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# Phytochemical Evaluation and Antioxidant Potential of *Garcinia indica* Fruits on $H_2O_2$ Induced Oxidative Stress in THP-1 Cell Line

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ABSTRACT
*Garcinia indica* have been used for centuries with claim for its medicinal value by traditional practitioners to treat numerous diseases. The anti-oxidant activity of *G. indica* fruit which were assessed by determining the total phenolic content, evaluating the 2, 2-diphenyl-1-picrylhydrazyl, (DPPH) activity, superoxide dismutase (SOD) activity, as well as its ability to inhibit Nitric Oxide (NO) and also their effect on $H_2O_2$ induced Reactive Oxygen Species (ROS) generation in human monocytic (THP-1) cells was investigated by tracking intensity of a cell permeable fluorescent dye. In addition, High Performance Liquid Chromatography (HPLC) was used to quantify the bioactive constituent of *G. indica* fruit extract responsible for its biological activity. The fruit extract of *G. indica* had the highest amount of hydroxycitric acid (HCA) and phenolic content showed potent antioxidant activity. The $IC_{50}$ values for DPPH, SOD and NO scavenging activities were 50.34, 37.08 and 34.15 μg $mL^{-1}$, respectively. *Garcinia indica* fruit extract (200 μg $mL^{-1}$) attenuated ≥60% of $H_2O_2$ mediated ROS generation in THP-1 cells. The above data provides evidence that the fruit extract of *G. indica* is rich in natural anti-oxidants and thus justify its use in folk medicine especially in the management of free radical-mediated disorders.

**Key words:** Antioxidant, *Garcinia indica*, hydroxy citric acid, nitric oxide, superoxide dismutase

## INTRODUCTION

Oxidative properties of oxygen play a vital role in aerobic life as well as in our metabolism. Oxygen with double-edged properties, being essential for life, can also damage the cells by oxidative stress (Kumar *et al*., 2012). There are evidences enumerating the key roles of Reactive Oxygen Species (ROS) and other oxidants in causing numerous disorders (Pisoschi and Pop, 2015). Hence, the importance of antioxidants has been discovered for prevention of diseases and maintenance of human health (Saeed *et al*., 2012). However, lipid peroxidation, hydroxyl radical and superoxide anions are often generated as byproducts from biological reactions/exogenous factors (Pisoschi and Pop, 2015). These reactive species exert oxidative damage by reacting with nearly every molecule found in living cells. Such oxidative stress are considered to be important causative factors in the development of various ailments such as aging, diabetes, stroke, atherosclerosis, cancer, cardiovascular diseases and neurodegenerative disorders (Campos *et al*., 2014;

Kizer *et al*., 2014). Antioxidants derived from natural sources either in the form of extract or its chemical constituents have been very effective to prevent the damage caused due to oxidative stress (Das *et al*., 2014). Almost all organisms are well protected against reactive species by enzymatic antioxidant defense such as superoxide dismutase (SOD), glutathione peroxidase, catalase etc. Non-enzymatic antioxidants are flavonoids, ascorbic acid, glutathione, α-tocopherol and carotenoids. When the mechanism of antioxidant protection becomes unbalanced, it leads to the massive production of free radicals, resulting in disease and aging (Sharma *et al*., 2012). In recent years, the use of natural antioxidants supplements present in dietary plants and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila *et al*., 2007). One among the plants is *Garcinia indica* Choisy that has been frequently used in a traditional medicine for centuries belonging to the family Clusiaceae commonly known as kokum (Padhye *et al*., 2009). These medicinal plants can be exploited to find out effective alternatives to the synthetic drugs. In the traditional Indian system of medicine like Ayurveda and other folk systems of medicine, the fruit rinds, seeds and leaves are used to treat various ailments. Many therapeutic effects of the fruit have been described in Ayurveda, such as allergies, skin burns, dysentery, diarrhea, tumors and heart diseases. The major phytochemical constituents present in *G. indica* are anthocyanins, hydroxycitric acid (HCA), garcinol, isogarcinol, ascorbic acid and polyphenols (Baliga *et al*., 2011). The HCA described as one of the major active constituent of kokum has been used as an anti-hyperlipidemic agent (Saadat and Gupta, 2012). Similarly garcinol, purified from the fruit rind is known to exhibit anti-oxidant properties (Padhye *et al*., 2009). Keeping in view the use of *G. indica* fruit as an effective remedy for oxidative stress associated diseases, the present study was under taken to evaluate the antioxidant activity of *G. indica* fruit.

