Original Article

Comparison of Antioxidant Potential of Ezenus Versus Marketed Herbal Products in India: an *In-vitro* Study

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ABSTRACT

The present investigation describes the comparative evaluation of Ezenus: a polyherbal, sugar free candy with twelve commercial products with respect to antioxidant potential. Total phenolics, flavanoids and thiol contents were estimated and antioxidant potential of Ezenus and commercially available products claiming to be anti-stress and detoxifier, were evaluated. Ezenus showed highest polyphenol content of 27.34±1.95 mg Gallic Acid Equivalent/100g was significantly higher (59.35%) than MCF-12 and flavanoid content was 291.20±10.05 µg Quercetin Equivalent/100g which is 60.34% higher than that for MCF-5. Total thiol of Ezenus was 1.09±0.08 µM/ml and showed highest percentage difference of 67.89% against MCF-6. Ezenus was found to be superior in inhibiting lipid peroxidation and Melondialdehyde content detected for Ezenus was 6.04±0.15 µmols MDA/g) whereas MCF-11 showed highest MDA level of 36.60±2.08 µmols MDA/g. IC_{50} value of Ezenus was found to be 423±28 µg/ml which was 3.5 times lower than MCF-6. Ezenus showed greatest inhibition with IC_{50} of 730±35 µg/ml followed by others. Highest reducing power was exhibited by MCF-9 (IC_{50} 414±52 µg/ml) followed by Ezenus (IC_{50} 440±21 µg/ml). Ezenus, MCF-1, MCF-3, MCF-6, MCF-7 and MCF-10 showed almost similar IC_{50} of 1000 µg/ml followed by MCF-9 and others. Highest hydroxyl radical inhibition was shown by Ezenus while MCF-12 showed minimum that is 1.6 times lower. The patented formulation Ezenus depicted greatest superoxide anion scavenging activity while MCF-4 showed lowest activity in terms of IC_{50} which was 2.7 times lower than that of Ezenus. The antioxidant assays results suggest that the patented formulation Ezenus demonstrated significant and higher antioxidant potential as compared to the other commercial formulation available in Indian market under same category. The formulation is very useful in combating the oxidative stress and maintaining general well being-

stress free life.

Keywords: Ezenus, anti-oxidant, oxidative stress, polyherbal formulation.

INTRODUCTION

Oxidative stress is a condition where human physiology is unable to defend against free radical radicals, reactive oxygen species and reactive nitrogen species, which are derived either from normal metabolic processes or from external sources leading to accumulation of harmful and toxic metabolites including reactive free radical and thus affect overall wellbeing[1,2,3]. This may lead to severe vital organ/tissue damages and initiate many other physiological disorders like diabetes mellitus[4]. Traditional system of medicines like Ayurveda describes various medicinal plants which are commonly used as food supplements for nutraceutical purpose. These plants are known to contain high amounts of anti-oxidants and the extracts of various parts of these plants including active constituents have been reported to possess potent anti-oxidant in-vitro and in-vivo in various disease models[2,3]. Anti-oxidants protect body from oxidative stress and are likely to involve several mechanisms of action, including inhibition of the generation of free radicals, enhancement of the scavenging capacity against free radicals, reducing capacity and metal chelating ability[5]. Furthermore the traditional system of medicine also describes the consumption of several plants combined together in defined amount to provide synergistic effects[6,7]. The present investigation is aimed to explore a formulation containing *Andographis paniculata, Rubia cordifolia, Tinospora cordifolia, Berberis aristata* and *Boerhaavia diffusa* in very low concentrations present in a sugar free candy form, herein after termed as Ezenus. The formulation was compared for various in-vitro parameters and was found to exert better anti-oxidant potential compared with various commercial formulations (Table 1) available in Indian market.

MATERIALS AND METHODS

Chemicals and reagents

DPPH (1, 1–diphenyl–1,2–picrylhy-drazyl), TPTZ (2,4,6,-tripyridy-s-triazine), potassium ferricyanide, trichloro-acetic acid (TCA), $FeCl_3$, sodium nitroprusside, sulphanilamide, napthylethy-lenediamine dihydrochloride, TPTZ (2,4,6,-tripyridy-s-triazine), NBT (nitroblue tetra-zolium), PMS (phenazine methosulfate), sulphuric acid (H_2SO_4), ammonium molybdate, ammonium persulphate, ascorbic acid, butylated hydroxytoluene (BHT), sodium nitrite, sodium EDTA folin-ciocalteu reagent, reduced glutathione (GSH), DTNB reagent, metaphosphoric acid, N-ethylmaleimide, O-phthalaldehyde, potassium ferricyanide, trichloroacetic acid (TCA), hydrogen peroxide, thiobarbituric acid (TBA), malondialdehyde (MDA), potassium hexa-cyanoferrat $[K_3Fe(CN)_6]$, potassium permanganate, etc 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. Ezenus candies were procured from Venus Remedies Ltd. Baddi and other commercial products (Table 1) were purchased from local market and given code name as MCF-1 to MCF-12 (marketed commercial formulation:MCF).

Plant extracts

All the standardized extracts of *Andrographis paniculata, Boerhaavia diffusa, Berberis aristata, Tinospora cordifolia and Rubia cordifolia* were procured from Moksha Lifestyle, New Delhi, India.

Quantification of anti-oxidant component content

Determination of total phenolics and total flavanoids

Total phenolics in the sample were determined according to Folin-ciocalteu procedure as described earlier[2,8]. Briefly 200 µl sample was oxidized with 0.1 ml of 10% Folin-ciocalteu reagent and neutralized with 1.5 ml of 7.5 % sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm. A calibration curve was prepared, using a standard solution of gallic acid (5, 10, 15, 25, 50 and 75 ug/L). Results were ex-pressed as mg gallic acid equivalents (GAE)/g of extract.

Flavanoid content was measured by aluminium chloride colorimetric assay described earlier with some modifications[2,9]. Briefly 1 ml of sample solution was mixed with 1 ml milli-Q water and 75 µl of 5% $NaNO_2$ solution and incubated for 5 min. Then 75 µl of 10% $AlCl_3.H_20$ solution was added and kept for 5 min. At sixth minute 0.5 ml of NaOH was added. The solution was mixed well and the absorbance was read against reagent blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/ 100 g extract. All samples were analyzed in triplicates.

Determination of total thiol

Total thiol contents were determined using method described earlier[10,11]. Briefly 1 g sample was homogenized with 10 ml 0.02 M EDTA and centrifuged for 10 min (8,000 rpm at 4°C). Resultant supernatant (0.5 ml) were mixed with 1.5 ml 0.2 M Tris buffer (pH 8.2) and 0.1 ml 0.01 M DTNB, to this 7.9 ml methanol was added. The mixture was allowed to incubate at room temperature for 10 min. Absorbance of resultant mixture was measured at 412 nm.

