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Ameliorative effect of *Ananus comosus* peel on 7, 12 dimethylbenz(α) anthracene induced mammary carcinogenesis with reference to oxidative stresss

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ABSTRACT

Objective: To *Ananus comosus* (*A. comosus*), has demonstrated a wide variety of biological activities which make it a good plant source for the treatment of many oxidative stress mediated diseases. The present study was aimed to evaluate therapeutic potential by assaying the activities of oxidative stress parameters in 7, 12 dimethylbenz(α)anthracene induced breast cancer rats. **Methods:** Animals were divided into five groups of six animals. Group I served as control, group II induced mammary carcinogenesis by 7, 12 dimethylbenz(α)anthracene, group III and IV are treatment groups (plant extract 250 mg/kg body weight and standard drug 10 mg/kg body weight) and group V served as animals treated with plant extract alone. All the animals were sacrificed after 30 days treatment and breast tissues are used for the analysis of protein content, enzymic and non-enzymic antioxidants using standard protocols. **Results:** The oral administrations of ethanolic peel extract of *A. comosus* (250 mg/kg body weight) to breast cancer bearing rats for 30 days demonstrated a significant ($P<0.05$) increased in body weight, tissue weight, protein content, enzymatic and non enzymatic levels. The altered activities of lipid peroxidation and tumor weight in breast tissue of control and experimental cancer bearing rats were significantly ($P<0.05$) were reverted to near normal levels by the administration of ethanolic extract suggesting that the extract have quenching capacity against free radicals thereby exhibiting anticancer potential of *A. comosus* in breast cancer bearing rats. **Conclusions:** Thus, modulatory effects of *A. comosus* on attenuating the lipid peroxidation and upregulation of oxidative stress key enzymes like enzymatic and non-enzymatic antioxidants, protein content afford a pledge for widespread use for the treatment of breast cancer in the future.

1. Introduction

Breast cancer is one of the leading second most prevalent malignant tumors in the world among women and its incidence continue to increase every year. In India, it is the second most frequently occurring cancer, varying from 22 to 28 per 10 000 women and is estimated to comprise 19.8% of total female cancers[1]. Annually, 910 000 new patients are diagnosed with breast cancer and 376 000 women die from the disease. According to the National Cancer Registry, the frequency of breast cancer in Indian women varies from 7% to 18% as recorded in six major cancer institutes that include, Ahmedabad (17%), Mumbai (18%), Chennai (16%), Hyderabad (18%), Kolkata (11%) and Kanpur (7%)[2]. Exposure to environmental pollutants such as polycyclic aromatic hydrocarbon (PAH) is associated with the development of numerous cancers in humans[3]. The etiology of breast cancer is multifactorial and the risk factors include early menarche, late menopause, nuliparity, and late age at first birth, postmenopausal obesity, extended use of oral contraceptives, hormone replacement therapy, family history and previous benign breast disease[4].

Polycyclic aromatic hydrocarbons (PAHs) are products formed by incomplete combustion of organic matter. PAHs universally present in the atmosphere, tobacco smoke and food are the most important class of carcinogens implicated in the development of mammary carcinogenesis in humans[5]. Enzymatic activation of PAHs leads to the

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generation of active oxygen species such as peroxides and superoxide anion radicals, which induce oxidative stress in the form of lipid peroxidation. The 7, 12-dimethylbenz[a] anthracene (DMBA) which comes under PAHs and acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress[6] and it has been widely used in various mammary cancer chemopreventive studies[7].

Human body is equipped with various enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), ascorbic acid (Vitamin C), α-tocopherol (Vitamin E) which can neutralize the deleterious action of ROS and protect from cellular and molecular damage.

ROS increases the permeability of blood brain barrier, as well as inhibit the mitochondrial respiration[8]. Free radicals generated in the brain have also been reported to influence gene expression, subsequently affecting apoptosis and neuronal death[9]. All of which is intimately linked to the degenerative processes in most neurological diseases.

Antioxidants are a chemical substances produced from natural sources like fruits and vegetables which may protect the body against ROS toxicity either by preventing the formation of ROS or by bringing interruption in ROS attack, by scavenging the reactive metabolites or by converting them to less reactive molecules. The antioxidant capacity gives information about the duration while the activity describes the starting dynamics of antioxidant action. Therefore, the uses of antioxidants, both natural and synthetic are gaining wide importance in prevention of diseases[10].

