Original Article

Estimation of Antioxidant and Cytotoxicity Activities of Extracts Obtained from the Leaves of Folk Medicinal Plant *Benkara malabarica* (Lam)

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ABSTRACT

Objectives: *Benkara malabarica* (Lam). is pharmacologically unexploited medicinal plant, used in the folklore Paliyar in India. Medicinal plant systems to treat many ailments such as cold, phlegm, stomach and body pains. The present investigation carried out with objective of finding an alternative antioxidant and cytotoxicity medicines from traditionally used medicinal plant and to provide ethnopharmacological information for its traditional usage. **Materials and Methods:** Petroleum ether, chloroform and methanol were used as solvents for the preparation of crude drug from the *B. malabarica* leaves. DPPH and O phenantroline reduction assay were used to estimate the antioxidant activities of the extracts, while MTT assay was used to determine the cytotoxicity of extracts using Hep G2 cell lines. Different concentration of extracts, in the range of 25, 50, 100, 150 and 200 μg/ml was used for the determination of antioxidant activities. In case of cytotoxicity studies the extract concentration were 25, 50, 100, 200 and 500 μg/ml. Ascorbic acid was used as standard in antioxidant studies and doxorubicin was employed as standard in the cytotoxicity studies. **Results:** In both antioxidant and cytotoxicity studies, the activities were increased with the increase the concentrations of extracts. The potential of three extracts were in the following order, methanol, chloroform and petroleum ether in both antioxidant and cytotoxicity studies. **Conclusion:** The remarkable antioxidant and cytotoxicity activities, assayed through various *in vitro* models in the present study. This may be due to the presence of phytochemicals, such as phenol, flavonoid, alkaloid and glycosides in the extracts. Scientific data generated from the present study supports the usage of *B. malabarica* in the folklore medicinal system.

Keywords: Paliyar, *Benkara malabarica, in vitro*, Antioxidant activity, Cytotoxicity.

INTRODUCTION

Cancer is a larger group of diseases that can affect any organ or tissue of the body. On average, 18 million new cases are reported annually around the world on account of this fatal disease. It is one of the leading causes of death globally, accounting for an estimated 9.6 million death in the year 2018. The estimated national expenditure for cancer care was $ 147.3 billion in US alone for the year 2017. The costs also likely to increase as new and expensive treatments are adopted as standard care.[1] Cancer is caused by many factors and they can be broadly classified into two main categories as genetic and non-genetic factors. Genetic factors are responsible for the 7% cancers. Normal body cells become cancerous cells due to the genetic mutations and epigenetic changes and they divide in an uncontrollable manner and invade the neighboring tissues and organs. Genetic predisposition of the autosomal dominant type imposes a high risk for many kinds of cancer. The remaining 93% of cancer is caused by the non-genetic factors like sunlight and ionizing radiation, organic and inorganic chemicals, microorganisms, diet, obesity and metabolic products such as highly reactive free oxygen species.[2-5] Reactive oxygen species is one of the metabolic product and said to be

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the reason behind many types of cancers.[6] Oxidative damage of DNA caused by reactive oxygen species can lead to the altered signal transduction and replication errors. It causes genetic instability and cancer.[7]

The normal clinical therapies followed in treating cancer are radiation, chemotheraphy, immunomodulation and surgery. These therapies have limited success in controlling the cancer.[8] The chemotherapy treatment shows unwanted side effects like fever, hallucinations, low blood pressure, bone marrow problems, paralysis, coma, liver problems and kidney problems.[9] Chemical compounds from herbal origin become the focal point now to improve the all-health needs include cancer. The drugs obtained from plants could be used to treat wide spectrum of diseases including cancer with less side effects.[10] Medicines from plant source are proved to be an alternative strategy to manage the cancer reported by Dai *et al.* (2010). Compounds from plant resources are reported to be effective in controlling all stages of cytotoxicity, including initiation, promotion and progression.[11] Many natural products or secondary metabolites form plants, such as alkaloids, coumarins, flavonoids, lignans, phenolics, quinines and terpenoids exhibit considerable antioxidant activities as well as anti-proliferation of activities.[12-15]

Indigenous peoples are using numerous plants as whole or their parts to treat various ailments including cancer. Medicinal plants used by the indigenous peoples are widely used as source of many modern drugs.[16] Many phytocompounds, obtained from ethnomedicinal plants, are directly used as a model for the synthesis of modern medicines.[17]

