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COMPARATIVE EVALUATION OF ANTIOXIDANT POTENTIAL OF EZENUS AND OTHER HERBAL PRODUCTS: AN IN-VITRO STUDY

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ABSTRACT

The aim of this investigation was to evaluate and compare the antioxidant potential of a patented poly herbal sugar free candy Ezenus with twelve other commercial products. Ezenus candy has Andrographolides, Polyphenols, Flavonoids, Vitamin C etc in its ingredients. Ezenus and different commercial products (MCF-1 to MCF-12) were checked for various parameters like metal chelating activity, Catalase activity, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase and Xanthine oxidase activity. Amongst the compared groups Ezenus showed the highest metal chelation with minimum IC_{50} at 730 ± 35 µg/ml which is 2.6 times more potent than MCF-1 where lowest chelating activity was recorded (IC_{50} 1923 ± 115 µg/ml). Catalytic activity is comparable for Ezenus MCF-3 and MCF-11 (1.5±0.05 µMH_2O_2/min/mg protein). followed by other products. Reduced glutathione content is comparable in Ezenus , MCF-4 and MCF-5 (1.9±0.06 GSH nmol/mg/protein) followed by other products. Ezenus showed the highest oxidized glutathione content (2.9±0.13 GSSH nmol/mg/protein) followed by MCF-12 which is 13% lower and MCF-11 which is 34% lower. The maximum activity of glutathione peroxidase is found to be present in Ezenus (1.1±0.07 nmol/min/mg protein) followed by MCF-6(9% lower) MCF-3 and MCF-12 which are 18% lower than Ezenus. Maximum Xanthine oxidase inhibitory activity was seen in Ezenus and MCF-4 with minimum IC_{50} of 110 µg/mL, followed by MCF-11, MCF-5 and MCF-12. From these observations it is concluded that out of 13 products compared for antioxidant potential, Ezenus scores in all of these parameters. MCF-12 is observed to be the second best product.

Keywords: Ezenus, detoxification, metal chelation, reduced glutathione, Xanthine oxidase.

INTRODUCTION

Stress has been called *"the silent killer"*. It is an established fact that mental, psychological and emotional stress combined with unhealthy lifestyle, faulty eating habits and exposure to air and noise pollution lead to series of biochemical reactions in the body and are associated with increased oxidant production and oxidative stress inside body. These same stress inducers also trigger a stress response in the liver leading to swelling of liver cells and subsequent inflammation of liver cells. Alcohol, fatty food, some drugs like paracetamol, food preservatives and pesticides sprayed on crops are the common components that cause liver damage due to lipid peroxidation, triggering inflammatory response. [1,2] Non alcoholic fatty liver is one of the most common and fatal problems of liver often termed as nonalcoholic steatohepatisis (NASH). More than 6 million children suffer from this. According to a report more than 15 million people in US alone suffer from alcohol abuse induced fatty liver. Herbal medicines derived from plant extracts are being used to treat a wide variety of clinical disease.[3] Natural antioxidants has been given more attention due to protective effects against drug-induced toxicity studies especially whenever free radical generation is involved.[4] Traditional systems of medicines have always played important roles in meeting global healthcare needs and will be catering the future needs. Natural products, including those from plants, animals, and minerals have been the basis of its treatment of disease. [5,6]

Therefore efforts have been made to study antioxidant potential of different commercial products which claim hepatic protection, blood purifier and anti-stress so as to identify which product can neutralize free radicals and prevent harm to liver and other vital organs, thereby reducing stress. Hence a comparative study was designed to evaluate thirteen marketed commercial formulations available in herbal category in India.

MATERIALS AND METHODS

Chemicals and reagents

Sodium EDTA, Ferric chloride, Ferrozin solution, hydrogen peroxide, sulphuric acid, potassium permanganate, DTNB reagent, glacial metaphosphoric acid, N-ethylmaleimide, reduced glutathione (GSH) were purchased from Sigma and Merck India. Other chemical and solvents were purchased from local supplier and were of highest purity grade. Milli-Q

water was used for all the assays. All commercial products (Table 1) were purchased from local market and given code name as MCF-1 to MCF-13.

