Abstract

The present study was designed to examine the ability of partially purified fractions of *Luffa acutangula* (Cucurbitaceae) fruit, to attenuate *t*-BHP induced oxidative damage in human erythrocyte as an *in vitro* model. Initially, we investigated the antioxidant property of the hexane, methanolic and aqueous extracts (FHE, FME and FAE, respectively) by DPPH free radical method and found that fruit methanolic extract was showing higher antioxidant activity (71.4±4.46% at 1 mg/ml) compared to other extracts (FHE & FAE were 13.93±1.3 and 51.84±3.76%, respectively). Hence, this extract was further partially purified chromatographically and out of these fractions (F1, F2, F3, F4, F2-1, F2-2, F2-3 and F2-4) F2-3 showed significant antioxidant activity (73.96±6.4% at 25 µg/ml). This fraction was further tested for its effect on lipid peroxidation, superoxide dismutase, catalase and glutathione in *t*-butyl hydroperoxide (*t*-BHP) treated-erythrocytes. Pretreatment with fraction F2-3 significantly inhibited lipid peroxidation in a dose and time dependent manner compared to control (40.6±3.2, 27.9±2.4 and 75.5±5.2 nmol MDA/gHb, at 30, 90 min and control, respectively). Catalase, SOD and GSH levels were also brought up in a dose and time dependant manner compared to control (treated and control were CAT: 100.7±4.7 and 51.3±3.2 µMH₂O₂/ gHb/min, SOD: 9.68±0.87 and 1.15±0.12 IU/g Hb, GSH: 21.3±1.23 and 6.0±0.91 µM/g Hb, respectively). These results establish that *L. acutangula* fruit aqueous fraction F2-3 possesses beneficial role in mitigating *t*-BHP induced oxidative stress in erythrocyte.

Key words: Fruit methanolic extract; Reactive oxygen species, Superoxide anion, Hydroxyl radical; Malondialdehyde; Superoxide dismutase

Introduction

Hydrogen peroxide (H₂O₂), organo peroxide, superoxide anion (O₂⁻) and hydroxyl radical (OH), etc. are reactive oxygen species (ROS) generated by aerobic metabolism in biological system and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution. Under normal conditions, generated ROS are neutralized by inbuilt and default antioxidant enzymes present in the body such as catalase, superoxide dismutase, glutathione peroxidase, etc. (1, 2). Non-enzymatic antioxidant molecules such as ascorbic acid, glutathione and uric acid also play a key role in detoxifying the free radicals (3). Antioxidants are a type of complex compounds found in our diet that act as a protective shield for our body against several acute diseases (4). Increased oxygen flux conditions (i.e. exercise) or failure of antioxidant

Role of *Luffa acutangula* in Oxidative damage
mechanism leads to over production of free radicals that may exceed system capacity to remove them. This situation ultimately culminates into damage of macromolecules such as proteins, lipids and nucleic acids followed by oxidative stress (5).

Several compounds such as phenolic, flavonoids etc. obtained from different plants have strong antioxidant capacity (4). Dietary phenols are present in plant foods as bioactive molecules and data supporting the proposal that health benefits associated with vegetables and fruits are probably linked to the phenol antioxidants they contain. Phenols are present in a variety of plants utilized as important components of both human and animal diets (6, 7, 8). A general consensus has been reached during the last few years that diet has a major role in the development of chronic diseases, such as cancer, coronary heart disease, obesity, diabetes type 2, hypertension and cataract (9, 10). This consensus suggests that a predominantly plant-based diet rich in fruits and vegetables, pulses and minimally processed starchy staple foods reduces the risk for development of these diseases significantly (11, 12).

