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ANTIOXIDANT ACTIVITY OF BARK EXTRACT OF *BRIDELIA RETUSA* SPRENG ON DMBA INDUCED MAMMARY CARCINOGENESIS IN FEMALE SPRAGUE DAWLEY RATS

CORDEIRO M.C. AND KALIWAL B.B.
P. G. Department of Microbiology and Biotechnology, Karnatak University, Dharwad, 580008, India
*Corresponding author. E-mail: b_kaliwal@yahoo.com

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Abstract- The present study was undertaken to investigate anticancer activity of ethanolic extract of the stem bark of *Bridelia retusa* S. in DMBA treated female Sprague Dawley rats. Adult healthy virgin Sprague Dawley female rats of 45 days old were selected for the experiment. The rats were treated with 20 mg of DMBA (7,12-dimethylbenz[*a*]anthracene) to induce mammary tumors. The bark of *Bridelia retusa* S. was powdered and subjected to sequential extraction based on polarity. Oral suspensions containing 50mg/kg, 100mg/kg and 150mg/kg (body weight) of ethanolic extract were administered to DMBA treated rats. The toxic effect of DMBA was observed by significant ($p<0.05$) decrease in the body weights while significant ($p<0.05$) increase in tumor size of rats when compared to negative control. Weight loss and increase in tumor size observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was almost that of standard drug. The toxic effect of DMBA was justified by the significant ($p<0.05$) decrease in the activity of SOD (superoxide dismutase) and CAT (catalase) while significant ($p<0.05$) increase in LPO (lipid peroxidation) in the mammary tissue, liver and kidney when compared to negative control. The antioxidant effect of extract was observed by significant ($p<0.05$) increase in the activity of SOD and CAT while significant ($p<0.05$) decrease in lipid peroxidation in the mammary tissue, liver and kidney when compared to DMBA positive control. The standard drug showed significant and similar antioxidant activity to 150 mg/kg extract treated group, which gave best result in comparison to other extract treated groups..
Keywords: *Bridelia retusa* Spreng, sequential extraction, DMBA, anticancer activity.

Introduction

According to WHO survey 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for their primary health care needs. The chemical constituents obtained from plants may be pharmacological screened for developing novel agents [1, 2]. Phytochemicals are compounds found in plants that are not required for normal functioning of the body, but have a beneficial effect on health or play an active role in amelioration of diseases. Phytonutrients have various health benefits, for example, they may have antimicrobial, anti-inflammatory, cancer preventive, antidiabetic and antihypertensive effects to mention but a few [3]. Carcinogenesis is a multistage (initiation, promotion and progression) process that encompasses multiple genetic and epigenetic events [4, 5]. One of the most serious problems in oncology is breast cancer which is the leading cause of death among women in many countries. According to American Cancer Society estimates in 2005, approximately 2,11,240 women were diagnosed with this disease and 40,410 died annually. Reactive oxygen species (ROS) are involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis. Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes, and their scavengers (SOD, CAT, etc) represent inhibitors at different stages of carcinogenesis. The enzymes are found in cyotosolic and mitochondrial functions mainly involved in the biotransformation and detoxification of carcinogens. The continuing severity and magnitude of the cancer problems make it imperative to develop chemopreventive strategies utilizing natural antioxidants to block the initiation, or arrest, or reverse the progression of pre-malignant cells. Antioxidants may protect against the toxicity of reactive oxygen species (ROS) by the prevention of ROS formation and neutralization of oxygen-free radicals [6].

