and they often appear in patients with liver cirrhosis and these nodules are simultaneous with the existence of HCC. The importance of chemopreventive efficacies of phytochemical agents lies in this context. The close observation and treatment of these precancerous lesions with phytoconstituents would improve the survival rates of HCC patients.[32] The same structural pattern of the development of nodules occurred in NDEA induced hepatocellular carcinogenesis in animals also. Gene expression studies of the cross-species comparison showed that NDEA-induced liver tumors in rodents closely mimic a subclass of human HCC, allowing for the extrapolation of potential clinical chemopreventive effects of candidate agents.[33] The present study has shown that treatment with BUGG successfully hindered the process of carcinogenesis as evident from the reduced number of liver nodules accompanied by the low level of nodule incidence and multiplicity when compared to the NDEA control rats. Alpha-fetoprotein is a widely used plasma marker for screening and diagnosis of HCC and most studies report elevated levels of AFP concentration in 70% of HCC patients. Its level was significantly increased in NDEA treated rats, whereas its level was markedly reduced in rats treated with BUGG and its level is comparable with silymarin treated rats.

The altered serum markers showed by the liver damage induced by the NDEA and CCl_4 administration reflect the variability of liver cell metabolism. The normal levels of serum transaminases, LDH, ALP, and GGT are indicators of liver function and their augmented levels in the serum denote liver damage. The most sensitive markers employed in diagnosing hepatic damage are the serum levels of ALT, AST, ALP, γ-GT, and bilirubin.[34] The most important markers to assess liver function are the serum hepatic leakage enzymes like ALT and AST.[35] In all the pathological conditions of the liver, the level of AST is often found to fluctuate along with ALT normally. The level of enzymes, GGT, and ALP specify pathological hepatobiliary conditions allied with variation in biliary flow.[35]

The liberation of GGT from the hepatocyte plasma membrane into serum indicates cellular damage to the liver hence the level of GGT is measured as one of the

best indicators of liver damage. To detect the biochemical alteration in hepatocellular foci, nodules, and tumors in rats, the membrane-bound enzyme GGT has been widely used as a marker. It is well known that induction of GGT in preneoplastic foci represents an early event in hepatocarcinogenesis, and GGT-positive foci appear to be the first discernible evidence for the occurrence of tumor initiation.[36,37] Due to blockage of bile ducts or reduced conjugation and diminished secretion from the liver, the concentrations of direct and total bilirubin were elevated in NDEA-treated rats.[38] The BUGG treatment ominously suppressed the raised levels of total and direct bilirubin in HCC rats. The use of the ratio of unconjugated (indirect) and conjugated (direct) bilirubin values comprising the total bilirubin concentrations have been employed historically to differentiate between hepatic and extrahepatic hyperbilirubinemia disorders.[39] Hepatocellular toxicity can be evaluated by measuring the serum LDH.[35]

The bioactive fraction of *Gardenia gummifera* BUGG was found effective in restoring enzymatic as well as non-enzymatic parameters employed to assess liver functioning in the present study of plant-derived fractions to alter or hinder the process of NDEA-induced hepatocarcinogenesis. Also, HRLC-MS analysis of the butanol fraction spectrum profile exhibited some polyphenolic compounds. Among these Berbamine, Chlorogenic acid, Gallic acid, ellagic acid, Norstictic acid pentaacetate, Mitoxantrone, and Pyrvinium are strong antioxidants and anticancer agents. Because of the presence of these anticancer compounds in the butanol fraction, the treatment with BUGG (butanol fraction) showed intense positive effects as evident in all the parameters of the study, when compared to the toxic rats, with a good efficacy level comparable to the standard drug, Silymarin.

