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# *IN-VITRO* ANTIOXIDANT POTENTIAL OF SELECTED NUTRACEUTICALS

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**ABSTRACT:** The current study involves evaluation of antioxidant potential of selected nutraceuticals like barley grass juice (*Hordeum vulgare* L.), ethanolic extracts of cashew apple (*Anacardium occidentale* L.) and sea lettuce (*Ulva reticulata* F.) in *in-vitro* experimental model using goat liver slices at different concentrations (100, 200, 400, 800 and 1600 mg/kg, p.o). The oxidative stress was induced using hydrogen peroxide, and the levels of lipid peroxidation (LPO), superoxide dismutase (SOD), catalase, and reduced glutathione (GSH) were measured. Rutin was used as a standard. Barley grass juice, ethanolic extracts of cashew apple and sea lettuce have dose-dependently ameliorated the LPO, an indicator of membrane damage caused by oxidative stress. The selected nutraceuticals have significantly improved the levels of antioxidant enzymes like SOD, catalase, and GSH. Although the present study reported the antioxidant potential of the selected nutraceuticals, it still needs to be explored in different test systems employing different inducers.

**INTRODUCTION:** The word nutraceutical is an amalgamation of nutrition and pharmaceutical. In a broader sense, it is defined as a food or a part of food/herb/diet which plays a significant role in modulating and maintaining normal physiological processes. The worldwide market of nutraceuticals was expected to grow from $221.58 billion in 2014 to $424 billion by 2017. [1] The main reasons that can be attributed to this tremendous growth of the nutraceuticals market were a high expansion of population and varied health trends. Most of the lifestyle diseases observed nowadays have been raised due to oxidative stress. This has led to a tremendous demand for nutraceuticals possessing antioxidant activity.

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Reactive oxygen species (ROS) had shown deleterious effects on the human body, due to their ability to donate oxygen to other substances [2]. The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. Antioxidants that can quench ROS are classified based on their mechanism. Few antioxidants can donate a hydrogen atom or react with initial radicals or inhibit the enzymes or minimize the oxygen levels without generating radical species. They act as catalytic systems that neutralize or divert ROS [antioxidant enzymes SOD (superoxide dismutase), catalase, and glutathione peroxidase] and chain-breaking antioxidants which scavenge and destroy ROS (ascorbic acid, tocopherols, uric acid, glutathione, flavonoids [3].

In recent years, the knowledge of free radicals and reactive oxygen species (ROS) in biology has been a medical revolution that promises a new age of

health and disease management [4]. Epidemiological studies have indicated that the consumption of a diet rich in fruits and vegetables leads to decreased risk of developing certain chronic diseases, including heart disease, muscular degeneration and cancer [5, 6]. Direct and indirect methods have been applied to determine the chain-breaking antioxidant activity of natural products. Direct methods are based on studying the effect of a tested product (*e.g*., food) containing antioxidants on the oxidative degradation of a testing system. There is also a considerable amount of evidence revealing an association between individuals who have a diet rich in fresh fruits and vegetables and the decreased risk of cardiovascular diseases and certain forms of cancer. This benefit is accredited to their antioxidant property [7, 8]. Earlier studies have shown that plant products including polyphenols, terpenes, and various plants extract exerts antioxidant activity [9, 10, 11]. We have selected a few nutraceuticals like barley grass, cashew apple and sea lettuce whose antioxidant potential has been less explored. The antioxidant activity of barley seeds and cashew nuts has been reported but not with the selected nutraceuticals [12, 13]. This prompted us to evaluate the antioxidant potential of nutraceuticals using goat liver slices as a test model.

## MATERIALS AND METHODS:

**Plant Material:** Barley seeds (*Hordeum vulgare* L., Family - Poaceae), was purchased from Apex International, Tilak Bhavan, Jaipur, India. The cashew apple fruit (*Anacardium occidentale* L. Family - Anacardiaceae), was collected from the local market. Sea lettuce (*Ulva reticulata* F. Family - Ulvophyceae) was acquired from Mandapam sea coast, Rameswaram, Tamil Nadu, India. All of them were authenticated by Dr. K. Madavachetty, Department of Botany, Sri Venkateswara University, Tirupati. The voucher specimens of barley grass, cashew apple and sea lettuce bearing no's (1768, 0976 and 0184) were deposited in the Department of Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh, India.