Environmental factors, of either biologic or chemical origin, may act as initiators, promoters, or both of carcinogenesis. Chemical carcinogens such as 7, 12-dimethylbenz[*a*] anthracene [DMBA], benz[a]pyrene [BP], 4-nitroquinoline- 1-oxide, and N-nitroso-N-methylurea are commonly employed to

initiate and promote neoplastic transformation in experimental animals. However, the most commonly employed chemical carcinogen for inducing experimental carcinogenesis is DMBA [7]. DMBA is well established as a highly potent carcinogen. It is used solely in carcinogenesis research. DMBA is absorbed through the skin and respiratory and intestinal tracts; and by intravenous and intraperitoneal injection, ingestion, and inhalation. It is carcinogenic and may irritate tissues and induce sensitivity [8]. There has been an increasing interest in carcinogenic chemicals such as 7,12-dimethylbenz(a) anthracene (DMBA) to induce mammary carcinoma in rat models for the study of human breast cancers [9]. The role of polycyclic aromatic hydrocarbons (PAH) is clearly implicated in the process of carcinogenesis especially 7,12-dimethylbenz[*a*]anthracene (DMBA), which is one of the most potent skin and breast carcinogens known [10]. Studies on chemopreventive and anticancer agents which may be due to antioxidant system, against mammary cancer induced by DMBA have been suggested. *Jasminium grandiflorum* flowers and black tea polyphenols have potent chemopreventive potential, the protective effect of *Operculina turpethum* and dietary fish oil in DMBA-induced mammary carcinogenesis, which may be due to their antioxidants properties [7].

Bridelia retusa Spreng is a moderate sized tree or a shrub belonging to *Euphorbiaceae* family found growing throughout India [10]. In Southeast Asia, these species are usually part of the primary and secondary forest vegetation either as big trees or as smaller trees or shrubs. Decoction of stem bark with country liquor is used for diarrhea, ear ache and prevents pregnancy. Pounded bark is mixed with gum of *Steroulia urines* Roxb. and the mixture is prescribed orally 2-3 days after menstruation for complete infertility [11]. Extract from the stem bark has antiviral, anticancer and hypotensive properties. Paste of the stem bark is applied to wounds and bark juice taken internally in case of snake bite [10]. Hence, the development of a new strategy possessing anti-neoplastic and free radical scavenging properties becomes important. Thus, the objective of this study was to evaluate the anticancer and free radical scavenging activity in stem bark extracts of *Bridelia retusa* S.

Materials and methods

Plant materials and preparation of bark extracts

The bark of *Bridelia retusa* S. was obtained from the jungles of Western Ghats (Amboli) under the guidance of Forest Officer. The plant material was cut into pieces and subjected to shade drying. On complete drying the pieces were powdered and stored in air tight containers at room temperature for future use. The powder (50 gm) was subjected to extraction in soxhlet apparatus using various solvents of petroleum ether (40-60^{o}C), chloroform (60-62^{o}C distillation) and ethanol in order to obtain organic extract while distilled water was used for aqueous extract which was carried out one after the other in a sequential manner based on their polarity. The ethanolic extract was filtered using Whatman filter paper no. 1 and concentrated. The filtered extract was evaporated and dried extract obtained weas labeled, weighed and stored at 4^{0}C in air tight containers.

Animals

Laboratory bred adult virgin Sprague Dawley rats aged 45 days weighing between 80-100 gm were used in the experiments. The rats were maintained in P.G. Deparment of Pharmacology and toxicology, KVAFSU, Veterinary College, Bangalore. Rats were permitted by the ethical committee, CPCSEA. They were housed in separate polypropylene cages containing sterile paddy husk as bedding material. Standard rats pellet diet "Gold Mohar' (Hindustan Lever company, Mumbai) was provided along with water *ad libitum*. The rats were maintained under normal day/night schedule (12L:12D) at room temperature 25$\pm$2^{0}C.

Ethical clearance

The animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethics Committee approved experimental design performed in this study for the use of Sprague Dawley rats as an animal model for anticancer activity.

Drug Formulation

Bridelia retusa S. stem bark extracts were administered at doses below acute LD_{50} level of intoxication according to the body weight of the rats. Oral suspensions containing 50 mg/kg, 100 mg/kg and 150 mg/kg (body weight) of ethanolic extract were prepared in carboxymethly cellulose (0.4%).