Plant extracts

All the standardized extract of *Andrographis paniculata, Boerhaavia diffusa, Tinospora cordifolia* and *Vitis vinifera* seeds were procured from Moksha Lifestyle, New Delhi, India.

Metal chelating activity

Metal chelating potential of the samples was estimated as described earlier.[7,8] Briefly, 50 µl of 2 mM $FeCl_2$ was added to the sample solution (1 ml). The reactions were initiated by the addition of 0.2 ml (200 µl) of 5 mM ferrozine solution and the mixture was vigorously shaken and kept at room temperature for 10 min. The absorbance of the solution was measured at 562 nm and kept in dark for 30 minutes and the absorbance was measured.

Assay for Catalase activity

Catalase activity was determined by the titerimetric method as described earlier.[9] Briefly, 1 ml sample was mixed with 5 ml of 300 µM phosphate buffer (pH 6.8) containing 100 µM hydrogen peroxide (H_2O_2) and incubated at 25°C for 1 min. The reaction was terminated by addition of 10 ml of 2 % sulphuric acid. The residual H_2O_2 was titrated with potassium permanganate (0.01N) till pink color was obtained. Enzyme activity was estimated by calculating the decomposition of µM H_2O_2 per min per mg protein. [9,10]

Assay for reduced glutathione (GSH)

The amount of reduced glutathione in the samples was estimated by method of Boyne and Ellman. [11] 1 ml of the sample extracts were treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100 ml water). After centrifugation (rpm = 5000) for 10 minutes, 2.0 ml of the protein□free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 (5.68 g/100 ml water) and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri-sodium citrate). Absorbance was noted at 412 nm within 2 minutes. GSH concentration was expressed as nmol/mg protein.

Assay for oxidized glutathione (GSSG)

An aliquot of 0.5 ml of the supernatant was incubated at room temperature with 200 µl of 0.04 M N-ethylmaleimide (125 mg/100 ml) for 30 min to interact with the GSH present in the

supernatant. To this mixture, 4.3 ml of 0.1 N NaOH (0.4 g/100 ml water) was added. A 100 µl portion of this mixture was taken for the measurement of GSSG, 100 µl of the diluted supernatant, 1.8 ml of 0.1 N NaOH and 100 µl of O-phthaldehyde (1mg/ml). After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the OD was measured after excitation at 350 nm. [12]

Assay for glutathione peroxidase

The reaction mixture consisting of 0.8 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.2 ml of 10 mM sodium azide (65 mg/100 ml water), 0.4 ml of 4 mM reduced glutathione (GSH), 0.2 ml of 2.5 mM H_2O_2, 0.4 ml of water and 1 ml of sample was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 1 ml of 10% trichloroacetic acid (TCA) and after centrifugation (500 rpm for 10 minutes); 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein. [13]

Assay for Xanthine oxidase activity

The xanthine oxidase activity with xanthine as the sub-substrate is measured spectrophotometrically, by the method of Noro et al. (1983). The extract (500 µl of 0.1 mg/ml) and allopurinol (100 µg/ml) (in methanol) is mixed with 1.3 ml phosphate buffer (0.05 M, pH 7.5) (0.13 g monosodium + 1.09 g disodium in 100 ml water) and 0.2 ml of 0.2 units/ml xanthine oxidase solution. After 10 min of incubation at room temperature (25°C), 1.5 ml of 0.15 M xanthine substrate solution (2.28 g/100 ml water) is added to this mixture. The mixture is again incubated for 30 min at room temperature (25 °C) and then the absorbance is measured at 293 nm using a spectrophotometer against blank (0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase). The solution of 0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase and 1.5 ml xanthine substrate is used as a control. [14]

Statistical analysis

The data were subjected to a one way analysis of variance and the significance of the difference between means was determined by Duncan's multiple range tests using the SPSS. Values expressed were mean ± SD.

