

# Orthosiphon pallidus, a Potential Treatment for Patients with Breast Cancer

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## Key Words

*Orthosiphon pallidus*, total phenol content (TPC), total flavonoid content (TFC), antioxidant activity, cytotoxicity assay.

## Abstract

**Objective:** *Orthosiphon pallidus* (*O. pallidus*), which belongs to the *Lamiaceae* family, is a popular garden plant that is widely used for the treatment of various diseases, such as urinary lithiasis, fever, hepatitis, cancer and jaundice. The objective of the present work was to investigate the antioxidant free-radical scavenging and the anticancer activities of *O. pallidus* against human breast-cancer cell lines.

**Methods:** The antioxidant activity of *Orthosiphon pallidus* aqueous extract (OPAE) was investigated using different models, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) models, as were the Fe<sup>+</sup> chelation, the hydroxyl radical and superoxide radical scavenging, and total reducing power activities. The anticancer activities of the extract were determined by using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) and the sulforhodamine (SRB) assays on the MCF-7 and the MDA-MB-231 cancer cell lines.

**Results:** The aqueous *Orthosiphon pallidus* extract showed potent activity in *in-vitro* models. It significantly inhibited the scavenging of hydroxyl and superoxide radicals, but induced a remarkable Fe<sup>+</sup> chelation activity. For both cell lines, the percent cytotoxicity was found to increase steadily with increasing OPAE concentration up to 240 µg/mL.

**Conclusion:** These results suggest that *Orthosiphon pallidus* has excellent antioxidant, antimicrobial, and anticancer activities against human breast-cancer cell lines.

## 1. Introduction

Medicinal phytoconstituents or herbal plants are used as folk medicines throughout the world. The flora is enriched with a variety of pharmacological activities, such as antimicrobial, anticancer, antioxidant, anti-inflammatory, antiulcer, antiviral, and many more activities, for the treatment of patients with various ailments. Free radicals cause harmful oxidative stress, which can cause serious human diseases [1]. Most free radicals, such as hydroxyl radicals, reactive oxygen species (ROS), hydrogen peroxide, and superoxide anions, play a vital role in preventing damage to tissues in living organisms and are used in practice to treat patients with ailments. Plants are widely used in modern medicine, and they provide a great number of phytoconstituents that are used as drugs [2, 3]. Natural products are substances derived from natural sources and possess biological activities. These products are used and are being implemented as alternative deliv-

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ery systems for treatments of patients with various disorders [4]. Plant-based antimicrobials are eco-friendly and safer to use. When the vast potential of medicinal plants as antibacterial, antifungal, and antifungal agents is taken into account, the need for systematic studies to further their applications is clear. Today, phytochemicals, mainly antioxidants, are widely used against carcinogenesis [5]. The World Health Organization (WHO) estimates that more than 80% of the population rely on traditional medicine and that most herbal products are used for primary healthcare. Furthermore, new techniques now exist for the evaluation and the standardization of phytoproducts, and those techniques can improve the quality of such studies [6].

Worldwide, cancer, which can affect any part of the body, is spreading; it is considered to have the third highest mortality rate, being preceded by only infectious and cardiovascular diseases. Moreover, high toxicity and multi-drug resistance hinder the complete treatment of patients with cancer. For those reasons, many plant parts and phytoconstituents are being

used to treat patients with malignant and non-malignant cancers, and *in-vitro* cytotoxicity studies using MTT (3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide) and SRB (sulforhodamine B) have been done to determine cell viabilities and to provide the responses of different cancer cell lines to various plant parts and phytoconstituents [7].

*O. pallidus* (*Lamiaceae*) is a medicinal herbaceous shrub that is widely distributed in South East Asia and is used to treat patients with different diseases, such as fever, hepatitis, edema, jaundice, rheumatism, etc. Previous research on *Orthosiphon* (*O. stamineus*) established that the genus is highly rich in polyphenolic compounds. However, in the current study, we focus on aqueous extracts of *O. pallidus* because to date, even though detailed information on the antioxidant activities and the cytotoxicities of ethanolic and methanolic extract of this medicinal herb have been reported, its antioxidant or anticancer activities have not [8, 9]. Thus, the objective of the present work was to investigate the antioxidant free radical scavenging and the anticancer activities of *Orthosiphon pallidus* aqueous extract (OPAE) at various concentrations against cancer cells from two human breast-cancer cell lines.