Paliyar tribes are one such indigenous people, who have been settled in the vicinity of Western Ghats of Tamil Nadu State, India. These indigenous peoples have distinct culture, food habit and rich knowledge of traditional medicines.[18-19] The use of herbal remedies is important among Paliyar tribal and they have accurate knowledge about plants and their medicinal properties.[20] Kalusalingam and Balakrishnan (2018) reported that an ethanobotanical survey with Paliyar Tribal from Sathuragiri Hills hamlet, Virudhunagar District, Tamil Nadu State, shows that they are using 83 plant species from 37 families for the treatment of 47 different ailments. *B. malabarica* from Rubiaceae family is one among the 83 species and medicines prepared from the leaves and bark of the plant is used by Paliyar Tribal to treat various ailments such as cough, phlegm, throat infection, tooth ache, skin disorder and body pain.[21]

Kalusalingam and Balakrishnan (2018) reported that many phytochemicals like alkaloids, flavonoids, phenols, glycosides and stilbenes are reported from the methanolic extracts of stem, leaf and roots of *B. malabarica*. The crude leaf extracts of *B. malabarica* are reported to be effective in the growth inhibition of many bacterial species like *Staphylococcus aureus* (MTTC 3160), *Escherichia coli* (MTTC 46), *Pseudomonas aeruginosa* (MTTC 1688) and *Bacillus subtilis* (MTTC 441).[22] Hence the present study was conducted to evaluate the antioxidant and cytotoxicity effects of *B. malabarica* leaf extracts, obtained with three different solvents such as methanol, chloroform and petroleum ether.

MATERIALS AND METHODS

Chemicals

Analytical grade solvents and chemicals were only used for the study. The solvents, used for the extraction process, were purchased from Merck Ltd, Mumbai. All the remaining chemicals, used for antioxidant and cytotoxicity studies, were procured from Hi-Media, Mumbai India.

Collection and preparation of sample

The fresh leaf samples of *B. malabarica* were collected from the natural habitat of Sathuragiri Hills in the month of November-December 2018. The collected samples were properly authenticated by a professional plant taxonomist. The voucher specimen of the plant sample (BOT-AAGAC–03/2018) with annotation of date and place of collection was deposited in Herbarium of the Post Graduate and Research Department of Botany, Arignar Anna Government Arts College, Namakkal, Tamil nadu State, India. Standard guidelines on herbal quality control were adopted to ensure the quality in processing of collected plant samples.[23] Freshly collected samples were properly processed to remove foreign materials, unhealthy and dried leaves. Later the samples were thoroughly cleaned with water twice to remove sand and oils from the surface. The duly processed leaves were shade dried for 20 days and ground with mixer grinder. The powdered samples were properly labeled and stored in dry cool place.

Preparation of Extract

Crude plant extracts from the fresh leaf sample was prepared according to known standard procedures.[24] The freshly prepared leaf powders were extracted with 900 ml of different solvents (methanol, chloroform and petroleum ether) in a Soxhlet apparatus at 78°C. The extracts were then filtered and evaporated to dryness on hot water bath. The dried extracts were stored in sterile bottles, properly labeled and refrigerated until further use.

Cell Lines and Cell Culture

Hep G2 cancer cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, Maharashtra, India. The cell lines were properly maintained at 37°C/5% CO_2 in a humidified incubator. Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotics was used for the maintenance of the cell lines. Exponentially growing cells were used for all the anticancer studies.[25-26]

Antioxidant Studies with Crude Extracts

Determination of DPPH Scavenging Assay

Standardized method was used in the present study to evaluate the DPPH free radical scavenging activities of different extracts obtained with *B. malabarica* leaves.[27] An aliquot of 0.5 ml of different concentration of sample solutions (25, 50, 100,150 and 200µg/ml) was mixed with 2.5 ml of 0.5 mM DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV spectrophotometer. Ascorbic acid was used as positive control. The experiments were performed in three replications and the results were averaged.

Determination of Ortho-Phenanthroline reduction assay

In the present study, Ferric reducing antioxidant power (FRAP) or iron chelating activity of crude extracts were determined according to standard method with slight modification.[28]

To determine the iron chelating activities of the extracts, a reaction mixture, containing 1 ml of 0.05% O-Phenanthroline in concerned test solvent, 2 ml ferric chloride (200µM) and 2 ml of various concentrations of crude extracts, ranging from 25, 50, 100, 150 and 200 µg/ml was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiments were performed in triplicates and results were averaged.