One of the factors which lead to hepatocarcinogenesis in chronic oxidative stress and acts as a driving force in the alteration of chronic liver ailments into HCC. The NDEA initiates hepatocarcinogenesis by generating reactive oxygen species, including DNA-binding ethyl carbonium ions, resulting in adducts and superoxide radicals via lipid peroxidation of phospholipid membrane fatty acids.[40] The relationship between oxidative stress and hepatocellular carcinoma is evident from a plethora of earlier works, where ROS, lipid peroxidation, and exhausted levels of tissue antioxidant mechanisms contributed to the development and progression of various chemically induced HCC models including NDEA-induced carcinogenesis.[41] NDEA leads to oxidative stress induced by the generation of ROS and associated oxidative damage of DNA, proteins, and lipids. The levels of ROS and lipid peroxidation are remarkably decreased in BUGG-treated animals. The concentration of lipid peroxidation in serum has been widely used as a marker of oxidative stress because membrane lipids are more prone to ROS.[42] The results also suggest the restoration of the tissue-level antioxidant system. A study by Dakshayani et al demonstrated that oxidative stress may be the reason for the elevated lipid peroxidation level in the liver of NDEA intoxicated rats and in the present study, BUGG was found especially effective in regulating the parameters mentioned above.[43]

GSH is an important low molecular weight antioxidant (L-glutamyl cysteinyl glycine), critical for detoxification of endogenous metabolic byproducts, including lipid peroxides, and xenobiotic compounds including heavy metals, pollutants, and drugs.[44] The depletion of GSH in tissues leads to the production of numerous oxidative and nitrosative reactive intermediates comprising superoxide, hydroxide, peroxide, and peroxynitrite radicals, which all can lead to impairment in the cellular macromolecules including lipid membranes and DNA adduct formation [44]. Hence, the improved level of GSH in the BUGG treated groups highlights its antioxidant potential. Similarly, a reduction in the activity of the antioxidant enzymes SOD, CAT, GPx, and GR was observed in the liver of NDEA -treated rats. The tissue level antioxidant enzymes SOD, CAT, GPx, and GR are critical in upholding cellular oxidative balance. SOD, CAT, and GPx play a significant role in maintaining the body's defense mechanism against the deleterious effects of ROS.[45] Previous reports show that increased oxidative stress along with reduced SOD levels may intensify the progression of HCC.[46] The observed decrease in the components of the tissue antioxidant defense system may be attributed directly to the excessive production of ROS in the NDEA-induced carcinogenesis. The treatment with BUGG exhibited prominent restoration of tissue antioxidant defense due to the plant's antioxidant activity. NDEA-induced hepatocarcinogenesis was substantiated by the highly pronounced alterations observed in the histopathological evaluation of the liver tissue. The histological alterations observed in the study were in good agreement with the biochemical findings. The histopathological patterns of BUGG-treated rats when compared with NDEA treated control rats showed significant tissue-level changes such as neoplastic lesions in a dose-dependent manner. Characteristic pathological changes such as the formation of hyperplastic nodules and atypical nuclei observed on the NDEA induced liver are the indicators of NDEA induced hepatocarcinogenesis. Remarkable pathological development was observed in rats treated with BUGG. The histopathological patterns of BUGG-treated rats compared with NDEA treated control rats showed significant tissue-level changes such as neoplastic lesions in a dose-dependent manner. Altogether, our biochemical and histological data clearly shows that bioactive constituents of butanol fraction of *G. gummifera L.f* exerts hepatoprotection against NDEA-induced hepatic damage in rats by its antioxidant and anticancer potential.

Conclusion

In a conclusion, the results of the present study clearly showed that BUGG offers protection against oxidative damage produced by NDEA induction in experimental rats.

The fraction reduced the amount of ROS formation, lipid peroxidation, and restored the levels of tissue antioxidants. So, the present study established the antiproliferative efficacy of the butanol fraction of *Gardenia gummifera L.f* owing to the synergistic and cumulative effect of several polyphenols found in the fraction. So, further studies of elaborate preclinical studies of longtime duration in proper animal models are required to assess the antioxidant and anticancer efficacy in detail.

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