The recommendations are mainly based on epidemiological studies, are thus, that fruits, vegetables and less processed staple foods provide the best protection against the development of disease with little or no merit in recommending vitamin or other micronutrient supplements for disease prevention. This is a safe principle that promises to provide for improved public health. In recent years, search for novel type of antioxidants from several plant parts has achieved huge attention. Management of diseases with minimal side effects is still a complicated medical challenge. There is an increasing demand to use the natural products to prevent the free radical induced diseases (13, 14). In this regard, we selected Lacutangula to evaluate its antioxidant property because it is the popular in Indian traditional medicine. Luffa acutangula is a tropical running vine with rounded leaves and yellow flowers belongs to Cucurbitaceae family. Tea of these leaves is used as a diuretic, seeds have laxative properties and juice of the fruit is used against internal hemorrhage in traditional medicine. The results suggest that free radicals might play a role in the development of brain injury following brain hemorrhage (4). The fruit of the Luffa acutangula is rich in phenolic contents. Presently we made a hypothesis that Luffa acutangula may have potential antioxidant activity due to which it is being used against internal hemorrhage in traditional medicine. None of its therapeutic potentials are scientifically evaluated except ribosome inactivating activity (15). Therefore we set out to determine the effect of various doses of Lacutangula fruit extract on lipid peroxidation, glutathione (GSH) and Superoxide dismutase (SOD), Catalase (CAT) activities in erythrocytes in a time and dose dependent manner using Tertiary butyl hydroperoxide t-BHP induction. It has been shown that human RBCs may compose up a potent system that can be used as an in vitro experimental model to investigate the antioxidant potential of dietary foods (16). t-BHP is a well known cytotoxin and oxidative stress inducer, induces oxidative damage in different organs like liver (17) testes (18), etc. mainly by mobilization of arachidonic acid (AA) from membrane phospholipids under cytotoxic conditions.

Materials and methods

Reagents : The medicinal plant Lacutangula was purchased from Dr. Madhava Shetty, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. Thiobarbituric acid (TBA), 1,1,3,3, tetraethoxypropane (TEP), t-butyl...
hydroperoxide (t-BHP), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), GSH, SOD, xanthine, xanthine oxidase, nitroblue tetrazolium, GSH-Px, reduced nicotinamide adenin dinucleotide phosphate (NADPH), bovine serum albumine and glutathione reductase (GR) were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA.)

**Extraction** : Fruit hexane (FHE), fruit methanolic (FME) and fruit aqueous (FAE) extraction procedure was performed as described by Reddy et al. (19). Briefly around 300g of fresh plant material (fruit) was washed with tap water, air dried and then chopped into small fragments which were shade dried and reduced to coarse powder with mortar and pestle. The powdered materials were extracted thrice times with hexane (2.5 l), then extracted three times with methanol (2.5 l) and followed by distilled and deionised water (1 l) at room temperature in cycle of 48 h each on orbital shaker. The combined methanolic extracts were then concentrated in a rotavapour at reduced pressure, below 40°C and pooled water extracts were concentrated by lyophilization.

**Partial purification** : Since methanolic extract had shown significant antioxidant activity, this extract was further fractionated chromatographically as described by Reddy et al., 2009(20). Initially, 15 g of FME was loaded on a silica column (column height and diameter were 24 and 2 inches, respectively) and was eluted with stepwise gradient elution of ethyl acetate-methanol (4:1→0.1, v/v). Four fractions were collected at a regular interval and named as F1, F2, F3 and F4. Antioxidant activity of these fractions was evaluated by 2, 2-diphenyl-1-pircrylhydrazyl (DPPH) assay and found that only fraction F2 was showing significant activity. Hence, this fraction was further fractionated with the solvent system methanol-water (4:1→0.1, v/v). Four fractions collected at a regular interval were named as F2-1, F2-2, F2-3 and F2-4.

**HPTLC (High Performance Thin Layer Chromatography) fingerprinting** : Fingerprinting of *Luffa acutangula* was performed on 5x10 cm HPTLC plates coated with 0.25 mm layer of silica gel 60F254. Before using, the plates were washed with methanol and activated at 110°C for 5min. Samples were applied as 4mm wide bands and 6mm apart by using a Camag (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100 µl syringe. A constant application rate of 6 µl/sec was used. The chromatograms were monitored at 600nm using benzene : acetone (60:40) as mobile phase and vanillin H₂SO₄ as reducing agent.

**DPPH free radical scavenging activity** : The DPPH free radical scavenging activity was measured using a method described previously by Reddy et al. (19). Briefly, the stock test extracts of hexane, methanolic and aqueous fruit were dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml, the methanolic fractions, (F2-1, F2-2, F2-3 & F2-4) were dissolved in DMSO at concentration of 25 µg/ml and ascorbic acid was dissolved in DMSO at a concentration of 100 µg/ml. DPPH was prepared freshly in absolute alcohol at a concentration of 4.9 mg/25ml. The reaction mixture consisting of 125 µl of DPPH, 100µl of freshly prepared 0.5mM tris buffer (pH 7.2) and 25 µl test extracts or standard was added to 96 well plates. The plates were incubated at room temperature for 10 min and then absorbance was measured at 517 nm by a UV--visible spectrophotometer (SPECTRA max PLUS®, Molecular Devices, USA). The percentage of free radical scavenging activity was determined from the following formula:

\[
\text{Radical scavenging (\%)} = \frac{(\text{CONTROL} - \text{SAMPLE})}{\text{CONTROL}} \times 100.
\]

**Erythrocytes preparation** : Erythrocytes were prepared as described in Betul et al. (21) Human
blood was collected from healthy donors (we don’t have any institutional review board protocol to draw the blood, it is done by our technician) and centrifuged at 600 g for 5 min. The clear plasma anduffy coat were discarded. Erythrocytes were washed thrice with phosphate-buffered saline (PBS; pH 7.4) by 600 g for 30 sec. The washed erythrocytes were used for subsequent antioxidant activity assay of *Luffa acutangula*.

