ANTIOXIDANT POTENTIALS OF ACTIVE PRINCIPLES FROM TERMINALIA BELLARICA FRUIT – AN IN-VITRO AND IN-VIVO APPROACH.

Manikkam Rajalakshmi* and Mary Selesty Sales

PG & Research Department of Biotechnology & Bioinformatics, Holy Cross College (Autonomous), Tiruchirappalli, India.

ABSTRACT

Introduction: Terminalia bellarica (T. bellarica) is a potential medicinal herb used from the ancient times to treat human diseases and disorders. The present study was designed to analyze the antioxidant efficacy of two active principles, octyl gallate (OG) and gallic acid (GA), isolated from the methanolic extract of T. bellarica fruit with reference to their action on free radicals, free radical scavengers using in vitro and in vivo approaches. Methods: Cells were cultured and incubated with OG & GA for 24 h at 37°C. The viability of the cells was measured using MTT assay. DMBA induced female Sprague-Dawley rats were used as animal models. Lipid peroxidation (TBARS), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) levels were determined with and without OG & GA treatment in both cell lines and breast and liver tissues.

Results: Cell viability analysis provided 40 μM OG and 80 μM GA as IC₅₀ concentrations of the compounds for both the cell lines. The lipid peroxidation levels estimated showed an increase in cancer groups and a significant decrease upon OG and GA treatment and a decrease in SOD, CAT and GPx levels in cancer groups which was found to be increased with OG and GA treatment. The above condition was noted in MCF-7, MDA-MB-231 cell lines and in breast and liver tissues of in-vivo models. Conclusion: The results obtained provides an insight for the anticancer and antioxidant potentials of OG and GA from T. bellarica on breast cancer models indicating its efficacy to be used for breast cancer therapies with further exploration on their mechanism of action.
KEYWORDS: Phytotherapy, antioxidant, apoptosis, free radicals, scavengers.

INTRODUCTION

Breast cancer is one among the most commonly occurring cancer worldwide and so in India. In India for each year there are more than 75,000 new cases of breast cancer reported.\(^1\) There is a regular generation of reactive oxygen species (ROS) in mammalian tissues with a normal balance between the free radicals and the antioxidant defense system. An imbalance between these two factors leads to oxidative stress resulting in various pathological conditions including cancer.\(^2\) Various study report validates the involvement of oxidative stress and lipid peroxidation in the development of tumor.\(^3\) In addition there are a set of antioxidant enzymes such as (SOD), catalase (CAT) and glutathione peroxidase (GPx) that acts as defense for ROS. Lack of required levels of these antioxidants also contribute for oxidative stress mediated occurrence of cancer.\(^4\)

Such alterations or imbalance in ROS and antioxidants can be balanced with the help of plants with antioxidant properties.\(^5\) Recent researches focus on plant based therapy as an efficient alternative therapy, which is more promising for all human ailments including cancer.\(^6\) *T. bellarica* is a widely used medicinal herb for its potential biological activity against numerous human disorders.\(^7\) Most of the traditional medicinal practice on the plant is with its fruit for its bioactive molecules and their potential activity.\(^8\) The methanolic fruit extract of the plant is identified to possess two active principles OG and GA with anti-diabetic property in STZ induced male Wistar rats.\(^9,10\) In general, OG also possess several therapeutic properties along with anticancer property via the oxidative stress induced apoptosis and anti-apoptotic protein expressions.\(^11\) Similarly, GA is also a potential stimulator of apoptosis and induces ROS-mediated cytotoxicity in cancer cell lines.\(^12,13\) GA is an effective antioxidant, anticancer and anti-inflammatory agent against human leukemia cells.\(^14\)

However there are very less explorations on the antioxidant and anticancer potentials of OG and GA isolated from the fruits of *T. bellarica* against breast cancer. Hence, the present study was designed to analyze the antioxidant and anticancer efficacy of these two active principles on MCF-7 and MDA-MB-231 cancer cell lines and DMBA induced female Sprague-Dawley rats as *in-vivo* models for breast cancer.
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. [9, 10]

Cell Line
Human breast cancer cell lines (MCF-7 & MDA-MB-231) were procured from ATCC and were cultured in DMEM culture medium with 10% FBS at 5% CO₂ and 37°C. Cells were passaged using trypsin-EDTA at 70-80% confluence.