Table 1: Specifications of marketed commercial products

S.No.	Code	Active ingredients	No of constituents	Total amount of constituentmg/ ml or g
	MCF-1	*Rubia cordifolia, Hemidesmus indicus, Acacia catechu, Azadirachta indica, Curcuma longa, Smilax china, Tinospora cordifolia* & Honey	8	260 mg/ml
	MCF-2	*Hemidesmus indica, Terminalia chebula, Swertia chirata, Anchrographic paniculata, Rheum emodi & Cassia fistula.*	6	375 mg/tablet
	MCF-3	*Bauhinia variegata, Canscora decussata, Cassia angustifolia, Chrozophora plicata, Curcuma caesia, Cuscuta reflexa, Dalbergia sissoo, Fumaria parviflora, Ipomoea turpethum, Lavandula stoechas, Melia azadirachta, Nymphaea lotus, Ocimum canum, Pterocarpus santalinus, Rosa damascena, Smilax china, Sphaeranthus indicus, Swertia chirata, Tephrosia purpurea, Terminaliachebula & Tinospora cordifolia*	21	10 mg/ml
	MCF-4	*Curcuma longa, Cassia fistula, Psoralea corylifolia, Saussurea lappa, Picrorhiza kurroa, Azadirachta indica, Tinospora cordifolia, Crataeva magna, Triphala, Embelia ribes, Eclipta alba & Andrographis paniculata*	14	400 mg/tablet
	MCF-5	*Embilica officinalis ,Ichnocarpus fructescene, picrorhiza kurroa, Rubia cordifolia, Terminalia Chebula , Crocus sativus, Tinospora cordifolia, Eclipt alba* ,Honey, *Swertia Chirata* & Wheat Germ Oil	11	150 mg/ml
	MCF-6	*Picrorhiza kurroa, Boherria diffusa, Andrographis paniculata, Achillea millefolium & Berberis aristata*	5	450 mg/ml
	MCF-7	*Chichorium intybs, Solanum nigrum, Terminalia arjuna & Achillea millefolium*	4	25 mg/ml
	MCF-8	*Withania somnifera, Asparagus adscendens, Rubia cordifolia, Terminalia chebula, Curcuma longa, Berberis aristata, Glycyrrhiza glabra, Pluchea lanceolata, Pueraria tuberosa ,Terminalia arjuna, Cyperus rotundus, Ipomoea turpethum, Hemidesmus indicus, Cryptolepis buchanani, Petrocarpus santalinus, Santalum album,*	28	34 mg/ml

		Acorus calamus, Plumbago zeylanica, Woodfordia fruticosa, Honey, Zingiber officinale, Piper nigrum, Piper longum, Cinnamomum zeylanicum, Cinnamomum tamala, Elettaria cardamomum, Callicarpa macrophylla & *Mesua ferea*		
	MCF-9	*Withania somnifera, Valeriana wallichi* & *Centella asiatica*	3	600 mm/capsule
	MCF-10	*Withania somnifera*	1	500 mg/capsule
	MCF-11	*Withania somnifera* root extract	1	300 mg/capsule
	MCF-12	*Withania somnifera*	1	250 mg/capsule
	MCF13 (Ezenus)	*Andrographis paniculata* ext., *Boerhaavia diffusa* ext., *Tinospora cordifolia* ext. & *Vitis vinifera* seed extract	4	17 mg/g

RESULTS

Metal chelating activity

One of the mechanisms of anti-oxidative action is chelation of transition metals and thus preventing catalysis of hydro peroxide decomposition and Fenton-type reactions. It has been well established that chelating agents stabilize transition metals and reduces their availability as catalysts, to inhibit the first few free radicals and consequently suppresses lipid peroxidation. The samples under test showed concentration dependent metal chelating potential and among these (Ezenus) MCF-13 showed greatest inhibition with minimum IC_{50} at 730 ± 35 µg/ml (Fig. 1) which was 2.6 times more potent than MCF-1 where lowest chelating activity was recorded (1923 ± 115 µg/ml). MCF-2 to MCF-12 showed IC_{50} in range of 744 ± 42 µg/ml to 1216 ± 98 µg/ml.

