

Original Article

ANTIOXIDANT, DNA DAMAGE PROTECTIVE AND HEPATOPROTECTIVE ACTIVITIES OF *AMORPHOPHALLUS CAMPANULATUS*

PANKAJ SINGH, RAM LAKHAN SINGH*, POONAM KAKKAR¹

¹Nutraceutical Laboratory, Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad 224001, India, ²Herbal Research Section, Indian Institute of Toxicology Research, P. O. Box 80, MG Marg, Lucknow 226001, India
Email: drrlsingh@rediffmail.com

Received: 02 Dec 2015 Revised and Accepted: 03 Feb 2016

ABSTRACT

Objective: *Amorphophallus campanulatus* (AC) is commonly known as *Suran*. It is used to cure piles, deranged digestion and liver diseases. The present study was carried out to evaluate AC plant as a new source of natural bioactive molecules having antioxidant potential and their protective role against hepatotoxicity induced by t-BHP.

Methods: Ethanolic extracts of AC leaf and fruit were subjected to assessment of its antioxidant potential by superoxide anion radical scavenging activity (SARSA), free radical scavenging activity (FRSA), ferrous ion chelation activity (FIC) and reducing power (RP). The study was further carried out to assay the DNA damage and hepatoprotective activity of AC leaf extract.

Results: Leaf extract of AC showed lower IC₅₀ for SARSA, FRSA and FIC (111.67 mg/ml, 11.58 mg/ml and 8.56 mg/ml, respectively) than fruit extract. HPLC analysis of leaf showed the presence of chlorogenic acid, caffeic acid, rutin, quercetin and kaempferol. Leaf extract showed potential to protect hydroxyl radical-induced DNA damage. Hepatoprotective effects of AC leaf was shown by increasing superoxide dismutase (SOD), glutathione content (GSH), catalase (CAT) and protein content and decreasing nitric oxide (NO) and MDA formation, when t-BHP treated hepatocyte cells were exposed to different concentration (1.0, 2.5, 5.0 and 7.5 µg) of extract.

Conclusion: The results suggest that AC leaf has antioxidant, cytoprotective and hepatoprotective properties which may be due to the presence of important bioactive constituents; hence it may be used in the form of dietary component and also in formulations against liver diseases.

Keywords: Hepatoprotective activity, Glutathione, Antioxidant enzyme, Lipid Peroxidation, Hepatocytes

© 2016 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

INTRODUCTION

Our body system has a unique balance between the generation of ROS and antioxidant defence system. When ROS generation becomes uncontrolled and dominates over the antioxidant defence system of the cell, free radicals start attacking the cell component such as lipid, protein and carbohydrate. This leads to the development of degenerative diseases such as ageing, rheumatoid arthritis, atherosclerosis, cancer, neurodegenerative and cardiovascular diseases, etc. Singh *et al.* [1] reported that phytochemicals having antioxidant activity in *Trigonella foenum-graecum* play an important role in the scavenging of free radicals and protection of DNA damage. The same group demonstrated that the extracts of *A. nilotica* pod exhibited strong and effective *in vitro* and *in vivo* antioxidant potential by chelating metal ions as well as scavenging free radicals and anti-quorum sensing activity [2]. The liver is an important organ which shows a promising effect in metabolism and excretion of xenobiotics from the body. Liver cell injury is caused by various toxic chemicals such as certain antibiotic, chemotherapeutic agents, carbon tetrachloride (CCl₄), t-BHP, etc. Consequently, this hepatic cell toxicity leads to a range of liver diseases such as ischemia-reperfusion injury/fibrosis and liver failure [3]. The available synthetic drugs to treat liver disorders in this condition may also cause further damage to the liver [4]. ROS generated while drug metabolism are detoxified by antioxidants present in the body or taken in the form of diet. Tripathi *et al.* [5] reported that ethanolic extract of *Fumaria parviflora* have protective activity against nimesulide induced hepatotoxicity and attenuated critical control points of apoptotic cell death. In another study, the same group reported that Glycyrrhizic acid (GA), the main bioactive ingredient of *Glycyrrhiza glabra* could be beneficial against liver diseases induced by oxidative stress [6]. A combination of terpenes *i.e.* camphene and geraniol was found to prevent oxidant-antioxidant imbalance and reduce the nimesulide-induced hepatotoxicity. Therefore, the use of these natural antioxidants may improve benefit to liver cells [7].

Antioxidants can offer protection against cellular oxidative damage under disease/stress conditions. Plant-derived antioxidants such as flavanoids and polyphenols are considered to be safe. Hence considerable attempts are being made to identify plants with antioxidant capacity.

Amorphophallus campanulatus (Roxb.) (Family: Araceae) is commonly known as *Suran*. The literature showed that forms of the *Suran* are used in various ayurvedic formulations. The tubers are used as an analgesic, liver tonic, thermogenic, antihelminthic and diuretic agent [8]. Roots are used to treat ophthalmia and boil [9]. Some important chemical constituents of the tubers include carbohydrates, sitosterol, stigmasterol, thiamine and riboflavin [10]. The studies reported in the literature about AC plant are not sufficient to arrive at a definite conclusion regarding their remedial powers. Also, the bioactive phytochemicals have not yet been fully identified. In view of increasing awareness about health and increased use of phytochemical in the prevention/treatment of common as well as serious diseases, there is a need to find out some safe alternative drug/nutraceuticals which are used in our day to day life in one or the other form. Therefore, the present study was designed to investigate the *in vitro* antioxidant activities of AC through parameters such as superoxide anion radical scavenging activity, free radical scavenging activity, ferric ion chelation activity and reducing power. Bioactive compounds were also detected along with an evaluation of the DNA damage protective and hepatoprotective activities of leaf extract.

MATERIALS AND METHODS

Materials

Plant samples were collected from vegetable growing areas of eastern Uttar Pradesh. Collected plant samples were washed under running tap water, dried, in sun shade, powdered in a grinder and stored in polythene bags at 4 °C. The plant identification was

confirmed by National Botanical Research Institute (NBRI), Lucknow, India. The voucher specimens were also deposited at the institute.

Chemicals and reagents

Gallic acid, quercetin and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich, St. Louis, USA. β -Carotene, Ascorbic acid, Folin Ciocalteu's phenol reagents were the product of E. Merk, Mumbai, India. Nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), Phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), RPMI media, fetal bovine serum, glutathione reduced, NADH, tertiary-butyl hydroperoxide (t-BHP), nitro blue tetrazolium (NBT), thiobarbituric acid (TBA), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich Co., USA. Potassium ferricyanide, trichloroacetic acid, ferric chloride and sodium dodecyl sulphate were purchased from SRL, India. All other reagents and chemicals used were of analytical grade.

Extraction procedure

Twenty grams of dried AC leaf and fruit sample were extracted with 70% ethanol until discoloration. The extracted solvent was evaporated at 40 °C in a rotary vacuum evaporator and lyophilized till dryness. The powdered form of plant extracts was stored at -4 °C and used for the activity determination.