Sample preparation
1mM stock solutions of the compounds (OG & GA) were prepared with DMSO. From the stock, the samples were prepared at different micromolar concentrations (10, 20, 30, 40 and 80) with serum free medium (SFM) for the test. The concentration of DMSO was aimed not to exceed 0.01%.

In-vitro analysis

Cell viability assay
Cells were seeded in 96-well plate (5×10³ cells/well) in medium containing 10% FBS and incubated for 24 h under 5% CO₂ at 37°C for attachment. The cells were then washed with 1X PBS, 100 µL of the prepared samples were added to the wells and 100 µL of SFM was added to the control well and incubated for 24 h. The medium was then removed and washed with PBS. Hundred microlitres of 0.5 mg/mL MTT solution was added to each well and incubated for 2-3h. After the incubation period 100 µL of DMSO was added for solubilization of cells and kept in dark for 1h. The intensity of the color developed was read at 570 nm using ELISA reader. The growth inhibition was then calculated as follows:

% of Cell viability = Absorbance of treated cells/Absorbance of control cells ×100
Five different observations were carried out and the IC$_{50}$ values were calculated.

**Treatment protocol**

Cells were seeded in 6-well plate (1.8 x 10$^6$ cells/well) in medium containing 10% FBS and incubated for 24 h under 5% CO$_2$ at 37°C for attachment. The cells were then washed with 1X PBS, OG (0 and 40 µM) and GA (0 and 80 µM) was added to the wells containing MCF-7 & MDA-MB-231 cells and incubated for 24 h. The medium was then removed and the cells were washed with PBS. Finally, the cells were collected by adding trypsin-EDTA, pelletized and the supernatant was used for further assays.

**Determination of TBARS (n mol/ml)**

Lipid peroxide levels were measured by the method developed by Ohkawa et al.$^{[15]}$ The assay mixture consisting of 0.1 mL of the cell/tissue lysate, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mol of 20% acetic acid (adjusted to pH 3.5 with NaOH) and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid was heated for 60 min at 95°C. Thereafter, the mixture was cooled and extracted with 5 mL of mixture of n-butanol and pyridine (15:1 volume/volume). After centrifugation at 4000 rpm for 10 min, the organic phase was assayed spectrophotometrically at 532 nm and the lipid peroxide levels TBARS was determined (n mol/ml).

**Determination of superoxide dismutase**

SOD level was estimated by the method described by Kakkar et al.$^{[16]}$ The assay mixture contained 0.1 mL of the cell/tissue lysate, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 mL phenazine methosulphate (186 µM), 0.3 mL of 300 µM nitroblue tetrazolium and 0.2 mL of NADH (750 µM). The reaction was started by the addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of 0.1 mL glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 mL of n-butanol, and the mixture was allowed to stand for 10 min. After centrifuging the mixture, the butanol layer was separated. Colour intensity of the chromogen in the butanol was measured at 560 nm spectrophotometrically, and the concentration of SOD was expressed as U/mg protein.

**Determination of catalase**

CAT level was measured by the method described by Aebi.$^{[17]}$ A volume of 0.1 mL of cell/tissue lysate was added to a cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). A reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H$_2$O$_2$. The
rate of decomposition of H$_2$O$_2$ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of CAT was expressed as μ. mol H$_2$O$_2$ metabolized/mg protein/min.

Assay of glutathione peroxidase (GPx)
To 0.2 mL of the cell/tissue lysate, 0.2 mL of 0.8 mM EDTA, 0.1 mL of sodium azide, 0.1 mL of 4 mM GSH, 0.1 mL H2O+ solution, 0.4 mL of 0.4 M phosphate buffer (pH 7.0) was added. The mixture was incubated at 37°C for 10 min and 0.5 mL of 10% TCA was added and centrifuged at 2000 rpm for 10 min. The supernatant was collected and 0.1 mL of 0.04% DTNB solution was added. Optical density was read at 420 against blank and the results were obtained.