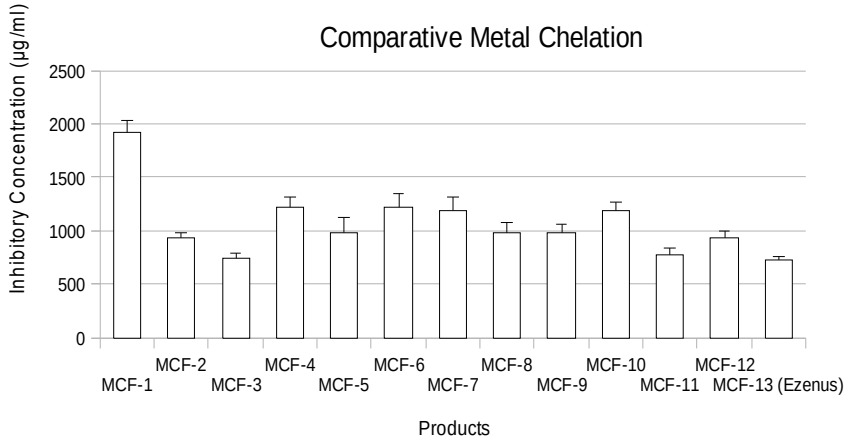

Figure 1: Metal chelating activity (IC_{50}) of MCF-13 (Ezenus) and commercial formulations MCF-1 to MCF-12.

Catalase activity

Catalase is an enzyme which converts hydrogen peroxide to water and oxygen in tissues and thus serves as an anti-oxidant. MCF-13 (Ezenus) shared highest catalytic activity (1.5±0.02 μMH_2O_2/min/mg protein) at par with MCF-3 (1.5±0.04 μMH_2O_2/min/mg protein) and MCF-11 (1.5±0.05 μMH_2O_2/min/mg protein). Rest all productsshowed very low activity in comparison to MCF-13, MCF 3 and MCF-11. MCF-5 showed (0.6±0.03 μMH_2O_2/min/mg protein), MCF-2 and MCF-12 showed same activity i.e., 0.5±0.02 μMH_2O_2/min/mg protein and 0.5±0.01μMH_2O_2/min/mg protein respectively. MCF-4 and MCF-10 showed lowest and same activity i.e., 0.4±0.03 μMH_2O_2/min/mg protein and 0.4±0.02 μMH_2O_2/min/mg protein respectively which were 3.75 times lower in activity than MCF-13 (Ezenus).

Determination of reduced glutathion

The reduced glutathion in the sample was expressed as GSH nmol/mg/protein. MCF-13 (Ezenus) showed the highest reduced glutathione content along with MCF-4 and MCF-5 i.e., 1.9±0.08 GSH nmol/mg/protein, 1.9±0.03 GSH nmol/mg/protein and 1.9±0.06 GSH nmol/mg/protein respectively followed by MCF-12 (1.8±0.09 GSH nmol/mg/protein) and MCF-10 (1.2±0.05 GSH nmol/mg/protein). MCF-1, MCF-6, MCF-7 and MCF-11 showed same activity i.e., 0.8±0.04 GSH nmol/mg/protein, 0.8±0.04 GSH nmol/mg/protein, 0.8±0.04 GSH nmol/mg/protein and 0.8±0.02 GSH nmol/mg/protein respectively. MCF-2 showed lowest reduced glutathione content (0.1±0.01 GSH nmol/mg/protein) which was 19 times less than that of MCF-13 (Ezenus).

Determination of oxidized glutathione (GSSG)

The oxidised glutathion in the sample was expressed as GSSG nmol/mg/protein. MCF-13 (Ezenus) showed highest oxidised glutathione content (2.9±0.13 GSSG nmol/mg/protein) followed by MCF-12 (2.5±0.13 GSSG nmol/mg/protein) which is 13% lower and MCF-11 (1.9±0.12 GSSG nmol/mg/protein) which is 34% lower than MCF-13.