Chemicals and Treatment

DMBA 7,12-dimethylbenz(*a*)anthracene (Sigma-Aldrich) toxicity was induced at 20mg diluted in sesame oil (0.5 mg in 0.5 ml sesame oil) administered by intra-mammary (subcutaneous) injections given ones per week for four weeks. Cyclophosphamide (Kidwai Cancer Hospital, Bangalore) was used at 1mg/kg in 0.4 % carboxymethly cellulose as standard anticancer drug. Rats were divided into six groups of six rats each as follows [6]. Sesame oil (0.5ml) was gavaged orally to group I with normal food and water (negative control) and DMBA treated rats were used for mammary cancer studies (positive control). The group III received 2mg/kg cyclophosphamide (CYC) and the remaining were

given three doses 50mg/kg, 100mg/kg and 150mg/kg of ethanolic extract to mammary cancer induced rats for 20 days. The rats were monitored for change in body weight during the experiment. The tumor size was measured by venier calipers after 90 days of DMBA exposure. All the rats were necropsied after 90 days of DMBA exposure by mild ether anesthesia and vital organs were dissected out. Antioxidant enzymes of SOD [13], CAT [14] and LPO [15] in mammary tissue, liver and kidney were estimated. The statistical significance between control and experimental data was subjected to analysis of variance (ANOVA) together with Dunnett's test ($p<0.05$) using SPSS package.

Results

Effect of *Bridelia retusa* stem bark extract on body weight of DMBA treated rats.

The toxic effect of DMBA was observed by significant ($p<0.05$) decrease in the body weights of rats when compared to negative control (Table 1). The protective activity of the ethanolic extract was justified by the significant ($p<0.05$) increase in the weights when compared to DMBA treated group. Weight loss observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was almost that of standard drug.

Effect of *Bridelia retusa S.* stem bark extract on tumor size of DMBA treated rats.

The carcinogenic effect of DMBA was observed by significant ($p<0.05$) increase in the tumor size of rats when compared to normal control (Table 1). The protective activity of the extract was justified by the significant ($p<0.05$) decrease in the size when compared to DMBA treated group. The decrease in tumor size observed in extract treated group was maximum in 150 mg/kg which approached that of standard drug.

Effect of *Bridelia retusa S.* stem bark extract on oxidative parameters in mammary tissue of DMBA treated rats.

The toxic effect of DMBA was justified by the significant ($p<0.05$) decrease in the activity of SOD and CAT while significant ($p<0.05$) increase in lipid peroxidation in the mammary tissue when compared to negative control (Table 2). The antioxidant effect of extract was observed by significant ($p<0.05$) increase in the activity of SOD and CAT while significant ($p<0.05$) decrease in lipid peroxidation in the mammary tissue when compared to DMBA positive control. The 150 mg/kg extract treated group showed significant and similar antioxidant activity to standard drug, which gave best result in comparison to other extract treated groups.

Effect of *Bridelia retusa S.* stem bark extract on the liver of DMBA treated rats.

The activity of SOD and CAT significantly ($p<0.05$) decreased while lipid peroxidation significantly ($p<0.05$) increased in the liver of DMBA treated group when compared to normal control (Table 3). The extract showed antioxidant effect by significant ($p<0.05$) increase in the activity of SOD and CAT while significant ($p<0.05$) decrease in lipid peroxidation in the liver when compared to DMBA positive control. The activity of SOD was brought to that of normal by 150 mg/kg of extract treated group which significantly ($p<0.05$) more than cyclophosphamide treated group. Among the extract treated group, 150 mg/kg showed significant and similar antioxidant activity of CAT and lipid peroxidation to that of standard drug.

Effect of *Bridelia retusa S.* stem bark extract on the liver of DMBA treated rats.