In-vitro anti-oxidant assay

Determination of lipid peroxidation

The lipid peroxidation inhibition was measured as described aerlier[12,13]. Briefly, 0.13 ml of linoleic acid and 10 ml of ethanol (99.5%) were mixed together. The sample (1.3 mg) was dissolved in 10 ml of phosphate buffer (50 mM, pH 7.0), and then added to the mixture above and the final volume was adjusted to 25 ml with distilled water. The resultant mixture was incubated at 40 ± 1°C in the dark. The degree of linoleic acid oxidation was evaluated by measuring the ferric thiocyanate (FTC) values (12,14). Briefly, One hundred microlitres o f the resultant mixture was mixed with 4.7 ml of ethanol (75%), 0.1 ml of ammonium thiocyanate (30%), and 0.1 ml of ferrous chloride solution (20 mM) in 3.5% HCl. The thiocyanate value was determined after 3 min by reading the absorbance at 500 nm following colour development with $FeCl_2$ and thiocyanate (12).

DPPH free radical scavenging activity

The antioxidant activity of the samples was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. Sample solutions (1 ml) in a concentration range of 10-100 µg/ml were added to 3 ml of 0.004 % ethanolic DPPH free radical solution and incubated for 30 min. The absorbance of resultant solutions were measured at 517 nm by a UV spectrophotometer and compared with standard ascorbic acid to calculate percentage inhibition[15,16].

Nitric oxide radical scavenging assay

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess reaction [30]. Preparing of Standard is by taking 1.5 ml [Sodium nitroprusside (10 mM) in Phosphate buffered saline 1 M (pH 7.4)] and 1.5 ml ethanolic Sodium Nitrite (0.25-2.5 mg/ml). Incubate at 370C for 60 minutes. Add 50 µL freshly prepared Griess solution 1.5 ml [Sodium nitroprusside (10 mM) in Phosphate buffered saline 1 M (pH 7.4)] + 1.5 ml sample (1 mg/ml to 50 mg/ml) = 3 ml reaction mixture. Incubate at 37 ^{0}C for 60 minutes. Add 50 µL freshly prepared Greiss reagent. (1% sulphanilamide, 0.1% naphthyl ethylene-diamine phosphoric acid). Absorbance was taken at 546 nm[12,17].

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity is measured on the basis of the ability to reduce ferric ions as described earlier with some modifications[12,18]. Sample solutions (0.75 ml) were mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), and incubated at 50°C in a water bath for 20 min. The reaction was stopped by addition of 0.75 mL of trichloroacetic acid (TCA) solution and the resultant mixture was centrifuged (3000 rpm/min) for 10 min. The supernatent (1.5 ml) was mixed with 1.5 ml of distilled water and 0.1 mL of ferric chloride solution (0.1%, w/v) for 10 min and absorbance was measured at 700 nm. Ascorbic acid was taken as reference standard.

Scavenging of hydrogen peroxide activity

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and concentration of hydrogen peroxide was determined by absorption at 230 nm. Sample solution (1.4 ml) was added to 0.6 ml of 40 mM hydrogen peroxide solution. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution. The percentage of hydrogen peroxide scavenging by the sample was calculated as compared to control. Ascorbic acid was used as a positive control[19,20,21].

Hydroxyl radical scavenging activity

Hydroxyl free radical scavenging activities were measured as described earlier with some modifications[2,22]. Briefly, 200 µL of 2.8 mM 2-deoxy-D-ribose, 100 µL sample (200 µg/ml), 400 µL of 200 µM $FeCl_3$, 100 µL 1.00 mM EDTA (1:1 v/v), 200 µL of H_2O_2 (1.0 mM) and 200 µL of ascorbic acid (1 mM) were mixed together and incubated for 1 hour at 37°C. After the incubation 1.5 ml of 2.8% TCA was added and incubated at 100°C for 30 min. The resultant pink colour solution was cooled to room temperature and absorbance was measured at 532 nm. Appropriate blank solutions were used. Percentage of inhibitions was calculated as compared to control.

Superoxide anion scavenging activity

The reaction mixture for deter-mination of superoxide anion scavenging activity consisted of 1 ml sample solution (0.75 mg/ml), 1ml 60 µM phenazine methosulphate (PMS) in phosphate buffer (0.1M, pH 7.4) and 150 µM 1 ml nitroblue tetrazolium (NBT) in phosphate buffer. The resultant mixture was incubated at for 5 minutes, and the resultant color was measured spectrophotometrically at 560 nm. Ascorbic acid was taken as positive control (0.75 mg/ml)[2,23].

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 means SD. *p*-values <0.05 were considered to be significant.

RESULTS

Total phenolics and total flavonoids

Total phenolic and flavonoid contents of Ezenus and other commercial preparations are given in Table 1. Ezenus showed highest polyphenol content of 27.34±1.95 mg/GAE /100g, which was found to be 12.58% higher than MCF-3 and MCF-11; 17.63% higher than MCF-4; 30-40% higher than remaining groups except MCF-12 to which Ezenus was 54.97% higher. All MCF-1,2,5,6,7,8,9 showed almost similar polyphenol content. Ezenus showed highest percentage difference of 59.35% for MCF-10 (Table 2).

Highest flavonoid content was found to be present in Ezenus (291.20±10.05 µg QE/100g), which was found to be 17.39% higher than MCF-11; 20.75% higher than MCF-3. Flavonoid content of Ezenus was 25 to 50% (27.8% to 48.98%) higher than remaining groups however, the highest percentage difference of 60.34% for MCF-5 (Table 2).

Total thiol

Thiols play an important role in defence against the Reactive Oxygen Species. Thiols are the organic compounds that contain sulfhydryl groups. The total thiol content of Ezenus was found to be 1.09±0.08 µM/ml which was found to be almost equal to MCF-3 and MCF-11. Ezenus expressed 11.93% higher thiol content to MCF-7 with highest percentage difference of 67.89% against MCF-6 (Table 2). Remaining all groups had response in range of 35 to 60% lower than Ezenus.

Lipid peroxidation

Lipid peroxidation (LPO) refers to oxidative damage of lipids results in cellular damage. Malondialdehyde is an end product of the lipid peroxidation and its quantitative estimation is related with the potential of the samples to protect against lipid peroxidation. The assay results showed that the Ezenus showed minimum lipid peroxidation i.e., MDA level (6.04 ± 0.15 µmols MDA/g) whereas both MCF-11 and MCF-3 showed similar response which was found to be around 6 times higher than that of Ezenus (Table 2). MCF-2 showed a similar LPO content to Ezenus.