Quantification of bioactive phytochemicals

The ascorbic acid content of vegetables was estimated by the method of Arlington and reported in terms of g/kg of fresh weight [11]. Carotenoids were estimated by the method of Jensen and reported in terms of $\mu\text{g/g}$ of fresh weight [12]. Total phenolic content (TPC) was measured by the method of Ragazzi and Veronese and presented in term of g/kg of Gallic acid equivalent [13]. Carbohydrate content was estimated by Anthrone method and reported in term of g/kg of dry weight [14]. Protein content was estimated by the method of Lowry *et al.* and reported in term of g/kg of dry weight [15].

Superoxide anion radical scavenging activity (SARSA)

This assay was based on the capacity of extract to inhibit the reduction of nitroblue tetrazolium (NBT) by the method of Nishikimi *et al.* [16]. Three ml reaction mixture containing a different aliquot of plant extracts (50, 100, 150 and 200 μl) with 0.1 M phosphate buffer (pH 7.8), 60 μM Phenazine methosulphate (PMS), 468 μM nicotinamide adenine dinucleotide reduced (NADH) and 150 μM NBT was incubated for 5 min at ambient temperature. Absorbance was read after 6 min at 560 nm using UV-Vis spectrophotometer. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample.

Free radical scavenging activity (FRSA)

FRSA of the extracts was measured by using DPPH stable radical according to the method of Yen and Duh [17]. Each extract (0.1 ml) was added to freshly prepared DPPH solution (6 x 10⁻⁵ M in HPLC grade 2.9 ml methanol) and mixed vigorously. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 515 nm until the stable value was obtained.

$$\% \text{ Inhibition} = \frac{[(\text{blank absorbance} - \text{sample absorbance}) / \text{blank absorbance}] \times 100}{100}$$

The IC₅₀ which represents the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, representing a parameter widely used to measure the antioxidant activity, was calculated from a calibration curve by linear regression.

Ferric ion chelation (FIC)

Ferric ions chelating properties of the extracts were determined by the method of Decker and Welch (1990) [18]. Results were expressed in the terms of inhibitory concentration (IC₅₀). Different aliquots of plant sample (25 μl , 50 μl , 75 μl , 100 μl) were taken, and the volume was made up to 100 μl in each tube with distilled water.

One ml KSCN and 200 μl FeCl₃ were added in each tube. The red colour developed after adding FeCl₃. 1.0 ml ethanol was added for dilution and absorbance was read at 535 nm.

Reducing power (RP)

Reducing power of the extracts was determined by using the slightly modified method of ferric reducing antioxidant power assay [19]. Each extract (1.0 ml) was mixed with 2.5 ml of phosphate buffer (0.1 M pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide and incubated at 50 °C for 20 min. After completion of the incubation period, 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) was added to terminate the reaction. The upper layer (2.5 ml) was diluted with equal volume of deionized water. Finally, 0.5 ml of 0.1% (w/v) FeCl₃ was added and after 10 min the absorbance was measured at 700 nm against a blank. Reducing power was expressed as ascorbic acid equivalents (1 ASE = 1 mM ascorbic acid). The ASE value is inversely proportional to reducing power.

DNA damage assay

The DNA damage assay was performed using supercoiled pBR322 plasmid DNA according to the method of Lee *et al.* with some modifications [20]. A reaction mixture containing 10 μl of plant extract of different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml), pBR322 DNA (0.25 μg) and 10 μl Fenton's reagent (30 mM H₂O₂, 500 μM ascorbic acid and 800 μM FeCl₃) was incubated for 40 min at 37 °C and analysed on 0.9% agarose gel by staining with ethidium bromide.

HPLC analysis

The dried extracts were dissolved in HPLC grade methanol (1.0 mg/ml), filtered through sterile 0.22 μm millipore filter and subjected to qualitative and quantitative analysis by using Shimadzu LC-10A (Kyoto, Japan) HPLC instrument. The instrument was equipped with a dual-pump LC-10AT binary system (Shimadzu, Kyoto, Japan) HPLC, a UV detector SPD-10A (Shimadzu, Kyoto, Japan), and a Phenomenex Luna RP, C18 column (4.6x250 mm). Data were integrated by Shimadzu Class VP series software. Separation was achieved with an acetonitrile/water containing 1% acetic acid linear gradient program, started with 18% acetonitrile, changing to 32% in 15 min and finally to 50% in 40 min. Results were obtained by comparison of peak areas ($\lambda_{\text{max}} = 254 \text{ nm}$) of the samples (g/kg dry extract) with that of standards [21].

Isolation of hepatocytes

Animal handling in all experimental procedures was approved by the Institutional Animal Ethics Committee, (ITRC/IAEC/20/2006). Sprague-Dawley male rats weighing 200±20 g from the Indian Institute of Toxicology Research (IITR) animal colony were used for the experiments. Rats were housed in an air-conditioned room at 25±2 °C temperature with 60–70% humidity and a controlled 12 h light/dark cycle. Rats were fed on a standard pellet diet (Ashir-wad Pellet Diet, Mumbai, India) and water *ad libitum*. Chloroform was used for euthanasia.

Primary cell culture

Hepatocytes were isolated from the liver of overnight fasted rat after subjecting it to two stage collagenase perfusion with HEPES buffer [22]. Cell viability was checked by MTT test. Only preparations with cell viability greater than 95% were used for subsequent experiments. Hepatocytes were maintained in RPMI-1640 media supplemented with heat-inactivated 10% fetal bovine serum and 1% of 10,000 units Penicillin, 10 mg Streptomycin, 25 μg Amphotericin B, 1 mM sodium pyruvate, 2 mM glutamine under an atmosphere of 5% CO₂-95% air in an incubator (Thermo-forma) with controlled humidity at 37 °C. The cells were seeded at a density of 1.0x10⁴ cells/well (counted on hemocytometer) in 0.1% collagen pre-coated 96 well plates and used for exposure drug experiments after being cultured for 24 h.

Treatment of cells

t-BHP (250 μM) was dissolved in distilled water and filtered through 0.22 μm filter and used for subsequent treatment.

Quantitative analysis of viable cells

Cell viability was determined by a colorimetric MTT assay, as described by Mosmann [23]. After the treatment, the culture medium was carefully aspirated and MTT was added to each well. After 4h incubation, 0.2 ml DMSO was added to dissolve the formazan crystal and incubated for 20 min. Absorbance was measured at 530 nm using Spectramax PLUS 384 microplate reader (Softmax pro version 5.1; Molecular Devices, USA). The data are expressed as a percentage of control viability measurement in untreated cells.

Superoxide dismutase (SOD)

SOD assay is based on the spectrophotometric assay of the inhibition of nitro blue tetrazolium-NADH and phenazine methosulphate (PMS) mediated formazan formation. Briefly 10 μ l of cell lysate, 90 μ l of 50 mM sodium tetra pyrophosphate buffer (pH 8.3), 30 μ l of 0.3 mM nitro blue tetrazolium, 10 μ l of 0.96 mM PMS and 40 μ l of double distilled water (DDW) was added. The reaction was initiated by addition of 20 μ l 0.72 mM NADH. After incubation at 30°C for 90s, the reaction was stopped by the addition of 50 μ l glacial acetic acids. Absorbance was measured at 560 nm. 50% inhibition of formazan formation under assay condition in 1 min is taken as one unit of enzyme activity/minute [24].