Several plants tend to have antioxidant potential among that *Ananas comosus* (*A. comosus*)(L.) is belonging to the family Bromeliaceae and it is broadly cultivated in the tropical areas of the world. Pineapple has also been known for a number of beneficial biological activities like antioxidative, anticancer, anti-browning, anti-inflammatory and anti-platelet activities. The enzyme complex of *A. comosus* called bromelain is known for its clinical applications particularly modulation of tumor growth, blood coagulation and anti-inflammatory effect[11]. Pineapple has been extensively used in foods or for health benefits. Hence, the present investigation was to assess the enzymatic and non-enzymatic antioxidants in mammary cancer bearing animals and compare with the tamoxifen and ethanolic extract of *A. comosus* treated animals.

2 Materials and methods

2.1 Collection of plant material

Fresh pineapple plant was collected from Coimbatore, Tamil Nadu, India. The plant was authenticated by Dr. P. Sathyanarayanan, Botanical survey of India, TNAU Campus, Coimbatore and the voucher specimen No.BSI/SRC/5/23/2011/Tech-515. Fresh peel part of the sample was washed under running tap water, air dried, and then homogenized to fine powder and stored in airtight bottles.

2.2. Sample extraction

100 g of dried plant powder was extracted in 500 mL of ethanol in a water shaker for 72 h. Repeatedly extraction was done with the same solvent till clear colorless solvent is obtained. Obtained extract was evaporated to dryness by using a rotary vacuum evaporator at 40-50 ℃ and stored at 0-4 ℃ in an airtight container.

2.3. Chemicals

7, 12 Dimethylbenz(α)anthracene, oxidized glutathione and reduced glutathione were purchased from Sigma Chemical Company, USA. All the other chemicals used were of analytical grade.

2.4. Animals

Female Sprague Dawley rats weighing (180 ± 10) g were purchased from Karpagam University, Coimbatore and housed in plastic cages. The animals wee maintained under controlled environmental condition on alternative 12 h dark/light cycle. Commercial pelleted feed and water ad libitum were given to animals. All the experiments were carried out according to the guidelines recommended by the committee for the purpose of control and supervision of Experiments on Animals (CPCSEA) and approved by IAEC, Government of India for the use of Sprague dawley rats as an animal model for cancer activity.

2.5. Experimental setup

The animals were divided in to five groups of 6 animals each. Group I animals served as control, Group II animals were treated with 25 mg of DMBA in 1.0 mL olive oil by gastric incubation, to induce mammary cancer. After 90 d of tumor induction Group III and Group IV animals were treated with ethanolic extract of *A. comosus* (250 mg/kg body weight) and standard drug tamoxifen (20 mg/kg body weight) for 30 d. Group V animals were treated with ethanolic extract of *A. comosus* alone (250 mg/kg body weight) for 30 d.

2.6. Collection of mammary tissue

After the experimental period, the animals were sacrificed by cervical decapitation. Breast tissues were immediately excised. A 10% homogenate was prepared in 0.1 M Tris-HCl buffer pH 7.4 using Potter Elvehjem homogenizer with Teflon pestle. The tissue homogenates of breast were used for the following parameters.

2.7. Body weight and tumor weight

Body weight, mammary tissue weight and tumor weight was calculated according to the method given by Geran *et al*.,[12]. The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axes. The two short axes were measured with vernier caliper. The tumor weight was calculated by using the formula: Weight (g) =length (cm) × width^2 (cm)/2.

2.8. Biochemical analysis

The mammary tissue was used to analysis the various biochemical parameters are as follows. The protein content was determined according to the method of Lowry *et al*.,[13]. Superoxide dismutase was estimated by Das *et al*.,[14]. Catalase was assayed by the method of Sinha,[15]. Glutathione peroxidase was assayed by the method given by Rotruck *et al*.,[16]. Glutathione reductase was estimated by Beutler[17]. Glutathione-S transferase was measured by Mannervik[18]. Glucose 6-phosphate dehydrogenase was assayed by the method of Balinksy and Bernstein[19]. Total reduced glutathione was determined by Moron *et al*.,[20]. Ascorbic acid was assayed by the method of Omaye *et al*.,[21]. Vitamin E was estimated by the method of Rosenberg[22] and Lipid peroxidation was predictable according to the method given by Buege and Aust[23].