DPPH free radical scavenging activity

DPPH is a stable free radical that possesses a characteristic absorption maximum at 517 nm, which is diminished in presence of antioxidants which reduces to hydrazine form. Free radical scavanging is one of the known mechanism by which antioxidants inhibit lipids peroxidation. The results obtained suggest that all the samples including Ezenus reduce the radical radicals to the corresponding hydrazine but the maximum potential was observed with Ezenus. Figure 1 shows the free radical scavenging activity of samples on DPPH radical assay, which is commonly used as a basic screening method. The radical scavenging activity of Ezenus on DPPH radicals increased with increasing concentrations (10-500 µg/ml). Inhibitory concentration (IC_{50}) value of Ezenus was found to be 423±28 µg/ml which was found to be 3.5 times lower (more potent) than MCF-6 where 1481±33 µg/ml IC_{50} value was recorded. MCF-3 and MCF-11 showed a nearing trend to Ezenus.

Nitric oxide scavanging activity

It is potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. The samples under test showed concentration

dependent metal chelating potential and among these Ezenus showed greatest inhibition with minimum IC_{50} at 730 ± 35 µg/ml (Figure 2) followed by MCF-3, MCF-11 and others.

Ferric reducing antioxidant power (FRAP) assay

This assay is commonly used to evaluate the anti-oxidant activity of phenolic compounds. They usually work by donating hydrogen atom to break free radical chain reaction. Highest reducing power was exhibited by MCF-9 (IC_{50} 414 µg/ml) followed by Ezenus (IC_{50} 440 µg/ml), followed by MCF-3, MCF-2, MCF-4, MCF-5, MCF-7, MCF-10, MCF-11, MCF-8, MCF-1 and MCF-12. Minimum ferric reducing power was shown by MCF-6 (IC_{50} 1218 µg/ml) which was almost 2.8 times lower than Ezenus (Figure 3).

Scavenging of hydrogen peroxide activity

Hydrogen peroxide is produced by phagocytes and human body is also exposed to hydrogen peroxide present in environment in various forms. This hydrogen peroxide is rapidly decomposed in human body in to oxygen and water and may also produce hydroxyl radical that can initiate lipid peroxidation and cause DNA damage. Ezenus, MCF-1, MCF-3, MCF-6, MCF-7 and MCF-10 showed almost same minimum inhibitory concentration of 1000 µg/ml followed by MCF-9. MCF-4, MCF-5, MCF-8 and MCF-11 also showed similar results. (Figure 4). Lowest hydrogen peroxide scavenging activity was exhibited by MCF-2 which was found to be 5 times lower in potency as compared to Ezenus.

Hydroxyl radical scavenging activity

Hydroxyl radical radicals are reactive free radicals which initiate many chain reactions. Hydroxyl radical scavanging activity of diferent marketed formulations along with Ezenus is shown in Figure 5. Highest hydroxyl radical inhibition was shown by Ezenus, followed by MCF-3, MCF-5, MCF-2 and MCF-11. MCF-1 and MCF-6 to MCF-11 showed almost similar range of activity. MCF-12 showed lowest inhibition of hydroxyl radical which was found to be 1.6 times lower that of Ezenus.

Superoxide anion scavenging activity

Numerous biological reactions generate superoxide radical anions, which is a highly toxic species. This radical is potential precursors of highly reactive species, such as hydroxyl radical, and thus study of the scavenging of this radical is important (Kanatt, Chander, & Sharma, 2007). The scavenging activities of different samples are presented in Figure 6. The patented formulation Ezenus depicted greatest superoxide anion scavenging activity followed by MCF-3, MCF-8 and MCF-11. MCF-1 and MCF-9 showed similar activity. MCF-2, MCF-10 and MCF-12 also showed activity in the same range. MCF-4 showed the lowest activity in terms of Inhibitory concentration which was found to be 2.7 times lower than that of Ezenus (Figure 6).

DISCUSSION

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and failure of biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage[1,2,3]. This imbalance may lead to interaction of these reactive species with various substances at cellular level resulting in vital organ damage and progression of disease like atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, malignancies and many other severe to fatal diseases. Therefore removal of these ROS and free radical from the body is considered as one of the most important defence against

progression of diseases[12]. Anti-oxidants provide defence against oxidative damage to the tissues by scavenging the free radical radicals and thus also freeze the free radical chain reaction (lipid peroxidation). Plants usually contain large number of related compounds which may show synergistic effect or help to potentiate effect of bioactive compound. This is also reported and observed from the ancient literature like Ayurveda and scientific studies that a combination of different plants provides better activities than a single plant extract or a pure constituent. This is also reported that the whole extract could be more beneficial than the isolated constituents; properties of a single bioactive constituent may be changed by the other constituents present in the extract. Furthermore additive and synergistic effects of phytochemicals in 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 antioxidant can substitute or replace a combination of natural phytochemical to ascertain the most potent bio-activities. Therefore in the present investigation attempts has been made to develop a patented low calorie chewable stress reliever herbal candy using five potent plants and the formulation has been compared with twelve marketed formulations for anti-oxidant potential. The result suggests it to possess significantly higher anti-oxidant activity *in-vitro* compared with other formulations.

Andrographis paniculata is one of the major constituent of Ezenus and it is well documented for its anti-oxidant and hepotoprotective activities. *A. paniculata* extract has been reported to be beneficial in lipid peroxidation[24], protect free radical induced damages to rat liver sub cellular organelles[25], ameliorate nicotine induced oxidative stress on liver and other vital body organs[26], protects nicotine induced superoxide mediated DNA damage[27], and many other biological activity. It is also reported to possess significant antioxidant activity in different in-vitro and in-vivo animal models[28,29,30]. These activities are mainly attributed to the major active constituent andographolide.

Rubia cordifolia Linn. (Rubiaceae) is a plant known as Indian Madder or Manjistha and is traditionally known to be rich in antioxidants. It is reported to protect against lead nitrate-induced immune response impairment and kidney oxidative damage[36], decrease ROS level and activate antioxidant gene expression[37], provides radiation protection[38], showed nitric oxide scavenging activity *in vitro* and ex vivo[39], prevents free radical generation and lipid peroxidation during oxygen-glucose deprivation in rat hippocampal slices[40], also exerted neuroprotection by modulating the antioxidant system in rat hippocampal slices[40], along with other important pharmacological activities. Constituents isolated from *R. cordifolia* also showed potent anti-oxidant activities.

Tinospora cordifolia commonly named as "Guduchi" is known for anti-oxidant[41], amelioration from oxidative stress[42], free radical scavenging[43], inhibits lipid peroxidation in mice[44], restore anti-oxidants in alloxan-induced diabetic Wistar rats[45], free radical generation and lipid peroxidation during oxygen-glucose depri-vation in rat hippocampal slices[46], nitric oxide scavenging activity[47], exert neuroprotection by modulating the antioxidant system in rat hippocampal slices[48], protects provides protection against radiation by free radical scavenging and metal chelation[38]. It also constitutes an important part of various formulations used as immunomodulatory[49]. Also isolated constituents show various health beneficial activities including antioxidant.