In-vivo studies
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, breast and liver tissues were dissected out and tissue homogenates were prepared in 0.1 M Tris-HCl buffer pH 7.4 which was stored at 80°C, until its use for further analysis.
Biochemical analysis

The breast and liver tissue homogenates were used for estimation of antioxidants such as Superoxide dismutase (SOD)\textsuperscript{[16]}, Catalase (CAT)\textsuperscript{[17]}, Glutathione Peroxidase (GPx)\textsuperscript{[18]} and lipid peroxidation (LPO)\textsuperscript{[15]} levels in breast and liver tissue homogenates were estimated.

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 cell viability

The effect of OG and GA on the cell viability of the human breast cancer cell lines (MCF-7 and MDA-MB-231) was determined by MTT assay (Fig: 1). The percentage of cell viability was significantly decreased with increase in the concentration of the compounds. In both the cell lines 50% inhibition in cell viability was found at 40 µM OG and 80 µM GA. These concentrations were used for further in-vitro studies in exploring the antioxidant effect of the compounds.

Effect of OG & GA on TBARS level

TBARS is a byproduct of lipid peroxidation and most cancers report excess lipid peroxidation. TBARS level in both the cancer cell lines was found to be in an elevated level and a significant decrease in the levels was examined upon 40 µM OG and 80 µM GA treatment in both MCF-7 & MDA-MB-231 cell lines (Fig: 2). In-vivo experiments on breast tissue and liver (Fig: 3) reported a significant increase in lipid peroxidation level in cancer induced rats (Group-IV) when compared with control (Group-I) animals. No such significant increase in TBARS level was observed in OG and GA treated (Group V & VI) DMBA induced animals. Group II and III compound treated control animals showed normal level of lipid peroxidation. Hence, the results suggested that both OG & GA regulate a normal lipid peroxidation level in both in-vitro and in-vivo conditions.

Effect of OG & GA on SOD, CAT and GPx levels

SOD, CAT and GPx are antioxidant enzymes, usually found at lower levels in cancer conditions were examined. A significant increase in all the antioxidant enzymes (SOD, CAT
and GPx) levels were observed in OG and GA treated MCF-7 & MDA-MB-231 cell lines, when compared with the cancer control cells (Fig:4). The above in-vitro study results were also confirmed with the results of in-vivo studies on breast (Fig: 5) and liver tissues (Fig: 6). Group-IV cancer induced animals exhibited a significant decrease in the antioxidant levels when compared with Group-I control animals. Group-V & Group VI cancer induced animals treated with OG & GA respectively no such significant increase in the levels of antioxidant enzymes. Normal control animals treated with OG & GA also showed no alteration in antioxidant enzyme level of the normal. Therefore, it is suggested that a normal level of SOD, CAT and GPx levels were maintained in both in-vitro and in-vivo condition with OG and GA treatments.

![Graph 1](image1.png)

**Fig 1:** Effect of OG and GA on breast cancer cell viability. A) Effect of the compounds on MCF-7 cell line; B) Effect of the compounds on MDA-MB-231 cell line. Each bar represents the mean ± SEM of five independent observations with statistical significance at p <0.05.

![Graph 2](image2.png)

**Fig 2:** Effect of compounds on TBARS levels in MCF-7 & MDA-MB-231 cells. A) Effect of OG on lipid peroxidation B) Effect of GA on lipid peroxidation. Each bar represents the mean ± SEM of five independent observations. Statistical significance at p <0.05, a - compared with MCF-7 control; b - compared with MDA-MB-231 control.
Fig 3. Effect of OG and GA on lipid peroxidation in the breast and liver of control and experimental animals. Each value is expressed as mean ±SEM for six animals in each group. Statistical significance at p <0.05.

Fig 4: Effect of the compounds on antioxidants in MCF-7 cell line. A) Effect of OG on MCF-7 antioxidants B) Effect of GA on MCF-7 antioxidants. Units—SOD: U/mg protein. CAT: µ mol H₂O₂ metabolized/mg protein/min. GPx: µg of GSH utilized/min/mg protein. Each bar represents the mean ± SEM of five independent observations. Statistical significance* p <0.05.

