Advance Research in Pharmaceuticals and Biologicals

(A Peer Reviewed International Journal for Pharmaceutical and Allied Research)

ISSN 2250-0774 USA CODEN: ARPBGZ

IN VITRO ANTIOXIDANT ACTIVITY OF KIRGANELIA RETICULATA STEM

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Received on 11/04/2013 **Revised on 21/04/2013** **Accepted on 12/05/2013**

ABSTRACT:
In present study we carried out systematic record of the Phytochemical and antioxidant properties of the medicinal plant *Kirganelia reticulata.* The different solvents extract of *Kirganelia reticulata* stems were screened for their in vitro Phytochemical and antioxidant activity. Stem were extracted with different polarities like petroleum ether, chloroform, ethyl acetate, methanol, and aqueous. The separation of main active constituent such as alkaloids, flavonoids, phenols, steroids, tannins, etc. present in the plant were analyzed. It was focused to determine the total flavonoid contents. Antioxidant potentials of extract, using different models, DPPH assay, Hydrogen peroxide, Super oxide scavenging activity.

Keywords: Antioxidant, *Kirganelia reticulata,* Phytochemicals, Antioxidant assay.

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INTRODUCTION

Oxygen radicals are continuously formed in all living organisms, with deleterious effects that lead to cell injury and death. Production of oxidative species occurs under physiological conditions at a controlled rate, but it is dramatically increased in conditions of oxidative stress. Exposure of biological systems to xenobiotics, pollutants, ionizing radiation or U.V. light and development of certain pathological conditions lead to oxidative stress, consequently increase Production of oxy radicals[1]. Oxygen is, no doubt, an indispensable part of aerobic life. However, under certain circumstances, it can seriously affect our well being through the formation of reactive oxygen species (ROS) representing both free radical and non-free radical species, and their potential deleterious effects such as atherosclerosis, ischemic heart disease, ageing, anemia, asthma, arthritis, inflammation, diabetes, immunosuppressant, neurodegenerative diseases, cancer and others[2]. Free radical implicated in the pathogenesis of at least 50 diseases. Free radicals formation controlled naturally by various beneficial compounds antioxidants. The availability of antioxidants is limited that this damage can become cumulative and debilitating. Mostly antioxidants are capable of deactivate, stabilize or scavenge free radicals before attacks cells. They are absolutely critical for maintaining optimal cellular and systematic health and well being. Plants are potent biochemical factories and have been components of phytomedicine since times immemorial. The plant based natural constituents can be derived from any part of plant like bark, leaves, stems, roots, fruits, etc i.e. any part of the plant may contain active components. The beneficial medicinal effects of plant materials typically result from the combinations of secondary product present in the plant. The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, betacarotene and tocopherol are known to possess antioxidant potential[3-6].

KR is also known as *Kirganelia reticulata* (Poir) and *Phyllanthus reticulatus* (Poir). is a large, often scandent, shrub of the family Euphorbiaceae, Commons names**:(**Sanskrit): Krishna-Kaamboji, **(**Hindi): Bhuinowla**,** **(**Tamil): Abirangi**,** **(**Telugu): Nallapuli**,**

(Marathi): Pavana, (Assam):Amluki.,[7] The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands. The leaves and bark are used as astringent and diuretic. Juice of leaves is used for the treatment of diarrhea in children. The stems are used to treat sore eyes and the powdered leaf is used in sore, burns, suppuration and chafing of skin. The bark is also used for variety of ailments including asthma, small pox, bleeding forms gums, showed significant antiviral and antiplasmodial activity. The biological work performed so far on the plant showed hypotensive effects in gastric complaints including colic constipations etc. chemical study demonstrated the presence of octacosonal, texerol acetate, berulin, sitosterol etc. The antibacterial potential of the leaf extracts of this plant has been evaluated recently. Pharmacognostic parameters like microscopy, quantitative leaf, stem microscopy, physicochemical properties are studied. Chemical compounds such as flavonoid, tannins, glycosides, and alkaloids serve as a sink for several bioactive compounds. So focus of present study is the identification of secondary metabolites by estimation of flavonoids, and antioxidants activity of *K. reticulata* using different *in-vitro* models[8-12].