Berberis aristata (family Berberi-daceae), is an ayurvedic herb used since ancient times. It is also known as Indian berberi, Daruhaldi, and Chitra. The plant is reported as potent anti-oxidant besides other activities[50].

Boerhaavia diffusa is a traditional herb extensively used in the Ayurveda to modulate oxidative stress[51], showed anti-oxidant potetial[52], plant root ameliorates ethylene glycol-induced hyperoxaluric oxidative stress and renal injury in rat kidney[53], protect against gamma radiation induced damage in mice[54], demonstrated nitric oxide scavenging activity[55] and free radicals scavenging enzymes were found to be present the plant[56]. The plant is reported to contain flavanoids and other constituents which are well known for their potential to combat oxidative stress.

Some of these herbs are also present in other formulations but the synergy which is expressed by Ezenus is not expressed by any other herb. Although the response to total thiol and DPPH free radical scavenging activity parameters were similar to MCF-3 and MCF-11, yet these formulations lag behind in other parameters to Ezenus. Surprizingly, formulation MCF-10, 11 and 12 have the same composition, yet they express different results. *Withania somnifera*, a well know anti-stress and immunomodulator, is the major constituet of MCF-9 to MCF-12 but they fail to compete the Ezenus probably due to synergistic effect of polyphenolic constituets of ingredients used in Ezenus.

CONCLUSION

Antioxidant supplements are required as a part of dietry deficiency. Our in-vitro findings indicate that the Ezenus has great potential to combat oxidative stress and will be helpful in maintaining good health and wellbeing. Our study also suggests it to have greater potential than other commercial formulations available in Indian market under similar category. Finally it can be concluded that consumption of Ezenus is good for health and to combat oxidative stress.

REFERENCES

1. Mayakrishnan V, Veluswamy S, Sundaram KS, Kannappan P, Abdullah N. Free radical scavenging potential of *Lagenaria siceraria* (Molina) Standl fruits extract. *Asian Pac J Trop Med* 2013; 6: 20-26
2. Senthilkumar R, Parimelazhagan T, Chaurasia OP, Srivastava RB. Free radical scavenging property and antiproliferative activity of *Rhodiola imbricata* Edgew extracts in HT-29 human colon cancer cells. *Asian Pac J Trop Med* 2013; 6: 11-19
3. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. *Curr Neuropharmacol* 2009; 7: 65–74
4. Ahmad S, Beg ZH. Alleviation of plasma, erythrocyte and liver lipidemic-oxidative stress by thymoquinone and limonene in atherogenic suspension fed rats. *J Func Foods* 2013; 5: 251–259
5. Zhuang H, Tang N, Yuan Y. Purification and identification of antioxidant peptides from corn gluten meal. *J Func Foods* 2013; http://dx.doi.org/10.1016/j.jff.2013.08.013
6. Ahmad W, Zaidi SM, Mujeeb M, Ansari SH, Ahmad S.HPLC and HPTLC Methods by Design for Quantitative Characterization and *in vitro* Anti-oxidant Activity of Polyherbal Formulation Containing *Rheum emodi*. *J Chromatogr Sci* 2013; doi: 10.1093/ chromsci/bmt123
7. Bae WJ, Ha US, Kim S, Kim SJ, Hong SH, Lee JY, Hwang TK, Hwang SY, Kim HJ, Kim SW.Reduction of oxidative stress may play a role in the anti-inflammatory effect of the novel herbal formulation in a rat model of hydrochloric acid-induced cystitis. Neurourol Urodyn 2013; doi: 10.1002/ nau.22507.
8. Siddhuraju P, Becker K. Antioxidant properties of various solvent extracts of total phenolic constituents from three different

agroclimatic orgins of Drumstick tree (*Moringa olifera* Lam.) leaves. *J Agric Food Chem* 2003; 51: 2144-2155.
9. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on super oxide radicals. *Food Chem* 1999; 64: 555-559.
10. Namdjyon S, Namdjyon S, Kermanian H. Induction of phytochelatin and responses of antioxidants under cadmium stress in safflower (*Carthamus tinctorius*) seedlings. *Turk J Bot* 2012; 36: 495-502
11. Singh SK, Prakash V. Screening of Antioxidant activity and phytochemicals strength of some herbal plants. *Int J Pharm pharm Sci* 2013; 5: 296-300.
12. Yazdi MM, Mahaki H, Zardini HZ. Antioxidant activity of protein hydrolysates and purified peptides from *Zizyphus jujuba* fruits. *J Func Foods* 2013; 5: 62-70.
13. Osawa T, Namiki M. Natural antioxidants isolated from Eucalyptus leaf waxes. J. Agric. Food Chem 1985; 33: 777–780.
14. Chandrasekara A, Shahidi F. Antioxidant phenolics of millet control lipid peroxidation in human LDL cholesterol and food systems. *Journal of the American Oil Chemists' Society* 2012; 89: 275–285.
15. Zhang T, Li Y, Miao M, Jiang B. Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium* L.) protein hydrolysates. *Food Chem* 2011; 128: 28–33.
16. Zhuang H, Tang N, Yuan Y. Purification and identification of antioxidant peptides from corn gluten meal. *J Func Foods* 2013; doi.org/10.1016/j.jff.2013.08.013
17. Jagetia GC, Rao SK, Baliga MS, S Babu K. The evaluation of nitric oxide scavenging activity of certain herbal formulations *in vitro*: a preliminary study. *Phytother Res.* 2004;18: 561-565.
18. Ahmadi F, Kadivar M, Shahedi M. Antioxidant activity of *Kelussia odoratissima* Mozaff. in model and food systems. Food Chem 2007; 105: 57-64.
19. Kumar RS, Rajkapoor B, Perumal P. Antioxidant activities of *Indigofera cassioides* Rottl. Ex. DC. using various *in vitro* assay models. *Asian Pac J Trop Biomed* 2012; 2: 256–261
20. Kumarappan CT, Thilagam E, Mandal SC. Antioxidant activity of polyphenolic extracts of *Ichnocarpus frutescens*. *Saudi J Biol Sci* 2012; 19: 349–355
21. Srinivasan R, Chandrasekar MJ, Nanjan MJ, Suresh B. Antioxidant activity of *Caesalpinia digyna* root. *J Ethnopharmacol* 2007; 113: 284-91
22. Klein SM, Cohen G, Cederbaum AI. Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical-generating systems. Biochemistry 1981; 20: 6006–6012
23. Beauchamp C, Fridovich I.Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; 44: 276-287.
24. Wang HW, Zhao HY, Xiang SQ. Effects of Andrographis paniculata component on nitric oxide, endothelin and lipid peroxidation in experimental atherosclerotic rabbits. Zhongguo Zhong Xi Yi Jie He Za Zhi 1997; 17: 547-549.
25. Tripathi R, Kamat JP. Free radical induced damages to rat liver subcellular organelles: inhibition by *Andrographis paniculata* extract. Indian J Exp Biol 2007; 45: 959-967.
26. Neogy S, Das S, Mahapatra SK, Mandal N, Roy S. Amelioratory effect of *Andrographis paniculata* Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. *Environ Toxicol Pharmacol*. 2008; 25: 321-328.
27. Chao WW, Kuo YH, Li WC, Lin BF. The production of nitric oxide and prostaglandin E2 in peritoneal macrophages is inhibited by *Andrographis paniculata*, *Angelica sinensis* and *Morus alba* ethyl acetate fractions. *J Ethnopharmacol*. 2009; 122: 68-75.
28. Lin FL, Wu SJ, Lee SC, Ng LT. Antioxidant, antioedema and analgesic activities of *Andrographis paniculata* extracts and their active constituent andrographolide. *Phytother Res*. 2009; 23: 958-964.
29. Dandu AM, Inamdar NM. Evaluation of beneficial effects of antioxidant properties of aqueous leaf extract of *Andrographis paniculata* in STZ-induced diabetes. *Pak J Pharm Sci*. 2009; 22:49-52.