Lipid peroxidation (LPO)

Lipid peroxidation is quantified by measuring malondialdehyde (MDA), a breakdown product formed from polyunsaturated fatty acids (PUFA) hydroperoxides. In this assay, the evaluation of end product malondialdehyde (MDA) formed due to membrane lipid peroxidation was measured. Briefly, to 10 μ l lysate, 10 μ l of DDW, 50 μ l of 50 mM phosphate buffer, 10 μ l of 1 mM butylated hydroxytoluene (BHT), 75 μ l of 1.3% thiobarbituric acid (TBA) was added. The lipids were isolated by precipitating them with 50 μ l of 50% trichloroacetic acid. The mixture was then incubated at 60°C for 40 min and then kept in ice for 15 min. The reaction was stopped by addition of 10 μ l of 20% sodium dodecyl sulphate. This assay measures the amount of pink coloured MDA-TBA adduct at 530 nm and to account for the interference of phytochemicals it is also read at 630 nm [25]. The concentration of thiobarbituric acid reactive substances (TBARS) was expressed as nM of MDA formation. Diluted 1, 1, 3, 3-tetraethoxypropane was used for the standard calibration curve.

Nitric oxide production

Accumulation of nitrite, the end product of NO metabolism in culture medium was determined using Greiss reagent. In brief 100 μ l of the cell supernatant (1×10^4 cells/100 μ l) was incubated with 100 μ l Greiss reagent consisting of 1% sulphanilamide, 0.1% naphthylethylenediamine-dihydrochloride and 2.5% H_3PO_4 at 37°C for 30 min and the absorbance was recorded at 542 nm. A range of concentration of sodium nitrite was used to generate the standard curve [26].

Reduced glutathione (GSH)

Total glutathione was measured using GSH reductase-DTNB recycling assay and the rate of color developed was measured at 412 nm. Total glutathione was measured by incubating 50 μ l of lysate with 2.5 μ l of 2-vinyl pyridine for 1 h at room temperature prior to recycling assay [27].

Catalase (CAT)

CAT was assayed spectrophotometrically using the method of Aebi *et al.* [28]. Briefly, the assay mixture of 1.0 ml contained 970 μ l of 50 mM sodium phosphate buffer pH 7.0 and 20 μ l of homogenate. The reaction was started by addition of 10 μ l hydrogen peroxide. The decrease in absorbance was then observed for 180s at every 15s interval at 240 nm. Catalase activity is expressed as Unit/mg of protein.

Total protein content

Protein estimation was done by using the standard protocol of Bradford *et al.* [29]. Bovine serum albumin was used as a standard, and the colour developed was read at 595 nm.

Statistical analysis

Statistical analysis was done by using prism software. Values from *in vitro* antioxidant activities were reported as mean \pm standard deviation (SD) of three determinations. Result were considered significant * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, when compared with untreated cell with treated (t-BHP+leaf extract at different concentration) cells.

RESULTS

Primary parameters

Leaf of AC plant contains more phenolics (35.78 g/kg of GAE) than fruit (23.58 g/kg of GAE). In the same manner leaf contain more vitamin C and protein (0.5714 g/kg of fresh weight (FW) and 295.36 g/kg of dry weight (DW), respectively) than fruit (0.0762 g/kg of FW and 35.96 g/kg of DW, respectively). But carotenoids and carbohydrate contents are more in fruit (3.68 μ g/g of FW and 165.17 g/kg of DW, respectively) than leaf extract (2.83 μ g/g of FW and 59.25 g/kg of DW, respectively) [table 1].

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity of the plant extracts has been presented in Fig.1. The IC_{50} value of ethanolic leaf and fruit extracts of AC were 111.67 and 123.89 mg/ml, respectively. The results showed that leaf extract of AC inhibited NBT reduction more significantly than fruit extract.

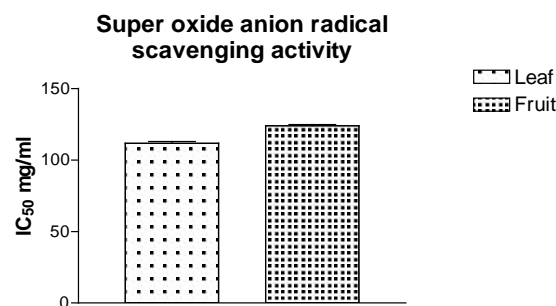


Fig. 1: Superoxide anion radical scavenging activity of leaf and fruit extracts of AC plant. Values are mean \pm SD (n=3)

Free radical scavenging activity

The bleaching action of DPPH is mainly due to the presence of phytochemicals having antioxidant activity. Free radical scavenging activity of AC leaf and fruit extracts is shown in fig. 2. The IC_{50} value of leaf and fruit were found to be 11.58 and 17.10 mg/ml, respectively.

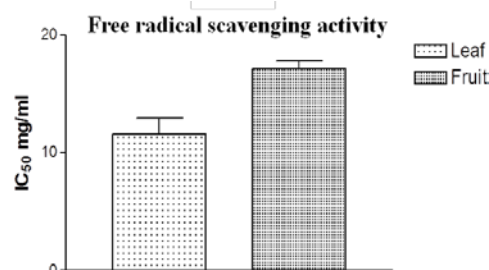


Fig. 2: Free radical scavenging activity of leaf and fruit extracts of AC plant. Values are mean \pm SD (n=3)

Ferric ion chelation activity

FIC activity in ethanolic leaf and fruit extracts are shown in fig. 3. FIC activity of leaf and fruit extract in terms of IC_{50} was found to be 8.56 and 20.63 mg/ml, respectively. It was clear that leaf extract of AC

showed maximum ferrous ion chelation activity. Fe^{2+} increased the formation of hydroxyl radicals through the Fenton reaction, which is responsible for the occurrence of many diseases [30].

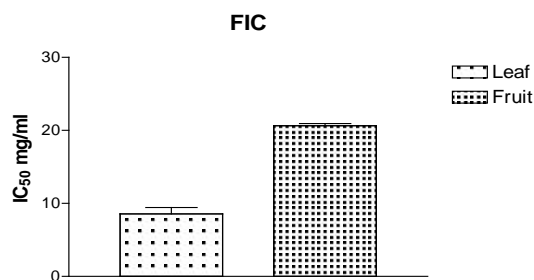


Fig. 3: Ferrous ion chelation (FIC) activity of leaf and fruit extracts of AC plant. Values are mean \pm SD (n=3)

Reducing power

RP is determined to measure the reductive ability of antioxidant, which is evaluated by transformation of Fe (III) to Fe (II) in the presence of the plant extracts. Reducing power of AC leaf and fruit extracts are shown in fig. 4. RP of leaf and fruit extract of AC was found to be 4.29 and 3.57 ASE/ml, respectively.

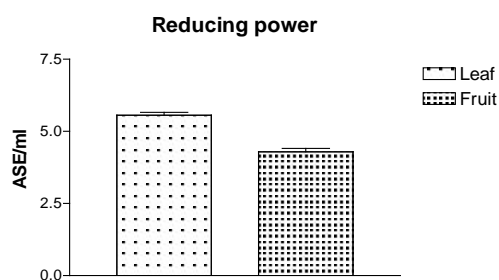


Fig. 4: Reducing the power of leaf and fruit extracts of AC plant. Values are mean \pm SD (n=3)

DNA damage assay

Hydroxyl radical scavenging activity of AC plant was further explored by the protection of plasmid pBR322 DNA against Fenton reagent-induced damage. The results obtained through gel

electrophoresis showed that leaf extract has significant DNA damage protective activity in comparison to fruit extract under *in vitro* condition [fig. 5]. The addition of Fenton reagent to a mixture containing DNA and different concentration (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) of leaf extract showed a significant reduction information of nicked circular form DNA and increased supercoiled or native forms of plasmid DNA with an increase in plant extracts concentration.