2.9. Statistical analysis

The results are expressed as Mean ± standard deviation (S.D). Difference between the groups was assessed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS 10.0 version software package for windows. The values were considered statistically significant if *P* value was less than 0.05 ($P<0.05$).

3. Results

The effect of *A. comosus* peel extract on mean value of body weight, mammary tissue weight and tumor weight in control and experimental animals were depicted in table 1 and 2. The body weight and tissue weight of mammary cancer induced group showed significantly depleted ($P<0.05$) when compared to group I animals. On the contradictory, administration with Ananus comosus and tamoxifen (standard drug) treated groups showed significantly increased body and tissue weight when compared with group II rats; whereas no significant changes were observed in plant extract treated alone group (group V) animals when compared to control animals.

Table 1

Effect of *A. comosus* on body weight and mammary gland weight of control and experimental animal.

Groups	Body weight		Mammary gland weight
	Initial	Final	
Group I	150.67±0.52^a	235.83±13.20^c	1.89±0.06^c
Group II	150.67±0.80^a	84.16±7.36^a	0.49±0.06^a
Group III	150.83±0.17^a	154.00±3.46^b	1.10±0.08^b
Group IV	150.33±0.31^a	166.67±5.28^b	1.61±0.11^{bc}
Group V	150.50±0.87^a	210.00±12.25^c	1.82±0.11^c

Values are expressed as Mean±SD for six animals. Values not sharing common Superscript letters (a-c) differ significantly at $P< 0.05$ (DMRT)

Table 2

Effect of *A. comosus* on tumor weight of control and experimental animals.

Groups	Tumor weight
Group I	0.00±0.00
Group II	6.15±0.32^a
Group III	1.18±0.26^b
Group IV	0.89±0.14^c
Group V	0.00±0.00

Values are expressed as Mean±SD for six animals. Values not sharing common superscript letters (a-c) differ significantly at $P< 0.05$ (DMRT).

On the other hand, significant increase in tumor weight was observed in cancer-induced group when compared with normal control animals. On treatment with plant extract and tamoxifen groups showed significant decreased in tumor weight when compared to group II animals. No significant changes occur in *A. comosus* alone treated group when compared with group I animals.

Figure 1 represents the activity of protein level in breast of control and experimental animals. Mammary tissue of group II cancer bearing animals shows a significant decrease in protein levels ($P<0.05$) when compared to group II control animals. However, the levels of protein were increased significantly in group III and IV animals ($P<0.05$) when compared with group II rats. No significant changes were found in drug control animals (group V) when compared to control animals.

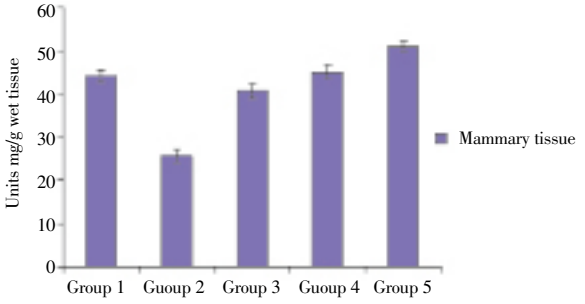

Figure 1. Effect of *A. comosus* on protein level in breast tissue of control and experimental animals.

The activities of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) in mammary tissue of control and experimental rats are shown in Table 3. The levels were significantly reduced in cancer bearing animals (group II). In drug treated animals (group III and IV) the activities were reverted to normal when compared to group II animals. The drug alone group were only plant extract was administered shows no significantly difference when compared to control rats.

Table 3

Effect of *A. comosus* on SOD, CAT and GPx levels in breast of control and experimental animals.

