EVALUATION OF ANTIOXIDANT PROFILE AND ACTIVITY OF AMALAKI (*EMBLICA OFFICINALIS*), SPIRULINA AND WHEAT GRASS

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ABSTRACT

Aqueous and alcoholic extracts of amalki (*Embllica officinalis*), spirulina and wheatgrass were prepared and analyzed for antioxidant vitamin content (vitamin C and E), total phenolic compounds. Antioxidant status, reducing power and effect on glutathione S-transferase (GST) activity were evaluated in vitro. Vitamin C content of crude amalaki powder was found to be 5.38 mg/g, while very less amount 0.22 mg/g was detected in wheat grass. Amalki was rich in vitamin E like activity, total phenolic content, reducing power and antioxidant activity. Total antioxidant activity of aqueous extract of amalki, spirulina and wheat grass at 1mg/ml concentration were 7.78, 1.33 and 0.278 mmol/l respectively. At similar concentrations the total antioxidant activity of alcoholic extract of amalaki, spirulina and wheat grass was 6.67, 1.73 and 0.380 mmol/l respectively. Amalki was also found to be rich source of phenolic compounds (241mg/g gallic acid equivalent). Alcoholic extract of wheat grass showed 50 % inhibition in $FeCl_2$ -ascorbic acid induced lipid peroxidation of rat liver homogenates in vitro. Both aqueous and alcoholic extracts of amalaki inhibited activity of rat liver glutathione S-transferase (GST) in vitro in dose dependant manner. Since GST acts as powerful drug metabolizing enzyme its inhibition by amalaki offers possibility of its use for lowering therapeutic dose of herbal preparations. The aqueous extracts of both amalki and spirulina also showed protection against t-BOOH induced cytotoxicity and production of ROS in cultured C_6 glial cells.

KEY WORDS

Amalki, *Embllica officinalis*, Spirulina, Wheat grass, Nutraceutical, Vitamin C.

INTRODUCTION

Various herbs and components of foods or other ingestible substances that have potentially beneficial effects on human health and function are used as nutraceuticals. In general, they are not viewed as essential nutrients, but as beneficial nutrients (1). Nutraceuticals include plant-derived factors (phytochemicals) and factors derived from animal sources as well as from microbial sources. The activities of nutraceuticals are broad and include antioxidants, modulators of enzyme activity and hormone actions (agonists and antagonist) or a substance which acts as precursors for one or more beneficial

molecules. Some of the recognized nutraceuticals are flavonoids, carotenoids, allyl compounds, protease inhibitors, saponins, licorice, fibers and omega 3 and omega 6 polyunsaturated fatty acids (2). Use of herbal medicines has increased significantly over the years (3-5). Dietary supplements are intended for ingestion in pil, tablet, capsule or liquid form and not for use as conventional diet. Some of the most commonly used herbal products are St John's wort, Echinacea, garlic, ginseng, soy, valerian, cranberry, black cohosh, ginkgo and saw palmetto (6). Amalaki (dried powder of fruits of *Embllica officinalis*), spirulina (dried powder of blue green algae) and wheat grass (dried powder of aerial part of 7 day old wheat grass) are among commonly used herbal preparations. These three nutraceuticals are available in the market as single herb preparations. There are several studies related to their biological activities and mainly claims are about their antioxidant activity (7-13). In an earlier study we observed antioxidant activity of wheat grass and spirulina in human volunteers during physical training (13). The present study was conducted to determine relative efficacy of three plant

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products on the basis of *in vitro* markers of antioxidant status.