MATERIALS AND METHODS

Plant material: Stem of *K.reticulata* was collected from local area of Pune, Maharashtra, India in December 2012, and identified by Botanical Survey of India, Pune. A voucher specimen (YOTPR-2) for this collection has been retained in the Pharmacognosy Laboratory, Modern College of Pharmacy, Moshi, Pune.

Chemicals: (DPPH 2, 2-diphenyl- 1-picrylhydrazyl), Hydrogen peroxide, Riboflavin, Ascorbic acid, Nitro blue tetrazolium, EDTA.

Extraction of plant material: The shade-dried stem were coarsely powdered and extracted successively with chloroform, ethyl acetate, methanol by a soxhlet apparatus. The solvent was completely removed by rotary evaporator and kept in desiccators to obtain gummy exudates. This crude extract was used for further investigation for potential antioxidant properties[13, 14].

Phytochemical screening: The freshly prepared extracts of *K.reticulata* were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorffs reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce foam. Gum was tested using molish reagents and concentrated sulfuric acid. These were identified by characteristic color changes using standard procedures[15, 16].

Determination of total flavonoids: Take 1 ml of test sample in test tube and add 4 ml of water. 0.3 ml of sodium nitrate, 0.3 m of aluminum chloride was added. For 6 min incubate solutions at room temperature, and then 2M of NaOH was added to the reaction mixture, made the volume up to 10 ml by adding distilled water. The absorbance of reaction was measured at 510 nm against bank by using spectrophotometer. Queracetin was used as standard (mg/ml in distilled water). Total flavonoids were expressed as Queracetin equivalent in milligrams[17].

Total antioxidant capacity: The antioxidant activity of extracts was determined by the phospho molybdenum method. The 0.3 ml of extract was combined with 3ml of reagent solution (0.6 M Sulphuric acid, 4mM ammonium molybdate, 28mM sodium phosphate). The reaction mixture was incubated at 95^0C for 90min and cooled at room temperature. Absorbance of solution was measured at 695 nm using spectrophotometer against blank. Ascorbic acid was used as standard (1mg/ml in distilled water); the total antioxidant capacity was expressed as the number of equivalents ascorbic acid (AAS) [18-20].

Antioxidant assay:

DPPH radical scavenging activity: The free radical scavenging capacity of the extracts was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% ethanol. Methanol, chloroform, ethyl acetate extract of *K. reticulata* was mixed with 95% ethanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and *K. reticulata* extracts was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 517 nm using a spectrophotometer (HACH 4000 DU UV–visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation: absorbance of the control minus absorbance of the test sample divided by absorbance of the control multiplied by 100[21].

$$\% \text{ Inhibitin} = \frac{A0 - A1}{A0} \times 100$$

Where, A0 = absorbance of the control and A1= absorbance in the presence of the sample of extract and standard

Hydrogen peroxide scavenging activity: Scavenging activity of Methanol, chloroform, ethyl acetate extract of K. reticulata was evaluated by hydrogen peroxide. 1ml of various concentrations of the extract and standards in ethanol was added to 2 ml of hydrogen peroxide solution in phosphate buffered saline (PBS, pH 7.4). Then finally the absorbance was measured at 230 nm after 10 min. Ascorbic acid were used as standard. Control sample was prepared containing the same volume without any extract and standard and the absorbance was read at 230 nm using a spectrophotometer[22]. The percentage inhibition was calculated according to the following equation:

$$\% \text{ Inhibitin} = \frac{A0 - A1}{A0} \times 100$$

Where, A0 = absorbance of the control and A1= absorbance in the presence of the sample of extract and standard.

