Quest Journals
Journal of Research in Pharmaceutical Science
Volume 8 ~ Issue 9 (2022) pp: 37-43
ISSN(Online) : 2347-2995
www.questjournals.org

Research Paper

Antioxidant Activity of Aqueous Extracts of Some Plants Estimated by Noninvasive Methods

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ABSTRACT: *Oxidative stress has been involved in several diseases includes cancer, atherosclerosis and neurodegenerative diseases. The aim of this study was to screen aqueous extracts of some plants to potent antioxidant activity in-vitro in order to find possible sources for future novel antioxidants in food and pharmaceutical formulations. Aqueous extracts of Cyperus esculentus, Punica granatum, Zingiber officinale and Embeliaribes were screened for possible antioxidant activities by scavenge free radicals and lipid peroxidation inhibiting activity. It was observed that radical scavenging activities were positively correlated to the concentration of the extract. The results suggested that antioxidant activity could be due to polyphenols, but mainly by different molecules or substances present in the extracts. Based on their IC_{50} % values, Zingiber officinale was found to be the potent among other aqueous extracts of plants in scavenging superoxide radicals, hydroxyl radical, lipid peroxidation inhibiting activity and nitric oxide scavenging effect comparing with ascorbic acid.*

KEYWORDS: *Antioxidant Activity, Plant Extracts, Natural Antioxidants, Lipid Peroxidation*

R*eceived 12 Sep, 2022;* ***R****evised 26 Sep., 2022;* ***A****ccepted 28 Sep., 2022 © The author(s) 2022.*
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I. INTRODUCTION

Ayurveda, the Indian system of medicine is gaining greater attention and popularity in many parts of the world. In recent times, focus on plant research has increased all over the world. There has been phenomenal rise in the interest of scientific community to explore the pharmacological actions of herbs and to confirm the claims made about them in official books of Ayurveda. The use of food supplements like vitamins, minerals, amino acids and natural products like beta-carotene, quercetin, herbs, phytonurtrients and probiotics collectively called as nutraceuticals has been increased[1]. Recent interest in phenolic compounds in general and flavonoids in particular, has increased greatly owing to their antioxidant capacity and their possible beneficial implications in human health. These include the treatment and prevention of cancer, cardiovascular disease and other pathological disorders. Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation[2].*Cyperus esculentusLinn* commonly known as Chufa (Cyperaceae), in Ayurveda it is used as digestive tonic and nervine tonic. Tiger nut was reported as healthy and it helps in preventing heart attacks, thrombosis and activates blood circulation. It was also found to assist in reducing the risk of colon cancer. The nut is rich in energy content, mineral and vitamins E and C[3]. The plant has screened for anti-inflammatory, antiarthritic, analgesic, anticonvulsant, in-vitro anti sickling property and antibacterial activity[4] and [5]. The tubers of *Cyperus esculentus Linn* have contain the various phytochemical constituents such as alkaloids, flavonoids, phenolics, tannins, glycosides and terpenoids[6].

The Pomegranate (*Punica granatum)* fruit is an ancient and rich source of bioactive compounds and also it has been used in folkcore medicine for centuries. Pomegranate bark and the rind of the fruit contain tannin and the root contains punico-tannic acid, mannite, sugar, gum, pectin and an active liquid alkaloid (pelletierine) and oil liquid (isopelletierine) and rich in polyphenols[7]. In Ayurveda it is commonly used as a febrifuge, during malaria and seasonal fevers, and in all bilious complaints as a cooling drink to ameliorate the action of bile. The plant has reported for various pharmacological activities includes, Antioxidant, Anti-ulcerogenic, Antidiarrhoeal, Antibacterial and Antifungal, Antimutagenic, Preventive effects on Alzheimer's

disease, Anti-erectile dysfunction, Improved learning, Anti-atherosclerotic, Anti-hypertensive, Inhibits prostatecancer cell growth[8], [9], [10] and [11]. *Punica granatum* has been used in folkcore medicine for centuries,the basic phenols of pomegranate fruit are gallic acid,chlorogenic acid, caffeic acid, coumaric acid and catechin in *Punica granatum*. *Zingiber officinale* Roscoe (Zingiberaceae), rhizomes have been employed as common condiment for various foods and beverages, and in folk medicine as a carminative, a diaphoretic, antispasmodic against intestinal colic and as an anti-emetic[12]. The ginger is used for the various actions in Ayurveda and Unani includes, Aromatic, carminative, stimulant to the gastrointestinal tract, stomachic, aphrodiasiac, sedative of pains, strengthens memory, remove obstruction in the vessels and used in nervous diseases. Ginger contains a number of pungent constituents and active ingredients sesquiterpene hydrocarbons, bisapolene, zingiberene, zingiberol, sesquiphellandrene, curcurmene. The lipophilic rhizome extracts, have yielded potentially active phenolic compound; gingerols[7].The *Zingiber officinale* has been used in folk medicinecontains biologically active constituents that include flavonoids, volatile oils which contain monoterpenes, sesquiterpenes and sesquiterpene alcohol zingiberol, gingerol and shagoals.

