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IN-VITRO ANTIOXIDANT ACTIVITIES OF DIFFERENT SOLVENT FRACTIONS FROM THE ETHANOLIC EXTRACT OF *HIBISCUS ROSA SINENSIS* PETALS

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Keywords:

Hibiscus rosa sinensis, Reactive oxygen species, Phenolic, and flavonoid content, Antioxidant activity, Reducing power

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ABSTRACT: Overproduction of free radicals can cause oxidative damage to biomolecules (*e.g.*, lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans. Plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites, which are rich in antioxidant activity. In the present study *in-vitro* antioxidant potential of various fractions of *Hibiscus rosa sinensis* petal (HRS) ethanolic extract was investigated. Fractions prepared from crude ethanolic extract of HRS were concentrated and used for various *in-vitro* assays. Ethyl acetate fraction of HRS (EHRS) had a very high content of total phenolics and flavonoids. DPPH radical scavenging activity (IC_{50}-9µg/ml) of EHRS was comparable with standard Quercetin. Superoxide, ABTS, and hydroxyl radical scavenging activities of EHRS were also found higher than other fractions. The total antioxidant activity and reducing power were also high in EHRS. So the study suggests that EHRS may be a potential source of bioactive substances with multifaceted activity.

INTRODUCTION: A free radical is any atom or molecule that has a single unpaired electron in an outer shell. Most biologically-relevant free radicals are highly reactive and contain oxygen, so called, as oxidants. For most biological structures, free radical damage is closely associated with oxidative damage. Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems.

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Also, several external agents can trigger reactive oxygen species (ROS) production. A sophisticated enzymatic and nonenzymatic antioxidant defense system, including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defense.

Similarly, increased ROS may also be detrimental and lead to cell death or acceleration in aging and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids, and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific

redox-sensitive signaling pathways [1]. Once activated, these diverse signaling pathways may have either damaging or potentially protective functions [2]. Thus, various endogenous and exogenous sources together with altered antioxidant defense systems contribute to an increased level of reactive oxygen species. The enhanced ROS alters normal physiological functions that cause aging, diseases and may ultimately lead to cell death. Several substances from natural sources have been shown to contain antioxidants and are under study. Antioxidant compounds like Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases [3]. Ethnomedical literature contains a large number of plants that can be used against diseases, in which reactive oxygen species and free radicals play an important role. There are a plethora of plants that have been found to possess strong antioxidant activity [4]. Recent reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human disease [5].

Hibiscus rosa sinensis Linn. (Malvaceae) is a glabrous shrub widely cultivated in the tropics. The plant is reported to possess hypoglycemic, cardioprotective, antitumor, anti-inflammatory, antifertility, anti ovulatory, antiulcer, antiviral, antifungal, antibacterial, hair growth promoting and antioxidant properties [6]. Mainly the flower petals, leaves, and roots are used for medicines. There are various reports showing the antioxidant properties of crude alcoholic extracts of HRS *in-vivo* studies [7-10]. In some regions, the flowers of HRS are eaten raw or cooked [11] and made into a kind of pickle or used as a dye for coloring foods, such as preserved fruits and cooked vegetables [12, 13]. As a traditional medicine fresh juice of flower is used to treat gonorrhea, and the infusion of the petals is used as a refrigerant drink in fevers [14, 15]. It is an important source of various types of compounds with diverse chemical structures as well as pharmacological activities. Presence of such a wide range of chemical compounds indicates that the plant could serve as "lead" for the development of novel agents having good efficacy in various disorders in the upcoming years. In the light of reports mentioned above, the present was carried out to evaluate the *in-vitro* antioxidant properties of various fractions of crude ethanolic extract of hibiscus that may help to isolate the bioactive principles from HRS.

MATERIALS AND METHODS:

Plant Material: *Hibiscus rosa sinensis* (HRS) flowers were collected from Trivandrum and authenticated by Dr. G. Valsaladevi, Department of Botany, the University of Kerala, where a voucher specimen (KUBH 5845) has been deposited. Petals were taken and shade dried.