## MATERIALS AND METHODS

**Chemicals:** The DMSO (dimethyl sulfoxide), DPPH (2,2-diphenyl-1-picrylhydrazyl), ferric chloride, Folin-Ciocalteu reagent, gallic acid, Griess reagent, MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide), $Na_2CO_3$ (sodium carbonate), NADH (nicotinamide adenine dinucleotide-hydrogen (reduced)), NBT (nitro blue tetrazolium), potassium ferricyanide, PBS (phosphate buffer saline), PMS (phenazinemethosulphate), sodium nitroprusside and trichloroacetic acid were purchased from Sigma Aldrich, India. RPMI-1640 (Roswell Park Memorial Institute), penicillin, streptomycin, gentamycin, human AB serum and RNase were purchased from HiMedia, India. DCFH-DA (2-7-diacetyl dichlorofluorescein), trypan blue, Fetal Bovine Serum (FBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen, USA.

**Preparation of extract:** The fruit rind of *G. indica* was collected from the natural habitat, at Konkan region of Maharashtra, India during November 2013. The samples were identified and authentication was done by the plant anatomist, for which a voucher specimen (ISISM/RES/B0016) has been preserved in the Interdisciplinary Institute of Indian System of Medicine (IIISM), department, SRM University for future reference. The plant materials were air dried under shade, powdered mechanically and stored in an airtight container for further use. Thirty grams of the powdered plant material was soaked in hydro-alcoholic extract (70 mL ethanol and 30 mL water) at room temperature with continuous shaking and the extraction was repeated three times and then the solvent was removed using rotary vacuum evaporator (Rotovac R-210, Buchi, Germany).

**Total Phenolic Content (TPC) of *Garcinia indica* fruit:** Total phenolic content in *G. indica* fruit extract was determined by spectrophotometric method using Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound (Shukla *et al*., 2012). The 1.0 mL of extract solution containing 1.0 g extract in a volumetric flask was diluted with 46 mL of distilled water. The 1.0 mL of Folin-Ciocalteau reagent was added and mixed thoroughly. Three minutes later, 3.0 mL of 2% sodium carbonate was added and the mixture was allowed to stand for 3 h with intermittent shaking. Later the absorbance was read at 760 nm with a UV-Vis Spectrophotometer (3200, Lab India Analytical, India). The concentration of total phenolic content was expressed as mg $g^{-1}$ of dry extracts.

**High Performance Liquid Chromatography (HPLC) analysis of *Garcinia indica* fruit extract:** *Garcinia indica* extract was dissolved in HPLC grade MeOH (1 mg $mL^{-1}$) and subjected to Reverse Phase (RP-HPLC) for the qualitative and quantitative analysis of HCA contents. The HPLC system consisted of Shimadzu LC 2020 (Shimadzu, Japan) equipped with binary solvent delivery system, column oven, Photo Diode Array Detector (PDA) and Lab solution software was used for all data analysis. The chromatographic separation was performed on Phenomenex-Luna (Torrance, CA, USA) $C_{18}$ column (i.d. 250×4.6 mm, 5 μm) and the column oven were set at ambient temperature. The isocratic mobile phase consisted of acetonitrile: water (0.03% $H_3PO_4$) with a flow rate of 0.8 mL $min^{-1}$ and the samples were injected using Hamilton micro liter syringe into a 20 μL injection loop.