## 2. Materials and Methods

The plant *O. pallidus* was procured from the region of Pratapgarh, Uttar Pradesh, India. The plant was identified and authenticated by Dr. B. K. Shukla, Scientist-D, Botanical Survey of India, Central Regional Centre, Allahabad, Uttar Pradesh, India. The freshly collected whole plants were air dried and then extracted in aqueous solvent by using Soxhlet for 8-10 h at 50-55°C. Whatman filter paper No.1 was used to filter the supernatant, which was then concentrated under reduced pressure (vacuum) at 44±1°C in a rotavapour apparatus, followed by lyophilization. The final powder was stored at 22°C.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid (ferrozine), nitro blue tetrazolium (NBT), phenazine methosul-

fate (PMS), nicotinamide adenine dinucleotide (NADH), MTT, fetal bovine serum (FBS), phosphate buffered saline (PBS), curcumin, Dulbecco's modified Eagle's medium (DMEM), catechin, ferrous chloride, gallic acid, ethylene diamine tetra acetic acid (EDTA), and Griess reagent were purchased from Sigma chemicals (USA). ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) was obtained from Roche Diagnostics Mannheim, Germany. Ferrozine was purchased from Himedia laboratories Pvt. Ltd, Mumbai, India. Propanol, aluminium and dimethyl sulfoxide (DMSO) were obtained as gift samples from E. Merck Ltd., Mumbai, India. Butylated hydroxyl toluene (BHT), glucose, trichloroacetic acid (TCA), acetic acid, antibiotics, ascorbic acid, and Folin-Ciocalteu reagent were received from Sisco Research Laboratories, Kolkata, India. The other reagents, chemicals, and solvents required for this study were of standard analytical grade.

Human breast-cancer cell lines, MCF-7 and MDA-MB-231, were used for the cytotoxicity study. Stock cells of the MCF-7 and MDA-MB-231 cell lines were cultured in DMEM. To the medium was added 10% inactivated FBS, amphotericin B (5 mg/mL), penicillin (100 IU/mL), and streptomycin (100 mg/mL) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The cells were then dissociated with 0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS (TPVG solution). The stock cultures were grown in culture flasks (25 cm<sup>2</sup>). The experiments were conducted in 96 microtitre plates. Both cell lines, MCF-7 and MDA-MB-231 cancer cells, were used in this study to confirm the efficacy of the OPAE against them.

In the cytotoxicity studies, each weighed test drug was dissolved separately in DMSO, to which DMEM supplemented with 2% inactivated FBS was added to get a stock solution with a 1-mg/mL concentration, after which the solution was sterilized by using filtration. Twofold serial dilutions were used to carry out the cytotoxicity studies. The Folin-Ciocalteu [FC] reagent method was used to estimate the total phenol content (TPC) [10]. Gallic acid was used as standard to obtain the calibration curve. The extract was evaluated using triplicate measurements, and the results were expressed in (mg GAE/100 g), i.e., mg of gallic acid equivalents per 100 gram of plant extract. The total flavonoid content (TFC) was estimated by using a spectrophotometric method [2], and the absorbance determined at 430 nm was evaluated for the samples, with quercetin being used as the standard for the calibration curve. The extract was evaluated using triplicate measurements, and the results were expressed as mg of quercetin equivalents per/100 gram of plant extract.

All potential antioxidant activities were expressed as IC<sub>50</sub> (The concentration of test extract needed to cause 50% of the effect). The free-radical scavenging activity of the plant extract was assessed by using different *in-vitro* methods, namely, the activities of the (DPPH), ABTS, hydroxyl, and superoxide radicals, the metal chelating activity, the total reducing power, etc. Gallic acid (GA), ascorbic acid (AA), BHT, EDTA, catechin, and curcumin, which are well-known standard anti-oxidant compounds, were used as standards. The antioxidant activity of the OPAE was determined in terms of hydrogen donating or radical scavenging ability by using stable DPPH [11, 12]. Gallic acid and

ascorbic acid were the reagents used as standard drugs. Different concentrations (120, 100, 80, 60, 40, 20 µg/mL) of the extract were added to a 0.1-mM aqueous solution of DPPH (3 mL). The mixture was shaken and allowed to stand in a dark room for 90 min. The absorbance was measured at 517 nm, and all the samples were assessed in triplicate. The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

where  $A_0$  is the absorbance of the control and  $A_1$  is the absorbance in the presence of the OPAGE. This activity was expressed in terms of the  $IC_{50}$ , i.e., the concentration of the sample required to inhibit 50% of the effect induced by DPPH.