### Preparation of Extracts:

**Barley Grass Juice (*Hordeum vulgare* L.):** The fresh barley grass, of approximately 15 days age was collected, washed and chopped into small pieces was blended using a mechanical blender. The barley grass juice (BGJ) was then squeezed using a muslin cloth. The collected juice was lyophilized, using a lyophilizer (Mini-Lodel, FD-10-R) and the obtained solid mass was preserved in storage vials at 4 ºC until further use.

**Ethanolic Extraction of Cashew Apple Juice (*Anacardium occidentale* L.) and Sea Lettuce (*Ulva reticulate* F.):** The collected cashew apple and sea lettuce were washed cleanly with water and cut into small parts. The shade dried material was pulverized to obtain coarse powder (sieve no. 20) and extracted with ethanol using a Soxhlet apparatus for 6 h. The collected solvent was filtered through filter paper, and filtrates were evaporated under reduced pressure at 40 ºC using a rotary evaporator to collect the solid mass. The residual collected ethanolic extracts of cashew apple (CAEE) and *Ulva reticulata* (UAEE) were stored in storage vials and stored at 4 ºC for further analysis.

**Antioxidant Assays:** The collected plant extracts were then dissolved in distilled water to make a concentration of 1 mg/mL and then diluted to prepare series of concentrations (100, 200, 400, 800, 1600 µg/mL) to measure *in-vitro* antioxidant activity and lipid peroxidation.

**Preparation of Tissue Homogenate:** The fresh liver tissue was collected from slaughterhouse in a sterile vessel containing phosphate buffer (pH 7.4). Liver lobes were dried using blotting papers and were cut into thin liver slices (1 mm) using the sterile scalpel. Liver slices (1 g) in 5 ml of PBS was treated with 100 µl of $H_2O_2$ (200 µM) and incubated at 37 ºC for one hour with mild shaking. The slices were incubated in different conditions as given below.

- Untreated tissue homogenate (Normal).
- Tissue homogenate + $H_2O_2$ (Disease control).
- Tissue homogenate + $H_2O_2$ + rutin at different concentrations (standard).
- Tissue homogenate + $H_2O_2$ + BGJ at different concentrations.
- Tissue homogenate + $H2O_2$ + CAEE at different concentrations.
- Tissue homogenate + $H_2O_2$ + UAEE at different concentrations.

After the incubation period, tissues were immediately homogenized in the same aliquot of PBS using homogenizer and centrifuged to remove the debris. The obtained supernatant was then used for the estimation of enzymatic antioxidants and the extent of lipid peroxidation in terms of formation of thiobarbituric acid reactive substances (TBARS).

**Determination of Enzymatic Antioxidants:**

**Assay of Superoxide Dismutase (SOD):** Superoxide dismutase activity was estimated spectrophotometrically by following the method [14]. The reaction mixture of 3 ml was prepared by adding 50mm potassium buffer, 45 µM methionine, 5.3 µM riboflavin, 84 µM NBT, 20 mm potassium cyanide and 500 µl of tissue homogenate. The mixture was maintained at 25 ºC equipped with 15W fluorescent lamps in an aluminum foil-lined box. After the incubation period for 10 min, reduced NBT was then measured using Spectrophotometer at 600 nm. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of NBT reduction. The enzyme unit can be calculated using the following equations:

Rate (R) = Final OD-Initial OD / 3 min

% of inhibition = (Blank OD-R / Blank OD) × 100

Enzyme unit (U) = (% of Inhibition / 50) × common dilution factor.
(50 % Inhibition= 1 U)

**Assay of Catalase:** The catalase activity was estimated by following the method 15.3 ml of $H_2O_2$ in phosphate buffer (pH 7.0) was taken into a quartz cuvette, and the baseline was adjusted at 240 nm followed by 500 µl of homogenate and thoroughly mixed. The time interval for the decrease in absorbance was recorded at 240 nm. The tissue homogenate and phosphate buffer without $H_2O_2$ served as control. Δt was noted till the absorbance reach to 0.45. If it is longer than 60 sec, the assay was repeated with concentrated tissue homogenate.