MATERIALS AND METHODS

Commercially available authenticated powders of Amalaki, Spirulina and Wheat Grass as single herb preparation were purchased from Himalaya Drug Company, Bangalore, India, M/s Nutraceutical Bio-Tech, Mumbai, India and M/s Sanat Products Ltd, Delhi, India respectively. The products were not characterized separately at our laboratory except for water-soluble and alcohol extractable portions, and variations between different batches was found to be less than 10%. The powders were used for the preparation of extract (14). Five grams of each powder was suspended in 50ml of water and kept for 24h with occasional shaking. Thereafter it was filtered and lyophilized and was treated as aqueous extract. Yield of aqueous extract of amalaki, spirulina and wheat grass were 34.9%, 25.5% and 12.6% respectively. Another five grams of powders were extracted in 50ml of dichloromethane: methanol (1:1) mixture. The extract was evaporated to dryness in a water bath at 40°C. After the solvent was evaporated the extract was weighed and dissolved in ethanol (10mg/ml) and used as alcoholic extract. Yield of alcoholic extract were 24.8%, 12.8% and 4.8% respectively in case of amalaki, spirulina and wheat grass.

The estimation of vitamin C was done by the method of Zannoni et al (15). The estimation of vitamin E using bathophenanthroline was carried out by the method of Desai (16). The amount of total phenolic compounds was determined with Folin & Ciocalteu's reagent according to method described by Slinkard and Singleton (17) using gallic acid as standard. Reducing power of extracts was determined by method described by Yen and Chen (18). In brief 0.5 ml of solution (1.0 mg/ml) was mixed with 2.5 ml of 0.1 M phosphate buffer pH 6.6. To this was added 2.5 ml of 1% Potassium ferricyanide (w/v) and tubes were heated at 50°C for 30 minutes. After incubation 2.5 ml of 10% TCA (w/v) was added, tubes were centrifuged at 1000 g for 10 min. Supernatant 2.5 ml was mixed with equal volume of distilled water and 0.5 ml of 0.1% ferric chloride solution (w/v) and optical density was measured at 700nm against reagent blank in Biorad Smart Spec 3000 spectrophotometer. Increase in OD was considered as reducing power of the test compound.

The total antioxidant activity was measured by the method of Miller et al (19) incubating ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonated]) with a peroxide (metmyoglobin) and hydrogen peroxide to produce the radical cation ABTS⁺. This has a relatively stable blue green color,

which is measured at 600nm. Antioxidants added to reaction mixture caused suppression of this color production to a degree, which was proportional to their concentration.

The rat liver homogenates (10% w/v) prepared in ice-cold 150mM KCl were used for *in vitro* assays. Homogenate was centrifuged at 1000 x g for 15 min and supernatants (cell free homogenate) were stored at -70°C until used. FeCl₂ – ascorbic acid stimulated lipid peroxidation in rat liver homogenates was determined using method described by Halici et al.(20).

The activity of Glutathione S-transferase was measured using 1-chloro 2, 4 dinitrobenzene as substrate initially in crude homogenate (21). The effect of different concentrations of amalaki extracts on GST activity was determined using partially purified enzyme preparation (21). Protein content of cell free homogenate was determined by method of Lowry et al (22). The effect of test substances was evaluated by their addition in assay mixture with 10 min incubation at 37° C along with suitable controls (for aqueous extract water and for alcoholic extract ethanol was used).

C₆ glioma cells were obtained from National Center for Cell Science, Pune were cultured in MEM supplemented with 10% fetal calf serum. Effect of extracts on t-BOOH induced cytotoxicity and production of reactive oxygen species (ROS) was investigated using methods as described by Gitika et al. (23). C₆ glial cells (1 x 10⁴/well in 96 well plate) were incubated with extract/ antioxidant 30 min prior to exposure to 100 mM t-BOOH for 3 h . After 3 h treatment, cells were assessed for cytotoxicity using neutral red assay (24). For cytotoxicity assay neutral red dye was added to a final concentration of 0.1% to each well and incubated further for 3 h at 37°C in a CO₂ incubator. Cells were washed three times with saline (0.9% sodium chloride solution) followed by addition of 200 µl of ethanol-acetic acid solution (50:1). The OD was measured at 570 nm using Plate reader. ROS generation was measured by incubating cells with 2, 7-dichloro fluorescein diacetate (DCFH-DA) fluorescent probe after t-BOOH treatment (25). In brief 10 µl of DCFH-DA solution (200 µM solution in DMSO) was added to each well containing 1 x 10⁴ cells and incubated at 37° C for 30 min. After incubation cells were washed three times with saline and lysed with 200 µl lysis buffer (10 m M Tris, 20 m M EDTA, 0.25 % Triton X-100, p H 8.0). Fluorescence of solution was measured at excitation and emission wavelength of 485nm and 530 nm respectively.