**Erythrocyte treatment:** Stock solutions of fruit methanolic fraction (F2-3) were prepared in DMSO then diluted with distilled water to obtain 12.5, 25 and 50 µg/ml (DMSO concentration lower than 1%). Erythrocytes at 5% hematocrit in PBS which are treated by 1.5 mM t-BHP for 1 h were incubated for different intervals (30, 60 and 90 min) at 37°C (with continuous mixing) with fruit sub fraction (F2-3) at different doses from 12.5 –50 µg/ml. Samples of erythrocytes without fruit sub fraction (F2-3) is used as controls. After incubation, the mixtures were haemolysed in -20°C. The mixtures were thawed the following day and then centrifuged at 3600 rpm for 15 min. All assays were performed in these supernatants. The concentration of haemoglobin (Hb) was determined using the method described by (22). All experiments described in these studies were reproduced with at least three separate isolates.

**Lipid peroxidation:** The concentration of the malondialdehyde (MDA) in blood was estimated as described by Stocks and Dormandy (23). The principle of this method is spectrophotometric measurement of pink color produced by the reaction of thiobarbituric acid with MDA. The results were expressed as nmol/ml. Briefly 1 ml of 28% trichloroacetic acid–sodium meta arsenite solution was added to 2 ml supernatant and centrifuged at 2000 rpm for 15 min. Then 0.5 ml of 1% thioobarbituric acid (TBA) was added to supernatants and placed in a boiling water bath for 15 min. After reaching room temperature, absorbance was measured using a spectrophotometer at 532 nm. MDA values in erythrocytes were determined from the standard curve using 1,1,3,3, tetraethoxypropane (TEP) as standard.

**Total glutathione (GSH):** Determination of total glutathione content in incubation solutions was done according to the method of Betul (21). The principle of this method is that the oxidized glutathione (GSSG) is converted into GSH in the presence of NADPH and glutathione reductase. The chromophoric product 2-nitro-5-thiobenzoic acid, resulting from reaction of the 5, 5 dithiobis-(2-nitrobenzoic acid) (Ellman reagent) with GSH possesses a molar absorption at 412 nm. 25 µl of the supernatant was added to standard assay mixture containing 0.6 µmol DTNB, 10 µg glutathione reductase and 0.2 µmol NADPH. The reaction was initiated by the addition of NADPH and the color development at 412 nm was followed for 6 min. The concentrations of total glutathione were calculated from a standard curve prepared with GSSG and were expressed as µmol glutathione/g Hb.

**Superoxide Dismutase (SOD):** Activity of the SOD enzyme in incubation solution was determined according to the method described by Sun et al. (24). Xanthine reacts with xanthine oxidase and generates superoxide radicals that react with nitroblue tetrazolium to form formazan dye. To analyze this, 400 µl of incubation solution was haemolysed by four times cold water and Hb was removed by adding mixture containing 0.6 ml chloroform (CHCl3) and 1 ml ethanol (EtOH) to haemolysates, mixed vigorously and centrifuged. A 600 µl of reaction mixture containing 0.1 mmol of xanthine, 0.1 mmol of EDTA, 50 mg of bovine serum albumin, 25 µmol of nitroblue tetrazolium per liter was added to 125 µl supernatant or 125 µl SOD standard solutions.
then 25 µl of 9.9 nmol xanthine oxidase solutions was added to each tube at 30 s intervals. Each tube was incubated for 20 min at room temperature and the reaction was terminated by adding 0.5 ml of 0.8 mM CuCl₂ solution per tube every 30 s. The production of formazan was determined at 560 nm. Under these conditions, the absorbance at 560 nm of the blank tube was about 0.25. SOD enzyme concentrations were determined from the standard inhibition curve with the x-axis being the logarithmic SOD concentrations and the Y-axis represent percent inhibition and expressed as IU/g Hb.