***In vitro* antioxidant study**

**DPPH radical scavenging activity:** The free radical scavenging activity of *G. indica* fruit extract was determined *in vitro* by slightly modified method using a 96 well microtiter plate (Sulaiman *et al*., 2011). Briefly, 100 μL of various concentrations of extract (6.25-200 μg $mL^{-1}$) or ascorbic acid were added in DMSO and 100 μL of DPPH (200 μM) solution

was added to each well. The plates were incubated at 37°C for 30 min without exposing to light. The absorbance was read at wavelength of 540 nm using a microtiter plate reader. And the percentage inhibition was calculated using equation (Abs-absorbance):

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100 \quad (1)$$

**Superoxide radical scavenging (SOD) activity:** The superoxide scavenging activity of *G. indica* fruit extract was determined by the method described by McCord and Fridovich (1969). Briefly, $O_2$ was generated by adding 62.5 µL of 468 µM NADH solution, 62.5 µL 150 µM NBT solutions and 62.5 µL of 60 µM PMS and different concentration of *G. indica* fruit extract (6.25-200 µg $mL^{-1}$) or standard (ascorbic acid) were added to a micro well plate and incubated at room temperature of 25°C for 5 min. After incubation, the optical density of the reaction mixtures was measured at 560 nm and the percentage inhibition was calculated. The percentage inhibition was determined by comparing the results of control and test samples (Eq. 1). The $IC_{50}$ value was calculated (Kumar *et al*., 2010).

**Nitric oxide scavenging (NO) activity:** Nitric oxide scavenging activity was measured by Griess reagent. Briefly, 150 µL of various concentrations of extract (6.25-200 µg $mL^{-1}$) or standard (ascorbic acid) were added in 130 µL of deionized water and 20 µL of Griess reagent was added to each well and incubated for 30 min at room temperature. The absorbance was read at wave length of 548 nm using a microtiter plate reader. The concentration which inhibited 50% of the cellular growth ($IC_{50}$ value) was determined (Eq. 1).

**Quantification of intracellular ROS induced by $H_2O_2$ in THP-1 cells:** Human monocytic (THP-1) cells were purchased from National Center for Cell Sciences, Pune, India. The cells were cultured under standard conditions in the RPMI media, supplemented with 10% FBS, 100 U $mL^{-1}$ of penicillin and 100 U $mL^{-1}$ of streptomycin in a humidified 5% $CO_2$ at 37°C. The cytotoxic effect of *G. indica* fruit extract was assessed by measuring the activity of mitochondrial dehydrogenase as described previously (Wang *et al*., 2010; Vasanth *et al*., 2014). The THP-1 cells were grown to $5\times10^3$ cells/well in 96-well plates and then incubated with various concentrations of *G. indica* fruit extract (7.8-500 µg $mL^{-1}$) at 37°C with 5% $CO_2$ for 24 h. After incubation the culture media was removed and the cells were incubated with 5 mg $mL^{-1}$ MTT in fresh medium at 37°C for an additional 4 h. After this period, the supernatants were removed and 100 µL DMSO was added to each well to dissolve the formazan crystals. The plates were read on a microplate reader at a test wavelength of 540 nm and a reference wavelength of 650 nm. Doses of *G. indica* at the concentration that gave cell viability of 80% or more were used in the further experiments.

The ROS production was determined by staining THP-1 cells with oxidation sensitive probe DCFH-DA as described previously with minor modifications (Zhang *et al*., 2014).The fluorescent intensity is proportional to the amount of ROS generation by the cells. THP-1 cells ($2\times10^5$ cells/well) were cultured in 12 well plate treated with indicated concentrations in the presence or absence of *G. indica* fruit extract (50, 100 and 200 µg $mL^{-1}$) and incubated at 37°C with 5% $CO_2$ for 24 h. The cells in 12 well plates were washed twice with HBSS and incubated in 1 mL working solution of 20 µM DCFH-DA at 37°C in the dark for 30 min. Then the cells were harvested, washed with ice-cold PBS. Cells were treated with 10 mM $H_2O_2$ to induce intracellular ROS. The fluorescence emission of DCF was analyzed in FL-1 channel on FACS Calibur flow cytometer (BD, USA) using excitation and emission wavelengths at 488 and 525 nm, respectively. The Mean Fluorescence Intensity (MFI) of $10^4$ cells acquired were quantified using Cell Quest Software.

**Statistical analysis:** The data was statistically evaluated using GraphPad prism (Windows version 5.01). Values were presented as Mean±SD of the three replicates of each experiment. The results with $p<0.05$ were considered to be statistically significant.

## RESULTS

**Total Phenolic Content (TPC) of *G. indica* fruit:** In this study, *G. indica* fruits extract possessed high phenolic contents 63.21 mg GAE $g^{-1}$ of extract. The TPC was calculated using standard curve of gallic acid (Fig. 1).