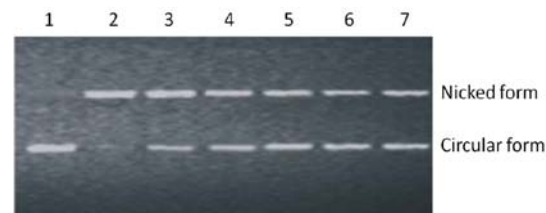


Fig. 5: Agarose gel electrophoretic pattern showing concentration dependent protective effect of AC leaf extract on supercoiled DNA, damage caused by Fenton's reagent. Lane 1: pBR322 DNA; Lane 2: DNA+Fenton; Lane 3, 4, 5, 6, 7: DNA+Fenton+Plant extract (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml)

HPLC analysis

AC leaf extract was also examined for their phenolics composition using HPLC. Determinations of phenolic compounds such as chlorogenic acid (CGA), caffeic acid (CA), rutin (RU), quercetin (Quer) and kaempferol (Kaem) in leaf extract were done by using HPLC. Results showed the presence of five phenolics CGA (0.3757×10^{-2} g/kg), CA (0.21499×10^{-2} g/kg), RU (0.18272×10^{-2} g/kg), Quer (0.20693×10^{-2} g/kg) and Kaem (0.21985×10^{-2} g/kg) [table 2] in AC leaf extract. Due to the presence of these phenolic compounds, AC leaf extract may have show maximum antioxidant activity.

Quantitative analysis of viable cells

Varying concentrations of AC leaf extracts ($0.5-7.5 \mu\text{g}/10^4$ cells) were tested to determine the effective concentration, at which no toxicity was observed. In order to evaluate the cytotoxic potential of t-BHP, the hepatocytes were treated with concentrations of $250 \mu\text{M}$ and cell viability was determined by MTT assay. To see the protective effect of plant extracts against t-BHP induced oxidative stress in cultured hepatocytes, cells were treated with $250 \mu\text{M}$ of t-BHP and AC leaf extracts at concentrations of 1.0, 2.5, 5.0 and $7.5 \mu\text{g}$. AC leaf increased the cell viability by 1.67 ($P<0.001$), 1.44 ($P<0.001$), 1.46 ($P<0.001$) and 1.64 fold ($P<0.001$) respectively as compared to t-BHP stressed cells [fig. 6].

Table 1: Chemical constituents of *Amorphophyllus campanulatus* (AC) plant parts

| Name of vegetables | Parts of vegetable | Vit. C (g/kg of FW) | Carotenoids ($\mu\text{g}/\text{g}$ of FW) | TPC (g/kg of GAE of DW) | Carbohydrate (g/kg of DW) | Protein (g/kg of DW) |
|--------------------|--------------------|----------------------|---|-------------------------|---------------------------|----------------------|
| AC plant | Leaf | 0.5714 ± 0.08 | 2.83 ± 0.01 | 35.78 ± 0.01 | 59.25 ± 0.01 | 295.36 ± 0.09 |
| | Fruit | 0.0762 ± 0.03 | 3.68 ± 0.08 | 23.58 ± 0.03 | 165.17 ± 0.02 | 35.96 ± 0.03 |

Values are mean \pm SD (n=3); FW: Fresh weight; TPC: Total phenolic content; DW: Dry weight; GAE: Gallic acid equivalent.

Table 2: HPLC analysis of *Amorphophyllus campanulatus* leaf extract

| Sample name | CGA (g/kg) | CA (g/kg) | RU (g/kg) | Quer (g/kg) | Kaem (g/kg) |
|-------------|-------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| AC leaf | 0.3757×10^{-2} | 0.21499×10^{-2} | 0.18272×10^{-2} | 0.20693×10^{-2} | 0.21985×10^{-2} |

CGA: Chlorogenic acid, CA: Caffeic acid, RU: Rutin, Quer: Quercetin, Kaem: Kaempferol

Effect on superoxide dismutase activity

Cultured hepatocytes when subjected to $250 \mu\text{M}$ of t-BHP induced oxidative stress, showed 23.94 Unit/min/ 1×10^4 cells activity

($P<0.001$) which was 1.84 fold less than the untreated cells. Treatment of hepatocyte cells with AC leaf extract at concentrations of 1.0, 2.5, 5.0 and $7.5 \mu\text{g}$ against t-BHP induced oxidative stress SOD activity was found to be 119.26 ($P<0.001$), 87.84 ($P<0.001$), 137.28

($P < 0.001$), 113.47($P < 0.001$) Unit/min/ 1×10^4 cells, respectively [fig. 7]. Results revealed that leaf extracts showed maximum SOD activity at a concentration of $5.0 \mu\text{g}$.

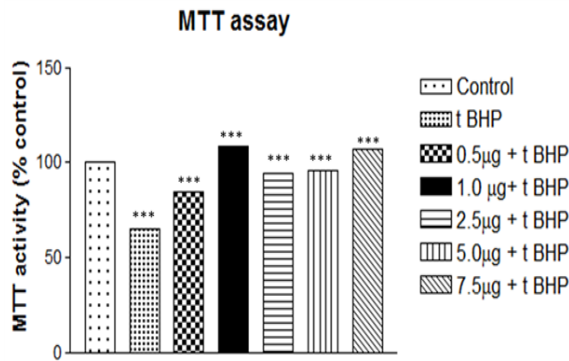


Fig. 6: Cytotoxic potential of t-BHP as well as the protective activity of ethanolic extract of AC leaf. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean±SD (n=3). * $P < 0.001$**

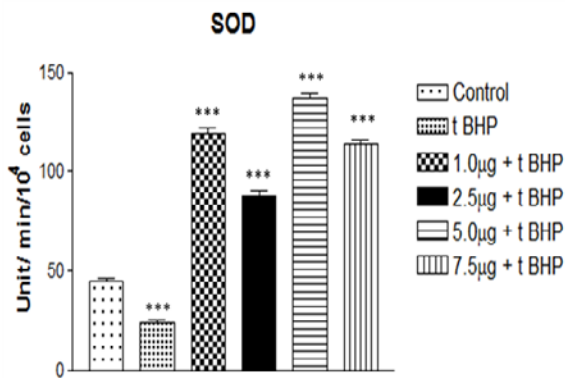


Fig. 7: Effect of ethanolic AC leaf extract on SOD activity against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean±SD (n=3). * $P < 0.001$**

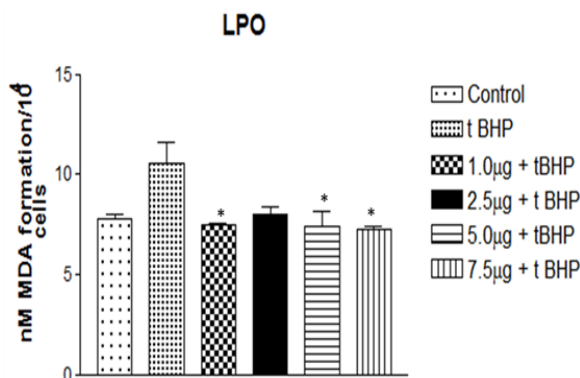


Fig. 8: Effect of AC leaf extract on nM MDA formation against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean±SD (n=3). * $P < 0.05$