Groups	SOD	CAT	GPx
Group I	1.27±0.39^{c}	2.22±0.11^{c}	1.76±0.20^{c}
Group II	0.84±0.22^{a}	0.96±0.12^{a}	1.14±0.14^{a}
Group III	0.99±0.23^{b}	1.41±0.44^{b}	1.28±0.26^{b}
Group IV	1.25±0.44^{c}	1.83±0.43^{c}	1.53±0.15^{c}
Group V	1.29±0.28^{c}	2.16±0.13^{c}	1.77±0.18^{c}

Values are expressed as Mean±SD for six animals. Values not sharing common Superscript letters (a-c) differ significantly at $P<0.05$ (DMRT)

Units:

SOD- Inhibition of 50% nitrite formation/min/mg protein

CAT- μ mole of H_2O_2 consumed/min/mg protein

GPx- μ g of glutathione oxidized/min/mg protein

Changes in antioxidants in breast tissues of rats induced with DMBA showed significant ($P<0.05$) depleted in glutathione reductase, glutathione-s transferase and glucose-6 phosphate dehydrogenase. Interestingly, the treatment with *A. comosus* extract and standard drug has restored normal levels of glutathione reductase and glutathione-s transferase as well as significantly upregulated glucose-6 phosphate dehydrogenase activity when compared with group II animals. Treatment with plant extract alone exhibited no significant changes on the enzyme activities compared to normal control group (table 4).

Table 4

Effect of *A. comosus* on GR, GST and G6PD levels in breast of control and experimental animals.

Groups	GR	GST	G6PD
Group I	4.50±0.77^{c}	68.41±2.4^{c}	9.65±0.19^{b}
Group II	2.50±0.72^{a}	35.76±2.41^{a}	7.24±0.47^{a}
Group III	3.25±1.13^{b}	49.75±2.27^{b}	8.22±0.23^{ab}
Group IV	3.50±1.22^{b}	59.08±2.3^{b}	9.24±0.22^{b}
Group V	4.25±1.13^{c}	67.63±3.90^{c}	9.70±0.62^{b}

Values are expressed as Mean±SD for six animals. Values not sharing common superscript letters (a-c) differ significantly at $P<0.05$ (DMRT)

Units:

GR- μ mole of glutathione utilized/min/mg protein

GST- μ moles of CDNB - GSH conjugate formed/min/mg protein

G6PD- nmoles of NADP+ reduced/min/mg protein

Table 5 represents the toxic effect of DMBA was justified by the significant ($P<0.05$) decrease in the non-enzymatic antioxidants such as Total reduced glutathione, Vitamin C and Vitamin E when compared to negative control group. The antioxidant effect of extract and standard drug was observed by significant ($P<0.05$) increase in the activity of those levels when compared to DMBA positive control. No changes were occurred in extract alone treated group (group V) when comp-ared with group I animals.

Table 5

Effect of *A. comosus* on non-enzymic antioxidants levels in breast of control and experimental animals.

Groups	GSH	Vitamin C	Vitamin E
Group I	10.95±0.68^{c}	1.55±0.12^{c}	3.28±0.15^{c}
Group II	5.82±0.61^{a}	0.89±0.17^{a}	1.25±0.11^{a}
Group III	7.30±0.51^{b}	1.29±0.11^{b}	2.88±0.51^{b}
Group IV	8.79±0.61^{b}	1.50±0.27^{c}	2.91±0.33^{bc}
Group V	10.82±0.98^{c}	1.55±0.10^{c}	3.27±0.19^{c}

Values are expressed as Mean±SD for six animals. Values not sharing common superscript letters (a-c) differ significantly at $P< 0.05$ (DMRT)

Units:

GSH, Vitamin C, Vitamin E - μ g/mg protein

A significant increase in LPO level was noted in the DMBA induced mammary cancer bearing rats (group II). On contrary, the formation of malondialdehyde, measured as index of lipid peroxidation level, revealed a significant ($P<0.05$) depleted in plant extract and tamoxifen treated animals (group III and group IV) as compared to cancer bearing animals indicating that treatment with drugs reverted the abnormal changes. Administration of *A. comosus* alone treated animals (group V) showed no significant changes ($P<0.05$) in the level of LPO when compared with normal group animals (Figure 2).

