ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 10, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

ANTIOXIDANT POTENTIAL OF POLYSACCHARIDE ISOLATED FROM METHANOLIC EXTRACT OF *TINOSPORA CORDIFOLIA* STEM BARK

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Received: 27 March 2017, Revised and Accepted: 04 July 2018

ABSTRACT

Objective: Investigation of the antioxidant efficacy of a novel polysaccharide isolated from the methanolic extract of *Tinospora cordifolia* stem bark with reference to its action as free radical scavengers using *in vivo* and *in vitro* approaches was performed in the study.

Methods: Lipid peroxidation (TBARS), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels were determined with and without polysaccharide treatment in the cell lines and breast and liver tissues. 7, 12- Dimethylbenz (α) anthracene-induced albino Wistar rats were used as animal models.

Results: The IC_{50} concentration of polysaccharide was 100 µM for both the cell lines. The lipid peroxidation levels were decreased in both breast cancer cell lines and liver and breast tissues. Polysaccharide treatment showed remarkably low levels of TBARS in breast cancer cells, and a significant reduction in the activity of SOD, GPx, and CAT levels in cancer groups was found to be increased with polysaccharide treatment. The alteration was found to be induced in MCF-7 and MDA-MB-231 cell lines and in breast and liver tissues of *in vivo* models.

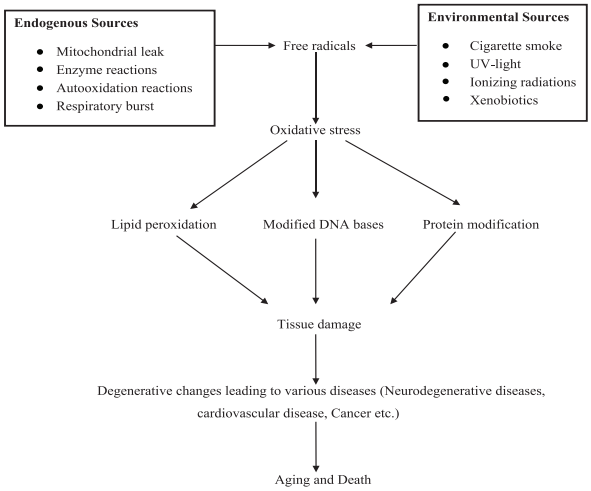
Conclusion: The results showed that the polysaccharide from *T. cordifolia* has antioxidant potential on human breast cancer cell lines and tissues with induction of apoptosis.

Keywords: Phytotherapy, Apoptosis, Antioxidant, Free radicals.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i10.19439

INTRODUCTION

Breast cancer is one of the most frequent cancers in women of the developed and developing countries. Oxygen free radicals are generated during various metabolic processes, and for the excess production of free radicals, an antioxidant defense mechanism is generated in the body [1]. Oxidative damage to cells and macromolecules is considered to be the cause of several diseases such as coronary heart disease, arthritis, and various neurodegenerative diseases including cancer [2]. Reactive oxygen species are involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis.



In addition, there are emerging evidences, suggesting that 7, 12- dimethylbenz (α) anthracene (DMBA) induces the production of reactive oxygen species (ROS) that result in lipid peroxidation, DNA damage, and depletion of cell's antioxidant defense systems [3]. Such alterations or imbalance in ROS and antioxidants can be balanced with the help of plants having antioxidant properties [4]. In recent days, plant-based therapies have emerged as a potential remedy for all human ailments including cancer [5]. Oxidative damage to biomolecules is caused due to the release of oxygen free radicals and oxidants which tend to generate oxidative stress [6,7]. The role of oxidative stress and lipid peroxidation in tumor development was studied *in vivo* [8]. There are certain detoxifying enzymes and small scavenger molecules at cellular levels acting as antioxidant defenses. The intracellular ROS-scavenging system includes superoxide dismutases (SOD), catalases (CAT), and glutathione peroxidase (GPx), which are potential free radical scavengers and are signified as cancer inhibitors at different stages of carcinogenesis [9]. *Tinospora cordifolia* is a widely used medicinal herb for its potential biological activity against numerous human disorders [10,11]. Various compounds explored from this plant have been recognized to possess several pharmacological activities [12], most importantly anti-diabetic [13] and anti-clastogenic [14] effects, and in such a way, the polysaccharide isolated from *T. cordifolia* has been experimentally reported to possess antidiabetic efficacy on type I diabetic animal models [15].