The radical scavenging activity of ABTS was assessed based on the decolorization of a stable free-radical cation by using the 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) method [13, 14]. Triplicate determinations were made at each dilution of the standard (GA or BHT), and the percentage inhibition was calculated against the blank absorbance at 734 nm by using a UV spectrophotometer (UV-1800, Shimadzu, Japan). The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

The hydroxyl (OH) radical scavenging activity of the OPAGE was determined by using the deoxyribose method, and the result was compared with that for catechin [10, 15]. The OH radical scavenging activity of the aqueous extract was determined by measuring the competition between deoxyribose and the compounds that produce OH radicals from the  $Fe^{3+}$ /ascorbate/EDTA/ $H_2O_2$  system. The absorbance of the solution was measured at 532 nm by using a spectrophotometer. The percent inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

The superoxide radical scavenging activity was measured by using the reduction in the slight modification caused by NBT [16, 17]. The non-enzymatic PMS/NADH system caused reductions of the PMS, NADH, and oxygen. In this assay, the superoxide anion was generated in phosphate buffer (100 mM, pH 7.4) containing 0.70 mL of NBT (300 µM) solution, 0.70 mL of NADH (936 µM) solution, and 0.3 mL of different concentrations of the plant extract. The reaction was initiated by adding 0.70 mL of PMS (120 µM) solution to the mixture. After 10 min of incubation at room temperature, the absorbance at 562 nm was measured using a spectrophotometer, and the percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

The ferrous ion ( $Fe^{2+}$ ) metal chelating activity was measured using ferrozine with a slightly modified standard method, and the result was compared with that of the EDTA standard compound [10, 18]. Briefly, different concentrations (100 - 600 µg/mL) of plant extract were added to a solution of 1-mM  $FeCl_2$  (0.05 ml). The reaction was initiated by the addition of 1- mM ferrozine (0.1 mL); then, 1 ml of that solution was added to an aqueous solvent. The mixture was shaken well and kept at room temperature for 15 min. After incubation, the absorbance of the solution was measured at 562 nm. All the tests were performed in triplicate. The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

The total reducing power of the OPAGE was determined according to the method slide modification [19, 20]. Different concentrations of the extracts (20 - 120 µg/mL) in corresponding solvents were mixed with phosphate buffer (1.5 mL) and potassium ferricyanide (1.5 mL). This mixture was kept at 50°C in a water bath for 30 minutes. After incubation, 1.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 2500 rpm for 25 minutes whenever necessary. The upper layer of the solution (1.5 mL) was mixed with distilled water (1.5 mL) and a freshly prepared ferric-chloride solution (0.5 mL). The absorbance was measured at 700 nm. Ascorbic acid and BHT were used as standard compounds. An increased absorbance of the reaction mixture indicates an increase in the reducing power. All tests were performed in triplicate, and the percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} * 100$$

The cytotoxicity was measured by using the MTT assay with minor modification [21, 22]. Briefly, human breast-cancer cells (MCF-7 and MDA-MB-231) were cultured in RPMI-1641 medium containing 10% FBS, 100-µg/mL penicillin, and 100-µg/mL streptomycin in a humidified atmosphere with 5%  $CO_2$  at 37°C. Cells were plated with 100 µL of the medium per well (8000 cells /well) in 96-well plates. After 48 h, the cells reached 70-80% confluency. The medium was removed from the plate, and the well was washed with PBS once. Serum free medium (100 uL) was added to each well, and the well was kept for 12 h at 37°C in an incubator with 5%  $CO_2$ . The plant extracts were diluted to obtain different concentrations (7.5, 15, 30, 60, 120, and 240 µg/mL), and the standard drugs were added in various concentrations (0.75, 1.5, 3.0, 6.0, 12, and 24 µg/mL), dissolved in 0.1% DMSO, and allowed to sit for 24 h at 37°C. After 24 h of treatment, 10 uL of the MTT solution were added to each well (stock 1 mg/mL in PBS). The wells were wrapped with foil and incubated at 37°C for 4 h, after which DMSO (200 uL) was added and kept on a shaker for 30 - 45 minutes. Readings were taken at 570 nm by using a ELISA plate reader. The % cytotoxicity was calculated from the absorbance values for the treated and the control groups.

The CTC<sub>50</sub> values for the sample were calculated from the dose response curves by using a linear regression analysis by using

$$\% \text{ cytotoxicity} = 100 - \frac{\text{Mean optical density of sample}}{\text{Mean optical density of control}} \times 100$$

