ABSTRACT

Introduction: Octyl gallate (OG) and gallic acid (GA) are molecules with significant biological activities and are widely present in several plants. The present study was aimed to determine the potentials of OG and GA isolated from the methanolic extract of *Terminalia bellarica* (*T. bellarica*) fruit against DMBA induced breast cancer in female Sprague-Dawley rats. Methods: The level of RBC, WBC and platelets in the normal, cancer and compound treated rats were analyzed using automated cell counter. Tumor markers CEA and CA 15.3 in the serum of all the experimental groups were analyzed using specific kits. Change in the tissue morphology and cell patterns in breast tissue was examined by histological studies. Results: All the blood parameters of study (RBC, WBC and platelets) were found to be in lowers levels compared to the normal. The tumor markers (CEA and CA 15.3) were found to be elevated in cancer controls. These changes in blood cell count and tumor markers in DMBA induced rats were found to be prevented and maintained in a normal range upon OG and GA supplementation. The compounds also inhibited the induction of breast cancer by DMBA showing normal tissue morphology and cell pattern when compared to the abnormal tissue morphology in cancer control. Conclusion: Hence the present study suggest OG and GA as effective molecules with chemopreventive property against breast cancer and these compounds with further studies can be used as chemopreventive agents against breast cancer.

KEYWORDS: chemoprevention, blood cells, cancer antigen, tissue morphology.
INTRODUCTION
Cancer is regarded as a complex genetic disease caused due to various abnormal conditions and also by carcinogens.[1] Among all, breast cancer is one of the most commonly occurring cancers both globally and also in India.[2] Approximately, 1.15 million new cases of breast cancer accounting for nearly one fourth of all malignancies are diagnosed among women worldwide.[3] There are also several therapies such as surgery, chemotherapy and radiotherapy, but all of these therapies mostly account for drastic side-effects or toxicities that result in high dropout rates and morbidity.[4] Hence generally it is necessary to explore effective agents that possess lesser side-effects to treat cancer. Plant or plant based medicines are examined from the past with the system of ayurvedha and medicinal herbs mediated therapeutic approaches are of current interest for their potential biological effect that is free from drastic side-effects.[5]

*T. bellarica* is known for its wide range of biological activity and is being used from the past as a therapeutic medicinal herb to treat human disorders.[6] Though all parts of the plant are of interest, study reports suggest that the plant fruit as an efficient source for various secondary metabolites with their potential biological activities.[7] OG and GA are two active principles reported for their presence in the methanolic extract of *T. bellarica* and these active principles are effective anti-diabetic agents.[8, 9] These compounds also act as mediators of apoptosis inducers as OG is an efficient anticancer agent by mediating apoptosis with reduction in ROS and the expression of Bcl-2 anti-apoptotic protein expression[10] and GA induces apoptosis in cancer cells with ROS-mediated cytotoxicity.[11,12] Reports also suggest that GA acid acts against oxidative stress and inflammations in human leukemia cells.[13]

With only few reports suggesting the effect of OG and GA as anticancer agents, the present study was designed to identify the effect of OG and GA isolated from the methanolic extract of *T. bellarica* fruit on the DMBA-induced female Sprague-Dawely rats. The compound induced effects on hematological parameters, cancer markers and histological studies were carried to explore their potential activities.

MATERIAL AND METHODS

*Plant material*
The fruits of *T. bellarica* were collected from the local market of Tiruchirappalli District, Tamil Nadu, India. The species was authenticated by Dr. Roseline, Department of Botany, Holy Cross College, Tiruchirappalli, India. The voucher specimen is being preserved in the
herbarium of the department. Fruits were dried under shade and mechanically reduced to moderate coarse powder and sieved.

**Isolation of octyl gallate & gallic acid**

The compounds OG and GA were isolated from the methanolic extract of *T. bellarica* fruit. Isolation of the compounds was carried out by the previous protocol reported from our laboratory.\[8, 9\]

**Animals**

Female Sprague Dawley rats aged between 50 and 55 days was procured from National Institute of Nutrition, Hyderabad and was housed in plastic cages. The animals were maintained under controlled environmental condition on alternative 12-h dark/light cycle. Commercial pelleted feed supplied by Sai enterprises Ltd., Chennai and water *ad libitum* were given to animals. This research work on Sprague Dawley rats was sanctioned and approved by the institutional animal ethical committee (IAEC No. 06/2013).