However, there are no proven evidences on the antioxidant potential of polysaccharide isolated from *T. cordifolia*, and hence, the present study was focused on identifying the antioxidant property of polysaccharide isolated from methanolic extract of *T. cordifolia* stem bark on MCF-7 and MDA-MB-231 cancer cell lines, and its *in vivo* activities on albino Wistar rat breast and liver tissues were also examined.

METHODS

Isolation of polysaccharide

Polysaccharide was isolated from the methanolic extract of *T. cordifolia* stem bark, by the protocol of Rajalakshmi *et al.* [15].

Cell line

Human breast cancer cell lines (MCF-7 and MDA-MB-231) were procured from ATCC and were cultured in DMEM culture medium with 10% FBS at 5% CO_2 and 37°C. Cells were passaged using trypsin EDTA at 70–80% confluence.

Compound preparation

1 mM stock solutions of the polysaccharide were prepared with dimethyl sulfoxide (DMSO). From the stock, the compounds were prepared at different micromolar concentrations (10, 20, 30, 40, 80, and 100 μM) with serum-free medium for the test. The concentration of DMSO was aimed not to exceed 0.01%.

In vitro studies

Treatment protocol

Cells were seeded in 6-well plate (1.8×10^6 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 ×1 PBS, and polysaccharide (100 μM) was added to the wells containing MCF-7 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

A lipid peroxide level was estimated by the method reported by Ohkawa *et al.* [16]. The assay mixture consisting of 0.1 mL of the cell and tissue lysate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5M 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 a mixture of n-butanol and pyridine (15:1 v/v). After centrifugation at 4000 rpm for 10 min, the organic phase was assayed spectrophotometrically at 532 nm and the lipid peroxide levels of TBARS were determined (mmol/mL).

Determination of SOD

SOD level was estimated by the method described by Kakkar *et al.* [17]. The assay mixture contained 0.1 mL of the cell and tissue lysate, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.025 M), 0.1 mL phenazine methosulfate (186 μM), 0.3 mL of 300 μM nitroblue tetrazolium, and 0.2 mL of NADH (780 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 1 mL glacial acetic acid. The reaction mixture was stirred vigorously with 4 ml of n-butanol, and the mixture was allowed to stand for 10 min. After centrifuging the mixture, the n-butanol layer was separated. Color intensity of the chromogen in n-butanol was measured at 520 nm spectrophotometrically, and the concentration of SOD was expressed as unit/min/mg of protein.

Determination of CAT

CAT level was measured by the method described by Aebi *et al.* [18]. A volume of 0.1 mL of cell and 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 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. The activity of CAT was expressed as μ. mol H_2O_2 consumed/min mg protein.

Assay of GPx

GPx level was measured by the method described by Rotruck *et al.* [19]. To 0.2 mL of the cell and tissue lysate, 0.2 mL of 0.4 mM EDTA, 0.1 mL of sodium azide, 0.2 mL of 2 mM GSH, and 0.1 mL $_H2_{O2}$ solution, 0.4 mL of 0.4M 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 to it. Optical density was read at 420 nm against blank, and the results were obtained. The activity of GPx was expressed as GSH utilized/min/mg of protein.

In vivo studies

Animals

Female albino Wistar rats aged between 50 and 55 days were procured from Tamil Nadu Veterinary Sciences, Madhavaram. The animals were maintained under controlled environmental conditions 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 albino Wistar rats was sanctioned and approved by the Institutional Animal Ethical Committee (IAEC No. 07/2013).

Experimental setup

The animals were divided into five groups of five animals each. Group I animals served as normal control, Group II was normal animals supplemented with polysaccharide (20 mg/kg body weight [bwt]), Group III was animals treated with 20 mg of DMBA in 1 mL corn oil to induce breast cancer, Group IV animals were treated with DMBA and simultaneously supplemented with polysaccharide (20 mg/kg bwt), and Group V animals were treated with DMBA and simultaneously supplemented with paclitaxel (1 mg/kg bwt). 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.

Antioxidant enzyme analysis

The breast and liver tissue homogenates were used for the estimation of antioxidant levels such as SOD [17], CAT [18], GPx [19], and lipid peroxidation (LPO) [16].

Statistical analysis

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

RESULTS

Effect of polysaccharide on TBARS level

Analysis of TBARS is the widely used method for the evaluation of lipid peroxidation. TBARS level in breast cancer cell lines was found to be altered, and a significant decrease in the levels was examined on treatment with 100 μM polysaccharide in both MCF-7 and MDA-MB-231 cell lines (Fig. 1). *In vivo* experiments on breast and liver tissues (Fig. 2) observed a significant increase in lipid peroxidation level in DMBA-induced rats (Group III) when compared to normal (Group I) animals. No such significant increase in TBARS level was observed in polysaccharide-supplemented DMBA-induced animals (Group IV). Group I and Group V (standard drug-paclitaxel supplemented DMBA-induced animals) showed normal level of lipid peroxidation. Hence, the results suggested that the polysaccharide regulates a normal lipid peroxidation level in both *in vitro* and *in vivo* conditions.