Superoxide scavenging activity: Scavenging activity of Methanol, chloroform, ethyl acetate extracts of K. reticulata were evaluated by superoxide.1ml of extract at various concentrations then added 0.1ml of NBT (1.5mM), adds a 0.2 ml of EDTA (0.1M), 0.05 ml of riboflavin and finally added 2.55ml of phosphate buffer. Control tube containing a DMSO instead of sample extract. After addition of incubated a sample solution for 30min. ascorbic acid used as standard. Moreover, absorbance was read at 560nm using a spectrophotometer.[23] the percentage inhibition was calculated according to the following equation:

$$\% \textit{ Inhibitin} = \frac{A0 - A1}{A0} \times 100$$

Where, A0 = absorbance of the control and A1= absorbance in the presence of the sample of extract and standard.

RESULT AND DISCUSSION

The Phytochemical analysis carried out on the plant revealed the presence of several medicinally active methanol and ethyl acetate extract has shown to contains large number of alkaloid, carbohydrates, phenols, tannins, flavonoids, saponin glycosides etc. the result of which are shown in **table 1.** It may be attributed to reasons that stronger extraction capacity of methanol and ethyl acetate could have extracted a greater number of constituents. These compounds are known to be biologically active and hence aid the investigation of several activities these observations therefore support use of K. reticulate in herbal cure remedies. Alkaloids were detected together with flavonoids; this may be responsible for the antioxidant activity observed in the crude extract. The preliminary phytochemical evaluation revealed the presence of several secondary metabolites, which are known to posses various pharmacological effects. In last four decades the scientists are keen to evaluate many plant drugs used in medicinal folk are due to their specific healing properties, health action and no toxic effect.

Table 1: Phytochemical investigations of *K. reticulata* stem extract

S. No.	Test	Pet Ether extract	Chloro-form extract	Ethyl Acetate extract	Methanol extract	Chlor-oform water
1	Alkaloids	_	+	+	+	+
2	Carbohydrates	_	+	+	+	_
3	Phytosterols	_	_	+	_	_
4	Saponin glycoside	_	_	+	_	+
5	Cardiac glycoside	_	+	+	_	_
6	Protein & amino acid	_	_	+	+	_
7	Tannins & Phenolic compound	+	_	+	+	_
8	Flavonoids	+	+	+	+	+

Total Flavonoids content: Human readily ingests the flavonoids and they seem to display important anti-inflammatory, antiallergic, anticancer activities. They are also found to the powerful antioxidant and researchers are looking into their ability to prevent cancer and cardiovascular diseases. Flavonoids activity as chain breaking antioxidant important with formation of free radicals in the process of formation of intracellular substance throughout body including collagen, bone matrix and tooth dentine. The quantitative determination of flavonoids in plant extract shows that they good source of flavonoids high quantity of it was found to be 3.07 mg/g in ethyl acetate extract, which is followed by chloroform extract 1.62 mg/g , where as in the methanolic extract it was recorded to have the least value of 0.92 mg/g. **fig. 1.**

A variety of intrinsic antioxidant (CAT, SOD, Peroxides, reduced glutathione) are present in the organism which protect them from oxidative stress, there by forming the first line of defense. The antioxidants activities of have been attributed to various mechanisms among these are prevention of chain initiation binding of transition mutation catalyst decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging as stated before numerous polyphenol are known to posses excellent antioxidant

ISSN 2250-0774

effect, especially In-vitro and the amount of polyphenol present in a plant extract has been suggested to correlate with antioxidant activity. In our study, however same correlation was found showing the total antioxidant present in extract was high ethyl acetate 1.80, followed by chloroform 1.63 and methanol 0.66 extracts compared to standard as shown in fig.2.