Embeliaribes is being used in Indian traditional herbal medicine for the treatment of mental disorders and as brain tonic. Embelin is active constituent of *Embeliaribes* [7] and[13].The ripe fruits of *Embeliaribes* are the most commercially important part of the plant; as they have been found to contain the active compound namely Embelin. Further phytochemical investigation resulted in three new compounds namely embelinol, embeliaribyl ester and embeliol[4].The present study was carried out to find out their antioxidant activity by *in-vitro* against superoxide radical scavenging, hydroxy radical scavenging, lipid peroxidation inhibition and nitric oxide scavenging activity of the aqueous extract of tubers of *Cyperus esculentus* L, fruit of *Punica Granatum* Linn, rhizomes of *Zingiber officinale* Roscoe and fruits of *Embeliaribes* Burm.

II. MATERIALS AND METHODS

Aqueous extracts of *Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* were obtained as a gift samples from M/s Laila Implex, Vijayawada and Kisalaya Herbals Ltd. Ratlam Koti, Indore, 452001. Ethylenediaminetetraacetic acid (EDTA), sodium cyanide(NaCN), nitro blue tetrazolium, deoxy-D-ribose, ferric chloride, sodium dodecyl sulphate, ascorbic acid, Tris HCl buffer were purchased from Sigma Chemicals Co., U.S.A. Acetonitrile (HPLC grade) was obtained from Qualigens, methanol (HPLC grade) from SRL, hydrochloric acid, ortho-phosphoric acid, potassium dihydrogen phosphate all from Merck. All other chemicals and reagents used were of analytical grade.

2.1 Preparation of extract and preliminary phytochemical screening:

*Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* dry extractwere obtained from the Laila Implex Laboratory (batch number: L242134, product code: C/AU/PILO-03). Aqueous extract was qualitatively analyzed for the presence of various phytochemical constituents. The aqueous extract of the *Punica granatum* was tested for the presence of carbohydrates, proteins, alkaloids, flavonoids, glycosides, saponins, tannins, and essential oils using the standard procedures [14].

2.2 Superoxide radical scavenging *in-vitro* antioxidant activity

Super oxide scavenging activity of the extracts was determined by the McCord and Fridovich method which looks at light induced superoxide generation by riboflavin and the corresponding reduction of nitro blue tetrazolium [15]. 100 mg of each of *Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* dry extract and 100 mg ascorbic acid were weighed accurately and dissolved in 100 ml of distilled water to give standard stock solutions of 1 mg/ml of the extract and ascorbic acid. From these stock solutions various dilutions of the extract as 50 µg, 100 µg, 150 µg, 200 µg and 300 µg were made with phosphate buffer. 0.05 ml of riboflavin (2 µM), 0.3 ml of extract and ascorbic acid of different dilutions of the extract were added. 0.2 ml of EDTA (6µM containing 3µg NaCN) and 0.1 ml of nitro blue tetrazolium (NBT 50 µM) were added to the above mixture to obtained the volume 3 ml with phosphate buffer (58 mM, pH 7.8). The tubes were uniformly illuminated for 15 min and thereafter optical density was measured at 560 nm. The percentage inhibition by the extracts of superoxide production was evaluated by comparing the absorbance values of the control to the experimental tubes.The percentage inhibited by the extracts of superoxide production was calculated by using the formula;

$$\text{Percentage inhibition} = \frac{\text{Average control O.D} - \text{Test sample O.D}}{\text{Average control O.D}} \times 100$$

The optical density obtained with each concentration of the extracts and the ascorbic acid was plotted on a graph taking concentrations on X-axis and percentage (%) inhibition on Y-axis. The graph was extrapolated to find the concentration needed for 50% inhibition.