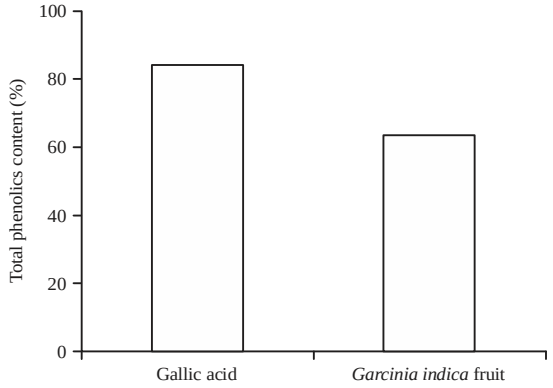


Fig. 1: Total phenolic content of *Garcinia indica* fruits extract

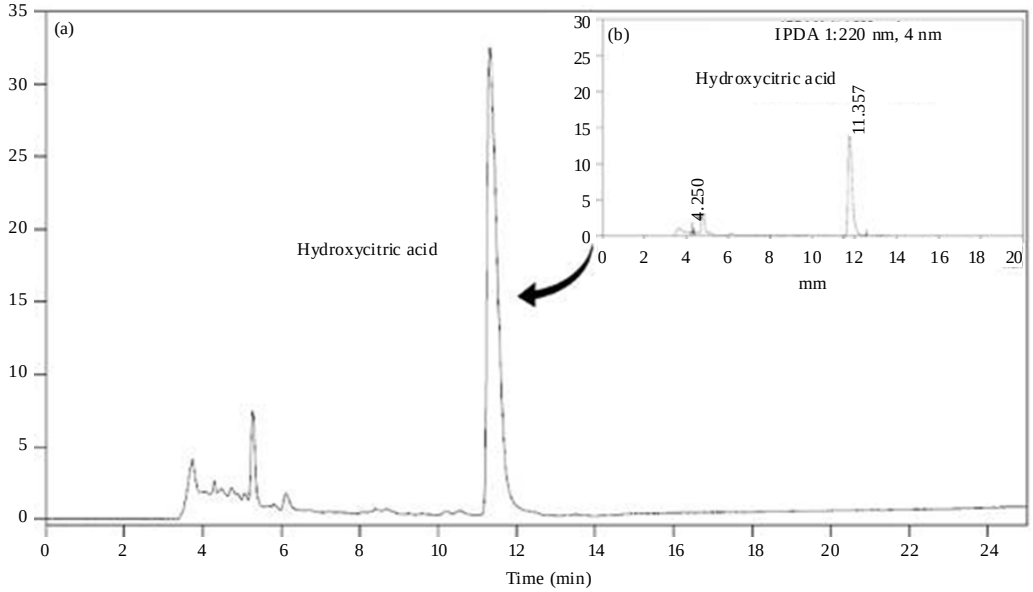


Fig. 2(a-b): HPLC chromatograms, (a) Hydro-alcoholic extract of *Garcinia indica* fruit extract at 220 nm, inset and (b) Hydroxycitric acid (standard marker compound)

**Quantitative High Performance Liquid Chromatography (HPLC) analysis of *Garcinia indica* fruit extract:** The HPLC chromatogram of reference standards like HCA detected at 220 nm is shown in Fig. 2. The presence of HCA, confirmed by comparing chromatographic peaks in hydro-alcoholic extract of *G. indica* fruits was 2.338% w/w with the retention time (Rt) 11.57 min.

**DPPH radical scavenging activity:** It is one of the most widely accepted methods used for screening of antioxidant activities in plant extracts. In the present study, *G. indica* fruit extract showed a significant effect in inhibiting DPPH reaching upto a concentration of 200 μg $mL^{-1}$. We observed a dose response curve of DPPH radical scavenging activity of *G. indica* compared with standard ascorbic acid. The $IC_{50}$ value of *G. indica* fruit extract was 50.34 μg $mL^{-1}$ while, the $IC_{50}$ value of standard ascorbic acid is 13.74 μg $mL^{-1}$ (Fig. 3a).