Fig 5: Effect of the compounds on antioxidants in MDA-MB-231 cell line. A) Effect of OG on MDA-MB-231 antioxidants B) Effect of GA on MDA-MB-231 antioxidants. Units—SOD: U/mg protein. CAT: µ mol H₂O₂ metabolized/mg protein/min. GPx: µg of GSH utilized/min/mg protein. Each bar represents the mean ± SEM of five independent observations. Statistical significance* p <0.05.
Fig 6: Effect of the compounds on antioxidants in female Sprague-Dawley rat breast tissue. Units—SOD: U/mg protein. CAT: μ. mol H₂O₂ metabolized/mg protein/min. GPx: μg of GSH utilized/min/mg protein. Each value is expressed as mean ±SEM for six animals in each group. Statistical significance at p <0.05.

DISCUSSION
Plants and plant based medicines have been used for the well being of the mankind for their better compatibility and lesser side effects. Several plant extract or their secondary metabolites are found to possess anticancer activity against breast cancer cell lines.¹⁹ There are also studies suggesting that antioxidant efficacy of plant directly contribute for their antiproliferative potentials indicating the importance of antioxidants in cancer cell growth inhibition.²⁰ In the present study antioxidant and anticancer efficacy of the two active
principles OG & GA isolated from the methanolic extract of T. bellarica fruit on MCF-7 & MDA-MB-231 cell lines and their effects on female Sprague-Dawley rat breast and liver tissues were examined.

Increase in lipid peroxidation with excessive ROS production with a lack of balanced antioxidant defense leads to oxidative stress contributing cancer development.[21, 22] The present study also revealed an analogous condition with excessive increase in lipid peroxidation levels and a significant decrease in the antioxidant enzyme levels in both in vitro and in vivo experimental cancer groups, when compared to the normal control or untreated groups.

The activity of SOD is found to be low in many types of cancers thereby indicating a reduction in its defense against ROS.[23, 24] Low levels of SOD support for the accumulation of superoxide anions and these in turn act as second messengers that promote cancer cell proliferation.[25] Increase in the SOD concentration in cancer conditions contribute for the inhibition of cancer development.[26] The present study results are in accordance to the above conditions, where in both in-vitro and in-vivo conditions there was a reduction in the SOD levels in cancer controls, whereas both the compounds increased the SOD level in the cancer cell lines and no abnormal change in SOD levels was observed in compound treated animal groups.

Activation of CAT is generally suggested to inhibit cancer progression and is also reported on the control of breast cancer.[27] Studies on breast cancer patients have reported a significant decrease in the CAT activity compared to their levels in normal groups.[28, 29] Inhibition of cancer cell proliferation and their migration have been examined on the increase in the CAT activities.[30] Both the compounds in the present study also increased the CAT levels in the cancer cell lines and no change in their normal expression levels were notice in the compounds treated DMBA induced groups.

In general, GPx act as an inhibitor of cancer initiation and metastasis in most of the cancers.[31] Their activity prevents the induction of cancer via ROS mediated DNA damage or can also inhibit the growth of the tumors.[32] In the present study, there was a significant increase in the GPx levels in OG and GA treated cancer groups in which GPx levels were found to be low originally. All the compound treated groups were found to exhibit a normal range of lipid peroxidation and antioxidant levels. These alterations brought about a normal
balance between the antioxidant defense system and the ROS generation thereby inhibiting the cancer cell proliferation of MCF-7 and MDA-MB-231 cell lines.

CONCLUSION
From the study it is evident that both the active principles octyl gallate and gallic acid isolated from the methanol extract of T. bellarica fruit works well on both in-vitro cell line and vi-vivo animal models as an efficient antioxidant and anticancer agent. Both the compounds normalized the elevated levels of lipid peroxidation and the decreased antioxidant enzymes in both NCF-7 and MDA-MB-231 cell lines. Similarly the elevated levels of lipid peroxidation and decreased activities of antioxidant enzymes in breast and liver tissues of DMBA induced breast cancer female Sprague-Dawley rats were prevented upon treatment with the compounds. With this it is evident that the balance in the ROS and the corresponding antioxidant enzymes obtained by the plant bioactive molecules contributed for the inhibition in cancer cell proliferation and tumor development making the conditions to be as in normal control groups. Hence, these compounds can be used as potential breast cancer therapeutic molecules with further explorations.

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REFERENCE