Effect on lipid peroxidation

Oxidative stress induced in the hepatocytes due to $250 \mu\text{M}$ of t-BHP, produces 10.57 nM MDA/ 1×10^4 cells. Treatment of hepatocytes cells with AC leaf extract at concentrations of 1.0, 2.5, 5.0 and $7.5 \mu\text{g}$ against t-BHP induced oxidative stress and produced 7.47 nM ($P < 0.05$), 8.02nM, 7.38 nM ($P < 0.05$) and 7.28 nM ($P < 0.05$) MDA/ 1×10^4 cells, respectively [fig. 8]. Results showed that there was a significant reduction in MDA formation in leaf extract treated cells. AC leaf has maximum anti-lipid peroxidation activity at a concentration of $7.5 \mu\text{g}$.

Cellular GSH level

In the current study, we found that 1h treatment with t-BHP caused reactive oxygen species (ROS) production which decreases GSH content. Cultured hepatocytes, when subjected to $250 \mu\text{M}$ of t-BHP treatment, showed $1.54 \mu\text{g}$ GSH/mg of protein ($P < 0.001$) which is 1.63 folds less than the untreated cells. Treatment of hepatocytes cells with AC leaf extract at concentrations of 1.0, 2.5, 5.0 and $7.5 \mu\text{g}$ against t-BHP induced oxidative stress protect the GSH depletion up to 1.66, 1.65, 1.55 and 1.59 fold, respectively as compared to t-BHP [fig. 9]. The results reflect that treatment of hepatocytes cells with AC leaf extracts efficiently blocked t-BHP-induced GSH depletion at a concentration of $1.0 \mu\text{g}$.

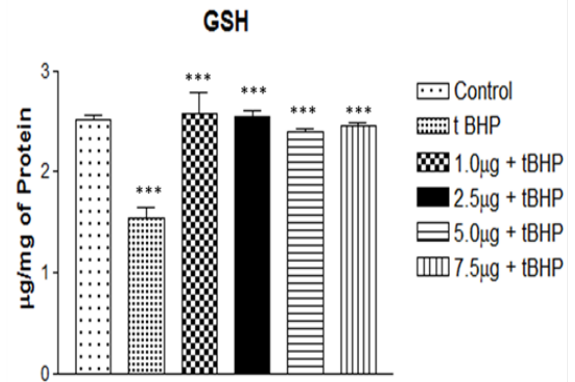


Fig. 9: Effect of AC leaf extract on total GSH content against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean±SD (n=3). * $P < 0.001$**

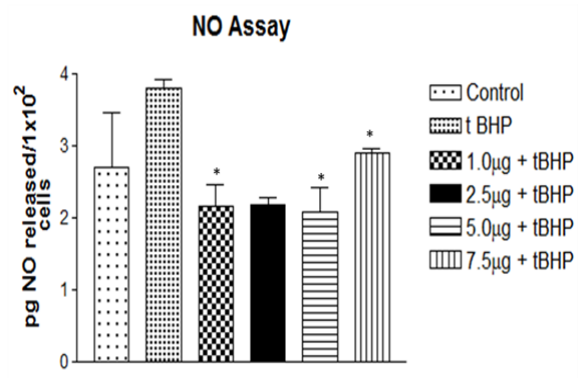


Fig. 10: Effect of AC leaf extract on NO release in pg against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean±SD (n=3). * $P < 0.05$

Effect on NO release

NO release in the hepatocytes by free radical generation due to $250 \mu\text{M}$ of t-BHP was $3.80 \text{pg}/1 \times 10^4$ cells. When stress induced

hepatocytes with t-BHP were treated with AC leaf extract at concentration of 1.0, 2.5, 5.0 and 7.5 μg , it released 2.15pg ($P<0.05$), 2.17pg, 2.07pg ($P<0.05$), 2.90pg $\text{NO}/1 \times 10^4$ cells ($P<0.05$), respectively [fig. 10]. Results showed that AC leaf extracts have best anti nitric oxide inhibition activity at a concentration of 5.0 μg .

Catalase activity

CAT is an antioxidant enzyme that protects the cells against oxidative stress from highly reactive free radicals. The activity of this enzyme gets induced when free radicals are generated in the cells. When cells were treated with 250 μM of t-BHP alone; CAT decreased 2.66 fold as compared to control. But when the cells treated with t-BHP were exposed to AC leaf extract at a concentration of 1.0, 2.5, 5.0 and 7.5 μg , extracts showed 0.787, 1.33, 3.60 and 2.31 fold increases in antioxidant enzyme activity which represented resistance against ROS production [fig. 11].

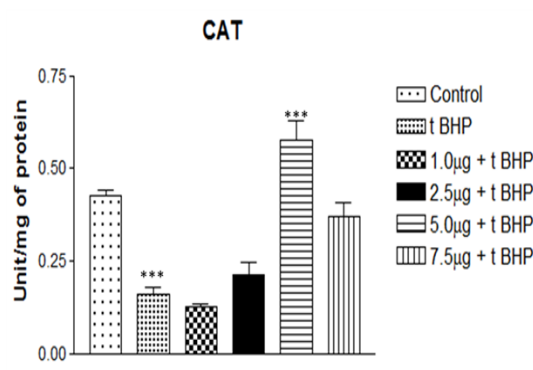


Fig. 11: Effect of AC leaf extract on Catalase enzyme activity against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean \pm SD (n=3). *P<0.001**

Total protein content

Treatment with 250 μM of t-BHP caused 1.54 fold decreases in total protein content. Exposure of t-BHP pretreated hepatocytes cells with AC leaf extract at a concentration of 1.0, 2.5, 5.0 and 7.5 μg protect the protein content depletion to the extent of 1.44, 1.41, 1.47 and 1.44 fold, respectively as compared to t-BHP [fig. 12].

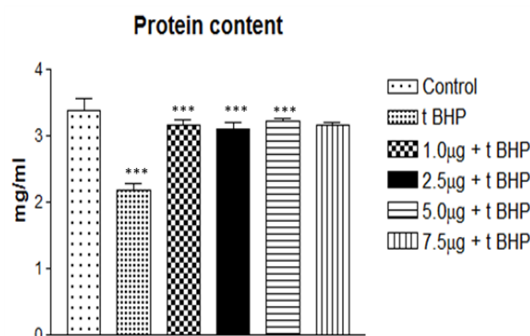


Fig. 12: Effect of AC leaf extract on total protein content against t-BHP induced stress in primary rat hepatocytes. Cells treated with t-BHP alone were compared to control cells, whereas cells treated with plant extracts followed by t-BHP were compared to cells treated with t-BHP alone. Values are mean \pm SD (n=3). *P<0.001**