2.3 Hydroxyl radical scavenging Activity for *in-vitro* antioxidant

Hydroxyl radical scavenging activity was competitive measured between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radical attacks deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances (TBARS)[16]. 100 mg each of *Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* dry extract and 100 mg ascorbic acid were weighed accurately and dissolved in 100 ml of distilled water to give standard stock solutions of 1 mg/ml of the extract and ascorbic acid. From these stock solutions various dilutions of the extract such as 50 µg, 100 µg, 150 µg, 200 µg and 300 µg were made with phosphate buffer. 0.2 ml of deoxyribose (2.8mM), 0.2 ml of each of ferric chloride (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), were added. To the above mixture 0.2ml of different dilutions of extract and ascorbic acid and 1.2 ml of phosphate buffer (20 mM, pH 7.4) were added and the tubes were kept in the incubator at 37°c for 1 h. The reaction mixture (1ml) was treated with sodium dodecyl sulfate (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8%) and acetic acid (1.5 ml, 20%, pH 3.5). The total volume was then made up to 5 ml by adding distilled water and kept in oil bath at 100°C for 1h. The percentage inhibition of the extracts on hydroxyl radicals was determined by comparing the absorbance values of the control, and experimental tubes as in the case of superoxide assay.

2.4 Lipid peroxidation inhibiting activity by induction by Fe^{2+}/ascorbate system

Inhibition of lipid peroxidation was determined by the thiobarbituric acid method[17]. 100 mg each of *Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* dry extract and 100 mg ascorbic acid were weighed accurately and dissolved in 100 ml of distilled water to give standard stock solutions of 1 mg/ml of the extract and ascorbic acid. From these stock solutions various dilutions of the extract such as 50 µg, 100 µg, 150 µg, 200 µg and 300 µg were made with phosphate buffer. 0.1 ml of rat liver homogenate (25% w/v) in tris-HCl buffer (40 mM, pH 7), 0.1 ml each of KCl (30 mM), ascorbic acid (0.06 mM) and ferrous ion (0.16 mM) and various dilutions of the extract and ascorbic acid were incubated for 1 h at 37°c. The reaction mixture (0.5 ml) was treated with sodium dodecyl sulfate (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8%) and acetic acid (1.5 ml, 20%, pH 3.5). The total volume was then made upto 4 ml by adding distilled water and kept in oil bath at 100°C for 1 h. After the mixture had been cooled 1 ml distilled water and 5 ml of butanol-pyridine mixture (15:1v/v) were added. Following vigorous shaking, the tubes were centrifuged and the absorbance of the organic layer containing the chromophore was read at 532 nm. The percentage inhibition of lipid peroxidation by the extract was determined by comparing the absorbance values of the control against the experimental tubes as calculated for superoxide radical assay.

2.4 Determination of Nitric Oxide Scavenging Activity

100 mg each of *Cyperus esculentus***,** *Punica granatum, Zingiber officinale* and *Embeliaribes* dried extract and 100 mg ascorbic acid were weighed accurately and suspended in 100 ml of distilled water to give standard stock solutions of 1 mg/ml of the extract and ascorbic acid. From these stock solutions various dilutions of the extract such as 50 µg, 100 µg, 150 µg, 200 µg and 300 µg were made with phosphate buffer. To 4 ml of different concentrations of plant extract, 1 ml 25 mM Sodium nitroprusside solution was added in the test tubes and incubated at 37°C for 3 hr. An aliquot of incubated solution (0.5 ml) was taken in to a test tube and 0.3 ml Griess reagent was added. The absorbance of the chromophore formed during diazotization of nitrate with sulphanilamide and subsequent coupling with napthylethylene diamine dihydrochloride was immediately measured at 570 nm. A control was prepared using 0.1 ml of respective vehicle in place of plant extract /ascorbic acid. The percentage of inhibition of nitric oxide scavenging activity was measured by comparing the above absorbance values of the control and the extract as calculated in superoxide radical assay[18].

2.5 Statistical analysis

Data were analyzed by SigmaPlot software. Equations for best fitted line to estimate IC_{50} values obtained by linear regression statistics based on least squares method. Following one way analysis of variance (ANOVA), treatment means were compared using post hoc comparisons tests.