Extraction and Fractionation of *Hibiscus rosa sinensis* Petals: Dried petals were extracted using 80% ethanol. The filtered extract was dried in a water bath at 60 ºC. Water suspension of crude ethanolic extract of HRS was fractioned successively using petroleum ether, ethyl acetate, butanol, and water, according to their increasing order of polarity. Fractions obtained were concentrated and used for various *in-vitro* antioxidant assays **Fig. 1**.

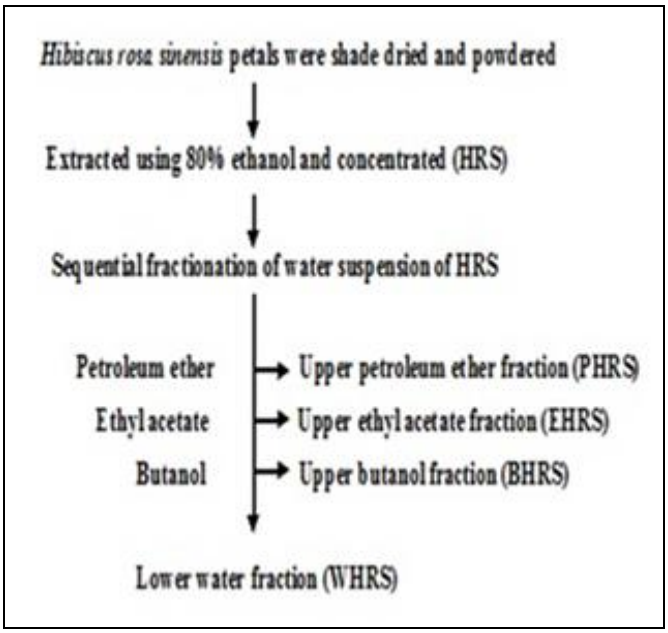

FIG. 1: EXTRACTION AND FRACTIONATION PROCEDURE OF HRS

Chemicals: The chemicals used in the present study were of analytical reagent grade. It was purchased from Sigma Aldrich, USA and SRL Pvt. Ltd., India.

Determination of Total Phenolic Content: Total phenolic content of different fractions was determined using the Folin-Ciocalteu method as described by Singleton *et al.* [16] 0.5 ml of the

fractions was added to 3 ml distilled water and 0.25 ml Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 2 min, and then 0.75 ml of 20% sodium carbonate was added to the mixture, and the volume was made up to 5 ml with distilled water. The absorbance was measured at 765 nm after standing for 2 hours. The content of phenolics was expressed as percent gallic acid equivalents (GAE).

Total Flavonoids Estimation: Aluminum chloride colorimetric technique was used for flavonoids estimation [17]. Fractions were mixed with 1.5 ml methanol, 0.1 ml 10% aluminum chloride, 0.1 ml 1 M potassium acetate, and 2.8 ml distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was plotted with quercetin, and results were expressed as a percentage of quercetin equivalents (QE).

1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay: The free radical scavenging activity of the extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH [18]. The reaction mixture contains 2.5 ml DPPH solution (0.1 mM in methanol), 0.2 ml fraction (25-150µg/ml) (adjusted to 3 ml by adding methanol) and absorbance recorded after 30 min at 517nm. The antioxidant activity of the compound was expressed as $IC_{50,}$ and it is defined as the concentration of fraction that inhibited the formation of DPPH radicals by 50%, and gallic acid (GA) was used as standard. The percentage of inhibition of the free radical was calculated using this equation:

% Inhibition = (Absorbance of control – Absorbance of sample × 100 / Absorbance of control