Effect of polysaccharide on SOD, CAT, and GPx levels

Variation in the activities of antioxidant enzymes such as SOD, CAT, and GPx that is usually found at lower levels in cancer conditions was examined. A significant increase in the activity of antioxidant enzymes was noted on polysaccharide-treated MCF-7 and MDA-MB-231 cell lines, when compared with the cancer control cells (Fig. 3). It is also proved through the *in vivo* studies on the breast (Fig. 4) and liver tissues (Fig. 5). Similarly, increase in the activity of these antioxidant enzymes was identified in polysaccharide-treated DMBA-induced animals and it was maintained near to normal, whereas DMBA-induced animals had a

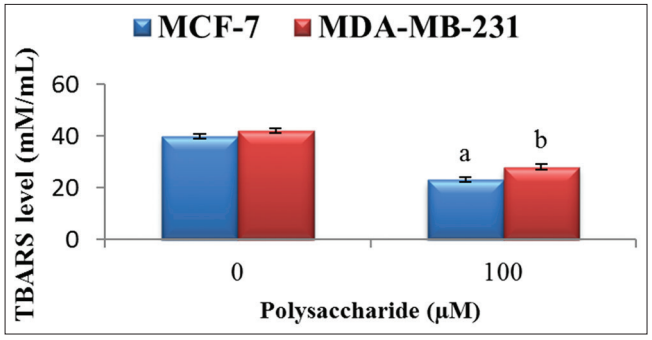


Fig. 1: Effect of polysaccharide on TBARS level of MCF-7 and MDA-MB-231 cells. Each bar represents the mean ± SEM of five independent observations. Statistical significance at p<0.05, (a) compared with MCF-7 control, (b) compared with MDA-MB-231 control

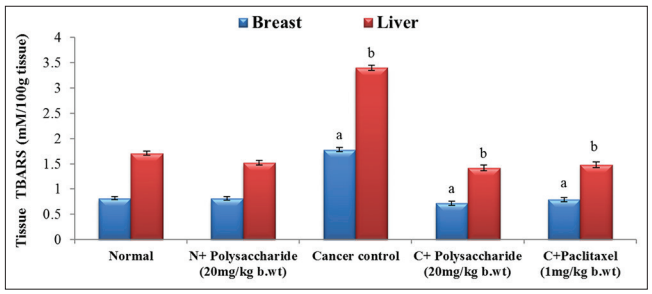


Fig. 2: Effect of polysaccharide on lipid peroxidation in the breast and liver tissues of control and experimental animals. Each value is expressed as mean ± SEM for five animals in each group. Statistical significance at p<0.05, (a) compared with breast control, (b) compared with liver control

reduction in the level of these enzymes. Therefore, it is suggested that normal levels of SOD, CAT, and GPx levels were maintained in both *in vitro* and *in vivo* conditions with polysaccharide treatment.

DISCUSSION

Natural antioxidants are characterized by their ability to scavenge free radicals. Medicines from herbal resources are preferred over the synthetic drugs for their better bioavailability and minimal side effects [20]. Plants that possess anticancer activity are also found to have a potential antioxidant activity, which is correlated with the cytotoxic efficacy of the molecules [21]. Many studies have found that the antioxidant property of a plant has a potential role in inhibiting the growth of cancer cells. In this study, the antioxidant potential of the polysaccharide isolated from the methanolic extract of *T. cordifolia* stem bark was studied on MCF-7 and MDA-MB-231 cell lines and on female albino Wistar rat breast and liver tissues.

Oxidative stress, which causes the development of cancer, is created by various underlying factors which include the excessive production of ROS that is responsible for the high level of lipid peroxidation and a deficit in antioxidant balance [22,23]. Antioxidant-rich plants are capable to inhibit the growth of several cancer cell lines, including breast cancer [24]. From the present study, it is found that, both in the *in-vitro* and *in-vivo* conditions, the level of lipid peroxidation was decreased and the level of antioxidant enzymes was increased in the polysaccharide-treated group of animals.