The *in-vitro* cytotoxicity short-term SRB method was to assess human breast-cancer cells from the MCF-7 and MDA-MB-231 cell lines incubated with different concentrations of the aqueous extracts of *O. pallidus* at room temperature for 2 - 3 h [23, 24]. Briefly, those human breast-cancer cells were cultured in RPMI-1641 medium containing 10% FBS, 100- $\mu\text{g}/\text{mL}$  penicillin, and 100- $\mu\text{g}/\text{mL}$  streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Cells were plated with 100  $\mu\text{L}$  of the medium per well (8000 cells/well) in 96-well plates. After 48 h, the cells reached 70-80% confluency. The medium was removed from each well, and the serum-free medium was added to each well after it had been washed with PBS once and kept at 37°C for 12 h. Compounds, including the standard drugs, were added in various concentrations (10, 20, 50, and 100  $\mu\text{g}/\text{mL}$  in 0.1% DMSO), and the solution was kept for 24 h at 37°C. After that, 50  $\mu\text{L}$  of chilled TCA (50% tri-chloro acetic acid) were added to each well, and the wells were incubated for 1 h at 4°C. The solution from each well was discarded, and the wells were washed with double-distilled water 3 - 4 times and completely air dried at room temperature (RT) overnight. SRB (100  $\mu\text{L}$ , 0.4%) was added to each well, and the wells were incubated at RT for 30 minutes. The cells were then washed with 1% glacial acetic acid 3-4 times and completely air dried at RT. Tris base solution (200  $\mu\text{L}$ , 10 mM, pH 10.5) was added to each well. After 20 minutes, a reading was taken at 510 nm. The % cytotoxicity was calculated from absorbance values of the treated and the control groups. The CTC<sub>50</sub> values for the sample were calculated from the dose response curves by using a linear regression analysis. The percent cytotoxicity was calculated using

$$\% \text{ cytotoxicity} = 100 - \frac{\text{Mean optical density of sample}}{\text{Mean optical density of control}} \times 100$$

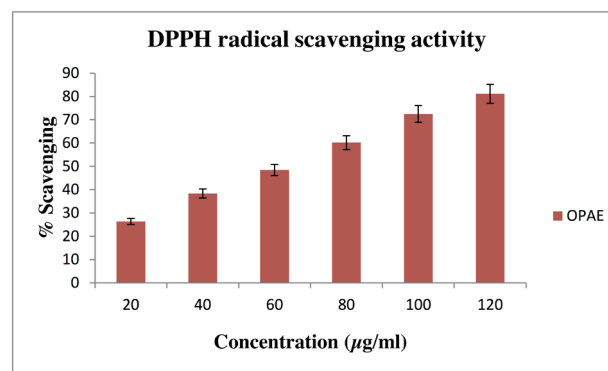
All results were expressed as means  $\pm$  standard deviations of triplicate measurements. For statistical analyses, the one way ANOVA (Analysis of Variance) and Duncan's test were applied using SPSS version 17 software. The significance level was set at  $P < 0.05$ .

### 3. Results

The key findings of the study showed that, based on the averages of triplicate measurements, the total phenol content (TPC) of the OPAE was  $506.64 \pm 1.09$  mg gallic acid equivalents/g plant extract and the total flavanoid content (TFC) was  $365.63 \pm 0.32$  mg quercetin equivalents/g plant extract. Thus, based on these results, one can conclude that *O. pallidus* aqueous extracts contain significant amounts of TPC and TFC compounds.

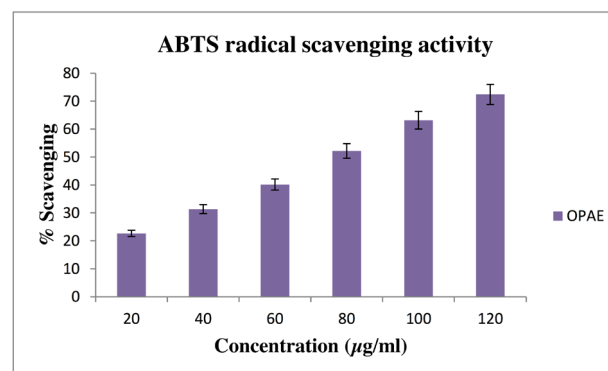
The DPPH radical scavenging activities of OPAE and the standard drugs were estimated for concentrations from 20

to 120  $\mu\text{g}/\text{mL}$ . The IC<sub>50</sub> value was found to be  $61.9 \pm 1.2$   $\mu\text{g}/\text{mL}$  for the extract, and the corresponding values for the standard compounds gallic acid and ascorbic acid were  $1.55 \pm 0.3$   $\mu\text{g}/\text{mL}$  and  $2.01 \pm 1.2$   $\mu\text{g}/\text{mL}$ , respectively. These results were found to be inversely proportional to the antioxidant potential. The free-radical scavenging activity was also found to increase with increasing concentration of OPAE up to 120  $\mu\text{g}/\text{mL}$ .