Determination of Cytotoxicity Effects of Crude Extracts

The viability of cells was assessed by MTT assay using Hep G2 cell lines.[29] MTT assay is based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide) into formazan. The intensity of reduction depends upon the metabolic activities of cells. Healthy and rapidly growing cells have NADPH-dependent cellular oxido reductase enzymes. When MTT (yellow colour) is added to healthy and rapidly growing cell culture, the oxidoreductase enzyme of cell cultures reduce the MTT into formazan (Purple colour), which is easily dissolved in DMSO at 10 %.

The viability of cells is connected with the quantification of formazan at 540 nm. High purple colour intensity indicates the higher cell viability, whereas the decrease in purple colour indicates the reduced viable cell number and thus cytotoxicity of the given substances.[30]

Drug Preparation

The crude extracts were dissolved in 10% Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 0.5% and did not affect cell survival.

Cell Viability Test

The Hep G-2 cells were plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 hr, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of test compound (25, 50, 100, 200 and 500 µg/ml) for 24 hr. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 hr at 37°C in a CO_2 incubator.

The MTT containing medium was then discarded and the cells were washed with 200 µl phosphate buffered saline (pH 7.4). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software. Doxorubicin was used as positive control.

Observation of Morphological Changes with Phase Contrast Microscope

The morphological changes in treated and control cell lines were observed in phase contrast microscope.[31]

Statistical analysis

All the experiments were carried out in triplicates. The observed data are presented as means with ± standard error. Two-way ANOVA test was performed to statistically analyze significant difference between samples using MS Excel Stat. The significant value of $p<0.05$ was considered.

RESULTS

Antioxidant Activities - DPPH Assay

The results of the DPPH assay are summarized in the Table 1. All the three extracts (Methanol, chloroform

Table 1: Antioxidant effects of crude extracts of *Bankara malabarica*-DPPH Assay and Orthophenanthrone activity.

Sl. No	Conc. of Extract µg/ml	DPPH Scavenging Activity and Orthophenanthrone activity (%)				
		Test	Methanol Extract	Chloroform Extract	Petroleum Ether Extract	Control Ascorbic acid/EDTA
1.	25	DPPH	15.1+1.2	7.7+0.4	6.7+0.8	46.3+0.6
		Orthophenanthrone activity	18.2+1.1	10.3+0.4	11.4+0.9	42.5+1.8
2.	50	DPPH	31.7+0.6	13.7+0.7	13.1+0.4	56.9+1.5
		Orthophenanthrone activity	23.1+1.4	17.8+0.6	17.5+1.7	60.7+1.3
3.	100	DPPH	51.9+2.0	32.1+1.3	24.1+1.3	68.4+0.4
		Orthophenanthrone activity	31.2+1.0	28.1+0.6	22.8+1.1	73.3+0.9
4.	150	DPPH	58.1+0.3	43.3+0.6	35.1+2.1	70.1+1.6
		Orthophenanthrone activity	40.8+1.3	35.7+0.5	29.6+2.8	75.3+2.4
5.	200	DPPH	69.1+0.7	58.2+0.4	49.4+1.1	79.6+0.9
		Orthophenanthrone activity	50.8+1.1	44.8+0.8	36.1+0.9	77.2+1.1
IC_{50} Orthophenanthrone activity		DPPH	121.60	170.41	206.73	22.07
			198.14	223.26	301.25	15.30

Values are represented as Mean ± Standard Error of triplicate analysis. All the values are statistically significant $p < 0.05$. The observed antioxidant activities of *Benkara malabarica* are found to be well comparable to that of ascorbic acid for DPPH assay and EDTA for Orthophenanthrone activity.

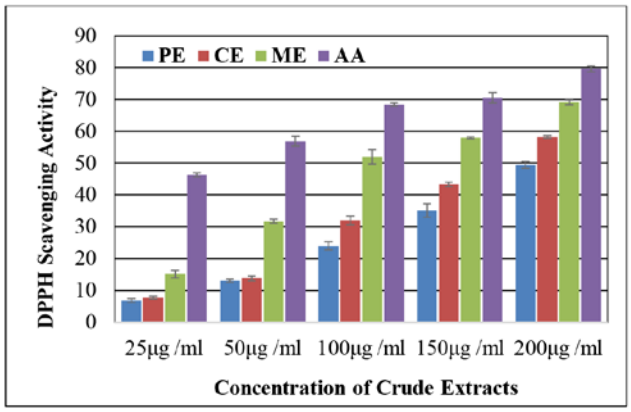

Figure 1: Antixoidant effects of crude extracts obtained from *Benkara malabarica* –DPPH Assay.PE – Petroleum Ether Extract, CE – Chloroform Extract, ME – Methanol Extract, AA – Ascorbic acid.