The SOD activity was very low in almost all types of cancer which indicates the reduction in the ROS defending mechanisms, and this low level of SOD leads to the increase of superoxide anions which in turn promotes cancer cell proliferation by acting as a second messenger. Inhibition of the development of cancer can be brought about by the increased level of SOD. The results of this study proved that the polysaccharide treatment increased the level of SOD in the cancer cell lines.

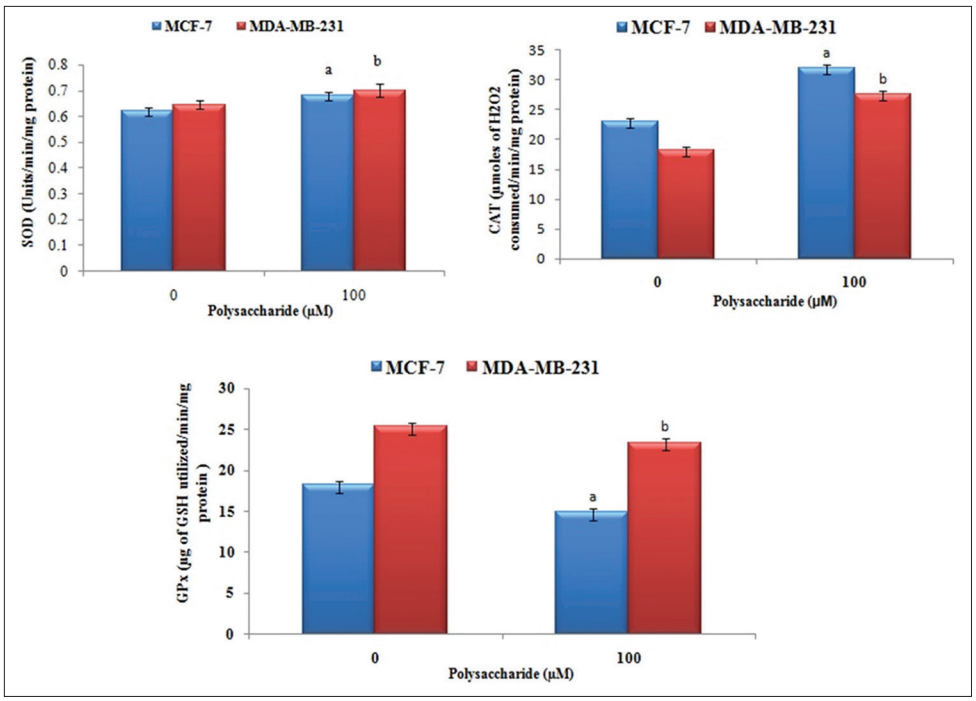


Fig. 3: Effect of the polysaccharide on antioxidants in MCF-7 and MDA-MB-231 cells. Units SOD: Unit/min/mg protein. CAT: µ.mol H2O2 consumed/min mg protein. GPx: µg of GSH utilized/min/mg protein. Each bar represents the mean ± SEM of five independent observations. Statistical significance at p<0.05, (a) compared with MCF-7 control, (b) compared with MDA-MB-231 control

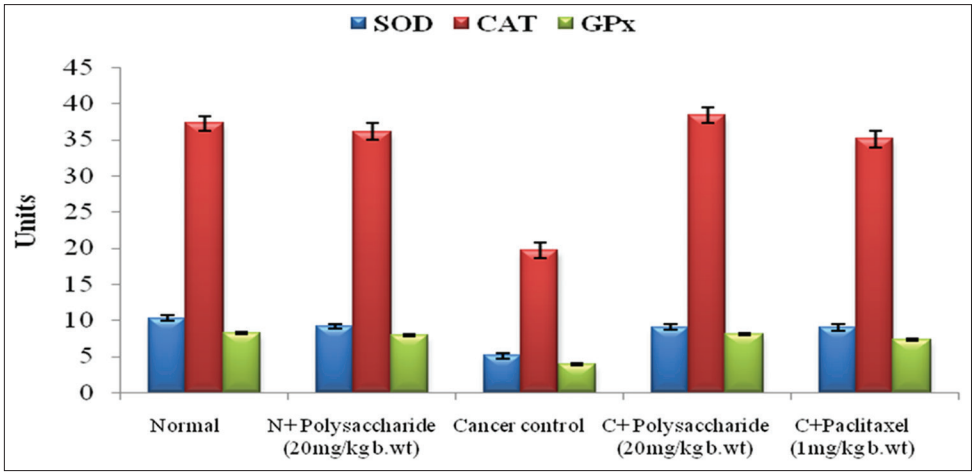


Fig. 4: Effect of the polysaccharide on antioxidants in female albino Wistar rat breast tissue. Units - SOD: Unit/min/mg protein. CAT: µ.mol H2O2 consumed/min mg protein. GPx: µg of GSH utilized/min/mg protein. Each value is expressed as mean ± SEM of five animals in each group