MCF-2 and MCF-10 showed same GSSG content i.e., 1.8±0.06 GSSG nmol/mg/protein) and 1.8±0.08 GSSG nmol/mg/protein) respectively followed by MCF-5 (1.7±0.07 GSSG nmol/mg/protein). Lowest oxidised glutathione content was shown by MCF-8 (0.2±0.01 GSSG nmol/mg/protein) which was 14.5 times lower than MCF-13 (Ezenus).

Table 1: Comparative Catalase activity, reduced glutathione, oxidized glutathione and glutathione peroxidase activity

Sample	Catalase activity (µMH2O2/min/mg protein)	Reduced glutathion (GSH nmol/mg/protein)	Oxidized glutathione GSSG nmol/mg/protein	Glutathione peroxidase (nmol/min/mg protein)
MCF-1	0.2±0.03	0.8±0.04	1.0±0.02	0.8±0.04
MCF-2	0.5±0.02	0.1±0.01	1.8±0.06	0.7±0.04
MCF-3	1.5±0.04	0.7±0.03	0.8±0.03	0.9±0.03
MCF-4	0.4±0.03	1.9±0.03	1.5±0.05	0.2±0.03
MCF-5	0.6±0.03	1.9±0.06	1.7±0.07	0.8±0.05
MCF-6	0	0.8±0.04	0.8±0.02	1.0±0.06
MCF-7	0	0.8±0.04	1.5±0.03	0.2±0.03
MCF-8	0	0.2±0.02	0.2±0.01	0
MCF-9	0	0.7±0.03	0.9±0.02	0.1±0.02
MCF-10	0.4±0.02	1.2±0.05	1.8±0.08	0.7±0.03
MCF-11	1.5±0.05	0.8 ±0.02	1.9±0.12	0.8±0.05
MCF-12	0.5±0.01	1.8±0.09	2.5±0.15	0.9±0.05
MCF-13 (Ezenus)	1.5±0.02	1.9±0.08	2.9±0.13	1.1±0.07

Values are Mean± SD of three readings each.

Determination of glutathione peroxidase activity

Glutathione peroxidase activity in the sample was determined and expressed in terms of nmol/min/mg protein. The maximum activity was found to present in MCF-13 (Ezenus) (1.1±0.07 nmol/min/mg protein) followed by MCF-6 (1.0±0.06 nmol/min/mg protein), MCF-3 (0.9±0.03 nmol/min/mg protein) and MCF-12 (0.9±0.05 nmol/min/mg protein). MCF-1, MCF-5 & MCF-11 shared same activity i.e., 0.8±0.04 nmol/min/mg protein, 0.8±0.05 nmol/min/mg protein and 0.8±0.05 nmol/min/mg protein respectively. Lowest glutathione peroxidase activity was shown by MCF-9 which was found to be 11 times less as compared to MCF-13 (Ezenus).

Inhibition of Xanthine oxidase activity

Xanthine oxidase in an enzyme which catalyses the conversion of xanthine to uric acid. All the assayed sample showed more or less xanthine oxidase inhibitory activity, where maximum activity was found to be present in MCF-13 (Ezenus) and MCF-4 with minimum

IC_{50} of 110 ± 9 µg/mL and IC_{50} of 110 ± 12 µg/mL respectively, followed by MCF-11, MCF-5 and MCF-12. MCF- 2 and MCF-8 shared same activity i.e., IC_{50} of 198 ± 10 µg/mL and 198 ± 8 µg/mL respectively. MCF-10 showed lowest Xanthine oxidase inhibition (IC_{50} of 348 ± 10 µg/mL) which was found to be 3.16 times lower than that of MCF-13 (Ezenus) (Fig. 2).