One unit catalase is equaled to the amount of enzyme that liberates half the peroxide oxygen from $H_2O_2$ solution of any concentration in 100 sec, and it was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

Moles of $H_2O_2$ consumed ⁄ min [units / (mg of tissue)] = $2.3/\Delta t \times \text{In (E initial / E final)} \times 1.63 \times 10^{-3}$

**Estimation of Non-Enzymatic Enzyme:**

**Reduced Glutathione:** Reduced glutathione levels were determined by the method proposed by Moron *et al*. [16] The liver homogenate of 0.5 ml was added to the 2.5 ml of 5% TCA, and the precipitated protein was centrifuged at 1000 rpm for 10 min. The supernatant of 0.1 ml was used for the estimation of GSH. 0.2 ml of collected sample was made up to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). 2 ml of freshly prepared DTNB solution (0.6 M in phosphate buffer) was added, and the intensity of the yellow color formed was read at 412 nm using spectrophotometer after 10 min. The amount of reduced glutathione was expressed as µg of GSH/mg of wet tissue.

**Lipid Peroxidation Assay:** After incubation, 200 µl of the tissue homogenate was taken in a tube containing 2.0 ml of 10% trichloroacetic acid. After 10 min, tubes were centrifuged, and the supernatant was separated and mixed with 1.5 ml of 0.67% thiobarbituric acid in acetic acid. The mixture was heated in a water bath at 85 ºC for 30 min, followed by heating in a boiling water bath to complete the reaction. The intensity of the pink colored complex formed was measured at 535 nm in a spectrophotometer [17]. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of control as per the following formula:

% Inhibition = (Control Absorbance - Test Absorbance) / (Control Absorbance) × 100

The TBARS concentration was calculated by using the following formula and expressed as nM/mg of tissue [18].

nM of TBARS ⁄ (mg of tissue) = OD × volume of homogenate × 100 × $10^3$ / (1.56 × $10^5$ ) × volume of extract taken

**Statistical Analysis:** One-way analysis of variance (ANOVA) using GraphPad Prism version 5 software was applied to test the significance of the data. All the values were expressed as mean ± Standard deviation (SD). $P<0.05$ was considered statistically significant.

**RESULTS:** Incubation of liver slices with $H_2O_2$ for one hour has caused a significant decrease in

the *in-vivo* antioxidant enzymes like SOD, catalase and reduced glutathione when compared to the normal conditions. This confirms the induction of oxidative stress with $H_2O_2$. Treatment of barley grass juice, cashew apple and sea lettuce extract at various concentrations had shown protection against the depletion of *in-vivo* antioxidant enzymes, noted as significantly high levels of SOD, catalase and reduced glutathione, when compared to the control. Similarly, upon the assessment of LPO levels in tissue homogenates a significant increase in LPO was noticed in the control samples when compared to the normal. Barley grass juice, cashew apple, and sea lettuce extract have significantly ameliorated the LPO levels when compared to the control. These results confirm the antioxidant activity of the selected test substances. The effect was found to be dose dependent and comparable to that of the standard rutin. When compared among the studied test compounds no significant difference in the activity was noticed among them. All the results were tabulated in **Table 1**.

**TABLE 1: EFFECT OF SELECTED NUTRACEUTICALS ON THE ANTIOXIDANT STATUS IN GOAT LIVER SLICES *IN-VITRO* EXPOSED TO HYDROGEN PEROXIDE**