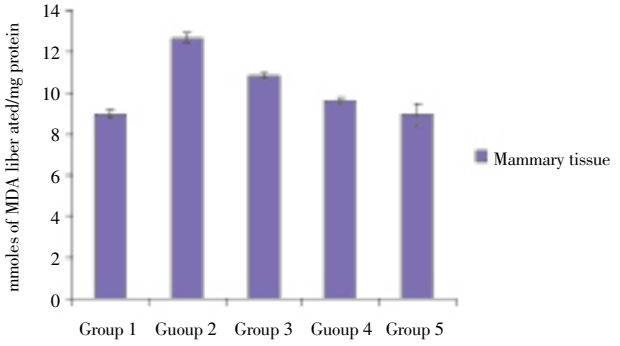

Figure 2. Effect of *A. comosus* on lipid peroxidation level in breast tissue of control and experimental animals.

4. Discussion

During the treatment of cancer, the chemicals used in the chemotherapy and radiation therapy may lead to the production of reactive oxygen species (ROS) which can damage healthy cells also[24]. Overindulgence generation

of reactive oxygen species, resulting in increased oxidative stress in the body of breast cancer patients which may affect treatment response and contribute to tumor recurrence[25]. Therefore, the importance of a diet rich in antioxidant food should be emphasized, not only as a way to protect against disease development and progression, but also to prevent the breast cancer, during and after treatment of supply of antioxidants[26]. Hence, an attempt to deals with that chemotherapeutic effect of *A. comosus* (Linn) Merr peel against the DMBA induced mammary gland carcinogenesis in Sprague dawley rats.

It is well known that during cancer condition excessive energy expenditure of the host, ultimately contributing to mechanisms that promote loss of weight. In this study, the food and intake of water in different experimental groups were found to be unaltered, because downgrading of nutritional supplement causing loss of body weight, tissue weight and increased in tumor volume. The total body weight and tissue weight of cancer bearing rats were deprived when compared with control groups clearly indicated that the change in energy metabolism takes place during tumor formation[27]. This is well in accordance with the study of Moselthy *et al*.,[28], changes in the energy metabolism results in the depletion of body weight in cancer condition. Earlier reports showed reduce in body weight and inhibition of tumor volume by administration of Bridelia retusa[29].

Proteins are macromolecular structures, which are reported to play significant role in the maintenance of cell structure and integrity. The disturbances in protein levels ultimately results in the metabolic imbalance and thus leads to severe deformities in cell, especially in cancer conditions. Tissues are an important site of protein metabolism and it has the highest rate of protein synthesis. Major protein mass of the organisms are severely affected in cancer cachexia. Protein waste implies the underlying metabolic imbalance, which is being expressed by an elevation in the apparent protein degradation rate with no change in the apparent synthesis rate. Reduced mammary protein level in DMBA induced cancer bearing animals because there is diminish in the recycling of amino acids in tumor conditions resulting in enhanced efflux of these amino acids from the tissues[30]. Thus, the host response to the tumor load results in tissue protein breakdown. Treatment with *A. comosus* might have prevented this protein breakdown thus bringing the protein levels to near normal condition and increases the total protein content by modulating protein synthesis, which inevitably proves that plant extract has some key modulatory effect by intervening in the synthesis of proteins and maintains its level in an appreciable amount. The results were concurrence with previous reports of Sivagnanam *et al*.,[31].

Superoxide dismutase (SOD) and Catalase (CAT) are the two major enzymes that are directly involved in the elimination of reactive oxygen species. The Superoxide dismutase is a first and foremost important line of antioxidant enzyme defense against reactive oxygen species[32]. It is present in cytosol and mitochondria, which in turn decline the superoxide anion to hydrogen peroxide and water. SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide that damages the membrane and biological structure.

Glutathione peroxidase (Gpx) is a cytoplasmic enzyme that catalyzes the detoxification of hydrogen peroxide to H_2O using the reducing equivalents of glutathione[33]. GPX is a seleno-enzyme two third of which (in liver) is present in the cytosol and one third in the mitochondria. It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. GPx is a selenium containing enzyme present in significant concentrations, detoxifies H_2O_2 through the oxidation of reduced glutathione[34].

The decreased activity of these enzymes in cancer bearing animals might be due to super saturation of SOD with a high concentration of ROS. The levels of free radicals overcome the saturation level due to increased lipid peroxides. The decline in SOD activity leads to downregulation of H_2O_2. Since H_2O_2 is the substrate for the enzymes CAT and GPx, they were also found to be decreased. Upon treatment with plant extract, the activity of SOD was restored to near normal level because it produces hydrogen peroxide, which in turn revert the activity of CAT and GPx to near normal levels. This observation of *A. comosus* treatment is supported by previous findings on mammary carcinoma where the reduced activities of SOD, CAT and GPx were replenished by Semicarpus anacardium[35].