**Experimental setup**

The animals were divided into six groups of 6 animals each. Group I animals served as normal control, Group II were normal animals supplemented with OG (20mg/kg body weight (bw)), Group III were normal animals supplemented with GA (40mg/kg body weight (bw)), Group IV were animals treated with 20 mg of DMBA in 1 mL corn oil to induce breast cancer. Group III animals were treated with DMBA and simultaneously supplemented with OG (20 mg/kg body weight (bw)). Group IV animals were treated with DMBA and simultaneously supplemented with GA (40 mg/kg body weight (bw)). The overall induction and treatment period was 3 months for all groups. After the experimental period, the animals were sacrificed by decapitation. Blood and breast tissues were collected and used for further studies.

**Hematological parameters**

The hematological parameters – RBC, WBC and platelet counts were measured using an automated cell counter.
Estimation of tumor markers

The tumor markers Carcinoembryonic antigen (CEA) and cancer antigen 15.3 (CA 15.3) were estimated by the method of Chemiluminescence immune assay (CLIA) by the method of Coombes et al. (1981).

Histological studies

Breast tissues from the untreated and the experimental groups were blotted free of mucus, wash in physiological saline and fixed in Bouin-Hollande fixative for 74 h. after fixation, the tissues were washed in 70% alcohol for 2 or 3 days to remove the excess picric acid and dehydrated in graded series of alcohol. The tissues were cleared using xylene. The cleared tissues were infiltrated with molten paraffin at 58-60ºC through three changes (20-30 min) and finally embedded in paraffin. Sections of the tissues were obtained using rotary microtome and stained in Ehrlich’s hematoxylin with eosin as the counter stain. The slides were mounted using DPX mountant.

Statistical analysis

The data were analyzed using the SPSS Windows Students version software. For all the measurement, one-way ANOVA followed by Student–Newman–Keul’s (SNK) test was used to assess the statistical significance of difference between control and treated groups. A statistically significant difference was considered at the level of p < 0.05.

RESULTS

Effect of OG & GA on RBC, WBC and platelet counts

The potential effect of OG and GA on RBC, WBC and platelet counts in DMBA induced breast cancer rats was analyzed in the present study. Among all the groups under study investigation the levels of RBC, WBC and platelet counts in the DMBA induced breast cancer (Group IV) animals were found to be decreased when compared to their levels in the normal control (Group I) animals. The RBC, WBC and platelet counts in the OG and GA treated normal animals (Group II and III) were found to be maintained as in normal and in the OG and GA supplemented DMBA animals (Group V and VI) also, theses levels were maintained in normal range without any significant decline as in the DMBA induced cancer groups [Fig: 1, 2 and 3]. These results suggest the effect of OG and GA as a preventive agent of depletion of blood cells in the breast cancer induced animals.
Effect of OG & GA on carcinoembryonic antigen

The present study involved the analysis of tumor marker CEA, in the serum of normal, cancer and treated animal groups. CEA levels were found to be significantly elevated in the DMBA induced cancer groups (Group IV) when compared to their levels in the normal groups (Group I). A normal condition in the levels of CEA was observed in the compound treated normal animals (Group II and III). Upon OG and GA supplementation, the DMBA induced rats did not show any significant elevation in CEA levels indicating the efficacy of the compounds (OG & GA) in preventing tumor development [Fig: 4].

Effect of OG & GA on cancer antigen 15.3

Breast cancer specific tumor marker CA 15.3 levels in the normal, cancer induced and compound treated groups were determined in the present study. The cancer antigen was found to be in increased levels in the cancer control groups (Group IV), whereas very low levels of the antigen was found in the normal control animals (Group I). The normal level of the antigen was maintained upon OG and GA on the normal control groups [Fig: 5]. In DMBA induced rats treated with OG and GA, a normal level of the antigen was observed indicating the effect of the compounds in preventing the tumor development.

Effect of OG and GA on breast tissue

Histopathological studies were carried out on the breast tissues of all the groups studied in the present study [Fig: 6]. Microscopic images of the normal breast tissue (Group I) section characterized the normal tissues with uniformly arranged cells with normal pattern with proper orientation and morphology. Similar pattern of tissue morphology and arrangement patterns of the cells were observed in OG and GA treated normal animal (Group II and III) tissues. The DMBA induced cancer animal (Group IV) tissues were found with very diverse cell arrangements with no proper orientation as in the normal tissues. The tumor cells were arranged as glandular structures, as nest, or cords of various sizes or as solid sheets foci of necrosis in some areas. Microscopic margins of the cancers were found to be infiltrating, pushing, circumcised or mixed. DMBA induced animals supplemented with OG and GA (Group V and VI) exhibited no such change in the cell arrangement or morphology as in the cancer induced animals and retained a normal condition as that in the normal animal groups confirming the ability of both OG and GA to prevent the induction of breast cancer in female Sprague-Dawley rats.
Fig 1. Effect of OG & GA on RBC, WBC and platelets in Sprague-Dawley rats. Each bar represents mean ± SEM of 6 animals. Significance at \( p < 0.05 \). a - compared with normal, b - compared with DMBA induced.