**Figure 1** DPPH radical scavenging activity of OPAE.

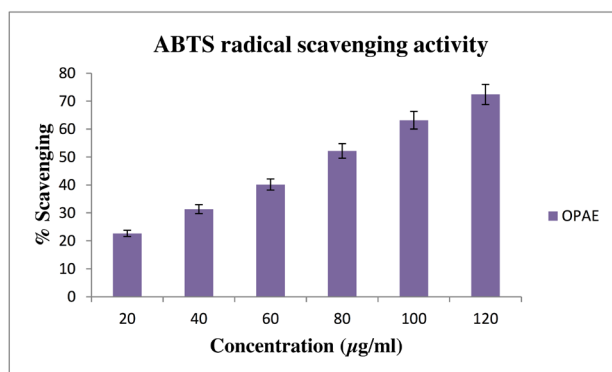
Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . OPAE- Orthosiphon Pallidus Aqueous Extracts



**Figure 2** ABTS radical scavenging activity of OPAE.

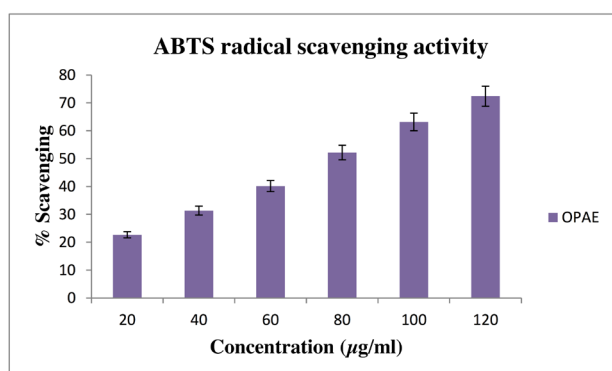
Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . OPAE- Orthosiphon Pallidus Aqueous Extracts

The ABTS antioxidant assay for the OPAE was based on the decolorization of (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS<sup>+</sup>. Figure 2 presents the ABTS radical scavenging activities of the OPAE and the standard drugs for various concentrations from 20 - 120  $\mu\text{g}/\text{mL}$ . The IC<sub>50</sub> values (inhibitory concentrations) were found to be  $75.9 \pm 1.5$   $\mu\text{g}/\text{mL}$  for the extract and  $1.12 \pm 0.9$   $\mu\text{g}/\text{mL}$  and  $3.25 \pm 1.5$   $\mu\text{g}/\text{mL}$  for the standard compounds gallic acid and BHT, respectively.



**Figure 3** Fe<sup>2+</sup> Chelation activity of the OPAE.

Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . OPAE- Orthosiphon Pallidus Aqueous Extracts



**Figure 4** Hydroxyl radicals assay of the OPAE.

Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . OPAE- Orthosiphon Pallidus Aqueous Extracts

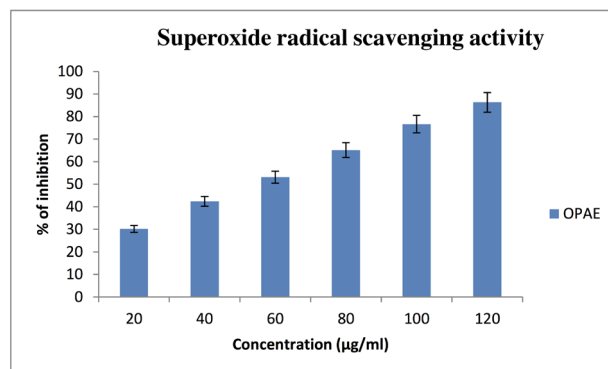
The Fe<sup>2+</sup> chelation activity for the OPAE is presented Figure 3. A violet color is produced when a complex is formed between Fe<sup>2+</sup> and ferrozine. The complex formation is interrupted upon the addition of a chelating agent. The absorbance of the ferrozine-Fe<sup>2+</sup> complex decreased linearly with increasing dose from 20 to 640 µg/mL. The OPAE showed a maximum metal chelating effect of  $73.26 \pm 1.6\%$  at 640 µg/mL, and the chelating activity of EDTA was  $15.61 \pm 1.9$  µg/mL.

A significant variation was observed in the hydroxyl radical assays in which the abilities of the OPAE and the standard drug to reduce hydroxyl radicals were experimentally analyzed at different concentrations from 20 to 120 µg/mL by using the standard catechin to inhibit hydroxyl-radical-mediated deoxyribose degradation in an Fe<sup>3+</sup>, EDTA, ascorbic acid, and H<sub>2</sub>O<sub>2</sub> reaction mixture (Figure 4).