DISCUSSION

Plants are a good source of the phenolic compound. These compounds are a major contributor of antioxidant and

hepatoprotective activity of plant [31]. Therefore, plants containing a high concentration of phenolic compounds could be a good source of natural antioxidants. Primary parameters of antioxidant activity such as ascorbic acid and total phenolic content were estimated in AC plant. The present study showed that these two parameters showed maximum content in this plant. Ascorbic acid can be oxidized by many species that have potential to be involved in human diseases [32]. Studies suggested that antioxidant activity of vitamin C showed the dose-dependent effect in human diseases such as atherosclerosis and cancer [33]. Ascorbic acid has the ability to enhance the body's antioxidant defence and is important in the healing of the ulcers and delay the onset of other diseases [34]. Phenolic compounds constitute a class of antioxidant agents acting as free radical terminators [35, 36]. The data demonstrate that polyphenols are widely distributed in the vegetable sample. According to Bravo *et al.*, polyphenols that are present in these vegetables is largely affected by genetic factors, environmental conditions, variety, etc [37]. In contrast, vitamin C is one of the most important water-soluble antioxidants in cells, and it scavenges reactive oxygen species such as hydroxyl radicals, peroxy radicals and singlet oxygen [38]. Consequently, when relating the antioxidant activities of plants sample to disease risk and health, it is important to consider the contribution of vitamin C in addition to that of phenolic compounds [39].

The SARSA of AC was monitored by PMS-NADH-NBT reduction system, a non-enzymatic method. In this method, O_2^- derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The decrease in color intensity showed that antioxidants present in the plant extracts scavenge the superoxide radical. The plant extract reduces the superoxide anion and inhibits the formation of blue formazan complex [40, 41]. The natural food items which are capable of quenching the O_2^- can prevent the oxidation of biomolecules of the body and may enhance the activity of endogenous SOD. Results showed that AC leaf extract showed maximum scavenging of superoxide anion radical when compared to fruit extract.

One of the proposed methods for assessment of antioxidative activity (AOA) is DPPH free radical colorimetric assay, in which colour changes from purple to yellow in the presence of antioxidants [42]. The kinetics of decolorization reactions directly relates to the types of antioxidants and to their concentrations [17]. Our studies showed that leaf extracts significantly reduced the DPPH radicals in dose-dependent manner. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant, its absorbance decreases [43]. Antioxidants react with DPPH radical and convert it into α, α -diphenyl-p-picryl hydrazine [44]. DPPH is stable nitrogen centred, lipophilic free radical widely used in evaluating antioxidant activities in a relatively short time compared to some other methods. The DPPH assay measures the ability of the plant extract to donate hydrogen to the DPPH radical resulting in bleaching of the DPPH solution. Greater the bleaching action, higher the antioxidant activity and this is reflected by higher ARP and lower value of IC_{50} [45]. Antioxidant activity results for this plant in this study are not in agreement with the results obtained by Ramesh *et al.* [46]. However, these contradictory results may be mostly due to differences in sample collection sites and the experimental conditions used in different studies.

The literature showed that chelating agents act as effective secondary antioxidants because they reduce the reduction potential stabilizing oxidized form of the metal ion [47]. In the FIC assay, ferrozine acts as a chelating agent and forms purple complex with Fe^{2+} giving maximum absorbance at 562 nm. However, in the presence of chelating agents, the complex formation is disrupted with the result that red colour of complex is decreased. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [48]. The present study showed that AC leaf contains strong ferric ion chelation activity than fruit.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [49]. Reducing power

assay measure the electron donating capacity of an antioxidant. The reduction of ferric ion to ferrous ion is measured by the intensity of resultant blue-green solution which absorbs at 700 nm and an increased absorbance is indicative of higher RP. Our result showed that leaf extract showed better reducing power than fruit extract.

The hydroxyl radical is the most reactive among reactive oxygen species. It can be formed by the Fenton reaction in the presence of transition metals such as Fe^{3+} and H_2O_2 , capable of damaging almost every molecule found in living cells such as strand breakage in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In the present study, plasmid pBR322 DNA was treated with Fenton reagent which interacts with the supercoiled DNA. Hydroxyl radical attacks on nitrogenous bases and sugar moiety of supercoiled pBR322 plasmid DNA, resulting in nicked circular form due to breakage of the sugar-phosphate backbone of nucleic acid. The damage was effectively minimized by treatment with AC leaf extract. The DNA damage may cause a number of genetic disorders [50]. The protection offered against DNA damage by AC was concentration dependent. It was clear from the study of HPLC analysis that AC leaf extract showed the presence of more phenolic compounds that is why it showed maximum protection of DNA damage against OH radical. On the basis of our results, we can conclude that AC leaf is promising sources of potential antioxidants. So, further studies were carried out to investigating the hepatoprotective activity of leaf extract.

t-Butyl hydroperoxide is widely used to study the effect of free radicals on cellular functions under *in vitro* condition [51]. It decomposes alkoxy or peroxy radicals which result in lipid peroxidation leading to the formation of various TBA-reactive substances, DNA damage as well as a decrease in GSH content [52-54]. The protective effect of test substances against oxidative damage was seen by measuring superoxide dismutase activity, lipid peroxidation, NO release, GSH content and catalase activity in the cell. In the present study, the toxic effect of t-BHP on cell membrane integrity was indicated by a significant decrease in the %viability of isolated rat hepatocytes. The decreased cell viability caused by hepatic damage is believed to be due to breakage of the structure of the cellular membrane [55]. Cell viability was reduced in t-BHP treated hepatocytes and on the other hand, AC leaf extract prevented the toxic effects induced by t-BHP in term of loss in the cell viability in a dose-dependent manner. Results revealed that AC leaf extracts showed better cell viability at concentration 1.0 and 7.5 μ g.

SOD is the first line of defence in the antioxidant system against the oxidative damage mediated by superoxide radicals [56]. SOD catalyzed the dismutation of superoxide radical into H_2O_2 . These radicals, if uncontrolled, cause damage to cellular macromolecules resulting in cellular dysfunctions [57]. The plant extracts in our study showed a protective effect of the cell from superoxide ions by enhancing the activity of superoxide dismutase enzyme as compared to t-BHP stressed cells.

Lipid peroxidation has been postulated as a major destructive process of liver injury due to t-BHP administration. The activated free radical binds covalently to the macromolecules and induces peroxidative degradation of membrane lipids of endoplasmic reticulum which are rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides which in turn generate products such as malondialdehyde (MDA) that cause damage to the membranes [58]. The results of the present study suggest that leaf extracts are capable of reducing hepatocyte lipid peroxidation caused by t-BHP. Sohn *et al.* reported that the peroxidation of membrane phospholipids result in increased peroxides which eventually lead to disruption of membrane structure, membrane fluidity and function [59]. The cell membrane contains polyunsaturated fatty acids which are more prone to free radical attack and may cause cellular destruction when there is a lack of adequate antioxidant defence [60]. So, the role of dietary antioxidants in the form of food ingredient can be an effective strategy to strengthen the immune system, healthy ageing and homeostatic balance during oxidative stress [61].