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity was measured by the method of Anusha *et al.* [19] Superoxide radicals are generated due to the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the superoxide radicals are generated in 3.0ml Tris-HCl buffer (16mM, pH 8.0) containing 1.0ml NBT (50 mM), 1.0ml NADH (78 mM) solution, and 1ml sample (25–150 µg/ml) in water. The reaction is started by the addition of 1.0ml PMS (10 mM) to the mixture. The reaction mixture was incubated at 25 ºC for 5 min, and the absorbance was measured at 560 nm against a blank. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. A system devoid of extract served as the control. The antioxidant activity of the fraction was expressed as $IC_{50,}$ and the percentage of inhibition of the free radical was calculated using this equation:

% Inhibition = (Absorbance of control – Absorbance of sample × 100 / Absorbance of control

2, 2'-Azinobis 3-ethylbenzothiazoline-6-sulphonate (ABTS) Radical Scavenging Activity: ABTS assay was done according to the method of Re *et al*., described by Ashafa *et al*. [20] The stock solutions included 7 mM ABTS solution and 2.4mM ammonium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowed to react for 12 h at room temperature in the dark condition. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm. Fresh ABTS solution was prepared for each assay. Different fractions (1 ml) were allowed to react with 1 ml ABTS solution, and the absorbance was taken at 734 nm after 7 min. The ABTS radical scavenging capacity of the extract was calculated as percentage ABTS radical inhibition and calculated using this equation:

% Inhibition = (Absorbance of control – Absorbance of sample × 100 / Absorbance of control

Hydroxyl Radical Scavenging Assay: The scavenging capacity for hydroxyl radical was measured according to the modified method described by Arunachalam *et al.* [21] The assay was performed by adding 0.1 ml EDTA (1mM), 0.01 ml $FeCl_3$ (10 mM), 0.1 ml H_2O_2 (10mM), 0.36 ml deoxyribose (10mM), 1.0 ml sample (25-150 µg/ml), 0.33 ml phosphate buffer (50 mM, pH 7.4) and 0.1ml ascorbic acid (1mM) in sequence. The mixture was then incubated at 37 ºC for 1hr. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml 10% TCA and 1.0 ml 0.5% TBA and measured at 532 nm after 20 min at 100 ºC. Percentage inhibition was calculated using this equation:

% Inhibition = (Absorbance of control – Absorbance of sample × 100 / Absorbance of control

Total Antioxidant Capacity: The antioxidant activity of fractions was evaluated by phosphor molybdenum method [22]. An aliquot of 0.1 ml of sample solution was mixed with 1 ml phosphor molybdenum reagent and incubated at 95 °C for 90 min. The tubes were cooled to room temperature, and the absorbance was read at 695 nm against a blank. The calibration curve was plotted with ascorbic acid, and the total antioxidant capacity was expressed as % ascorbic acid equivalents.

Reducing Power Assay: Reducing ability was performed by using potassium ferricyanide- ferric chloride system [23]. Different concentrations (25-150 µg/ml) of fractions (1 ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50 °C in a water bath for 20 min. After incubation, 2.5 ml TCA (10%) was added to terminate the reaction. The upper portion of the solution (2.5 ml) was mixed with 2.5 ml distilled water, and 0.5 ml $FeCl_3$ solution (0.01%). The reaction mixture was left for 10 min at room temperature, and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed six times. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical Analysis: Statistical analysis was done as described by Bennet and Franklin [24]. All analysis was performed on a computer using the statistical package SPSS 17. Data were analyzed by one-way analysis of variance (ANOVA). All the results were expressed as mean value ± SD.

RESULTS AND DISCUSSION: Phenolic compounds are known for their antioxidant activity. Such activity is related to their redox properties in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [25, 26]. They are also believed to have an inhibitory effect on carcinogenesis. Flavonoids, one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolics [27].

These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [28], such properties are especially distinct for flavonols. The results of the present study showed that EHRS contained the highest amount of total phenolics (50.66 mg % of GAE) and flavonoids (12.87 mg % of QE), followed by BHRS **Fig. 2**. This may provide a very good antioxidant capacity to EHRS.