The estimated IC<sub>50</sub> value of the OPAE is  $49.09 \pm 1.9$  µg/mL, and that of catechin is  $7.1 \pm 0.5$  µg/mL. The OPAE in the reaction mixture exhibited hydroxyl radical scavenging activity in a dose-dependent manner for doses ranging from

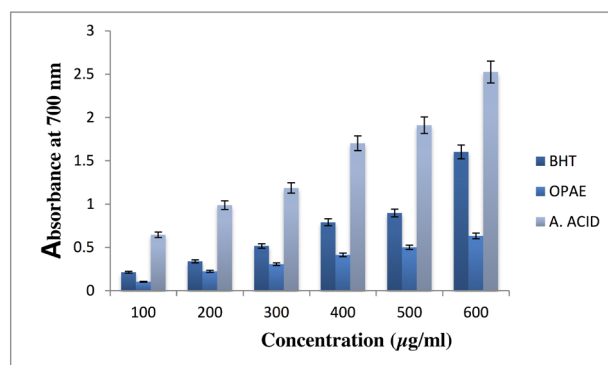
20 to 120 µg/mL, with  $88.21 \pm 1.4\%$  scavenging at a concentration of 120 µg/mL. The superoxide radical activities of the OPAE and the standard drugs at different concentrations were observed and found to be dose dependent (Figure 5). The estimated IC<sub>50</sub> values of the superoxide radical activities for the OPAE and BHT were  $69.18 \pm 2.8$  µg/mL and  $122.10 \pm 1.4$  µg/mL, respectively, clearly showing that the inhibitory concentration (IC<sub>50</sub>) of the OPAE is more potent than that of the standard drug.

The total reducing power (TRP) is used to measure the reductive ability of potential antioxidants and is mainly estimated by using the transformation of Fe<sup>3+</sup> (ferric) iron to ferrous Fe<sup>2+</sup> iron in the presence of the OPAE [25]. In this present study, the TRPs of the OPAE and standard compounds such as BHT & ascorbic acid were found to increase steadily with increasing concentration in the order of ascorbic acid > BHT > OPAE. The extract exerts its reducing power by breaking the radical chain by donating a hydrogen atom (Figure 6).



**Figure 5** Superoxide radical scavenging activity of the OPAE.

Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . OPAE- Orthosiphon Pallidus Aqueous Extracts

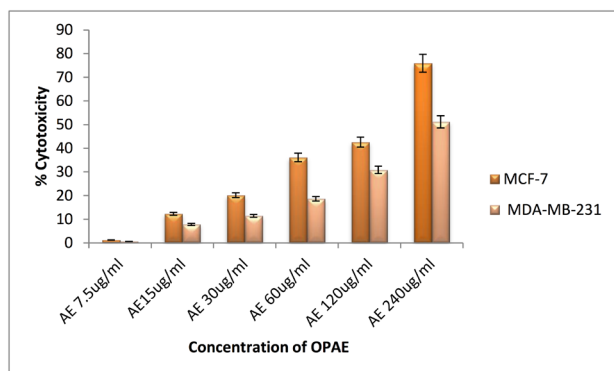


**Figure 6** Reducing power of the OPAE.

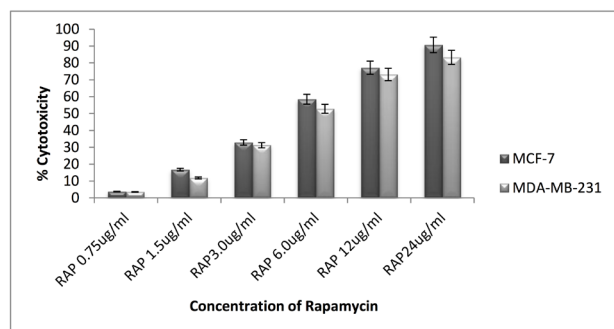
Each value is expressed as mean  $\pm$  standard deviation (SD) of triplicate measurements and the significance accepted at  $P < 0.05$ . BHT- Butylated hydroxyl toluene OPAE- Orthosiphon pallidus Aqueous extracts A. Acid- Ascorbic Acid

Cytotoxicity assays were carried out on cancer cells from the MCF-7 and the MDA-MB-231 cell lines in the presence of the OPAE at various concentrations, and the  $CTC_{50}$  values were determined by using MTT assays. The results are presented in Figure 7A. The figure clearly shows that for both cell lines, the percentage of cytotoxicity steadily increases with increasing concentration up to 240  $\mu\text{g}/\text{mL}$ . The  $CTC_{50}$  values of the OPAE for the MCF-7 and the MDA-MB-231 cell lines were found to be  $143.39 \pm 1.025$  and  $225.42 \pm 0.154$   $\mu\text{g}/\text{mL}$ , respectively, while those for rapamycin, which was used as the standard drug (Figure 7B), were found to be  $3.68 \pm 1.47$  and  $3.92 \pm 2.369$   $\mu\text{g}/\text{mL}$ , respectively; this difference was statistically significant ( $P < 0.05$ ). The experimental results demonstrate that the OPAE exhibits more than 50% cytotoxicity on the MCF-7 cell line; however, it had much less effect on the MDA-MB-231 cell line.