30. Akowuah GA, Zhari I, Mariam A, Yam MF. Absorption of andrographolides from *Andrographis paniculata* and its effect on CCl(4)-induced oxidative stress in rats. *Food Chem Toxicol*. 2009; 47: 2321-2326.
31. Guan SP, Tee W, Ng DS, Chan TK, Peh HY, Ho WE, Cheng C, Mak JC, Wong WS. Andrographolide protects against cigarette smoke-induced oxidative lung injury via augmentation of Nrf2 activity. *Br J Pharmacol*. 2013; 168:1707-1718.
32. Krithika R, Verma RJ, Shrivastav PS. Antioxidative and cytoprotective effects of andrographolide against CCl4-induced hepatotoxicity in HepG2 cells. *Hum Exp Toxicol*. 2013; 32: 530-543.
33. Radhika P, Annapurna A, Rao SN. Immunostimulant, cerebroprotective & nootropic activities of *Andrographis paniculata* leaves extract in normal & amp; type 2 diabetic rats. *Indian J Med Res*. 2012;135: 636-641.
34. Woo AY, Waye MM, Tsui SK, Yeung ST, Cheng CH. Andrographolide up-regulates cellular-reduced glutathione level and protects cardiomyocytes against hypoxia/ reoxygenation injury. *J Pharmacol Exp Ther*. 2008; 325: 226-235.
35. Kamdem RE, Sang S, Ho CT. Mechanism of the superoxide scavenging activity of neoandrographolide - a natural product from Andrographis paniculata Nees. *J Agric Food Chem*. 2002; 50: 4662-4665.
36. Lodi S, Sharma V, Kansal L. The protective effect of *Rubia cordifolia* against lead nitrate-induced immune response impairment and kidney oxidative damage. *Indian J Pharmacol*. 2011; 43: 441-444.
37. Shkryl YN, Veremeichik GN, Bulgakov VP, Gorpenchenko TY, Aminin DL, Zhuravlev YN. Decreased ROS level and activation of antioxidant gene expression in Agrobacterium rhizogenes pRiA4-trans-formed calli of Rubia cordifolia. *Planta*. 2010; 232: 1023-1032.
38. Tripathi YB, Singh AV. Role of *Rubia cordifolia* Linn. in radiation protection. *Indian J Exp Biol*. 2007; 45: 620-625.
39. Basu S, Hazra B. Evaluation of nitric oxide scavenging activity, *in vitro* and ex vivo, of selected medicinal plants traditionally used in inflammatory diseases. *Phytother Res*. 2006; 20: 896-900.
40. Rawal A, Muddeshwar M, Biswas S. Effect of *Rubia cordifolia*, *Fagonia cretica* linn, and *Tinospora cordifolia* on free radical generation and lipid peroxidation during oxygen-glucose deprivation in rat hippocampal slices. *Biochem Biophys Res Commun*. 2004; 324: 588-596.
41. Sarala M, Velu V, Anandharamakrishnan C, Singh RP. Spray drying of *Tinospora cordifolia* leaf and stem extract and evaluation of antioxidant activity. *J Food Sci Technol*. 2012; 49: 119-122.
42. Sarkar R, Mandal N. Hydroalcoholic extracts of Indian medicinal plants can help in amelioration from oxidative stress through antioxidant properties. *J Complement Integr Med*. 2012; 9: 7.
43. Gacche RN, Dhole NA. Profile of aldose reductase inhibition, anti-cataract and free radical scavenging activity of selected medicinal plants: an attempt to standardize the botanicals for amelioration of diabetes complications. *Food Chem Toxicol*. 2011; 49: 1806-1813.
44. Singh RP, Banerjee S, Kumar PV, Raveesha KA, Rao AR. *Tinospora cordifolia* induces enzymes of carcinogen/drug metabolism and antioxidant system, and inhibits lipid peroxidation in mice. *Phytomedicine*. 2006; 13: 74-84.
45. Prince PS, Kamalakkannan N, Menon VP. Restoration of antioxidants by ethanolic *Tinospora cordifolia* in alloxan-induced diabetic Wistar rats. *Acta Pol Pharm*. 2004; 61: 283-287.
46. Rawal A, Muddeshwar M, Biswas S. Effect of *Rubia cordifolia*, *Fagonia cretica* linn, and *Tinospora cordifolia* on free radical generation and lipid peroxidation during oxygen-glucose deprivation in rat hippocampal slices. *Biochem Biophys Res Commun*. 2004; 324: 588-596.
47. Jagetia GC, Baliga MS. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants *in vitro*: a preliminary study. *J Med Food*. 2004; 7: 343-348.
48. Rawal AK, Muddeshwar MG, Biswas SK. Rubia cordifolia, Fagonia cretica linn and

Tinospora cordifolia exert neuroprotection by modulating the antioxidant system in rat hippocampal slices subjected to oxygen glucose deprivation. *BMC Complement Altern Med.* 2004; 4: 11.