**Superoxide radical scavenging (SOD) activity:** The SOD radical activity illustrates that *G. indica* fruits at 200 μg $mL^{-1}$ exhibited maximum 85.11±3.2 μg $mL^{-1}$ SOD scavenging activity. The $IC_{50}$ value of *G.* indica fruits was found to be 37.08 μg $mL^{-1}$ and that of ascorbic acid is 6.72 μg $mL^{-1}$ (Fig. 3b).

**Nitric oxide scavenging (NO) activity:** The HA extract of *G. indica* fruits was investigated for its inhibitory effect on NO production. It inhibited the NO production in a dose dependent manner. The $IC_{50}$ value of *G. indica* fruits was 34.15 μg $mL^{-1}$ and that of the standard ascorbic acid was 19.04 μg $mL^{-1}$ (Fig. 3c).

**Cell viability assay and quantification of intracellular ROS induced by $H_2O_2$ in THP-1 cells:** The concentration that gave more than 80% viability were selected and chosen for further experiments (Fig. 4). Different concentration of the extracts (50, 100 and 200 μg $mL^{-1}$) as determined from the MTT assay were chosen to find its inhibitory potential on ROS generation compared to control and $H_2O_2$ induced in THP-1 cells (Fig. 5).

## DISCUSSION

Many synthetic antioxidants such as butylated hyzdroxyanisole (BHA), butylated hydroxytoluene (BHT) are being used in order to retard the oxidation process, but have proven to have certain health hazards. This has led to the use of alternative antioxidants from natural sources (Sikora *et al*., 2008). Drugs from natural sources are the only alternative to synthetic drugs by decreasing the side effects in counteracting the free radicals associated diseases. A large number of naturally occurring herbal based medicines have been recognized possessing antioxidant abilities. Therefore, in the present study *G. indica* fruits were evaluated for their free radical scavenging activity by various *in vitro* methods. *Garcinia indica* fruits extract have considerably high phenolic compounds in a dose dependent manner. These results suggest that the higher level of free radical scavenging activity was due to the presence of phenolic components because their hydroxyl groups confer scavenging ability (Yamaguchi *et al*., 2000). Several studies have reported the importance of estimating the phenolic content in plants and the relationship between phenolics and anti-oxidant activity