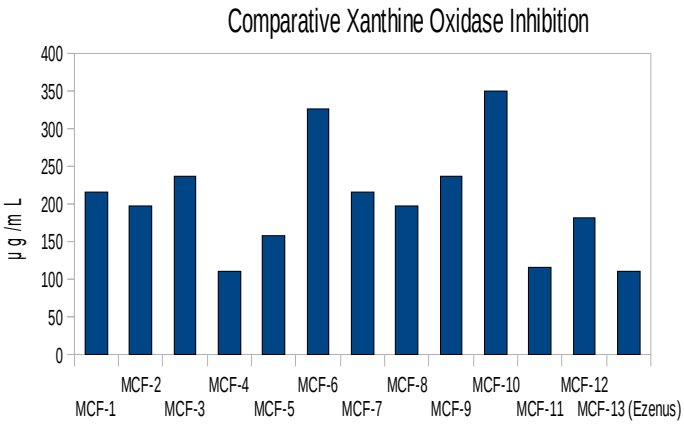

Figure 2: Xanthine oxidase inhibitory activity (IC_{50}) of MCF-13 (Ezenus) and commercial formulations MCF-1 to MCF-12.

DISCUSSION

Our body is made up of more than a trillion cells and a billion reactions occurs in our body cells every second. Oxidation is one of these reactions commonly occurring in all the cells to produce energy. Free radicals produced as a result of this oxidation reactions lead to extensive damage to cells and their components. The accumulated toxic free radicals form a vicious cycle called as *"oxidative stress"* which increases the risk of lifestyle diseases like cirrhosis, cancer, arteriosclerosis, neurological diseases and other chronic disorders. Detoxification is a process by which toxins are removed from our body and Liver is the major organ contributing towards the detoxification of general metabolites and other xenobiotics including drugs. This is generally achieved through redox reactions, conjugation and excretion of molecules from cells or tissues and these reactions are catalysed by various enzymes. Whenever there is a disturbance in this mechanisms, various disease start growing and affect the human body.[2,15]

Flavonoids interact with metal ions and form chelates. This chelation of metals is crucial to prevent free radical generation, which damage target biomolecules and cause toxicity/ stress on body organs. There is an increased demand for liver detoxifier as food additives,

nutraceutical or medicinal products to combat the oxidative stress induced harmful effects. Our result suggests that MCF-13 (Ezenus) possesses significant antioxidant and detoxifying potential.

Ezenus contains herbs such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Tinospora cordifolia* and *Vitis vinifera* which have been demonstrated to possess natural therapeutic components such as Andrographolides, Flavonoids, Ascorbic acid, Polyphenols etc. Responsible for antioxidant, immunomodulatory, detoxifying and stress relieving properties. It is also reported that additive and synergistic effects of phytochemicals in medicinal plants are responsible for their potent bioactive properties and benefit of a combination or a formulation is usually attributed to the complex mixture of phytochemicals present in formulation. This strongly suggests that no single herb can substitute or replace a combination of natural phytochemical to a certain the most potent detoxifying potential. [16,17,18]

Andographis paniculata possesses andrographolides which is reported to possess hepatoprotective and antioxidant property in BHC induced liver damage in mice,[19] ameliorate liver, kidney, heart, lung and spleen during nicotine induced oxidative stress,[20] prevent against free radical induced damages to rat liver subcellular organelles. [21] The major active constituent andrographolide induces autophagic cell death in human liver cancer cells,[22] also protect against carbon tetrachloride-induced acute liver injury in mice.[23] Phytochemicals such as alkaloid, saponin, terpenoids, coumarin, flavonoids, tannin,glycoside and steroid in *Boerhaavia diffusa* protects liver against acetaminophen-induced liver damage in rats.[24] *Tinospora cordifolia* is reported to restore antioxidant defence in in alloxan-induced diabetic liver and kidney,[25] it is also reported as hepatoprotective and immunomodulatory properties in CCl_4 intoxicated mature albino rats. [26]

Vitis vinifera seed extract contains oligomeric proanthocyanidins that show antioxidant and free radical-scavenging activities and are neuroprotective, hepatoprotective and acts as an immunomodulator and detoxifier also. Hence, MCF-13 (Ezenus) is the most potent antioxidant product as candy which acts by multiple modes to detoxify liver, body and thereby may be helpful in stress reduction.

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