49. Goel HC, Prem Kumar I, Rana SV. Free radical scavenging and metal chelation by *Tinospora cordifolia*, a possible role in radioprotection. *Indian J Exp Biol*. 2002; 40: 727-734.
50. Singh J, Kakkar P. Antihyperglycemic and antioxidant effect of *Berberis aristata* root extract and its role in regulating carbohydrate metabolism in diabetic rats. *J Ethno-pharmacol*. 2009; 123: 22-26.
51. Prathapan A, Vineetha VP, Abhilash PA, Raghu KG. Boerhaavia diffusa L. attenuates angiotensin II-induced hypertrophy in H9c2 cardiac myoblast cells via modulating oxidative stress and down-regulating NF-κβ and transforming growth factor β1. *Br J Nutr*. 2013; 110: 1201-1210.
52. Gulati V, Harding IH, Palombo EA. Enzyme inhibitory and antioxidant activities of traditional medicinal plants: potential application in the management of hyperglycemia. *BMC Complement Altern Med.* 2012; 12: 77
53. Pareta SK, Patra KC, Mazumder PM, Sasmal D. Aqueous extract of B*oerhaavia diffusa* root ameliorates ethylene glycol-induced hyperoxaluric oxidative stress and renal injury in rat kidney. *Pharm Biol*. 2011; 49: 1224-1233.
54. Manu KA, Leyon PV, Kuttan G. Studies on the protective effects of B*oerhaavia diffusa* L. against gamma radiation induced damage in mice. *Integr Cancer Ther*. 200; 6: 381-388.
55. Jagetia GC, Baliga MS. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants *in vitro*: a preliminary study. *J Med Food*. 2004; 7: 343-348.
56. Mandal M, Mukherji S. A study on the activities of a few free radicals scavenging enzymes present in five roadside plants. *J Environ-Biol.*2001;22:301-305.

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Table 1. Specifications of marketed commercial products

S.No.	Code	Active ingredients	No of constituents	Total amount of constituentmg/ml or g
1.	MCF-1	*Rubia cordifolia, Hemidesmus indicus, Acacia catechu, Azadirachta indica, Curcuma longa, Smilax china, Tinospora cordifolia & Honey*	8	260 mg/ml
2.	MCF-2	*Hemidesmus indica, Terminalia chebula, Swertia chirata, Anchrographic paniculata, Rheum emodi & Cassia fistula.*	6	375 mg/tablet
3.	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
4.	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
5.	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
6.	MCF-6	*Picrorhiza kurroa, Boherria diffusa, Andrographis paniculata, Achillea millefolium & Berberis aristata*	5	450 mg/ml
7.	MCF-7	*Chichorium intybs, Solanum nigrum, Terminalia arjuna & Achillea millefolium*	4	25 mg/ml
8.	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, Acorus calamus, Plumbago zeylanica, Woodfordia fruticosa, Honey, Zingiber officinale, Piper nigrum, Piper longum, Cinnamomum zeylanicum, Cinnamomum tamala, Elettaria cardamomum, Callicarpa macrophylla & Mesua ferea*	28	34 mg/ml
9.	MCF-9	*Withania somnifera, Valeriana wallichi & Centella asiatica*	3	600 mm/capsule
10.	MCF-10	*Withania somnifera*	1	500 mg/capsule
11.	MCF-11	*Withania somnifera root extract*	1	300 mg/capsule
12.	MCF-12	*Withania somnifera*	1	250 mg/capsule
13.	Ezenus	*Andrographis paniculata ext., Boerhaavia diffusa ext., Berberis aristata ext., Tinospora cordifolia ext.& Rubia cordifolia ext.*	5	10 mg/g

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Table 2. Comparative parameters of Ezenus and other commercial formulations for anti-oxidative potential

Sample	Total phenolics (mg GAE/100g)	Total Flavonoids (µg QE/100g)	Total thiols (µM/ml)	LPO (µmols MDA/g)
Ezenus	27.34 ± 1.95	291.20 ± 10.05	1.09 ± 0.08	6.04 ± 0.15
MCF-1	17.15 ± 0.25	155.65 ± 9.15	0.51 ± 0.07	16.5 ± 2.09
MCF-2	15.39 ± 1.08	197.89 ± 10.18	0.7 ± 0.02	7.87 ± 1.21
MCF-3	23.90 ± 2.54	230.78 ± 12.48	1.07 ± 0.1	33.6 ± 3.25
MCF-4	22.52 ± 1.48	210.25 ± 13.17	0.70 ± 0.04	12.7 ± 1.48
MCF-5	18.64 ± 0.94	115.48 ± 8.47	0.47 ± 0.02	18.1 ± 2.74
MCF-6	16.32 ± 1.05	148.56 ± 9.83	0.35 ± 0.01	15.6 ± 3.85
MCF-7	16.45 ± 1.15	165.23 ± 5.65	0.96 ± 0.03	12.7 ± 1.50
MCF-8	17.50 ± 1.90	148.57 ± 3.75	0.43 ± 0.07	19.4 ± 2.81
MCF-9	16.88 ± 1.12	210.59 ± 12.68	0.61 ± 0.05	19.16 ± 3.04
MCF-10	11.12 ± 0.54	203.45 ± 10.46	0.64 ± 0.01	16.5 ± 2.38
MCF-11	23.90 ± 1.61	240.56 ± 13.04	1.04 ± 0.10	36.6 ± 2.08
MCF-12	12.31 ± 0.84	195.38 ± 6.78	0.58 ± 0.07	20.7 ± 4.00

Values are Mean± SD of three readings each.

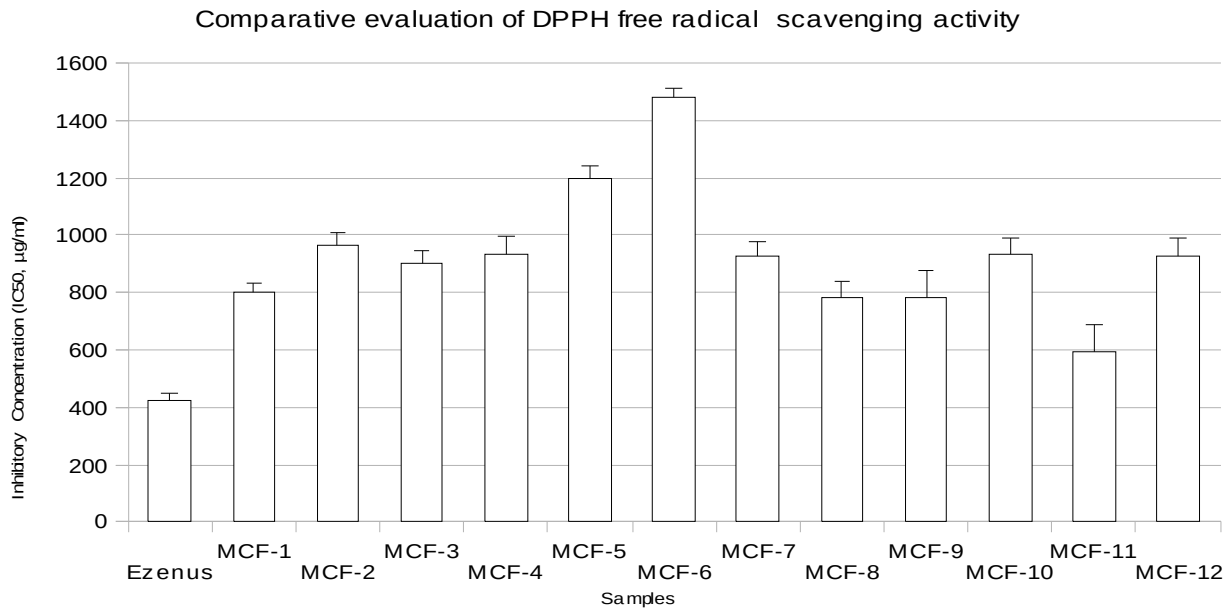

Figure 1. DPPH radical scavenging activity: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12