Fig 2. Effect of OG & GA on RBC, WBC and platelets in Sprague-Dawley rats. Each bar represents mean ± SEM of 6 animals. Significance at \( p < 0.05 \). a - compared with normal, b - compared with DMBA induced.

Fig 3. Effect of OG & GA on platelets in Sprague-Dawley rats. Each bar represents mean ± SEM of 6 animals. Significance at \( p < 0.05 \). a - compared with normal, b - compared with DMBA induced.
**Fig 4.** Effect of OG & GA on CEA in Sprague-Dawley rats. Each bar represents mean ± SEM of 6 animals. Significance at $p < 0.05$. a - compared with normal, b - compared with DMBA induced.

**Fig 5.** Effect of OG & GA on CA 15.3 in Sprague-Dawley rats. Each bar represents mean ± SEM of 6 animals. Significance at $p < 0.05$. a - compared with normal, b - compared with DMBA induced.

**Fig 6.** Photomicrographs of histopathological changes in the mammary tissues of control and experimental animals on OG and GA treatment (Hematoxylin and eosin staining showed at 20x).
DISCUSSION

The present scenario is of great interest in search for novel drugs, especially of plant origin to develop effective drug molecules with potential activities. There are several studies reported suggesting the potential roles of plant based naturally occurring molecules as effective anticancer agents under various systems of analysis including animal models.\textsuperscript{[14]} The present study was also aimed to identify the efficacy of two active principles, OG and GA isolated from methanolic extract of \textit{T. bellarica} fruit on DMBA induced female Sprague-Dawley rats. In general, the number of circulating erythrocytes under various physiological conditions is maintained by the process of erythropoiesis and various pathological conditions including malignancy is associated with any alteration in this process.\textsuperscript{[15]} In the present study we examined the levels of RBC, WBC and platelets in normal, cancer and OG and GA treated experimental groups. Alterations in the normal levels of RBC, WBC and platelets were observed in the cancer groups and such type of alterations were prevented by both OG and GA supplementation in DMBA induced animals. These results are in accordance to earlier studies with similar alterations in blood cell counts and their reversal upon treatment with plant source.\textsuperscript{[16]}

CEA and CA 15.3 are the most widely used breast cancer markers.\textsuperscript{[17]} These antigens are found to be at different levels breast carcinoma based on the various stages of cancer.\textsuperscript{[18]} In the present study these two tumor markers were studies for their differential levels in the normal, cancer and treated groups of female Sprague-Dawley rats. CEA is a glycoprotein, generally expressed in fetal condition and its expression stops before birth. In normal adults only a minute levels of the antigen is observed, but several reports suggest that the level of CEA to be significantly elevated from normal levels in breast cancer conditions.\textsuperscript{[19-21]} In the present study also, the DMBA induced breast cancer animals showed elevated levels of CEA in their serum and both OG and GA prevented such elevations in the DMBA induced groups.

CA 15-3 is also a serum marker for breast cancer that generally helps in cell adhesion and their elevation to high levels is observed in cancer condition.\textsuperscript{[22]} An increase in the levels of CA15-3 was noticed in the DMBA induced animal groups of the present study compared to the normal. But both OG and GA prevented such elevations in these animal groups. The above results focused on the tumor marker is in accordance to the report suggesting the chemotherapeutic effect of tangeretin, a plant derive compound on DMBA induced mammary carcinoma in rats, where the compound effectively reduced the elevated levels of CEA and
CA 15.3 to a normal range of these antigens.\cite{23} The histopathological study result also confirms the development of tumor in DMBA induced animals and the chemoprevention of the occurrence of tumor development by the compounds OG and GA and these results are also in accordance to the report.

CONCLUSION

It is evident from the present study that both OG and GA isolated from the methanolic extract of *T. bellarica* fruit effectively acted as potential chemopreventive agents against breast cancer. Both the compounds supported the maintenance of normal range of RBC, WBC and platelets in the cancer induced female Sprague-Dawley rats. The compounds also acted as better preventers of elevation in the levels of tumor markers CEA and CA 15.3 in the cancer induced animals. The histopathological studies also confirmed the efficacy of OG and GA as better chemopreventive agents preventing the induction of breast cancer by DBMA, with the breast tissues showing normal morphological and cellular patterns. Hence, with further studies on the compounds both OG and GA can be used as phytomolecules to prevent the occurrence of breast cancer.

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REFERENCE