and petroleum ether) and control ascorbic acid were exhibited dose dependent antioxidant activities in DPPH assay (Figure 1). The intensity of antioxidant activities of three extracts were in the following order, methanol, chloroform and petroleum ether. Highest scavenging activity of 69.14%, was observed in methanol extract at 200 µg/ml concentration. The IC_{50} values are in methanolic extract 121.60, Chloroform extract 170.41, Petroleum ether extract 206.73 and control ascorbic acid 22.07. The control ascorbic acid had an activity of 79.6% at 200 µg/ml concentration.

Orthophenanthroline Chelation Test

The orthophenanthroline chelating ability of three extracts and the standard EDTA at different concentration are furnished in Table 1. The iron chelating activities of leaf extracts and control EDTA were found to be increased with the increase of the concentration (Figure 2). The percentage of iron chelating activity was 50.8, 44.8, 36.0 and 77.2 % in methanol, chloroform, petroleum ether extracts and EDTA respectively at 200µg/ml concentration. Methanolic extract exhibited higher iron chelating activity than the chloroform and petroleum ether extracts at all concentration. The lowest chelating activity 11.4 % was observed in petroleum ether extract at 25µg/ml concentration. The IC_{50} values are in methanolic extract 198.14, Chloroform extract 223.26,

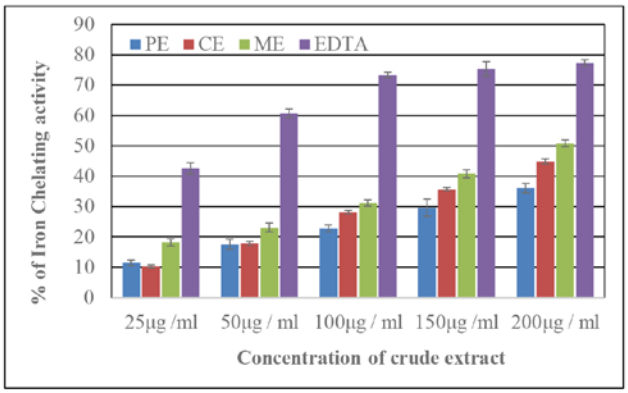

Figure 2: Orthophenanthrone activity of crude drugs from *Benkara malabarica*.PE – Petroleum Ether Extract, CE – Chloroform Extract, ME – Methanol Extract, EDTA (Ethylene Diamine Tetra Acetic Acid) – Standard.

and Petroleum ether extract 301.25 control ascorbic acid 15.30.

MTT Assay

Dose dependent cytotoxicity activities were observed in all the three extracts and control doxorubicin. Results are presented in Table 2 and 3. The lowest viability of Hep G2 cells, 50.85% were observed in methanolic extract at the concentration of 500µg/ml, whereas the viability was 66.10% and 73.10% in chloroform and petroleum ether extracts respectively at the same concentration (Figure 3).

Methanolic extract were found to be more efficient than the chloroform and petroleum ether extracts. The positive control doxorubicin exhibited exorbitant cytotoxic activities even at low concentrations. The viability percentages of cells were only 32.97% at 250 µg/ml (Figure 4). The IC_{50} values are in methanolic extract 540.11, Chloroform extract 770.90, Petroleum ether extract 1009.50 and control ascorbic acid 155.50.

DISCUSSION

Medicinal plants used in the folklore medicinal system have been drawing wide attention in the recent years.

Table 2: Cytotoxicity activities of crude extracts from *Benkara malabarica.*

Sl. No	Conc. of extract (µg/ml)	Percentage of cell viability		
		Methanol Extract	Chloroform Extract	Petroleum Ether Extract
1.	25	89.8±0.8	96.1±1.4	98.0±0.7
2.	50	80.3±1.4	81.5±0.7	86.1±2.3
3.	100	72.8±1.8	75.5±0.7	78.2±1.2
4.	200	59.7±1.1	73.2±0.6	73.7±0.2
5.	500	50.8±1.4	66.1±1.9	73.1±0.5
	IC_{50}	540.11	790.90	1009.50

Values are represented as Mean ± Standard Error of triplicate analysis. All the values are statistically significant $p < 0.05$.