In order to confirm the above results based on more precise data, we used SRB assays to screen the OPAE at various concentrations for its cytotoxicity against the MCF-7 and the MDA-MB-231 cell lines to determine the  $CTC_{50}$  value. The percent cytotoxicity was found to increase linearly with increasing concentration steadily up to 7.5  $\mu\text{g}/\text{mL}$  for both cell lines (Figure 8A), and the  $CTC_{50}$  values for the OPAE against the MCF-7 and the MDA-MB-231 cell

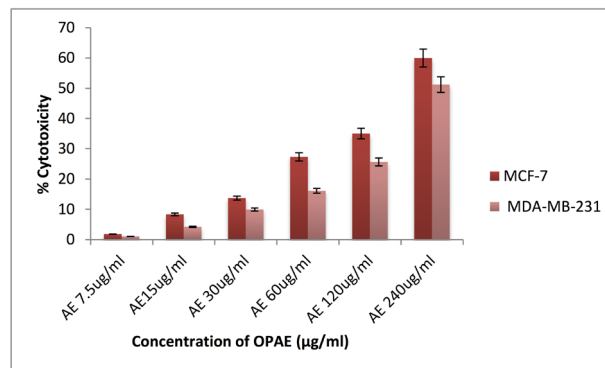


**Figure 7A** Cytotoxicity of OPAE on MCF-7 & MDA-MB-231 cell line by MTT Assays

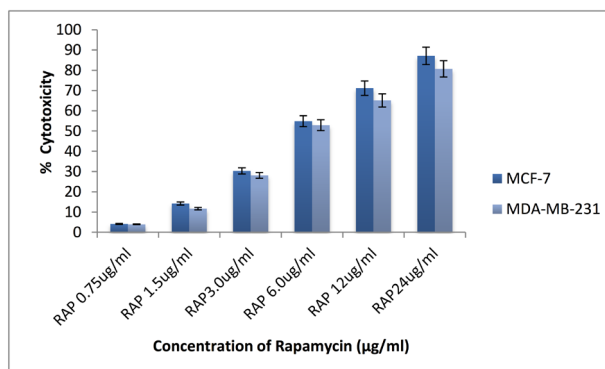


**Figure 7B** Cytotoxicity of Rapamycin on MCF-7 & MDA-MB-231 cell line by MTT Assays

lines were  $188.43 \pm 2.34$  and  $234.28 \pm 0.187$   $\mu\text{g}/\text{mL}$ , respectively. Here again, rapamycin was taken as the standard drug, and the percent cytotoxicities for both cell lines are presented in Figure 8B. The  $CTC_{50}$  values for the MCF-7 and the MDA-MB-231 cell lines were 3.86 and 4.091  $\mu\text{g}/\text{mL}$ , respectively. Statistically, these differences between the effects of the OPAE and of the control drug were significant ( $P < 0.05$ ). The results demonstrate that the OPAE has potent activity against the MCF-7 cell line, but a less potent activity against the MDA-MB-231 cell line.



**Figure 8A** Cytotoxicity of OPAE on MCF-7 & MDA-MB-231 cell line by SRB Assays



**Figure 8B** Cytotoxicity of OPAE on MCF-7 & MDA-MB-231 cell line by SRB Assays

## 4. Discussion

The present research work focused on *O. pallidus* Polyphenols were the main constituents used for quantification and were estimated using phytochemical analyses. Phenolic compounds are a group of secondary metabolites mainly synthesised by plants throughout the biotic under biotic conditions like a diseased state, water stress, exposure to visible light, and low temperature stress [26]. The results showed that the TPC and the TFC of the OPAE were  $506.64 \pm 1.09$  mg gallic acid equivalents/g plant extract and  $365.63 \pm 0.32$  mg quercetin equivalents/g plant

extract, respectively. This is the first work to show such a high content of phenol compounds in the OPAE. These polyphenols exhibit higher antioxidant activities [27]. Phenolics exhibit antioxidant activity by preventing the decomposition of hydroperoxides into free radicals and by directly inactivating those free radicals. These phenolic antioxidant compounds form relatively stable intermediate compounds due to the distribution of charge throughout the aromatic ring system [28, 29].

The DPPH assay is based on the reduction of DPPH in an organic solvent to a non-radical form in presence of a hydrogen-donating antioxidant. The quenching ability of the DPPH assay results in a color change from purple to yellow when measured spectrophotometrically and is achieved in a short time span as compared to other methods. The disappearance of the purple color is monitored at 517 nm [30]. The IC<sub>50</sub> value was found to be  $61.9 \pm 1.2$  µg/mL for the extract, and those for the standard compounds gallic acid and ascorbic acid were  $1.55 \pm 0.3$  µg/mL and  $2.01 \pm 1.2$  µg/mL, respectively. The plant extracts show significant radical scavenging effect with increasing concentration. The antioxidant potential of the aqueous extract of *Orthosiphon pallidus* may be due to the presence of phenolic and polyphenolic compounds. These compounds decrease the level of free radicals, thereby causing oxidative stress [31].