III. RESULTS AND DISCUSSION

Oxidative stress may be defined as a state where oxidation exceeds antioxidant system, oxidative stress contributes to many pathological conditions, including cancer, neurological disorders, atherosclerosis, hypertension, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis and asthma[19]. Due to increased oxidative stress in biological system involved in generation of ROS and RNS. The ROS and RNS includes superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (-$OH^{\cdot}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) respectively [20]. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals. Antioxidants may offer resistance against oxidative stress by scavenging free radicals. Superoxide is produced from

molecular oxygen due to oxidative enzymes of mitochondria[21]. There is an increasing evidence that free radicals induced oxidative stress damage has been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiac disorders, neurological disorders, in process of aging and dementia. The antioxidants scavenge the free radicals and inhibit the lipid peroxidation. The different concentrations of extracts were tested for antioxidant activity by in-vitro models namely superoxide, Hydroxy radical, lipid peroxidation inhibition and nitric oxide scavenging in comparison with standard (ascorbic acid) antioxidant[22].Superoxide radical is considered a major biological source of reactive oxygen species. Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [23] and [24].The scavenging activity towards superoxide radicals is measured in terms of inhibition of superoxide radicals.The extract of *Cyperus esculentus, Punica granatum, Zingiber officinale and Embeliaribes* were found to scavenge the superoxides generated by photo reduction of riboflavin.*Cyperus esculentus, Punica granatum, Zingiber officinale, Embeliaribes* and ascorbic acid at quantities of 50-300 µg produced dose dependant inhibition of superoxide radicals (Figure 1).

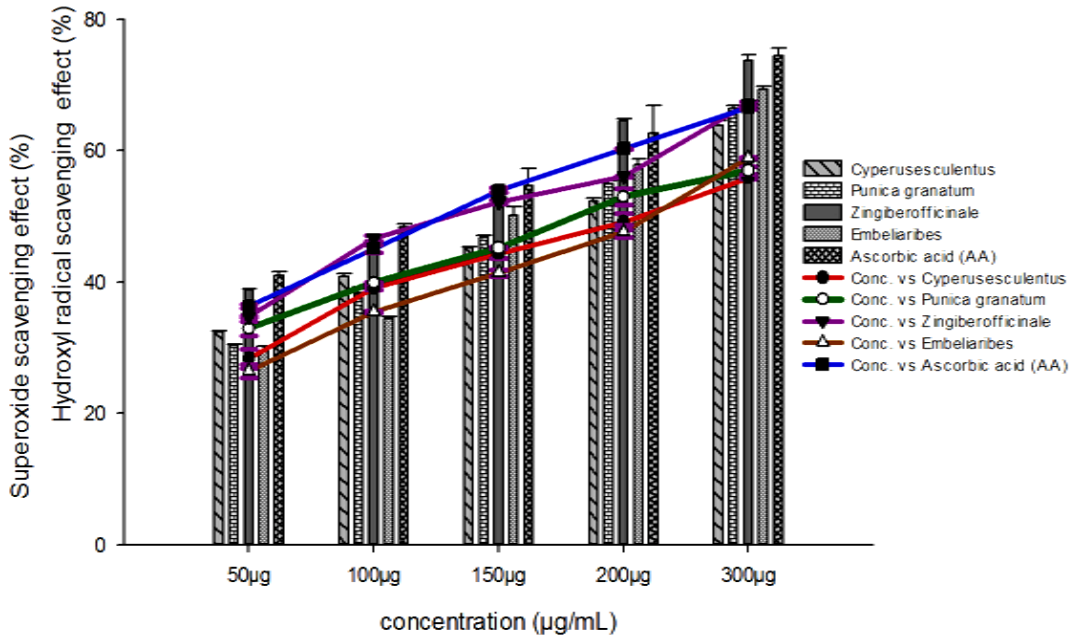

Figure1: Superoxide radical scavenging and hydroxyl radical scavenging effect vs. final concentration of extracts and Ascorbic acid (AA).

The quantity of the extract of *Cyperus esculentus, Punica granatum, Zingiber officinale, Embeliaribes* were needed for 50% scavenging of superoxides was found to be 135.7 µg, 179.2 µg, 114.00 µg and 172.00 µg respectively. The quantity needed for the same effect by the known antioxidant, ascorbic acid was 114.00 µg. This indicates that the aqueous extract of *Cyperus esculentus; Punica granatum, Zingiber officinale* and *Embeliaribes* possess antioxidant activity respectively. Figure 1 shows that the scavenging effects of samples on superoxide radicals and were in the following order: Ascorbic acid≥*Zingiber officinale*>*Cyperus esculentus*>*Embeliaribes*>*Punica granatum.*The EC_{50} values of scavenging superoxide radicals for the selected plants extract is given in Table 1. Though the antioxidant potential of fractions was found to be low ($P<0.05$) than those of ascorbic acid, the study revealed that *Zingiber officinale, Cyperus esculentus, Embeliaribes,* and *Punica granatum* have prominent antioxidant activity; the presence of phenolic compounds (containing phenolic hydroxyls) are mainly found in these fractions and could be attributable to the observed high antiradical properties of these fractions. Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals generated by the Fenton reaction.