Statistical comparisons between different concentrations and respective controls were made using one -way ANOVA.

RESULTS

The concentrations of Vitamin C in crude powder and aqueous extracts of amalaki and wheat grass are given in Table 1. Vitamin C was not detected in spirulina. The Vitamin E like activity was more in case of amalaki while lowest in spirulina and there was an enrichment of this activity in alcoholic extract. Content of total phenolic compounds expressed as gallic acid equivalent was found to be 241 ± 8.0 , 15.8 ± 0.9 and 10.7 ± 1.0 mg/g respectively in case of crude amalaki, spirulina and wheat grass.

Aqueous and alcoholic extracts of amalaki have shown very high reducing power. Reducing power of alcoholic extracts of spirulina and wheat grass was more in comparison to their

aqueous extracts. The total antioxidant activity of solutions containing 1 mg/mL extracts was evaluated and pattern of activity was essentially similar to reducing power (Table 2).

Table 2: Total reducing power and total antioxidant activity in aqueous and alcoholic fractions of Amalki, Spirulina and Wheat Grass at concentration of 1mg/ml

Variable	Extract	Amalki	Spirulina	Wheat Grass
Total reducing power	Aqueous	1.688 ± 0.031	$0.093 \pm 0.002^+$	$0.253 \pm 0.005^{+,#}$
(OD 700 nm)	Alcoholic	1.410 ± 0.100	$0.250 \pm 0.007^+$	$0.307 \pm 0.019^{+,#}$
Total Antioxidant	Aqueous	7.78 ± 0.17	$1.33 \pm 0.25^+$	$0.278 \pm 0.024^{+,#}$
Activity (mmol/L)	Alcoholic	6.67 ± 0.11	$1.73 \pm 0.03^+$	$0.380 \pm 0.015^{+,#}$

Values are mean $\pm$ SD of five determinations. Values are significantly different in comparison with Amalki (+ $p < 0.001$) and Spirulina (# $p < 0.001$).

Both aqueous and alcoholic extracts of amalaki were found to decrease glutathione S-transferase activity in dose dependent manner (Fig 1). However, spirulina and wheat grass have shown no marked effect on glutathione S-transferase activity (Table 3). FeCl_2 – ascorbic acid stimulated lipid peroxidation in rat liver homogenates was inhibited by only alcoholic extract of wheat grass (Table 3).

Table 1. Vitamin C, vitamin E and total phenolic content in Amalki, Spirulina and Wheat Grass

Variable	Extract	Amalki	Spirulina	Wheat Grass
Vitamin C	Crude Powder	5.38 ± 0.73	Not detected	$0.22 \pm 0.05^+$
(mg/g)	Aqueous	10.65 ± 1.12	-	$1.14 \pm 0.28^+$
Vitamin E *	Crude powder	245.6 ± 8.02	$0.10 \pm 0.01^+$	$0.66 \pm 0.05^{+,#}$
(mg/g)	Alcoholic	986.2 ± 15.6	$0.52 \pm 0.03^+$	$13.59 \pm 1.01^{+,#}$
Total Phenolic	Crude powder	241 ± 8.0	$15.8 \pm 0.9^+$	$10.7 \pm 1.0^{+,#}$
Content**				
(mg/g)				

Values are mean $\pm$ SD of five determinations. * α tocopherol equivalents, ** gallic acid equivalents. Values are significantly different in comparison with Amalki (+ $p < 0.001$) and Spirulina (# $p < 0.001$).