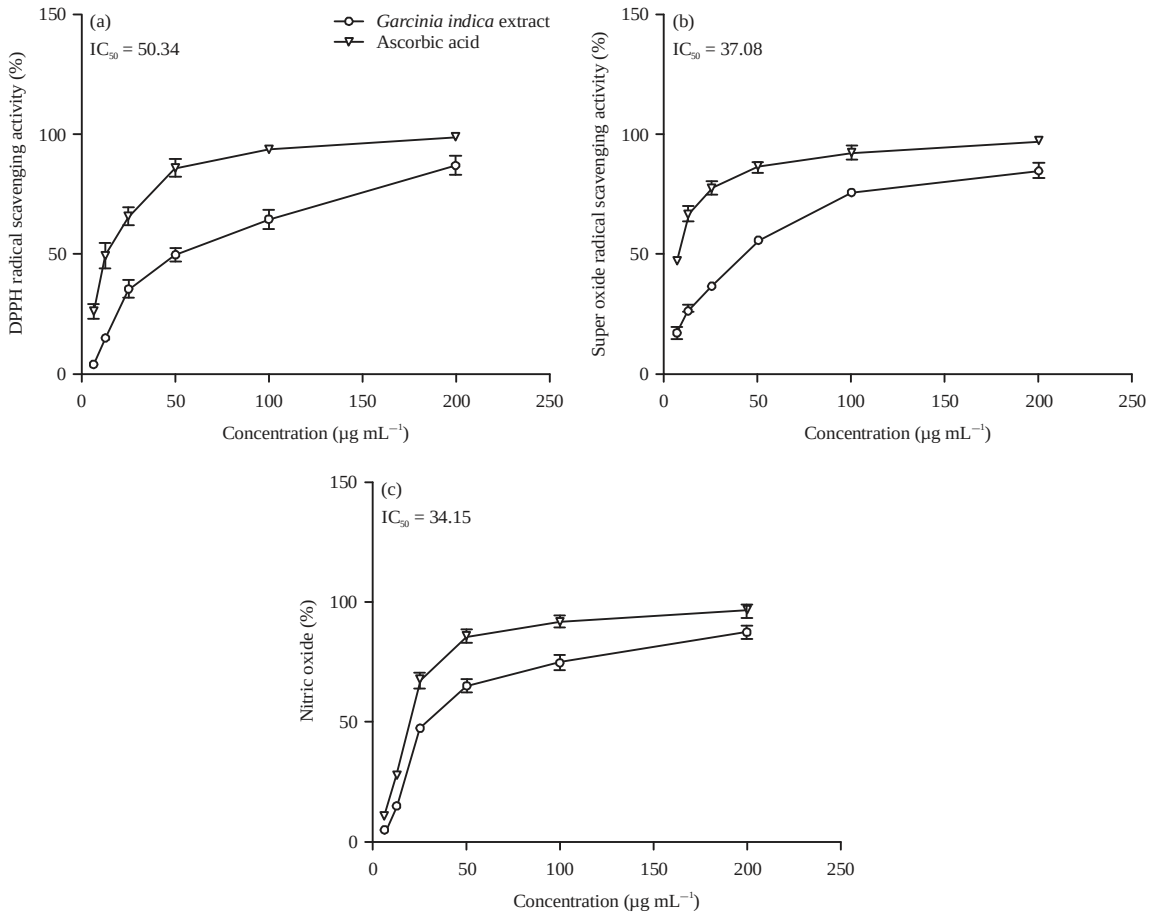


Fig. 3(a-c): Percentage of (a) DPPH, (b) SOD and (c) Nitric oxide of *Garcinia indica* fruit extract. Data expressed as Mean±SD (n = 3)

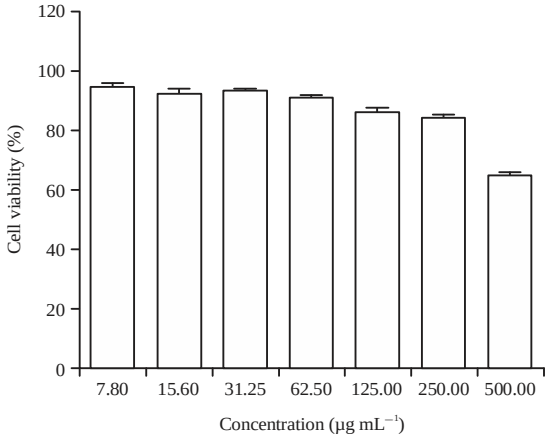


Fig. 4: Effect of *Garcinia indica* fruit extract on cellular viability of THP-1 cells was examined by the MTT assay

(Sadeghi *et al*., 2015). It should be noted that the extract of *G. indica* fruits has higher level of phenolic content. Thus, the fruits extract might be an alternative agent for treatment of free radical associated diseases.

The HPLC has been utilized to standardize many medicinal plant extracts (Saidan *et al*., 2015). Standardization of herbal products is more challenging than synthetic drugs due to the presence of various compounds in a single plant. In the present study *G. indica* fruits extract was subjected to HPLC analysis to find out the presence of biomarkers which could be due its synergistic pharmacological activities. Compounds are reputed for their health promoting properties due to their high antioxidant capacity. It is this reputation of the HCA that have received much attention in the mainstream of pharmaceutical research especially in weight management (Onakpoya *et al*., 2011). The HCA has been known to be present in considerable amounts in different species of *Garcinia* such as *G. cambogia*, *G. atroviridis* and *G. indica*. The safety of HCA as an anti-obesity dietary supplement has been extensively reviewed by *in vivo* and clinical studies (Chuah *et al*., 2013). Over the last few decades, the anti-obesity properties of this plant have been confirmed specifically due to its HCA content. It has been known to reduce dietary intake and also proven to play a beneficial role in obesity associated complications such as oxidative stress, inflammation (Asghar *et al*., 2007) and lipid abnormalities