The effect of DMBA was observed to be toxic due to the significant ($p<0.05$) decrease in the activity of SOD and CAT while significant ($p<0.05$) increase in lipid peroxidation in the kidney when compared to normal control (Table 4). The antioxidant effect was observed by significant ($p<0.05$) increase in the activity of SOD and CAT while significant ($p<0.05$) decrease in lipid peroxidation in the kidney of extract treated group when compared to DMBA control. The antioxidant activity of 150 mg/kg extract treated group was significant and similar to standard drug, which gave best result in comparison to other extract treated groups.

Discussion

DMBA is highly carcinogenic in experimental animals. Large single and multiple doses produce tumors of the skin, breast, and stomach or leukemias regardless of route of administration. Skin of mice is particularly sensitive to low, topically applied doses [8]. This carcinogen reacts with rapidly proliferating cells in the terminal end buds forming DNA adducts which transform normal terminal end buds to malignant pathways. The susceptibility of Sprague Dawley rats to DMBA is maximal at 55 to 60 days of age and is abolished by ovarietcomy, suggesting the inducible action of carcinogen depends on ovarian secretions. The estrogenic properties of DMBA molecule may be due to long lasting effects that the carcinogen exerts on the plasma membrane of estrogen sensitive neurons. DMBA can interact with estrogen receptor and partially mimic both positive and negative feedback of estradiol [16]. DMBA induced experimental carcinogenesis is preceded by a sequence of hyperplasia, dysplasia, and carcinoma. DMBA mediates carcinogenesis through formation of DNA adducts, DNA damage, generating excess reactive oxygen species and by producing chronic inflammation. Several studies suggested that DMBA mediated molecular, biochemical, genetic and histopathological changes were analogous to those

observed in human cancers. DMBA-induced experimental carcinogenesis might therefore be used as an ideal model to study the chemopreventive potential of medicinal plants and their active constituents. The evaluated results reported in DMBA induced carcinogenesis may assist the clinician in the diagnosis, prognosis and treatment monitoring of the cancer patients [7].

In the present investigation of toxic effects of DMBA there was a significant decrease in the body weights and increase in tumor size of rats when compared to negative control. Weight loss and tumor size observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was similar to that of cyclophosphamide (standard drug). Similar increase in loss of body weight by berberin (berberine is an isoquinoline alkaloid isolated from the roots and bark of several herbs including *Berberis vulgaris, Coptis chi-nensis, Hydrastis Canadensis* and *Berberis aquifolium*.) from earlier studies have been reported [17].There was a severe body weight loss observed at the experiment's end versus the control rats in previous study. The body weight increased and tumor size decreased due to treatment with luteolin (hydroxylated flavone derivative) and cyclophosphamide in DMBA-induced mammary tumors in rats [6]. On treatment with Kalpaamruthaa (a siddha formulation contain *Semecarpus anacardium* Linn., *Emblica officinalis* and honey), the gradual increase in body weight in earlier studies reported the counteractive property towards DMBA [18]. In mammary carcinoma bearing animals, there was a sharp drop in their body weight which may be due to the cancer cachexia. Cancer cachexia results in progressive loss of body weight, which is mainly accounted by wasting of host body compartments such as skeletal muscle and adipose tissue [19]. Similar inhibited tumor growth by methanolic extract of *Ganoderma lucidum* was observed in previous studies [10].