| Group | Concentration (µg / mL) | Catalase (µmoles of $H_2O_2$ decomposed / min / mg tissue) | SOD (U / mg tissue wet) | GSH (µg of GSH / mg of wet tissue) | LPO (TBARS) (nM/mg of tissue) |
|---|---|---|---|---|---|
| Normal | - | 148 ± 0.88 | 6.98 ± 0.08 | 6.23 ± 0.05 | 0.41 ± 0.08 |
| Disease control | - | 51.23 ± 0.55[a] | 3.01 ± 0.02[a] | 3.33 ± 0.01[a] | 1.76 ± 0.34[a] |
| BGJ | 100 | 59.87 ± 0.57[b] | 2.86 ± 0.02[b] | 2.19 ± 0.01[b] | 1.56 ± 0.28[b] |
| | 200 | 65.89 ± 0.53[b] | 3.23 ± 0.02[b] | 2.57 ± 0.03[b] | 1.34 ± 0.22[b] |
| | 400 | 74.87 ± 0.65[b] | 4.28 ± 0.02[b] | 3.89 ± 0.02[b] | 0.83 ± 0.20[b] |
| | 800 | 119.56 ± 0.75[b] | 6.89 ± 0.06[b] | 4.56 ± 0.04[b] | 0.46 ± 0.22[b] |
| | 1600 | 153.86 ± 0.88[b] | 7.34 ± 0.08[b] | 6.37 ± 0.05[b] | 0.32 ± 0.07[b] |
| CAEE | 100 | 47.98 ± 0.52[b] | 2.89 ± 0.02[b] | 2.07 ± 0.02[b] | 1.98 ± 0.22[b] |
| | 200 | 62.32 ± 0.52[b] | 3.18 ± 0.02[b] | 2.96 ± 0.02[b] | 1.78 ± 0.26[b] |
| | 400 | 86.89 ± 0.62[b] | 4.18 ± 0.02[b] | 3.99 ± 0.02[b] | 0.76 ± 0.24[b] |
| | 800 | 111.77 ± 0.74[b] | 5.96 ± 0.05[b] | 4.76 ± 0.02[b] | 0.63 ± 0.18[b] |
| | 1600 | 159.83 ± 0.85[b] | 7.86 ± 0.05[b] | 6.96 ± 0.04[b] | 0.33 ± 0.05[b] |
| UAEE | 100 | 48.00 ± 0.34[b] | 2.98 ± 0.02[b] | 2.34 ± 0.01[b] | 1.45 ± 0.25[b] |
| | 200 | 57.34 ± 0.51[b] | 4.13 ± 0.02[b] | 2.98 ± 0.02[b] | 1.23 ± 0.22[b] |
| | 400 | 72.67 ± 0.54[b] | 5.56 ± 0.05[b] | 3.34 ± 0.02[b] | 0.73 ± 0.20[b] |
| | 800 | 130.34 ± 0.62[b] | 6.11 ± 0.05[b] | 4.22 ± 0.02[b] | 0.62 ± 0.20[b] |
| | 1600 | 161.23 ± 0.87[b] | 7.99 ± 0.05[b] | 6.99 ± 0.05[b] | 0.42 ± 0.06[b] |
| Rutin (standard) | 100 | 58.22 ± 0.44[b] | 2.98 ± 0.02[b] | 2.95 ± 0.02[b] | 1.65 ± 0.22[b] |
| | 200 | 74.65 ± 0.54[b] | 3.67 ± 0.02[b] | 3.98 ± 0.04[b] | 1.22 ± 0.20[b] |
| | 400 | 91.22 ± 0.61[b] | 4.56 ± 0.02[b] | 4.23 ± 0.02[b] | 0.72 ± 0.20[b] |
| | 800 | 139.77 ± 0.67[b] | 6.99 ± 0.05[b] | 4.93 ± 0.05[b] | 0.55 ± 0.20[b] |
| | 1600 | 165.32 ± 0.87[b] | 7.01 ± 0.05[b] | 6.88 ± 0.05[b] | 0.22 ± 0.20[b] |

The values were represented as Mean ± S.D. of triplicates. a=statistically significant ($p<0.05$) compared with normal group,b= statistically significant ($P<0.05$) compared with disease control group.

**DISCUSSION:** Reactive oxygen species, unstable molecules have potent oxidative effects on many cellular constituents like proteins, lipids, and DNA which leads to impairment of various cellular functions. The antioxidant substances and enzymes like SOD, catalase and reduced glutathione present in the body offer resistance against oxidative stress by scavenging the free radicals, inhibiting both oxidation and lipid peroxidation thereby preventing the diseases [19]. However, when ROS generation overwhelms the antioxidant capacity, leads to oxidative stress and contributes to the development of many pathological conditions [20]. In the current study, hydrogen peroxide was selected as an oxidizing agent as it was regularly synthesized in physiological conditions causing oxidative damage. It can easily cross the cell membrane and attack different sites causing DNA damage which is believed to be the initial step in the induction of various diseases. Hence, we have planned to investigate whether the selected nutraceuticals could be able to decrease the toxicity produced by $H_2O_2$ in goat liver slices in the *in-vitro* conditions.