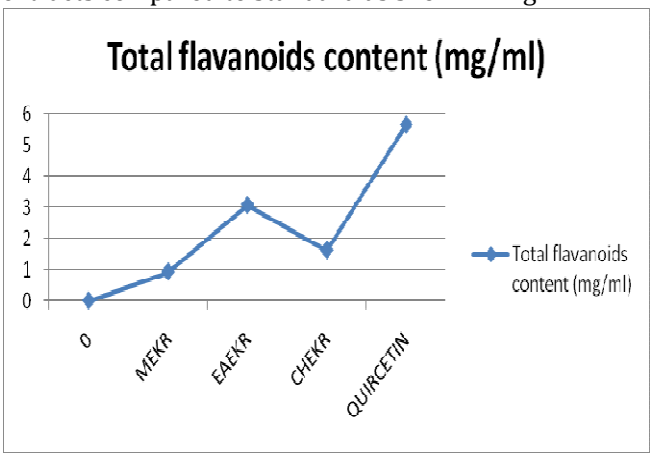

Fig.1 Total flavonoids content present in stem extract of *K. reticulata*

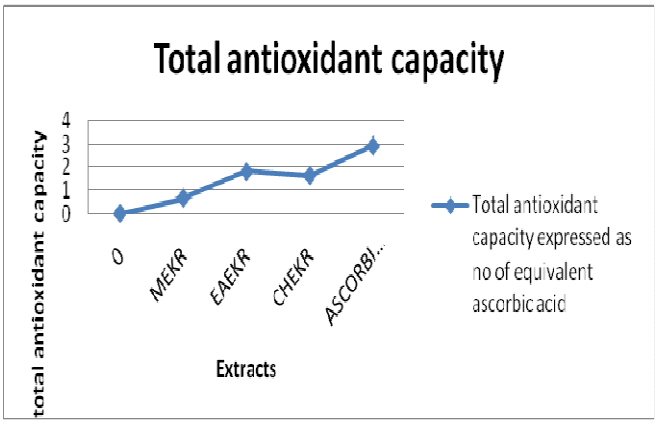

Fig.2 Total antioxidants present in the stem extract of *K. reticulata*

DPPH radical-scavenging activity assay:

From the dose dependent response curve of DPPH radical scavenging activity of different plant extracts of *K. reticulata,* it was observed that the chloroform extract had higher radical scavenging activity than ethyl acetate and methanol. At a concentration of 13.40 µg/ml, the scavenging activity of chloroform extract (CHEKR) reached 55.95%, and at concentration 15.51 µg/ml, the scavenging activity of ethyl acetate extract (EAEKR) reached 48.33%, which was comparable to that of standard drug ascorbic acid. The values obtained were plotted in graph. The ethyl acetate extract of *K. reticulata* showed excellent antioxidant and free radical scavenging activity Fig. 3. In considering this, the ethyl acetate plant extract was chosen for further study.

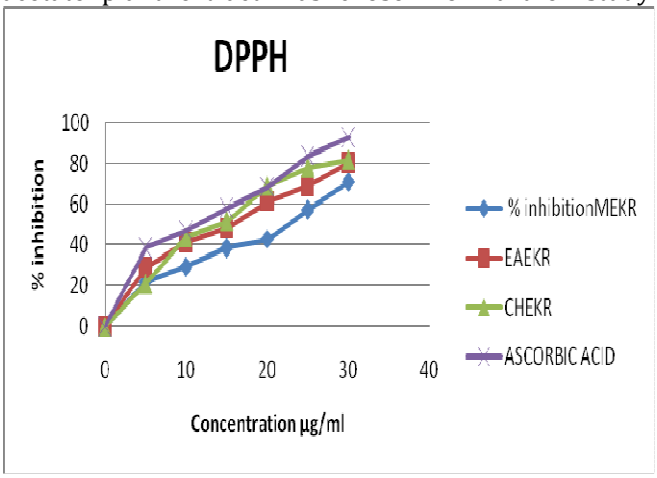

Fig.3 DPPH Free radical scavenging

Hydrogen Peroxide scavenging activity:

The scavenging ability of extracts of *K. reticulata* on hydrogen peroxide is shown Fig 4 Compared with ascorbic acid as standards. The *K. reticulata* extracts were capable of scavenging hydrogen peroxide in an amount dependent manner. 20 µg of methanol, ethyl acetate, and chloroform extracts of *K. reticulata* exhibited 33.12 to 85.88% scavenging activity on hydrogen peroxide. On the other hand, using the same amounts, ascorbic acid exhibited 48.20 % hydrogen peroxide scavenging activity. Results show that the scavenging activity values on hydrogen peroxide of 20µg of the extracts of *K. reticulata* decreases than that of ascorbic acid. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells Thus, the removing of hydrogen peroxide is very important for antioxidant defense in cell or food systems.

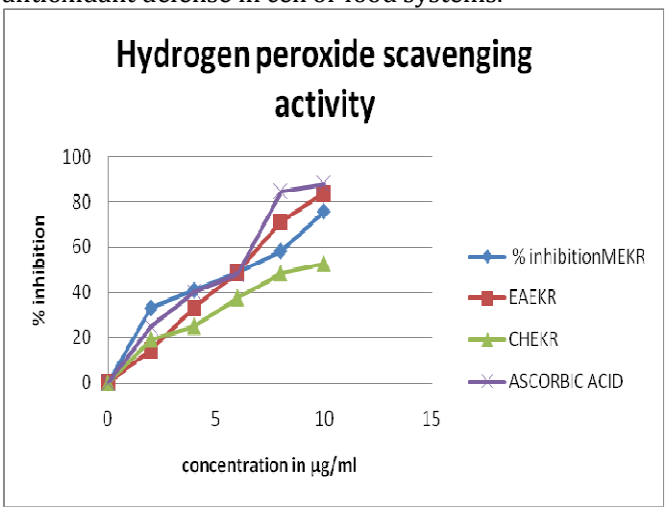

Fig.4 Hydrogen peroxide scavenging activity

Superoxide scavenging activity:
Superoxides are produce from molecular oxygen due to oxidative enzymes of body as well as via nonenzymatic reactions such as auto oxidation by catecholamine. The methanolic extract of *K. reticulata* and ascorbic acid at 50µg/ml, inhibited NBT reduction by 60.68% and 66.53% respectively. IC50 values were obtained 3.55 µg/ml in extract and 4.31µg/ml for the standard.(Fig.5)

CONCLUSION
In conclusion, we might say that our results further support the view that the four chosen medicinal plants are promising sources of natural antioxidants. Total Flavonoids content and values for different antioxidant assays differ significantly among ethyl acetate and methanolic extracts of *K. reticulata* plants. With the above results we can say that plants used in this study possess good antioxidant potential and it is possible that this high antioxidant potential could contribute in their folk medicinal properties.

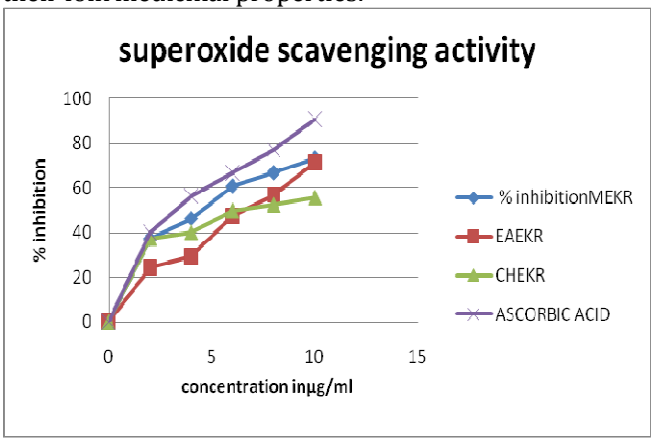

Fig. 5 Superoxide scavenging activity

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