Table 1: The IC_{50} values of scavenging and peroxidation effect of aqueous extracts (µg/ml).

Extracts/AA	Superoxide radical scavenging (IC_{50} µg)	Hydroxyl radical scavenging (IC_{50} µg)	Lipid peroxidation (IC_{50} µg)	Nitric oxide scavenging (IC_{50} µg)
Cyperus esculentus	135.7±0.12	212.9±0.19	321.4±0.82	251.5±0.24
Punica granatum	179.2±0.23	266.2±0.12	278.7±0.42	247.1±0.25
Zingiber officinale	114±0.12	169.4±0.22	198.5±0.72	135.4±0.57
Embeliaribes	172±0.5	210.1±0.42	279.3±0.25	226.2±0.82
Ascorbic acid (AA)	114±0.42	140.7±0.92	196.5±0.98	139.4±0.52

Degradation of deoxyribose mediated by hydroxyl radicals generated by Fe^{3+}/EDTA/H_2O_2 system was found to be inhibited by the extracts.The quantity of extract of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* were needed for 50% scavenging of hydroxyl radicals 212.9, 266.2, 169.4 and 210.1 and the same effect by the known antioxidant, ascorbic acid was 140.7 µg. The hydroxyl radical scavenging activity of *Cyperus esculentus***,** *Punica granatum*, *Zingiber officinale* and *Embeliaribes*can be ranked as Ascorbic acid≥ *Zingiber officinale*>*Embeliaribes*>*Cyperus esculentus*>*Punica granatum* (Figure 1).All extracts showed antioxidant activity in dose dependent manner at concentration 50-300 µg/ml (Figure 1). In the present investigation, the EC_{50} value of hydroxyl radical scavenging activity for the extracts was in between 169.4±0.22 and 266.2±0.12 µg/ml while for AA was 140.7±0.92 µg/ml (Table 1).The markedly strong ($P < 0.05$) antioxidant response of extracts in comparison with ascorbic acid might be helpful in characterizing the significant sources of natural antioxidant reaction. Lipid peroxidation generated by the induction of Fe^{2+}/ascorbate on rat liver homogenate was found to be inhibited by the addition of these extracts. *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* at quantities of 50-300 µg produced dose dependant inhibition of lipid peroxides. The quantity of the extract of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* were needed for 50% scavenging of lipid peroxides was found to be 321.4, 278.7, 198.5 and 279.3 µg. The quantity needed for the same effect by the known antioxidant, ascorbic acid was 196.5 µg. This indicates that the aqueous extract of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* were possessing potential antioxidant activity (Table 2). The order of potency of selected plant extract was Ascorbic acid≥*Zingiber officinale*>*Punica granatum* >*Embeliaribes*>*Cyperus esculentus.*

Table 2: Lipid peroxidation and Nitric oxide scavenging activity of *Cyperus esculentus, Punica granatum, Zingiber officinale and Embeliaribes.*

Extracts/AA	Lipid peroxidation inhibiting	Nitric oxide scavenging	Lipid peroxidation inhibiting	Nitric oxide scavenging	Lipid peroxidation inhibiting	Nitric oxide scavenging	Lipid peroxidation inhibiting	Nitric oxide scavenging	Lipid peroxidation inhibiting	Nitric oxide scavenging
	50 µg		**100 µg**		**150 µg**		**200 µg**		**300 µg**	
Cyperus esculentus	12.9±0.2	25.7±2.5	24±1.3	28.2±2.35	31±0.15	36±1.8	36.8±0.25	43.2±1.1	46.8±0.25	57.2±1.5
Punica granatum	19.1±0.25	23.3±2.35	30.7±0.55	34.2±1.95	37.4±0.5	39±2.2	44.3±0.15	46.7±1.85	55.3±0.15	53.3±1.80
Zingiber officinale	31.7±.1	26.7±2.1	39.8±2.12	34.2±0.2	46.4±1.85	42.5±1.55	51.2±3.25	49.4±1.2	60.2±0.1	56.3±1.25
Embeliaribes	18.6±0.4	22.1±5.35	31.2±0.45	36.8±1.30	34.7±0.55	37.3±0.55	45.2±0.25	44.5±1.85	49.5±1.2	54.7±1.05
Ascorbic acid (AA)	30.1±0.5	35.9±0.50	38.6±0.2	43.31±0.75	43±0.25	50.95±0.85	56.6±0.05	61.5±0.35	59.8±0.25	70.25±0.35