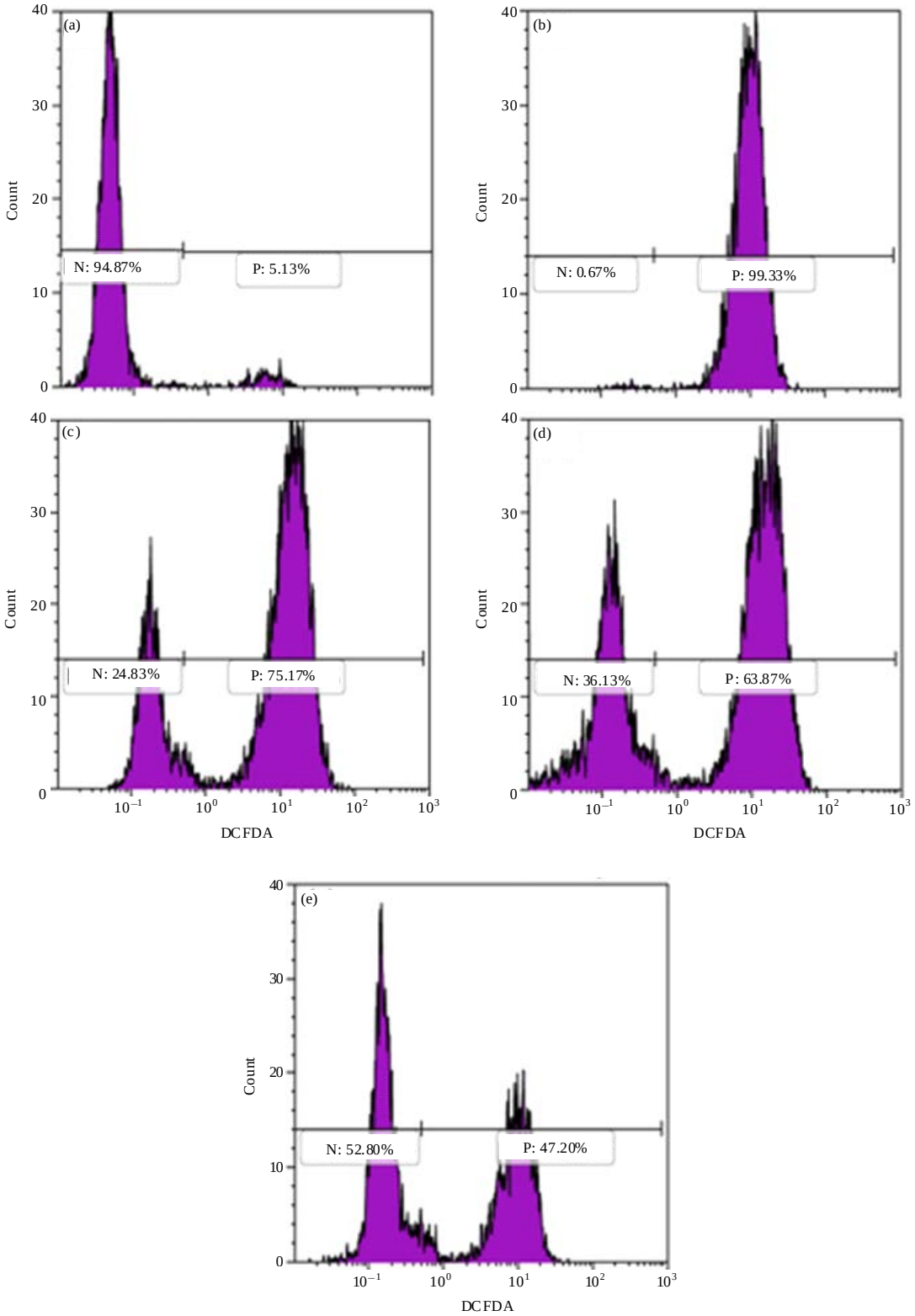


Fig. 5(a-e): Effect of *Garcinia indica* fruit extract on the $H_2O_2$ induced ROS production in THP-1 cells. Cells were stained with DCFH-DA and analyzed using FACS Calibur flow cytometer, (a) Control, (b) $H_2O_2$ induced cell, (c) Cells treated with 50 μg $mL^{-1}$, (d) 100 μg $mL^{-1}$ and (e) 200 μg $mL^{-1}$ of the extract

(Jena *et al*., 2002). The HCA found to be an active ingredient in the fruit rinds has been known to inhibit ATP citrate lyase (Sullivan and Triscari, 1977). In human clinical trials, HCA was found to be the cause of loss of appetite and plasma leptin levels (Preuss *et al*., 2004) and has been used as an anti-hypercholesterolemic agent.