Fig 1: Effect aqueous and alcoholic extracts of Amalki on purified rat liver glutathione S-transferase activity at different concentrations in vitro. ** $p < 0.01$ & *** $p < 0.001$ in comparison with control or 0 mg.

Fig 2. Activity of different extracts, vitamin C and α -tocopherol against t-BOOH induced cytotoxicity in C₆ glioma cells. Cells were incubated with extract/ antioxidant 30 min prior to exposure to 100 mM t-BOOH for 3 h. (1) Control without test compound (2) t-BOOH 100mM (3) Vitamin C 10mg /ml (4) Vitamin E 50 mg /ml (5) Amalki aqueous 50mg /ml + t-BOOH (6) Amalki alcoholic 50mg /ml + t-BOOH (7) Spirulina aqueous 50mg /ml + t-BOOH (8) Spirulina alcoholic 50mg /ml + t-BOOH (9) Wheat Grass aqueous 50mg /ml + t-BOOH (10) Wheat Grass alcoholic 50mg /ml + t-BOOH. *** $p < 0.001$ in comparison with control, + $p < 0.05$, +++ $p < 0.001$, ns= $p > 0.05$ comparison with t-BOOH

Table3. Effect of aqueous and alcoholic extracts of Amalaki, Spirulina and Wheat Grass on FeCl₂-ascorbate induced lipid peroxidation and Glutathione S-transferase activity in homogenates of rat liver in vitro

Variable	Extract	Control	Amalaki	Spirulina	Wheat Grass
Lipid	Aqueous	1.15 ± 0.07	1.24 ± 0.03	1.12 ± 0.04	1.14 ± 0.01
Peroxidation (m mol/ mg protein) ^a	Alcoholic	1.07 ± 0.03	1.15 ± 0.07	1.05 ± 0.02	0.54 ± 0.09 ⁺
Glutathione	Aqueous	0.38 ± 0.01	0.19 ± 0.01 ⁺	0.42 ± 0.01	0.37 ± 0.01
S-transferase (m mol/min/ mg protein) ^b	Alcoholic	0.41 ± 0.03	0.18 ± 0.01 ⁺	0.31 ± 0.05	0.36 ± 0.01

Values are mean ± SD of five determinations. Concentration in assay mixture: a = 50 mg, b= 100 mg. Aqueous and alcoholic extracts were dissolved in water and ethanol respectively to give required concentration in 10 µl solution and similar amount of solvent was added to respective controls. + Values significantly different in comparison with control (p <0.05)

Fig 3: Activity of different extracts, vitamin C and α-tocopherol against t-BOOH induced ROS generation in C₆ glioma cells. Cells were incubated with extract/ antioxidant 30 min prior to exposure to 100 mM t-BOOH for 3 h. (1) Control without test compound (2) t-BOOH 100mM (3) Vitamin C 10mg /ml (4) Vitamin E 50 mg /ml (5) Amalaki aqueous 50mg /ml + t-BOOH (6) Amalaki alcoholic 50mg /ml + t-BOOH (7) Spirulina aqueous 50mg /ml + t-BOOH (8) Spirulina alcoholic 50mg /ml + t-BOOH (9) Wheat Grass aqueous 50mg /ml + t-BOOH (10) Wheat Grass alcoholic 50mg /ml + t-BOOH. *** p<0.001 in comparison with control, + p<0.05, +++ p<0.001 in comparison with t-BOOH

Both aqueous and alcoholic extract of amalaki showed protective activity against t-BOOH induced cytotoxicity and ROS generation in cultured C₆ glioma cells at dose of 50 mg/ml (Fig 2). Significant cytoprotective activity and inhibition of t-BOOH induced ROS generation was also noted in case of aqueous extract of spirulina. In case of wheat grass only aqueous extract showed significant decrease in ROS generation (Fig 3).