Table 3: Cytotoxic activities of control doxorubicin.

Sl. No	Concentration of Doxorubicin (ng/ml)	Percentage of cell viability
1.	50	69.56±1.5
2.	100	61.81±1.5
3.	150	49.65±0.8
4.	200	41.17±0.8
5.	250	32.97±0.8
	IC_{50}	155.50 ng/ml

Values are represented as Mean ± Standard Error of triplicate analysis. All the values are statistically significant $p < 0.05$.

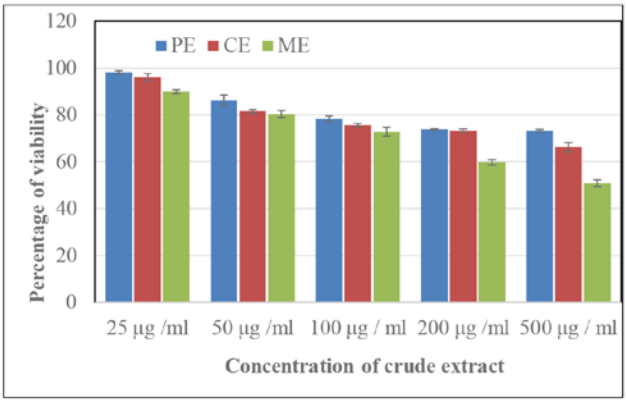

Figure 3: Cytotoxic activities of crude extracts obtained from *Benkara malabarica* PE – Petroleum Ether Extract, CE – Chloroform Extract, ME – Methanol Extract.

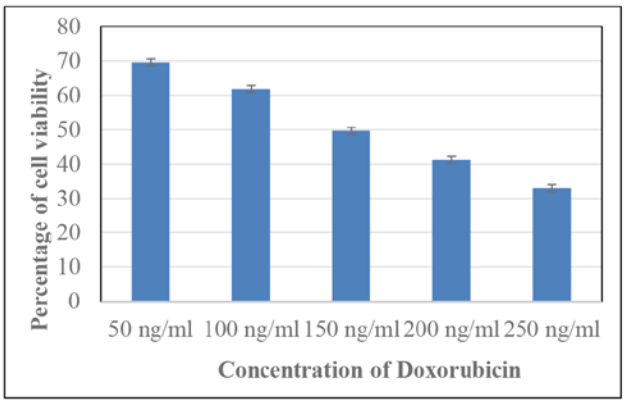

Figure 4: Cytotoxic activities of control doxorubicin

They have emerged as source of medicines for many ailments including cancer.[32] Presence many phytochemicals such as phenols, alkaloids and glycosides and significant antimicrobial activities are also reported in the leaf extracts of *B. malabarica* (Kalusalingam Balakrishnan, 2019). Hence the present study was undertaken to evaluate the antioxidant and anticancer potential of *B. malabarica* leaf extracts, obtained using three different solvents such as methanol, chloroform and petroleum ether.

In the present study, dose dependent DPPH free radical scavenging activities and iron chelating activities were observed in all the extracts. It shows the increased ability of extracts to donate an electron or hydrogen ions.[33-34] Many phytochemicals such as phenols, alkaloids and glycosides are reported in the stem, leaf and root extracts of *B. malabarica*. The redox properties of phytochemicals make them to act as hydrogen donors, reducing agents, singlet oxygen neutralizer and metal chelators.[35] In most of the studies, the antioxidant

activities of plant extracts are correlated with phenols and flavonoids. These phytochemicals have hydroxyl group in their structure, which makes them to act as good radical scavengers and metal chelators.[36-37] The presence of such phytochemicals in the leaf extracts of *B. malabarica* and their synergistic interactions with free radicals may be inferred with the strong antioxidant potentials of plant extracts. However the free radical scavenging and iron chelating activities of extracts were found to be lower than the positive controls, which are similar many previous studies with other plants.[38-39]

The capacity of the plant extract to react and neutralize the different radicals is depend upon the solubility of extracts in diverse solvents and stereo selectivity of the radicals.[40]