SOD acts as an anti-carcinogen inhibitor during initiation and promotion/transformation stages of carcinogenesis [6]. Superoxide radicals may be reduced by the enzyme superoxide dismutase to form H_2O_2 and oxygen. Catalase is an enzyme which converts H_2O_2 to neutral products O_2 and H_2O_2. The formation of Malondialdehyde is considered as an index of lipid peroxidation that causes cell injury. Elevation of Lipid Peroxides, as indicated by increased MDA was observed in breast cancer bearing animals. Significant increase in LPO in carcinogenic process may be due to abnormal levels of reactive oxygen species (ROS). ROS production in excess of cellular antioxidant capacity may result in damage to lipid, protein, RNA and DNA or other effects [20]. The stress caused due to DMBA in the present study showed a significant decrease in the activity of SOD and CAT while significant increase in lipid peroxidation in the mammary tissue, liver and kidney when compared to negative control. The antioxidant effect of extract was observed by significant increase in the activity of SOD and CAT while significant decrease in lipid peroxidation when compared to DMBA positive control. Similarly in previous study, the activities of SOD and CAT in mammary tissue were significantly reduced while lipid peroxides level were higher in cancer bearing animals but in taxol treated animals the activities of SOD and CAT were significantly increased when compared to cancer bearing animals. The drug (fungal taxol) treated and commercial drug treated (paclitaxel) rats showed decreased LPO levels when compared to DMBA administrated rats [20]. The naturally occurring free radical scavenger of the ethanolic extract in the present study lowered the MDA level suggesting reduced LPO. Due to the free radical scavenging property, helps to improve the antioxidants defense system and prevent the damage induced by free radicals. In earlier studies, CAT and SOD activity of liver and kidney was lower in animals with breast cancer, higher levels of CAT and SOD activity were recorded on treatment. The lipid peroxide levels were increased more in the breast cancer bearing animals; whereas, little reduction was found in the rats treated with luteolin as well as the cyclophosphamide individually treated groups of earlier studies [6]. Similar effects have been reported by ethanolic extract of *Symplocos racemosa* on SOD, CAT and LPO was observed in acute experimental liver injury induced by administration of DMBA [21]. Antioxidants exert various kinds of effects on rodents in addition to radical scavenging and/or decomposing activity. One of the most interesting effects is modification of chemical carcinogenesis. In general, antioxidant application simultaneously or prior to carcinogen treatment results in reduced tumor development [22].

Anbuselvam *et al.* [23] have demonstrated the protective effect of *Operculina turpethum* against DMBA-induced oxidative stress with reference to breast cancer in experimental rats. They suggested that the antioxidant activity of *Operculina turpethum* played a protective role against DMBA induced breast cancer. Kumaraguruparan *et al.* [23] have reported that the chemopreventive potential of black tea polyphenols in DMBAinduced mammary carcinogenesis is due to their modulating effect on xenobiotics metabolizing enzymes, oxidative stress, cell proliferation, apoptosis and angiogenesis. Although the exact mechanism of action of the ethanolic extract is unclear, its anti-lipid-peroxidative, antioxidant and modulating effect on detoxification cascade could play a possible role

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Table 1- Effect of Bridelia retusa stem bark extract (BSBE) on body weight and tumor size in DMBA treated rats.

Groups	Treatment (mg/kg/day)	Body weights (g)	Tumor size (mm)
Group I	Normal control	46.17+0.401	0.00+0.000
Group II	DMBA control	33.83±0.477 [a]	24.42±0.089 [a]
Group III	DMBA + CYC	38.84±0.523 [a,b]	6.40±0.091 [a,b]
Group IV	DMBA + BSBE (50)	34.34±0.334 [a,c]	7.78±0.066 [a,b]
Group V	DMBA + BSBE (100)	35.84±0.307 [a,b,c]	6.96±0.021 [a,b,c]
Group VI	DMBA + BSBE (150)	38.17±0.307 [a,b]	6.63±0.039 [a,b,c]

Superoxide dismutase expessed in units/mg proteins
Catalase expessed in μmoles of H_2O_2/min/mg proteins
[a] Significant (<0.05) when compared to group I.
[c] Significant (<0.05) when compared to group III.
Lipid peroxidation expessed in nmol MDA/min/mg protein
Values are mean ± SEM of 6 animals
[b] Significant (<0.05) when compared to group II.

Table 2- Antioxidant effect of Bridelia retusa stem bark extract (BSBE) on oxidative parameters in mammary tissue of DMBA treated rat.

