ANTIOXIDANT AND CYTOTOXIC POTENTIAL OF THE PLANT

**HOLIGARNA ARNOTTIANA** HOOK.F. BARK ETHANOLIC EXTRACT

Ayana Ravi* and Oomen P. Saj

Dept. of Botany, University College, Thiruvanathapuram.

**ABSTRACT**

The present study was aimed to reveal the antioxidant potential and cytotoxic activity of the ethanolic bark extract of *Holigarna arnottiana* plant. The in vitro antioxidant potential was determined by DPPH scavenging activity, Hydroxy radical scavenging activity, singlet oxygen scavenging activity and superoxide radical scavenging activity. The cytotoxic activity was detected by MTT assay and DLA assay. The results showed that the extract has a high DPPH radical scavenging activity with an IC50 value of the extract was 15.6±0.1µg/mL and that of the standard ascorbic acid was 24.92±0.03 µg/mL. In superoxide radical scavenging method the IC50 value was 49.2±0.2µg/mL and that of the standard was 95.1±0.3. The IC50 value in singlet oxygen analysis was 39.1±0.1µg/mL and that of standard was 38.6µg/mL. For Hydroxy radical analysis the IC50 value was 73.6±0.1µg/mL. In tryphan blue exclusion assay of cytotoxicity, the extract showed a CT50 value of 2.8µg/mL. In MTT assay the MCF cells showed a CT50 value of 50µg/mL and MCF 15 cells the CT50 value is 30µg/mL.

**KEYWORDS:** Antioxidant, cytotoxic, *Holigarna*, DPPH, MTT.

**INTRODUCTION**

The life on earth largely depends on the green plants as they are the producers of food, oxygen, fodder, medicine etc. The trees are the shelters of animals which represent life and maintain the ecological balance. Humans categorized the trees as medicinal, sacred, toxic, life sustaining etc. The Indian philosophy, thought, values and ethics have always had reverence for all that exists in nature. Several trees are worshipped in India and they are thus protected from exploitation (Malla, 2000, Sharma and Gogoi, 2006).
Free radicals are molecules having unpaired electrons and they damage cell membrane and cell organelles including DNA. The free radicals include reactive oxygen species such as super oxide anion, hydroxyl, hydroperoxyl, peroxy, alkoxyl radicals, hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen. There are reactive nitrogen species such as nitric oxide, peroxy nitrite and nitrogen dioxide. Antioxidants are compounds that reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells prevent damage to lipids, proteins, enzymes and carbohydrates. There are enzymatic and non-enzymatic antioxidants present. The enzymatic antioxidants are produced endogenously include superoxide dismutase, catalase, glutathione peroxidase. The non-enzymatic antioxidants include tocopherols, carotenoids, ascorbic acid, flavonoids and tannins.

Antioxidants are molecules having the capacity to scavenge the free radicals and thereby or preventing the oxidative or damage from oxygen process caused by free radicals. Several drugs in Ayurveda derived from medicinal plants have been reported as rich sources of antioxidants and the use of such natural resources will help in reduces the incidence of many diseases related to oxidative stress. Literature revealed that plant material such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs are rich sources of antioxidants.[1,2]

The uncontrolled division of cells is known as cancer and is characterized by loss of normal control mechanisms that govern cells survival, proliferation and differentiation. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Most common cancers are lung, breast, colorectum, prostate and cervix uteri cancers.[3]

The present study is to find out the antioxidant and cytotoxic capacity of the plant Holigarna arnottiana Hook.F.

**MATERIALS AND METHODS**

**Plant material**

The material used for the study was fresh bark of *H.arnottiana* which was collected from local area of Pathanamthitta District of Kerala.
Preparation of Drug
The fresh bark extract was crushed in a mortar and pestle using the polar solvent ethanol and sieved through a mesh and dried in open air. The extract was used for further studies.

1. **INVITRO ANTIOXIDANT ACTIVITY STUDIES**

1.1. **Hydroxyl radical scavenging**
The assay was performed by the method of Elizabeth and Rao[4], with a slight modification. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe$^{3+}$-ascorbate-EDTA-H$_2$O$_2$ system. The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM) in phosphate buffer (20 mM, pH 7.4), FeCl$_3$ (100 μM), EDTA (100 μM), H$_2$O$_2$ (1.0 mM); ascorbic acid (100 μM) and various concentrations (0–200 μg/mL) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. The Oxygen scavenger Mannitol was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

Scavenging activity (%) = \(\frac{1 - \text{Absorbance of sample}}{\text{Absorbance of control}}\) X control

1.2. **Superoxide radical scavenging**
Superoxide radical scavenging activity was measured by the reduction of NBT. The non-enzymatic phenazinemetho sulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (0–20 μg/ml) of the test sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. All tests were performed six times. Quercetin was used as positive control.