DISCUSSION

Nutraceuticals are the products derived from both plants as well as animal species, which have some beneficial effects

as dietary components. Thousands of biologically active compounds have been identified from vegetables and fruits. A diet rich in vegetables and fruits provides protection against cardiovascular and other chronic diseases originating from oxidative stress (26-27). Suitable antioxidant therapies to control oxidative damage have already attracted the worldwide attention in recent years. Extensive studies on phytochemicals in cell culture system and animal models have provided a wealth of information on the mechanism by which such nutraceuticals show their beneficial effects.

Amalaki is well known for its antioxidant activities. The vitamin C content of amalaki was found to be 5.38 mg/g. This amount is almost similar to reported levels of 600 mg per 100 g edible portion (28). However, Khopde et al (29) have reported vitamin C content as high as 44.65 mg/g of Amla by using titrimetric method. Khopde et al (29) have also estimated vitamin content by using HPLC and by this method content was found to be 32.5 per g of Amla. We have found significant level of vitamin E like activity in Amalaki. Term vitamin E like activity is being used as other compounds can also interfere with colorimetric assay using bathophenanthroline method. Vitamin C was not detected in spirulina while vitamin E like activity was found to be present though levels were lesser than of wheat grass and amalaki.

In amalaki several other compounds like polyphenols, ellagic acid, gallic acid and tannins are also present in very high amount along with vitamin C (7, 30, 31). Fruits and berries are known to have very high amount of phenolic compounds in comparison to herbs and barks of the trees (32). Total phenolic content of spirulina is reported to be 15.4 mg/ g of dry matter of alga by Miranda et al (8). The phenolic compounds such as salicylic, trans-cinnamic, synaptic, chlorogenic, quimic and caffeic acids found in the methanolic extract of alga may be responsible for its antioxidant activity,

individually or by a synergistic action (33). The total antioxidant activity evaluated using ABTS assay (19) was found to be very high in both aqueous as well as alcoholic fractions. This indicates that antioxidant properties are due to less identified compounds. Using ABTS assay total antioxidant activity has been reported to be 6.23 ± 0.15 mmol/L by Khopde et al (29) who have concluded high total antioxidant activity is due to polyphenols that are capable of scavenging oxidizing radicals.

Reduction in MDA by providing amla in diet has also been reported by Anilakumar et al (34) in rats challenged with dimethylhydrazine. However, in present study no protective effect of amalaki against FeCl_2 – ascorbic acid stimulated lipid peroxidation was observed. On the other hand wheat grass in spite of low content of antioxidants has shown protective effect against lipid peroxidation. Wheat grass supplementation to healthy volunteers during physical training for one month have been shown to reduce lipid peroxidation level in blood and efficiency was found to be better than spirulina (13).

The *in vitro* assay system using (t-BOOH) induced oxidative stress in C_6 glial cells is useful for evaluating water soluble antioxidants as reported earlier (23). Using this assay system significant protection against ROS generation and cell death has been observed in case of both spirulina and amalki. Various other plant extracts like green tea, blueberry, spinach, strawberry, *Allium sativum*, *Glycyrrhiza glabra*, *Withania somnifera*, *Convolvulus pleuricavus* and *Aloe vera* are reported to inhibit lipid peroxidation under various assay conditions and in most of the cases activity is attributed to presence of phenolic antioxidants (35-38).

Inhibition of GST activity in dose dependant manner by amalaki extracts is worth while to be explored because this enzyme acts as powerful drug metabolizing enzyme by its conjugation reactions with glutathione (39). Inhibition of activity offers a possibility of combining amalaki with drugs to enhance their potential in case of drug resistance or reduction of dose. Various Ayurvedic formulations use amalki as main ingredient of three herb preparation called triphala and mainly to enhance therapeutic value.

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