In the search for new alternatives, effective and safe anti-oxidants is a major thrust area in the mainstream of drug discovery. Medicinal plants play a vital role for the development of new anti-oxidant drugs (Eshwarappa *et al*., 2015). A robust survey in the recent past have enumerated the role of medicinal plants and its derivatives in the management of free radical associated diseases (Lobo *et al*., 2010). *Garcinia indica* is one of the traditional plants that has been used frequently as a folk medicine for various ailments (Liu *et al*., 2015). Different *in vi*tro methods namely DPPH assay, SOD, NO and ROS were employed. The present investigation has shown that the extract exhibited DPPH radical-scavenging activity in a concentration dependent manner and results were compared with the standard ascorbic acid, indicating their abilities to act as radical scavengers. The induction of oxidative damage by the free radical was suggested to be the cause of cancer, cardiovascular diseases and neurodegenerative disease. Hence, the DPPH activity was assessed which is based on a scavenging ability of anti-oxidants towards a free radical. It is considered to be one of the easiest and widely applied methods for estimating the antioxidant activity (Kedare and Singh, 2011). Superoxide anion radical is one of the most important ROS and is expressed in most of the diseases like cancer, inflammation and neurodegenerative disorder. The results suggest that the *G. indica* fruits extract are more potent to superoxide radical scavenging activity. The NO is an important physiological mediator involved in various pathological conditions like neurotransmission, vasodilation, platelet aggregation, inflammation and apoptosis. Thus, it is an important bio regulatory molecule with various physiological effects. However, the increasing expression of inducible nitric oxide synthase (iNOS) enzyme generates excess amounts of NO which leads to pathological complications during chronic diseases (Chen *et al*., 2015). Thus, much importance is being given to the development of drugs which can be which can be potent inhibitors of NO production in relation to the pathogenesis of various diseases such as chronic inflammatory disease (Joo *et al*., 2014). The fruit rind of the extract reduced the generation of NO *in vitro* in a concentration dependent manner.

Flow cytometric analysis by staining with a specific fluorescent probe DCFH-DA were used for measuring hydrogen peroxides and hydroxyl radicals revealed an increase in DCFH-DA positive cell population indicating generation of ROS (Zhang *et al*., 2014). The FACS analysis revealed that the pretreatment of cells with *G. indica* fruits extract showed significantly reduction in the intracellular ROS production in a dose-dependent manner. The ROS is a secondary messenger in multiple signaling pathways and plays an important role in pathological complications by regulating the activity of certain enzymes involved in various pathways that causes diseases (Poole *et al*., 2015). The study was conducted to investigate the inhibitory potency of *G. indica* fruit extract by measuring the oxidative stress *via* the mitochondrial pathway. Collectively, these findings provide *in vitro* evidences for the antioxidant activity of *G. indica* fruit for effective utilization of herbal drugs as an antioxidant agent. These antioxidants act as protection agents for reducing the oxidative damage of human body from ROS and thus the progression of many chronic diseases as well as lipid peroxidation is retarded (Wolfe *et al*., 2003). These results indicated that *G. indica* could serve as important frontiers in the development of future anti-oxidant drugs. It can be used in the pharmaceutical products as a source of natural antioxidants. These findings thus lay the foundation for the exploration, isolation and identification of novel antioxidant agents with different phytochemical and pharmacological properties.

## CONCLUSION

Ethnopharmacological knowledge may be beneficial to know the potentiality of different medicinal plants to yield anti-oxidant properties. It might have been brought by the acknowledgment of the significance of the medicinal plants as potential sources of new compounds of therapeutic interest and as sources of lead compounds in the drug development. In the present findings, we found that hydroalcoholic extract of *G. indica* fruit contains high amounts of hydroxycitric acid and also contains significant levels of phenolic compounds, which may be responsible for exhibiting high antioxidant activities. Thus based on a wide spectrum of activities exhibited by *G. indica*, the plant can be considered as an effective antioxidant resource for preventing oxidative stress mediated disorders and thus may serve as a good source for isolating new compounds for treatment against cancer or other neurodegenerative disorders.

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