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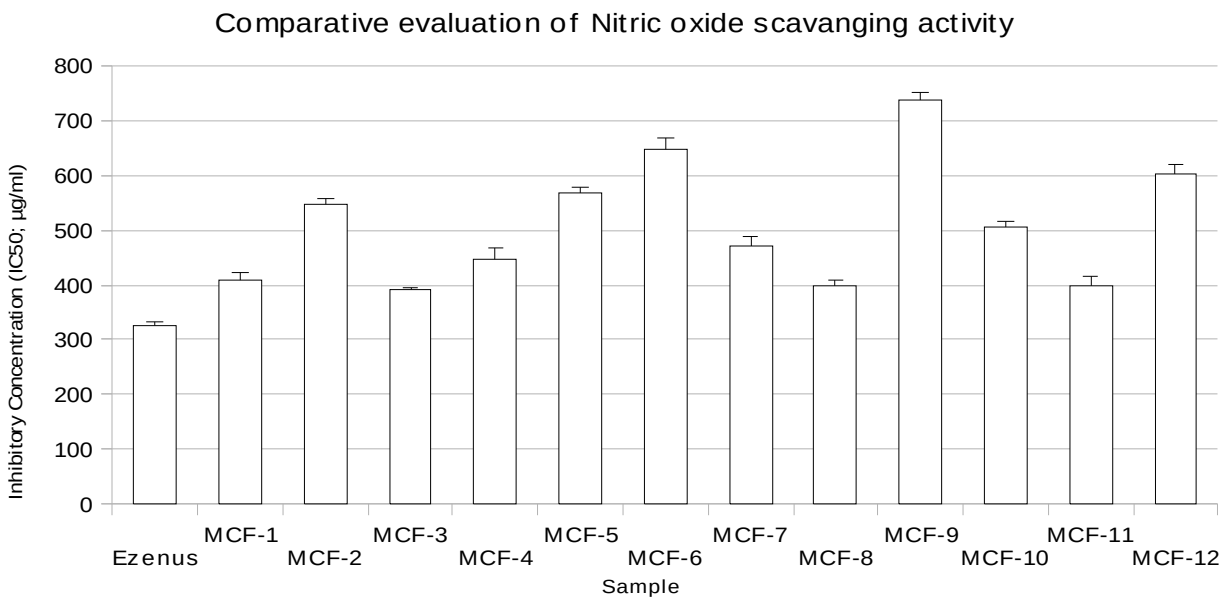

Figure 2. Nitric oxide scavanging activity: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12

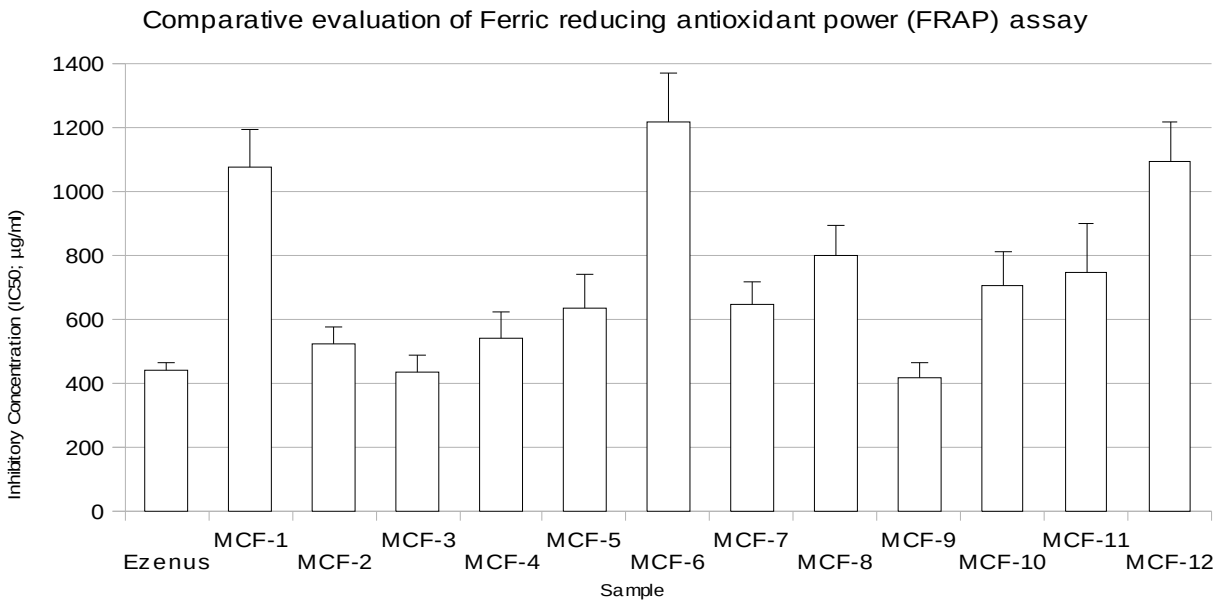

Figure 3. Ferric reducing antioxidant power (FRAP) assay: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12