Glutathione reductase (GR) is another major antioxidant enzyme that catalyzes the NADPH-dependent reduction of glutathione disulfide to glutathione, thus maintaining GSH levels in the cell[36]. This enzyme is critical in preventing high levels of oxidative stress because its activity can counteract oxidation. In addition GR is also important in the synthesis of DNA precursors as well as proton transport across membranes[37].

Glutathione-S transferase (GST) is an enzyme involved in antioxidant defense and involved in detoxification. It is used as a tumor marker in certain cancers such as breast and oral cancer. Alterations in GST levels in tumor tissue have also been reported by various studies. Most of the carcinogens are lipophilic and have a tendency to be converted into water soluble hydrophilic compounds that are easily removed from the body through the excretory system. This conversion or detoxification of these carcinogens is achieved through phase-II enzymes of which Glutathione S-transferase (GST) is an important constituent[38].

Glucose-6-phosphate dehydrogenase (G6PD) enzyme involved in the pentose phosphate pathway, especially important in red blood cell metabolism. It supplies reducing energy to cells by maintaining the level of the co-enzyme NADPH[39]. The entire antioxidant system, as well as other reductant- requiring processes, relies on an adequate supply of NADPH because it is the principal intracellular reductant for all cells. G6PD is the principle source of NADPH[40].

Significant elevation in the activity of GR following *A.*

comosus and standard drug treatment was evident, thereby helping the cell to maintain the basal level of GSH, which is important for many other GSH dependent detoxification reactions. The inauspicious alterations are reverted back in treatment groups which are due to the membrane stabilizing potential of secondary metabolites present in plant extract which may also significantly increase in GST and G6PD levels[41].

Total reduced glutathione serves as substrate for GPx, an enzyme that function to remove hydrogen peroxide, which oxidizes it to form reduced glutathione (GSSG). Vitamin E has an operative role in cancer, which acts as a good lipid soluble chain breaking antioxidant in the presence of the co-operative antioxidant like ascorbic acid, carotenoids. Activities on non enzymatic antioxidants such as total reduced glutathione (GSH), Vitamin C and Vitamin E have shown to ameliorate adverse effects associated with free radical damage to normal levels in cancer therapy and to decline the recurrence of mammary cancer[42].

Altered levels were seen in these antioxidants are not sufficient to counter higher reactive oxygen stress, leading to cellular and molecular damage thereby resulting in cell proliferation and malignant conversion[43]. In addition, decreased levels of GSH, Vitamin C and E in cancer bearing animals were indicator of oxidative stress leading to cellular damage and loss of functional integrity of cell membrane. Our investigation have also shown that decreased levels of non enzymatic antioxidants in DMBA induced animals indicates severity of tissue damage and altered activities of GSH, Vitamin C and Vitamin E in mammary tissue of cancer bearing rats which were brought back to near normal after treatment with *A. comosus*[44].

Malandialdehydes are produced from free radical attack on polyunsaturated fatty acids. It is known to play a role in lipid peroxidation and in the modulation of antioxidants during the progression of mammary cancer. This is naturally a balance between the amount of free radicals generated in the body and antioxidant defense system that scavenge them and thereby protect the body against pathogenesis[45]. It is evident from the results of Amin,[46] who reported the increased LPO levels in cancer bearing animals which were brought back to normal was observed in plant extract treated group animals indicating that ethanolic extract *A. comosus* peel may act as a potent free radical scavenger against DMBA induced mammary carcinogenesis.

In summary, it can be inferred that ethanolic extract of *A. comosus* peel positively modulated the antioxidant activity by quenching and detoxifying the free radicals induced by 7, 12-dimethylbenz(α)anthracene. Considering the antioxidant property of this extract, the bioactive compounds derived from the *A. comosus* peel can be supplemented with anticancer medicines in future. Further investigations on these extract on anticancer mechanisms are currently in progress.

Conflict of interest statement

We declare that we have no conflict of interest.

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