In our study, leaf extracts significantly reduced the NO production by the cells and thus protected them from the damaging effects of excess NO production. Nitric oxide (NO) is lipophilic and greatly

diffusible solute that forms within the cell. The production of NO by the activated macrophages against pathogens may cause cellular injury to macrophages as well as to the neighbouring tissues. Excess NO can react with superoxide radicals leading to the formation of harmful peroxynitrite radicals that are responsible for the damage to macromolecules resulting in protein nitration, DNA strand breakage and guanine nitration, etc. Jeong *et al.* revealed that the excess NO generated in body system causes tissue damage and inflammation [62]. The approach to reducing NO production is emerging as an important therapeutic strategy for treatment of inflammatory disorders. In our study the treatment with extracts reduced the NO concentrations which indicate the better antioxidant status of cells against t-BHP induced oxidative stress. Results also showed that AC leaf extracts have best anti nitric oxide inhibition activity at a concentration of 5.0 μ g.

GSH is an important intracellular antioxidant and redox potential regulator that provides efficient detoxification effects and protects cells from damage by free radical and peroxide. The thiol-based antioxidant system plays the second line of cellular defence against reactive free radical and another oxidant species-mediated oxidative damage. GSH with its-SH group functions as a catalyst in disulphide exchange reaction. Its function is to reduce free radicals. So the decrease in the concentration of GSH is related to the neutralization of free radicals [63]. In the present study, we found that cells were sensitive to t-BHP challenge as GSH levels were depleted. Administration of AC leaf extracts along with t-BHP inhibited GSH depletion. It is suggested that during oxidative stress GSH depletion occurs frequently and can also alter mitochondrial membrane fluidity [64]. Reports also showed that t-butyl hydroperoxide causes DNA damage as well as a decrease in GSH content [53, 54].

Catalase is an essential cytosolic antioxidant enzyme which converts H_2O_2 to H_2O (56). The extracts showed the enhanced activity of enzyme CAT ensuring better antioxidant and protective effect on the stimulated cells against the deleterious effects of the t-BHP and generation of other radicals.

The probable adverse reactions and undesirable side effects of synthetic drugs have led to the belief that natural products are safer, as herbal drugs are considered to be more harmonious with biological systems than the synthetically derived drugs. It is believed that plants with antioxidant property can prevent or protect tissues against the damaging effect of free radicals. In the present study, the reversal of altered antioxidant enzymes status and peroxidative activity due to free radical generated by t-BHP suggests that AC leaf extract has significant antioxidant and antiperoxidative property and hence reveals its potential to play a crucial role in defence against free radicals.

CONCLUSION

Amorphophallus campanulatus evaluated in this study had varied levels of phytochemical constituents which may be essential for good human health. In this study, leaf extracts of AC showed better antioxidative potential which may be due to the presence of phenolic compounds. The leaf extract also showed maximum protection of DNA damage caused by hydroxyl radicals in a dose-dependent manner. Induction of ROS caused depletion in antioxidant enzyme activity and GSH level. The protective activity of AC leaf extracts against t-BHP induced cell death observed was confirmed by increased cell viability; increased antioxidant enzyme activity, reduced NO release and MDA formation. The reversal of altered antioxidant enzyme status and peroxidative activity caused by t-BHP suggests that AC leaf extracts have antioxidant and hepatoprotective properties. Results showed that AC leaf is a rich source of many biomolecules and showed potential utility of AC leaf for use in herbal drug system or as a nutritional supplement.

ACKNOWLEDGEMENT

PS is grateful to University Grants Commission, New Delhi for the award of Junior Research Fellowship under "Research Fellowship in Science for Meritorious Students (RFSMS)" scheme.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest

REFERENCES

- Singh P, Vishwakarma SP, Singh RL. Antioxidant, oxidative DNA damage protective and antimicrobial activities of *Trigonella foenum-graecum* plant. J Sci Food Agric 2014;94:2497-504.
- Singh BN, Singh BR, Singh RL, Prakash D, Sarma BK, Singh HB. Antioxidant and anti-quorum sensing activity of green pod of *Acacia nilotica* L. Food Chem Toxicol 2009;47:778-86.
- Novo E, Parola M. Redox mechanisms in hepatic chronic wound healing and fibrogenesis. Fibrog Tissue Repair 2008;1:1-5.
- Sherwin ER, Brancn AL, Davidson PM, Salmincn S. Food Additives. Marvel Dekker Inc., New York; 1990. p. 139-93.
- Tripathi M, Singh BK, Mishra C, Raisuddin S, Kakkar P. Involvement of mitochondria-mediated pathways in hepatoprotection conferred by *Fumaria parviflora* Lam. extract against nimesulide induced apoptosis *in vitro*. Toxicol Vitro 2010;24:495-508.
- Tripathi M, Singh BK, Kakkar P. Glycyrrhizic acid modulates t-BHP induced apoptosis in primary rat hepatocytes. Food Chem Toxicol 2009;47:339-47.
- Singh BK, Tripathi M, Chaudhari BP, Pandey PK, Kakkar P. Natural terpenes prevent mitochondrial dysfunction, oxidative stress and release of apoptotic proteins during nimesulide-hepatotoxicity in rats. PLoS One 2012;7:e34200. Doi: 10.1371/journal.pone.0034200. [Article in Press]
- Arya VS. Indian medicinal plants-A compendium of 500 species. Madras: Orient Longman Ltd; 1994. p. 132-4.
- Kapoor LD. Handbook of Ayurvedic medicine plants. CRC Press: Herbal Reference Librar; 2005.
- Rastogi RP, Mehrotra BN. Compendium of Indian medicinal plants. Lucknow: Central Drug Research Institute, 1995. p. 40-1.
- AOAC. Official methods of analysis of the association of official analytical chemists. 14th edition. Association of Official Analytical Chemists, Arlington Virginia; 1984.
- Jensen A. Chlorophyll and Carotenoids. In: Helibust A, Cargei JS. Eds. Handbook of phythological methods. Cambridge, Cambridge university press: London; 1978. p. 5-7.
- Ragazzi E, Veronese G. Quantitative analysis of phenolic compounds after thin layer chromatographic separation. J Chromatogr 1973;77:369-75.
- Thomas G, Ludwig Hyman JVG. The Anthrone method for the determination of Carbohydrates in foods and in oral rinsing. J Dent Res 1956;35:90-4.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biochem 1951;193:265-75.
- Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. Biochem Biophys Res Commun 1972;46:849-64.
- Yen GC, Duh PD. Scavenging effect of methanolic extracts of peanut hulls on free radical and active oxygen. J Agric Food Chem 1994;42:629-32.
- Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. J Agric Food Chem 1990;38:674-8.
- Apati P, Szentmihalyi K, Kristoz T, Papp I, Vinkler P, Szoke E, et al. Herbal remedies of Solidago-correlation of phytochemical characteristics and antioxidative properties. J Pharmacol Biomed Anal 2003;32:1045-53.
- Lee JC, Kim HR, Kim J, Jang YS. Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. *Saboten*. J Agric Food Chem 2002;50:6490-6.
- Prakash D, Singh BN, Upadhyay G. Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). Food Chem 2007;102:1389-93.
- Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29-83.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
- Kakkar P, Das B, Viswanath PN. Modified spectrophotometer assay of SOD. Indian J Biochem Biophys 1984;95:51-8.
- Wallin B, Rosengren B, Shertzer HG, Camejo G. Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of antioxidants. Anal Biochem 1993;208:10-5.
- Feelisch M, Noack E. Nitric oxide (NO) formation from nitro vasodilators occurs independently of hemoglobin or non-heme iron. Eur J Pharmacol 1987;142:465-9.
- Schneider JC, Card GL, Pfau JC, Holian A. Air pollution particulate SRM 1648 causes oxidative stress in RAW 264.7 macrophages leading to production of prostaglandin E2, a potential Th2 mediator. Inhalation Toxicol 2005;17:871-7.
- Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121-6.
- Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
- Juntachote T, Berghofer E. Antioxidative, properties and stability of ethanolic extracts of holy basil and galangal. Food Chem 2005;92:193-202.
- Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J Nutr Biochem 2002;13:572-84.
- Halliwell B. Vitamin C: poison, prophylactic or panacea? Tandem Integrated Business Solutions 1999;24:255-9.
- Sebastian JP, Arie K, Wang Y, Peter E, Oran K, Lee JH, et al. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr 2003;22:18-35.
- Verma VK, Ramesh V, Tewari S, Gupta RK, Sinha N, Pandey CM. Role of bilirubin, vitamin C and ceruloplasmin as antioxidants in coronary artery disease (cad). Int J Clin Biochem 2005;20:68-74.
- Dhakarey R, Upadhyay G, Singh BN, Singh HB, Prakesh D, Kumar S, et al. Phenolic content and antioxidant potential of Rhododendron species. Indian J Agric Biochem 2005;18:40-3.
- Singh BN, Singh BR, Singh RL, Prakesh D, Singh DP, Sarma BK, et al. Polyphenolics from various extracts/fractions of red onion (*Allium cepa*) peel with potent antioxidant and antimutagenic activities. Food Chem Toxicol 2009;47:1161-7.
- Bravo L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutr Rev 1998;56:317-33.
- Halliwell B. Vitamin C: antioxidant or prooxidant *in vivo*? Free Radical Res 1996;25:439-54.
- Williams C. Healthy eating: clarifying advice about fruit and vegetables. Br Med J 1995;310:1453-5.
- Cos P, Ying LY, Calomme M, Hu JH, Cimanga Van Poel KB, Pieters L, et al. Structure activity relationships and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. J Nat Prod 1998;61:71-6.
- Parejo I, Viladomat F, Bastida J, Schmeda-Hirschmann G, Burillo J, Codina C. Bioguided isolation and identification of the nonvolatile antioxidant compounds from fennel (*Foeniculum vulgare* Mill.) waste. J Agric Food Chem 2004;52:1890-7.
- Qian H, Nihorimbere V. Antioxidant power of phytochemicals from *Psidium guajava* leaf. J Zhejiang Univ Sci 2004;5 Suppl 6:676-83.
- Gulcin I, Koksak K, Elmastas M, Aboul-Enein HY. Determination of *in vitro* antioxidant and radical scavenging activity of *Verbascum oreophilum* C. Koch Var Joannis (Fam. Scrophulariaceae). Res J Biol Sci 2007;2:372-82.
- Singh RP, Murthy CKN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* model. J Agric Food Chem 2002;50:81-6.
- Lim YY, Lim TT, Tee JJ. Antioxidant properties of guava fruit: comparison with some local fruits. Sunway Acad J 2006;3:9-20.
- Ramesh CK, Raghu KL, Jamuna KS, Joyce GS, Mala RSV, Vijay BR. Comparative evaluation of antioxidant property in methanolic extracts of some common vegetables of India. Ann Biol Res 2011;2 Suppl 2:86-94.
- Kumar KS, Ganesan K, Rao PVS. Antioxidant potential of solvent extracts of kappa phycusalvarezii (Doty)-an edible seaweed. Food Chem 2008;107:289-95.
- Aboul-Enein AM, El Baz FK, El-Baroty GS, Youssef AM, Abd El-Baky HH. Antioxidant activity of algal extracts on lipid peroxidation. J Med Sci 2003;3:87-98.
- Bhandari MR, Kawabata M. Organic acid, phenolic content and antioxidant activity of wild yam (*Dioscorea* spp.) tubers of Nepal. Food Chem 2004;88:163-8.