1.3. **DPPH radical scavenging assay**
The antioxidant capacity of the bark extract was confirmed by the DPPH scavenging assay according to Mahakunakornet al[5], with slight modification. Different concentrations (0-100 μg/ml) of the extracts and the standard ascorbic acid were mixed with equal volume of
ethanol. Then 50μl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for 2 minutes before the optical density (OD) was measured at λ = 517 nm. The measurement was repeated with six sets. The percentage radical scavenging activity was calculated from the following formula

\[
\text{% scavenging [DPPH]} = \left[ \frac{(A0 - A1)}{A0} \right] \times 100
\]

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of the samples and standard.

1.4. Singlet oxygen scavenging

The production of singlet oxygen (\(^1\text{O}_2\)) was determined by monitoring N, N-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported spectrophotometric method. Singlet oxygen was generated by a reaction between NaOCl and H\(_2\)O\(_2\), and the bleaching of RNO was monitored at 440 nm. The reaction mixture contained 45mM phosphate buffer (pH 7.1), 50mM NaOCl, 50mM H\(_2\)O\(_2\), 50mM histidine, 10μM RNO and various concentrations (0–200μg/ml) of sample in a final volume of 2 ml. It was incubated at 30°C for 40 min and the decrease in RNO absorbance was measured at 440nm. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times.

2. CYTOTOXIC ASSAY

For the cytotoxic study *H. arnottiana* ethanol bark extract was selected. The extract was evaporated in room temperature and then subjected to cytotoxic assay according to the accepted methodology.

2.1. MTT Assay

Estrogen-dependent BCa cells MCF-7 and estrogen-independent BCa cells MDA-231 were cultured. MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purpleformazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. MDA-231 and MCF-7 cells were seeded in 96 well
plates in the presence of growth medium and incubated for 24 hours for the cell to adhere to the surface of the well. The cells were then treated with different fractions of the extract dissolved in DMSO (2 µM, 4 µM, 6 µM and 8 µM). The cell culture suspension was washed with PBS and then added with 200 µl MTT solution to the culture (MTT -5 mg/volume dissolved in PBS) and incubated at 37°C for 3 hours. Removed all MTT by washing with PBS and added 300 µl DMSO to each culture. Incubated at room temperature for 30 minutes until all the cells get lysed and a purple colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. OD was read at 540 nm using DMSO as blank.[6,7]

2.2. DLA assay
The cell viability was estimated by trypan blue assay. The test compounds were studied for short term in vitro cytotoxicity using Dalton’s Lymphoma Ascites cells (DLA). The tumour cells were aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with normal saline and checked for viability using trypan blue dye exclusion method. The cell suspensions (1x10⁶ cells in 0.1 ml) was added to the tubes containing various concentrations 2, 4, 6, 8 and 10 µg/mL of the test compounds and the volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37°C and percentage of dead cells were counted by using a haemocytometer.[8,9]

RESULTS
1. Invitro Antioxidant Assay
1.1. Hydroxy Radical Assay
The results showed the ability to inhibit hydroxyl radical mediated deoxyribose degradation in a Fe³⁺-EDTA-ascorbic acid and H₂O₂ reaction mixture.

The Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals generated by the Fenton reaction. Mannitol was used as the positive control. The IC₅₀ value of the extract for hydroxyl radical scavenging activity was 146.8±0.4 µg/mL. A dose dependent activity was observed and the extract has more pronounced activity than the positive control mannitol which showed 38% of inhibition at 461.8± 9.94 µg/mL concentration but the extract possesses 63% of inhibition (Fig.1).
1.2. Superoxide Radical Scavenging
Super oxides are highly active anion with the chemical formula \( O_2^- \). The superoxide generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract and the reference compound Quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. The IC50 value of the extract is 13.8±0.2 µg/mL and the standard is 23.9± 0.2 µg/mL. At 25µg/mL the percentage of inhibition of plant extract is 75.4% (Fig.no.2).