Groups	Treatment (mg/kg/day)	Oxidative Stress Parameters		
		Enzymes		Byproducts
		SOD	CAT	LPO
Group I	Normal control	3.47±0.028	48.41±0.033	0.70±0.024
Group II	DMBA control	1.50+0.027 [a]	12.49+0.017 [a]	1.86+0.010 [a]
Group III	DMBA + CYC	3.56+0.005 [a,b]	34.38+0.024 [a,b]	1.26+0.017 [a,b]
Group IV	DMBA + BSBE (50)	2.61±0.022 [a,b,c]	27.55±0.008 [a,b,c]	1.74±0.004 [a,b]
Group V	DMBA + BSBE (100)	2.84±0.008 [a,b,c]	30.49±0.025 [a,b,c]	1.59±0.021 [a,b,c]
Group VI	DMBA + BSBE (150)	3.04+0.009 [a,b,c]	34.96+0.011 [a,b,c]	1.30+0.020 [a,b]

Superoxide dismutase expessed in units/mg proteins
Catalase expessed in μmoles of H_2O_2/min/mg proteins
[a] Significant (<0.05) when compared to group I.
[c] Significant (<0.05) when compared to group III.
Lipid peroxidation expessed in nmol MDA/min/mg protein
Values are mean ± SEM of 6 animals
[b] Significant (<0.05) when compared to group II.

Table 3-Antioxidant effect of Bridelia retusa stem bark extract (BSBE) oxidative parameters in the liver of DMBA treated rat.

Groups	Treatment (mg/kg/day)	Oxidative Stress Parameters		
		Enzymes		By products
		SOD	CAT	LPO
Group I	Normal control	6.14±0.007	51.40±0.033	0.55± 0.009
Group II	DMBA control	5.07+0.007[a]	28.16+0.009[a]	1.85+0.007[a]
Group III	DMBA + CYC	5.96+0.005[a,b]	45.37+0.027[a,b]	0.96+0.009[a,b]
Group IV	DMBA + BSBE (50)	5.57±0.043[a,b,c]	36.44±0.028[a,b,c]	0.94±0.004[a,b]
Group V	DMBA + BSBE (100)	5.84±0.008[a,b,c]	36.95±0.007[a,b,c]	0.93±0.002[a,b,c]
Group VI	DMBA + BSBE (150)	6.03+0.009[a,b]	37.56+0.007[a,b,c]	0.92+0.003[a,b,c]

Superoxide dismutase expessed in units/mg proteins
Catalase expessed in μmoles of H_2O_2/min/mg proteins
Lipid peroxidation expessed in nmol MDA/min/mg protein
Values are mean ± SEM of 6 animals
[a] Significant (<0.05) when compared to group I.
[b] Significant (<0.05) when compared to group II.
[c] Significant (<0.05) when compared to group III.

Table 4- Antioxidant effect of Bridelia retusa stem bark extract (BSBE) oxidative parameters in the kidney of DMBA treated rat.

Groups	Treatment (mg/kg/day)	Oxidative Stress Parameters		
		Enzymes		By products
		SOD	CAT	LPO
Group I	Normal control	4.24±0.007	46.64±0.003	0.75± 0.002
Group II	DMBA control	2.07+0.007[a]	30.54+0.012[a]	1.95+0.007[a]
Group III	DMBA + CYC	4.06+0.005[a,b]	44.38+0.027[a,b]	1.04+0.009[a,b]
Group IV	DMBA + BSBE (50)	3.03±0.005[a,b,c]	34.43±0.014[a,b,c]	0.98±0.002[a,b,c]
Group V	DMBA + BSBE (100)	3.07±0.003[a,b,c]	35.74±0.006[a,b,c]	0.95±0.003[a,b,c]
Group VI	DMBA + BSBE (150)	3.15+0.004[a,b,c]	36.86+0.008[a,b,c]	0.94+0.002[a,b,c]

Superoxide dismutase expessed in units/mg proteins
Catalase expessed in μmoles of H_2O_2/min/mg proteins
Lipid peroxidation expessed in nmol MDA/min/mg protein
Values are mean ± SEM of 6 animals
[a] Significant (<0.05) when compared to group I.
[b] Significant (<0.05) when compared to group II.
[c] Significant (<0.05) when compared to group III.