The aqueous extract of our plant also showed good ABTS•+ scavenging ability. The IC<sub>50</sub> value was found to be  $75.9 \pm 1.5$  µg/mL for the extract; those values for the standard compounds gallic acid and BHT were  $1.12 \pm 0.9$  µg/mL and  $3.25 \pm 1.5$  µg/mL, respectively. This is also true for other plant members of the Lamiaceae family [32, 33]. The ABTS scavenging ability is produced by the reaction between ABTS and potassium persulfate and leads to the appearance of a blue-green color [16].

In the metal chelating activity, ferrozine can form a complex with ferrous ion (Fe<sup>2+</sup>). This quantitative complex is formed in the presence of a chelating agent. When the formation of the complex is disrupted, the red color of the complex decreases [11]. The bond formed between the chelating agent and the metal causes the complex to be effective as a secondary antioxidant as it reduces the redox potential and stabilizes the oxidized form of the metal ion [34].

The scavenging effects on the hydroxyl radical of the OPAE significantly increased with increasing concentration from 20 to 120 µg/mL. The estimated IC<sub>50</sub> value of the OPAE was  $49.09 \pm 1.9$  µg/mL, and that of catechin was  $7.1 \pm 0.5$  µg/mL. The hydroxyl radicals, OH• ions, chelate with the antioxidants, thereby inhibiting and suppressing of the peroxidation processes of biological molecules [35]. The superoxide radical scavenging activity reduces the molecular oxygen that is produced by the enzymatic system during an auto-oxidation reaction [36]. The estimated IC<sub>50</sub> values of the plant extract and the BHT for superoxide radical activity were  $69.18 \pm 2.8$  µg/mL and  $122.10 \pm 1.4$  µg/mL, respectively. The present data suggest that the OPAE is a more potent than the standard drug.

The reducing power of a compound serves as a potential indicator of its antioxidant activity. In the assay to determine the reducing power the color of the test solution changes from yellow to green, depending on the reducing power of the test compound [37]. According to the litera-

ture, most synthetic drugs possess significant side effects, and any strains of microorganisms are resistant to antibiotics. Due to this, investigations of anti-microbial drugs obtained from natural sources are the utmost priority of today's researchers and scientists [38].

*In-vitro* cytotoxicity studies provide basic information about the nature and the effect of herbal formulations on cancer cells. The MTT assay, an enzyme-based assay, mainly works on the principle of the reduction of a coloring reagent during colorimetry and can be used to estimate cell viability. The MTT assay is the best-known method for determining the cytotoxicity through mitochondrial dehydrogenase activities in the cells of living plants. This method is useful for measuring the growth of cells, growth factors, responses of cells to mitogens, membrane stability, and growth curves [39].

Cancer is one of the most dreadful diseases worldwide and is increasing at a progressive rate. Phytopharmaceuticals or herbal formulations are gaining in importance and public awareness day by day. Most novel drug delivery systems of phytoconstituents act as therapeutic agents and provide a defensive mechanism [40]. In every system of medicine, Siddha, Ayurveda, Unani, allopathic, and others, most secondary metabolites of herbal origin are used for the treatment of various diseases, such as cancer, diabetes, psoriasis, arthritis, etc. Thus, herbs and phyto-products will continue to be used as prominent sources for anti-cancer drugs. Cytotoxicity assays by using the MTT and the SRB methods were used to assess the cytotoxicity potential of the OPAE. Results from those assays revealed that the OPAE exhibited good percent cytotoxicity and that the cytotoxicity increased with increasing concentration of the active component in the plant extract.

## 5. Conclusion

In conclusion, the results obtained in this study indicate that aqueous extracts of *Orthosiphon pallidus* possess high amounts of phenol and flavanoid constituents and exhibit significant and potent antioxidant free-radical scavenging and anticancer activities. The results of this study also provide a clear indication that the aqueous extracts of *O. pallidus* are rich in antioxidants. These findings show the potential of this indigenous drug and draw our attention towards its anticancer activity against cells in various cancer cell lines. The *in-vitro* evaluations revealed significant anticancer activity against the cells in the MCF-7 cell line.

Anticancer activity against the cells in the MDA-MB-231 cell line was also observed, but to a lesser degree. Therefore, our results demonstrate that aqueous extracts of *Orthosiphon pallidus* exhibit potential carcinogenic free-radical scavenging and potent anticancer activity.

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