All the three extracts, methanol, chloroform and petroleum ether, of *B. malabarica* exhibited noticeable and dose dependent anticancer activities in the MTT assay. The presence of phytochemicals such as phenols, alkaloids and glycosides in the extracts of *B. malabarica* may be inferred with the anticancerous activities of the extracts. The role of phytochemicals as anticancer agents is reported in many previous studies.[41-44] Doxorubicin was used as positive control in the MTT assay of the present study. It had exhibited very high cytotoxicity activity even at low dosage. Doxorubicin is widely used and effective medicine for in cancer treatments. It directly inhibits the topoisomerase II enzyme of the cells and thus causing the cell death or cytotoxicity.[45-46]

Methanol extracts of *B. malabarica* exhibited higher antioxidant and anticancer activities, when compared with chloroform and petroleum ether extracts. Highest extraction power and polarity of methanol can be inferred for the higher activities of methanol extracts.[47] The type of phytochemicals, present in the extracts varies with use of different solvents. Each solvent has specific influence on the extraction of phytochemicals from plant parts. The extraction of phytochemicals from plant parts is depending on the nature of solvent and its interface with specific compounds.[48-50]

The oxidation is generally required to many living things for the production of energy to fuel in several biological processes.[51] Chemical substances which are able to perform antioxidant reactions and radical scavengers.[52] Free radical scavengers or antioxidant has the potent to prevent, delay or ameliorate many of disorders.

Previous reports on the cytotoxicity and antioxidant activity of *B. malabarica* are very little or none in the literature. This made the comparison of our results with those of previous studies very difficult. The highest cytotoxicity showed that in 25 µg/ml of plant leaves extract such as 89.8 %, 96.1 %, 98 % in methanol, chloroform and petroleum ether extracts respectively.

CONCLUSION

The present investigation sheds light on the antioxidant and cytotoxicity effects of extracts obtained from the leaves extracts of *B. malabarica* obtained using different solvents such as methanol, chloroform and petroleum ether. The usage of this under exploited plant may play a vital role in preventing human diseases, such as cancer, cardiovascular and aging problems, where free radicals are involved. Though the preliminary results are promising, further studies such as isolation and identification of individual compounds are essential to demonstrate the mechanism of observed pharmacological activities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DPPH: 2,2,Diphenyl picryl Hydrazyl; **OPhenantroline:** Orthophenanthroline; **MTT:**3-(4,5, dimethylthiazol-2-yl)-2-5,diphenyl-2H-tetrazolium bromide; **Hep G2:** Liver Hepatocellular Carcinoma; **WHO:** World Health Organization; **DNA:** Deoxyribonucleic Acid; **NCCS:** National Centre for Cell Sciences; **CO_2:** Carbondi oxide; **C:** Carbon; **DMEM:** Dulbecco's Modified Eagle's Medium; **FBS:** Fetal Bovine Serum; **%:** Percentage; °C: Degree Celsius; **mM:** Milli Molar; **UV:** Ultra violet; **FRAP:** Ferric Reducing Antioxidant Power; µM: Micromolar; µg: Microgram; **min:** Minutes; **EDTA:** Ethylenediamine tetra acetic acid; **NADPH:** Nicotinamide Adenine Dinucleotide Phosphate; **DMSO:** Dimethyl sulfoxide; **nm:** Nanometer; **hr:** Hour; µl: Microlitre; **ANOVA:** Analysis of variance; **IC_{50}:** The Half maximal Inhibiting Concentration.

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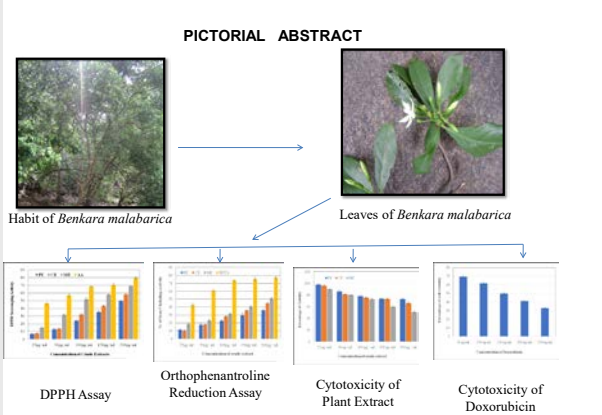
PICTORIAL ABSTRACT



SUMMARY

The present study is to determine the antioxidant and cytotoxicity activities of the leaves extracts using different assays. The extracts were further used to analyze their antioxidant and cytotoxicity potential using different methods like Orthophenanthroline, DPPH and MTT assay. The results of all the three assays indicate that the extracts possess considerable antioxidant and cytotoxicity properties. The activities were found to be dose dependent.

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