Nitric oxide generated by the diazotization of nitrate with sulphanilamide was found to be inhibited by the addition of these extracts. The quantity of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* extract needed for 50% scavenging of nitric oxide was found to be 251.5, 247.1, 135.4 and 226.2 µg. The quantity needed for the same effect by the known antioxidant, ascorbic acid was 139.4 µg. This indicates that the aqueous extract of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* posses potential antioxidant activity (Figure 2).

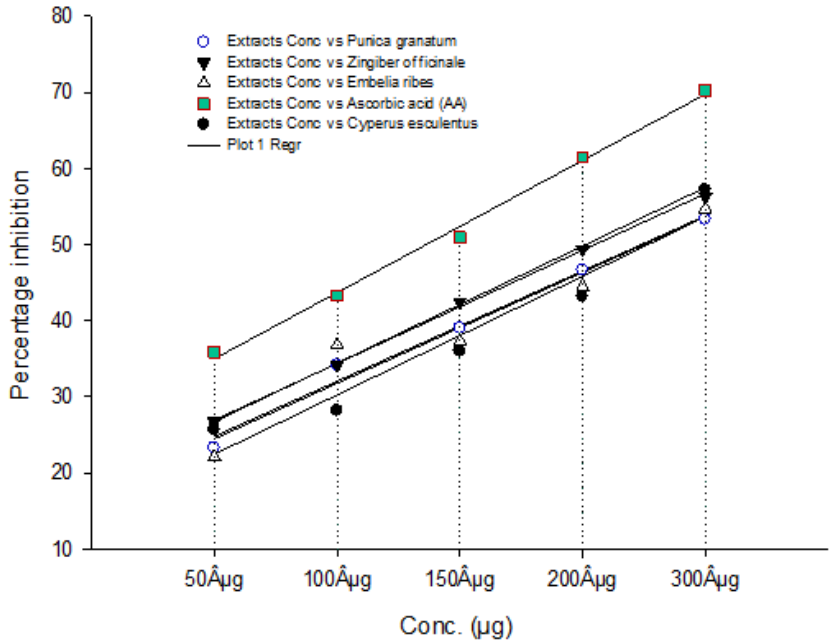

Figure 2: Nitric oxide scavenging activity of *Cyperus esculentus, Punica granatum, Zingiber officinale and Embeliaribes*

The order of potency of selected plant extract was *Zingiber officinale*≥Ascorbic acid *>Embeliaribes>Punica granatum>Cyperus esculentus.* The EDRF is nitric oxide involved in various physiological processes. The oxygen reacts with excess nitric oxide (NO) to generate nitrite and peroxy nitrite ($ONOO^-$) which act as free radicals[21]. In this study, it is concludes the aqueous extract of Cyperus *esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes* showed better activity in competing with oxygen to react with nitric oxide and prevention of generation of anions.

IV. CONCLUSION

Cyperus esculentus, Punica granatum, Zingiber officinale, Embeliaribes exhibited antioxidant activity estimated by *in-vitro* methods namely superoxide radical scavenging activity, hydroxyl radical scavenging activity, nitric oxide and lipid peroxidation inhibition effect. Based on their IC_{50}% values, *Zingiber officinale* was found to be the potent among other aqueous extracts of plants in scavenging superoxide radicals, hydroxyl radical, lipid peroxidation inhibiting activity and nitric oxide scavenging effect comparing with ascorbic acid. *Cyperus esculentus, Punica granatum* and *Embeliaribes* showed variation in potency. The presence of flavonoids, phenolic compounds in the selected plant extract may be responsible for the antioxidant activity.

Acknowledgments

The authors are very thankful to Rajiv Memorial Education Society's College of Pharmacy Gulbarga; Karnataka for providing facilities to carried out the research work. The authors gratefully acknowledge M/s Laila Implex, Vijayawada and Kisalaya Herbals Ltd. Ratlam Koti, Indore, India for supported by providing gift samples of aqueous extracts of *Cyperus esculentus, Punica granatum, Zingiber officinale* and *Embeliaribes.*

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