1.3. DPPH Assay
The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. In this test DPPH is used as substrate to evaluate the antioxidant activity the scavenging activity of the tested drug towards the stable DPPH is measured. The activity of the extract was measured and compared with the positive control ascorbic acid. The IC50 values of extract and ascorbic acid are 17.2±0.08 and 24.92±0.03 respectively and showed that the tested extract has high potential than the standard used. The extract showed dose dependent activity (Fig.3).

1.4. Singlet Oxygen scavenging Activity
Singlet oxygen is the highly reactive species of oxidant. Ethanol bark extract of *H. arnottiana* is an effective singlet oxygen scavenger with an IC50 value of 47.6±0.5 and which is comparable with that of the standard, 44.6±0.4. The tested drug showed a concentration based activity (Fig.4).

2. Cytotoxic activity
Estimation of cytotoxicity was done by trypan blue exclusion method. Viable cells which remained unstained by trypan blue were counted in a haemocytometer. The various concentration of laccol and ethanol bark extract used were 2,4,6,8 and 10 µg/mL and a decrease in cell count was observed with increase in concentration of the drug. There was a dose dependent increase in cytotoxic activity with CT50 value observed in a concentration of 2.8µg/mL (Fig.5). The cytotoxicity of both ethanol extract of *H. arnottiana* bark extract and was determined by using breast cancer cells MCF-7 and MDA 231 by a method of MTT assay. In this the percentage of cells inhibited by the extract was determined. When tested with ethanol extract, the CT 50 value for MCF-7 was 50µg/mL and for CT 50 value for MDA 231 was 30µg/mL. and the ethanol extract showed more activity against MDA -231 cells. The extract showed dose dependent activity and 100% inhibition was observed in 10µg/mL.
of extract (Fig.6). The antioxidant activity result showed that higher concentration of the ethanol *H. arnottiana* bark extract have higher inhibition percentage than the standards used in four different methods of study. It was reported by Sumithira *et al.*,\(^{[10]}\) that the TLC fraction of the methanol extract of the nuts of *Semicarpus anacardium* showed potent antioxidant activity when compared with known synthetic standard of Butylated HydroxyTolune by the method of DPPH radical scavenging activity. Similarly Kim *et al.*,\(^{[11]}\) had reported that the DPPH radical scavenging capacity of allergen removed ethanol extract of *Rhus verniciflua*. Similar antioxidant activity studies were done in *Rhus verniciflua* by Kim and Kim,\(^{[12]}\) and it was reported that100% ethanol extract of the plant showed stronger antioxidative activity when studied by DPPH method. It was reported that petroleum ether and ethanol extracts of nuts of *Semecarpus anacardium* showed higher antioxidant activity when tested by DPPH method and which was dose dependent.\(^{[13]}\) The results indicate that the ethanol bark extract of *H. arnottiana* has a potent anticanerous activity. The capacity may be attributed by the chemical components like phenolic acids. Similar anti-cancerous activity of the allergic plant *Semecarpus anacardium* on Hep 2 and Vero cell lines were reported.\(^{[14]}\) Similarly isolated active compound from the kernel of *Semecarpus anacardium* is cytotoxic to tumor cell lines and also induced apoptosis in human leukemia cell lines in a dose dependent manner.\(^{[15]}\) Similar urushiol congeners were reported to cytotoxic activites.\(^{[16]}\) Thus the results indicated that the ethanol bark extract of *H. arnottiana* is a potent anticancer drug and several such results of allergic compound supports the results. Thus the plant may be used as a new drug after further research.

![Fig.1. Hydroxy radical scavenging assay](image)

*Fig.1. Hydroxy radical scavenging assay*

Data represent the percentage Hydroxy radical inhibition. All data are expressed as mean ± S.D. (n = 5). **,** **,** p < 0.00.
Fig. 2: Superoxide Radical Scavenging Activity
The data represent the percentage superoxide radical inhibition. All data are expressed as mean ± S.D. (n = 5). ***p < 0.001.

Fig. 3: DPPH Assay
The data represent the percentage DPPH inhibition. All data are expressed as mean ± S.D(n = 5). ***p < 0.001
Fig. 4. Singlet Oxygen scavenging Activity
The data represent the percentage singlet oxygen scavenging. All data are expressed as mean ± S.D. (n = 5). ***p < 0.001.

Fig. 5. MTT Assay of ethanol extract

Fig. 6. DLA Assay of ethanol extract
REFERENCE