**Catalase (CAT):** CAT activity was measured according to the method described by Aebi (25). The principle of the assay is based on the determination of the rate constant of hydrogen peroxide decomposition by CAT enzyme. A mixture containing 50 mM phosphate buffer (pH 7.0), 20 mM H₂O₂ and 500 l of supernatant was incubated at room temperature for 2 min. The change in absorbance at 240 nm in 2 min was calculated and the decrease in H₂O₂ was measured spectrophotometrically at 240 nm, for 3 min at 25°C. The catalase activity was expressed as mmole H₂O₂ consumed /min/g Hb.

**Statistical Methods:** All the data were expressed as mean ± S.D. using Microsoft XL 2007 software.

**Results and Discussion**

The percentage extraction yield of hexane, methanol and aqueous extract of *L. acutangula* fruit was 3.2 %, 15.02 % and 9.98 %, respectively. HPTLC fingerprinting of *L. acutangula* fruit methanolic extract is presented in Figure 1 (20).

In this study, all the three crude extracts, Fruit Hexane Extract (FHE), Fruit methanolic Extract (FME) and Fruit Aqueous Extract (FAE), were initially tested for their antioxidant activity. FME was showed higher antioxidant activity compared to FHE and FAE (Fig. 2). Hence this extract was selected for further purification using column chromatography. Fractions were collected at regular intervals and named as F1, F2, F3, F4 and F2-1, F2-2, F2-3, F2-4. Fraction F2 demonstrated higher antioxidant activity (Fig. 2) than the other fractions (F1, F3 and F4). Fraction F2 was further fractionated chromatographically and named as F2-1, F2-2, F2-3 and F2-4. Out of these four fractions, F2-3 was found to possess antioxidant activity (Fig. 2). Hence, fraction F2-3 was selected to evaluate the oxidative stress inhibitory activity.
in the human erythrocyte model by measuring different parameters such as lipid peroxidation, SOD, catalase and GSH levels.

Lipid peroxidation is an autocatalytic process, which may lead to an oxidative stress, inflammation, cancer, tissue damage, DNA damage and aging (26). Since, MDA is an end product of the lipid peroxidation, we evaluated the effect of F2-3 on MDA as an index for the lipid peroxidation. When erythrocytes were challenged with \( t\)-BHP, all the oxidative stress parameters, GSH, SOD, and CAT were significantly reduced but MDA levels were increased drastically (Fig.4, Fig.5, Fig.6 and Fig.3, respectively) indicating \( t\)-BHP induces oxidative stress in the erythrocyte. In experiments, where erythrocytes were pre-incubated with fraction F2-3 for one hour and following exposure to \( t\)-BHP, MDA levels were significantly reduced in a dose and time dependent manner. MDA levels in 12.5 \( \mu g/ml \) treated group at the time intervals of 0, 30, 60, 90 min and \( t\)-BHP-control and control were 68.1, 51.8, 46.5, 40.4, 70.34 and 21.2 nmol/g Hb, at the concentration of 25 \( \mu g/ml \) MDA levels were 70.6, 46.7, 42.6 and 30.8 and at the concentration of 50 \( \mu g/ml \) MDA levels were 75.5, 40.6, 29.9 and 27.9 nmol/g Hb, respectively (Fig.3). These data suggested that the fraction F2-3 of \( L.\) \( \text{acutangula} \) fruit possesses lipid peroxidation inhibition properties.

GSH, one of the most potent biological molecules, it's can prevent occupation including free radicals reactions in the erythrocytes. The main defensive roles of glutathione against oxidative stress are: glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionetransferase etc. (27). GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase.

In this study effects of F2-3 fraction on cellular GSH levels in \( t\)-BHP-treated cells were represented in Fig.4. GSH levels in 12.5 \( \mu g/ml \) treated group at the time intervals of 0, 30, 60, 90 min, \( t\)-BHP-control and control were 6.7, 9.0, 13.2, 13.6, 7.8 and 28.77 \( \mu mol/g \) Hb, at the concentration of 25 \( \mu g/ml \) GSH levels were 7.2, 12.3, 19.4 and 19.5 and at the concentration of 50 \( \mu g/ml \) GSH levels were 7.1, 13.2, 20.6 and 21.3 \( \mu mol/g \) Hb, respectively (Fig.4). The obtained results clearly indicating that fraction F2-3 has ability to replenish cellular redox buffer with GSH. Since GSH levels are increased, the co-factor for glutathione related enzymes, glutathione

![Fig. 3. Effect of methanolic fraction of \( L.\) \( \text{acutangula} \) fruit (F2-3) on \( t\)-BHP induced lipid peroxidation in erythrocyte was monitored by measuring the malonaldehyde (MDA). Values are mean ± SD.](image)

![Fig. 4. Effect of methanolic fraction F2-3 on cellular redox buffer (GSH) was monitored in \( t\)-BHP treated erythrocytes. Values are mean ± SD.](image)
peroxidase and glutathionetransferase, is readily available to neutralize the free radicals generated by $t$-BHP.