Catalase is a widely distributed antioxidant enzyme in all animal tissues including red blood cells and

liver. It is also one of the most important intracellular enzymes in the detoxification of the oxidant hydrogen peroxide. The ability of tissues to catalyze the decomposition of hydrogen peroxide, with the release of oxygen, was reported by Thenard in 1818 [21] and the responsible activity named as catalase by Lowe in 1901 [22]. Additionally, the level of catalase was lowered under oxidative stress which will predispose the living system to the hydrogen peroxide toxicity. Modulation of catalase is one of the methods by which the cell combats the potential toxicity of $H_2O_2$. Hence, we have assessed the catalase levels in the current investigation. In the present study, treatment of $H_2O_2$ treated liver homogenate with selected nutraceuticals at different concentrations improved catalase activity similar to the normal and standard group when compared to disease control indicating the protective nature of nutraceuticals. These results also were similar with the earlier studies reported by Krishnaraju *et al*., [23].

Superoxide dismutase (SOD), widely known detoxification enzyme which acts as a component of a first-line defense system against reactive oxygen species (ROS). It is an endogenous antioxidant enzyme that responsible for catalyzing the two molecules of superoxide anion to hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$), by dismutation, therefore rendering the toxic superoxide anion to less hazardous. SOD is a metalloenzyme which requires metal cofactors like iron (Fe), zinc (Zn) copper (Cu) and manganese (Mn) for its activity [24, 25]. Hence, superoxide dismutase is a potent antioxidant enzyme in the cell which helps to protect against destruction by superoxide radical anion ($O^{2-}$) generated during oxidative stress [26]. Studies have reported the role of decreased SOD promoting various diseased conditions like vascular hypertrophy, vascular dysfunction in hyperhomocysteinemia [27].

It has also been suggested that taking natural sources as SOD supplementation can protect the immune system can reduce the occurrence of many diseases [28]. In the current study the level of SOD was found to be increased in the treatment groups with nutraceuticals and standard rutin, when compared to H2O2 alone, treated group indicating protective nature of SOD activity in the liver slices during oxidative stress.

GSH is an antioxidant found in high concentration in the liver, protected cells from reactive oxygen species (ROS) such as free radicals and peroxides and known to have a key function in various protective process [29]. Reduced glutathione also constitutes the first line of defense against free radicals produced during oxidative stress [30]. It acts directly as a free radical scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radical. GSH, apart from being a strong antioxidant by itself also acts as a substrate for antioxidant enzymes like GPx and GST. It also reduces peroxides and maintains protein thiols in the reduced state [31]. GSH is the major cytosolic thiol compound and is required to maintain the normal reduced state of the cells and to counteract ROS, thereby reducing the oxidative stress. In the current study, treatment with nutraceuticals and standard antioxidant rutin has significantly increased the GSH levels compared with oxidative stress induced group.

Lipid peroxidation (LPO) is a free radical-mediated process leading to oxidative deterioration of polyunsaturated lipids. LPO is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation. Malondialdehyde (MDA) is the major oxidative product of peroxidized polyunsaturated fatty acids, and its increased content is an indicator of lipid peroxidation. It is connected with various disease processes, such as carcinogenesis, atherosclerosis, and hypertension. To control LPO, there is a defensive system consisting of antioxidant enzymes that play an important role in scavenging reactive oxygen species [32]. In the present study, treatment of $H_2O_2$ treated liver homogenate with nutraceuticals such as barley grass juice, ethanolic extracts of cashew apple and sea lettuce showed a significant decrease in MDA content compared to disease control indicating the prevention of rising in lipid peroxides.

Though the exact mechanism of ROS producing and scavenging systems in different organelles was not able to discuss, the current investigation attests the antioxidant effect of selected nutraceuticals by combating the oxidative stress induced in the *in-vitro* conditions using goat liver slices which can establish the *in-vivo* mechanism of antioxidant system in the normal physiological process.

**CONCLUSION:** In conclusion, selected nutraceuticals including barley grass juice, cashew apple, and sea lettuce showed potent antioxidant capacities in $H_2O_2$ induced free radicals in goat liver model. These nutraceuticals could be potentially naturally rich sources of natural antioxidants which could be developed into functional foods or drug for prevention and treatment of diseases caused by oxidative stress.

In the future, the specific components present in them could be isolated and explored for their health effects on oxidative stress.

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