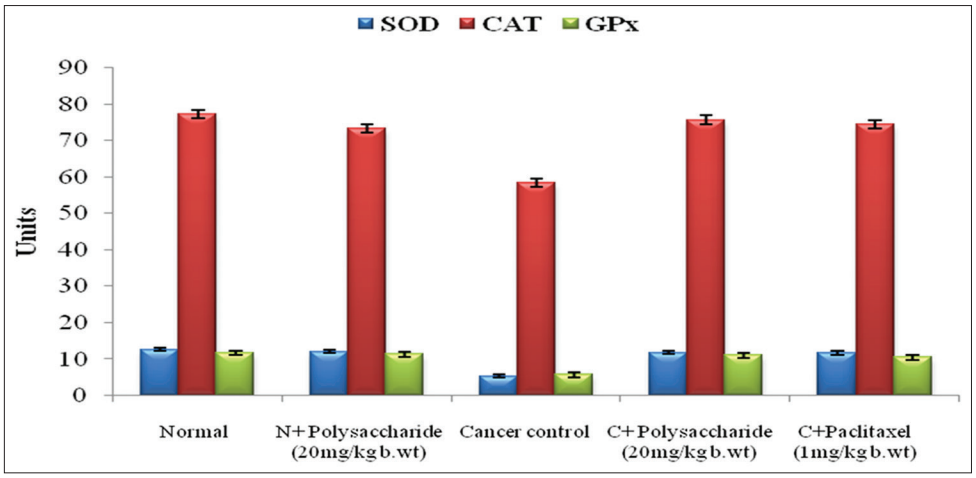


Fig. 5: Effect of the polysaccharide on antioxidants in female albino Wistar rat liver tissue. Units - SOD: Unit/min/mg protein. CAT: µ.mol H2O2 consumed/min mg protein. GPx: µg of GSH utilized/min/mg protein. Each value is expressed as mean ± SEM of five animals in each group

An anticancer agent with antioxidant activities balances the ROS levels, inhibits further proliferation of cancer cells, and allows apoptosis to occur. Besides, polysaccharide-treated MCF-7 and MDA-MB-231 cells enhanced the levels of the antioxidant enzymes and thereby decreased the oxidative stress. Increase in SOD and CAT is reported to inhibit tumor progression with less proliferation and migration of the cancer cells [25,26]. In the present study, there was a significant increase in the CAT levels in the polysaccharide-treated cancer cell lines when compared to DMBA-induced cancer groups.

GPx inhibits cancer initiation and metastasis in many of the cancers [27]. This may be due to the reduction of ROS-mediated DNA damage or mutations that induce carcinogenesis. Thus, the increase in the antioxidant enzymes activity is attributed to the induction of cell apoptosis. In this study, GPx levels in the polysaccharide-treated cancer groups were increased which were initially low.

Thus, the polysaccharide-treated groups exhibited normal range of lipid peroxidation and antioxidant levels. These alterations maintained 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

Thus, the present study proved that the polysaccharide isolated from the methanolic extract of *T. cordifolia* stem bark has an efficient antioxidant property on both *in vitro* cell lines and *in vivo* animal models. Elevated levels of lipid peroxidation and decreased activities of antioxidant enzymes in both MCF-7 and MDA-MB-231 cell lines and in breast and liver tissues of DMBA-induced breast cancer female albino Wistar rats were prevented on treatment with the compound. Thus, it is evident that the abnormality with ROS and antioxidant enzymes was normalized by the polysaccharide which subsequently resulted in the inhibition of cancer. Hence, the novel polysaccharide can be used as an antioxidant agent with further explorations.

ACKNOWLEDGMENT

The work was supported by the grant from the University Grants Commission (UGC) (F. No. MRP- 5094/14 (SERO/UGC) and DBT-BIF, Ministry of Science and Technology, Government of India, New Delhi, for infrastructure facility.

AUTHOR'S CONTRIBUTION

Research guidance - Manikkam Rajalakshmi. Laboratory and experimental work and writing the article - Antony Ludas. Manuscript writing - Sabapathy Indu. Laboratory work - Sekar Hinduja. Laboratory work - Anthonisamy Kumari Nirmala.

CONFLICTS OF INTEREST

All authors have none to declare.

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