SOD catalyses the depletion of the superoxide radical and protects oxygen-metabolizing cells against harmful effects of superoxide free radicals. Some types of SOD like MnSOD which contains a manganese prosthetic group, resides in the mitochondria, perhaps because of the need to protect mitochondrial proteins, membranes, and DNA from $O_2^-$ generated as a result of the respiratory chain. Figure 5 showing total SOD levels in human erythrocytes with reference to sub fraction F2-3. SOD levels in 12.5 $\mu$g/ml treated group at the time intervals of 0, 30, 60, 90 min, $t$-BHP-control and control were 1.2, 1.9, 3, 3.23, 1.85 and 11.02 IU/g Hb protein at the concentration of 25 $\mu$g/ml MDA, SOD levels were 1.22, 2.5, 5.2 and 5.2 and at the concentration of 50 $\mu$g/ml SOD levels were 1.1, 5.3, 9.4 and 9.7 IU/g Hb, respectively (Fig.5).

CAT, a soluble protein in erythrocytes, plays a role in the decomposition of hydrogen peroxide to give $H_2O$. In humans, the highest levels of catalase are found in liver, kidney and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition. Figure 6 showing catalase activity in human erythrocytes with reference to FME sub fraction F2-3. CAT levels in 12.5 $\mu$g/ml treated group at the time intervals of 0, 30, 60, 90 min, $t$-BHP-control and control were 50.1, 55.4, 61.8, 62.4, 53.9 and 99.78 $\mu$mol $H_2O_2$/g Hb/min. at the concentration of 25 $\mu$g/ml MDA levels were 52.8, 60.9, 72.3 and 76.6 and at the concentration of 50 $\mu$g/ml MDA levels were 51.1, 62.5, 98.3 and 100.7 $\mu$mol $H_2O_2$/g Hb/min., respectively (Fig.6). Since both the enzymes SOD and CAT are directly involved in the neutralization of free radicals, these enzymes play a pivotal role in the oxidative stress. These enzyme levels were significantly reduced in $t$-BHP treated groups indicating that antioxidant ability of the erythrocytes are reduced in $t$-BHP treated group. Since these enzymes are replenished in response to F2-3 fraction of the fruit, erythrocytes again gained the antioxidant potentials to combat against the free radicals generated in the various metabolic reactions.

The present findings show that *L. acutangula* fruit aqueous fraction F2-3 pretreatment attenuated $t$-BHP induced lipid peroxidation in human erythrocytes. Specifically, fraction F2-3 prevented $t$-BHP induced increases in MDA levels, and concomitantly restored GSH content, SOD and CAT activity in erythrocytes, though to a different degrees. These effects may reflect the ability of fraction F2-3 to enhance the scavenging and inactivation of $H_2O_2$ and hydroxyl radicals. In addition, fraction F2-3 may also terminate lipid peroxidation by induction of enzymatic and non-enzymatic antioxidants, such as GSH, SOD and CAT (30). Accordingly, the

**Fig. 5.** Effect of fraction F2-3 on $t$-BHP induced oxidative stress in erythrocytes was monitored by measuring the superoxide dismutase activity. Results are mean ± SD.
protection afforded by fraction F2-3 against t-BHP induced ROS generation is likely attributable to its antioxidant effects.

In conclusion, L. acutangula methanolic extract fraction F2-3 showed significant antioxidant activity in human erythrocytes and further studies are required to elucidate the fraction components and their molecular mechanism. Systemically, superoxides could be produced in huge amounts by various metabolic and physiological processes (31, 32, 33). The formation of superoxide radical leads to a cascade formation of other ROS in the cell, whenever the antioxidant system fails to combat with ROS that can cause to lethal damage to the system (34). Hence, our data for the first time reports the oxidative stress inhibitory property of the L. acutangula fruit. So that it may help to prevent diseases caused by the ROS. However, one thing we should consider that antioxidant activity may differ from organism to organism because of the genetic configurations to respective antioxidant mechanism (16). So we can expect less or more activity from different organism.

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Reference

![Fig. 6. Effect of fraction F2-3 on t-BHP induced oxidative stress in erythrocytes was monitored by measuring the activity levels of catalase. Results are Mean ± SD.](image-url)


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Role of Luffa acutangula in Oxidative damage