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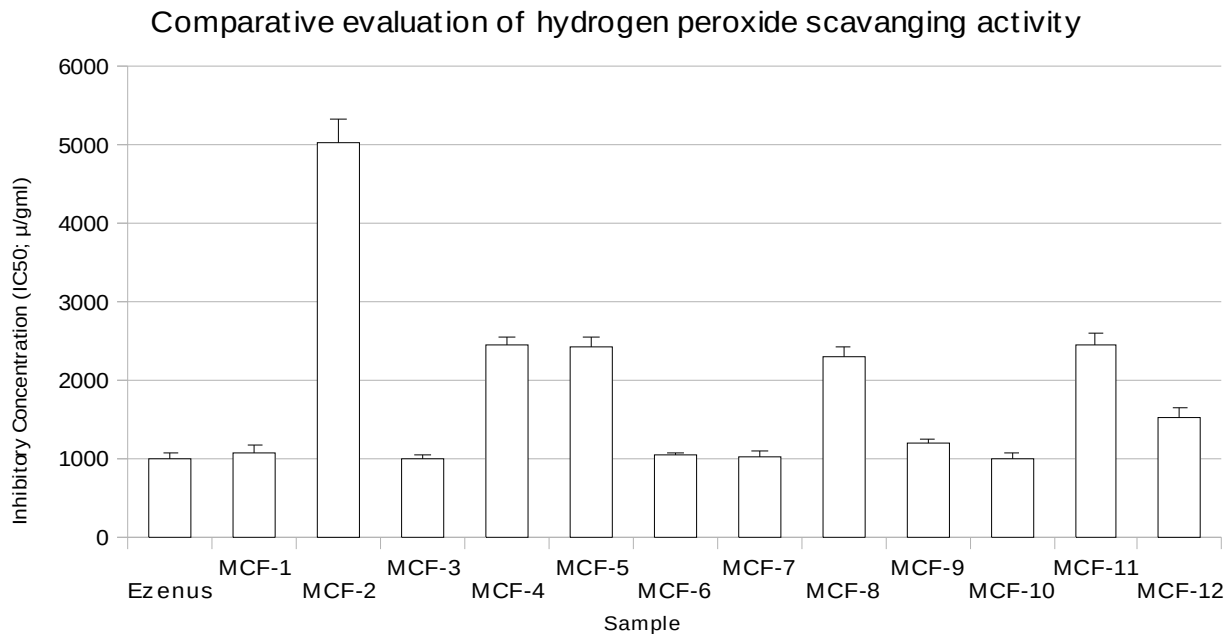

Figure 4. Scvenging of hydrogen peroxide activity: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12

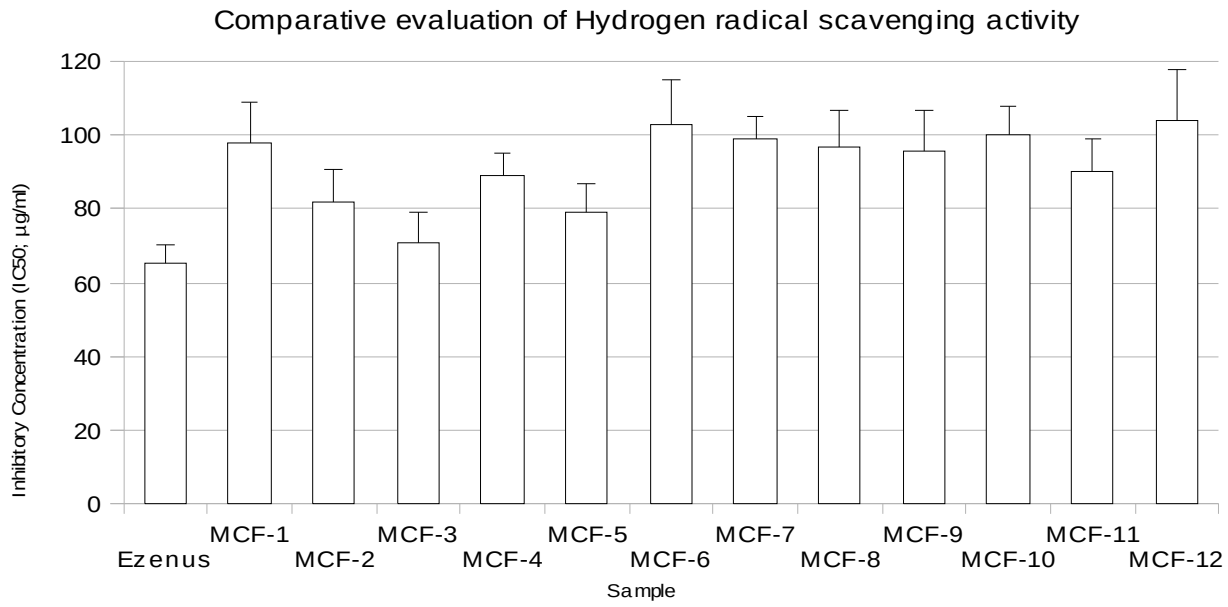

Figure 5. Hydrogen radical scavenging activity: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12

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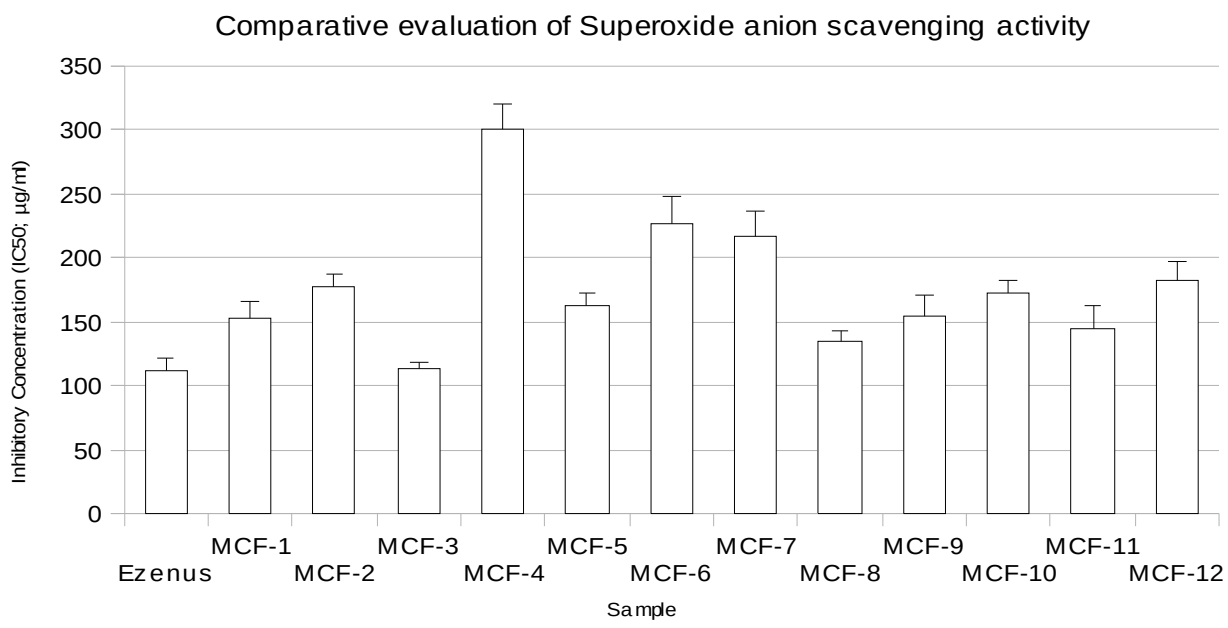

Figure 6. Superoxide anion scavenging activity: IC_{50} of Ezenus and commercial products MCF-1 to MCF-12