50. Moskovitz J, Yim KA, Choke PB. Free radicals and disease. *Arch Biochem Biophys* 2002;397:354-9.
51. Alonso JG, Ros G, Periago MJ. Antiproliferative and cytoprotective activities of phenolic-rich juice in HepG2 cells. *Food Res Int* 2006;39:982-91.
52. Baker MA, He SQ. Elaboration of cellular DNA breaks by hydroperoxides. *Free Radical Biol Med* 1991;11:563-72.
53. Dubuisson B, DeWergifosse A, Trouet A, Baguet F, Marchand-Brynaert J, Rees JF. Antioxidative properties of natural coelenterazine and synthetic methyl coelenterazine and synthetic coelenterazine in rat hepatocytes subjected to tert-butyl hydroperoxide induced oxidative stress. *Biochem Pharmacol* 2000;60:471-8.
54. Rosa RM, Moura DJ, Melecchi MIS, Dos-Santos RS, Richter MF, Camarao EB, *et al.* Protective effects of *Hibiscus tiliaceus* L methanolic extract to V 79 cells against cytotoxicity and genotoxicity induced by hydrogen peroxide and tertbutyl hydroperoxide. *Toxicol In Vitro* 2007;2:1442-52.
55. Ou CC, Chiu YH, Lin SL, Chang YJ, Huang HY, Lin MY. Hepatoprotective effect of Lactic Acid Bacteria in the attenuation of oxidative stress from tert-Butyl hydroperoxide. *J Food Drug Anal* 2012;20:101-10.
56. Li S, Yan T, Yang JQ, Qberley TD, Oberly LW. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. *Cancer Res* 2000;60:3927-39.
57. Afonso V, Champy R, Mitrovic D, Collin P, Lomri A. Reactive oxygen species and superoxide dismutases: role in joint diseases. *Jt Bone Spine* 2007;74:324-9.
58. Halliwell B. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? *Cardiovasc Res* 2000;47:410-48.
59. Sohn JH, Han KL, Lee SH, Hwang JK. Protective effects of panduratin A against oxidative damage of tertbutyl hydroperoxide in human HepG2 cells. *Biol Pharm Bull* 2005;28:1083-6.
60. Kanupriya DP, Sharma SK, Sairam M, Ilavazhagan G, Sawhney RC, Banerjee PK. Flavanoids protect U-937 macrophages against tert-butyl hydroperoxide induced oxidative injury. *Food Chem Toxicol* 2006;44:1024-30.
61. Han SH, Espinoza LA, Liao H, Boulares AH, Smulson ME. Protection of antioxidants against toxicity and apoptosis induced by the sulphur mustard analogue 2-chloroethylethylsulphide (CEES) in jurkat T cells and normal human lymphocytes. *Br J Pharmacol* 2005;141:795-802.
62. Jeong MY, Lee JS, Lee JD, Kim NJ, Kim JW, Lim S. A combined extract of *Cinnamomi ramulus*, *Anemarrhenae rhizoma* and *Alpiniae officinari* rhizoma suppresses production of nitric oxide by inhibiting NF kB activation in RAW 264.7 cells. *Phytother Res* 2008;22:772-7.
63. Jones DP. Redox potential of GSH/GSSH couple: assay and biological significance. *Methods Enzymol* 2002;348:511-24.
64. Schattner A, Sokolovskaya JC. Fatal hepatitis and renal failure during treatment with nimesulide. *J Intern Med* 2000;247:153-5.