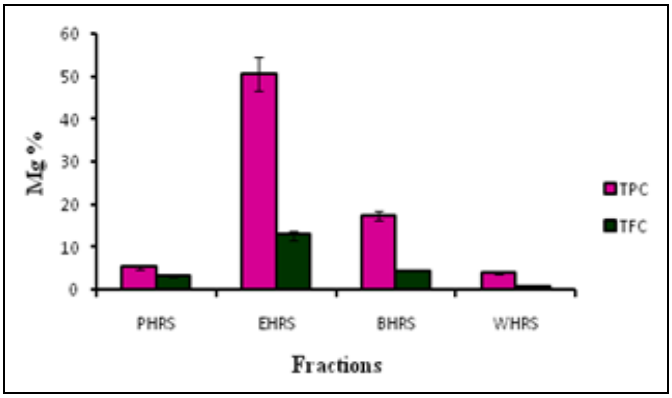

FIG. 2: TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT

It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. The DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 490 nm and also for visible deep purple color [29].

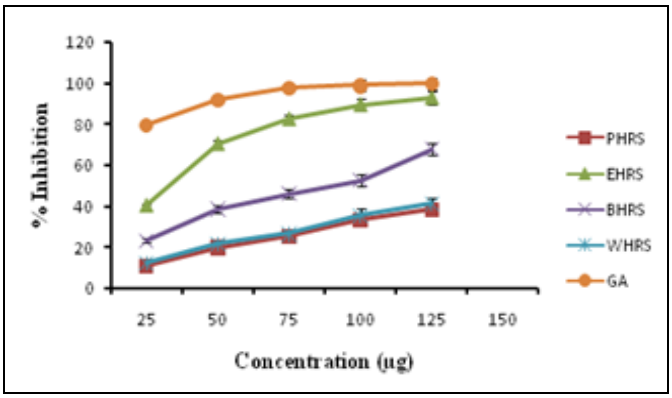

FIG. 3: DPPH RADICAL SCAVENGING ACTIVITY

When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Comparison of the DPPH scavenging activity of the extracts and ascorbic acid are shown in **Fig. 3**. In the present study, the percentage of DPPH radical scavenging was higher in ethyl acetate fraction than other fractions.

The IC_{50} value of EHRS (9 µg/ml) was comparable with that of standard quercetin, indicating the better antioxidant potential of EHRS. Superoxide radical, ABTS radical, and hydroxyl radicals are highly reactive molecules that react with various substances produced through metabolic processes. Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxide dismutase enzymes present in aerobic and anaerobic organisms catalyzes the breakdown of superoxide radical [30]. EHRS showed encouraging a response in quenching radical. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy- D-ribose into fragments that on heating with TBA at low pH form a pink chromogen [31]. The HRS fractions added to the reaction mixture removed the hydroxyl radicals and prevented it from degradation. The highest scavenging activity was shown by EHRS. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm that decreases with the scavenging of the proton radicals [32]. The fractions from HRS were fast and effective scavengers of $ABTS^{+}$ radical. Based on the IC_{50} values and percentage scavenging capacity obtained from the study, it was found that EHRS has the maximum free radical scavenging potential than others **Table 1**. Appropriate standards were used for each radical scavenging assay.

TABLE 1 RADICAL SCAVENGING ACTIVITY

IC_{50} values (µg/ml)	Standard	PHRS	EHRS	BHRS	WHRS
Super oxide radical	Quercetin- 60.7±5.2	215.25±19.64	82.00±7.48	106.60±9.72	256.25±23.38
Hydroxyl radical	Ascorbic acid- 5.0±0.53	153.75±14.03	25.62±2.33	41.00±3.74	141.45±12.90
ABTS radical	Gallic acid- 2.2±0.17	92.25±8.41	10.25±0.93	46.12±4.20	97.37±8.88

Total antioxidant capacity mainly concentrates on the thermodynamic conversion and measures the number of electrons or radicals donated or quenched by a given antioxidant molecule and measure the capacity of biological samples under defined conditions. The phosphor-molybdenum method was based on the reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH with maximal absorption at 695 nm [33]. As shown in **Fig. 4,** all the sample fractions exhibited antioxidant activity.

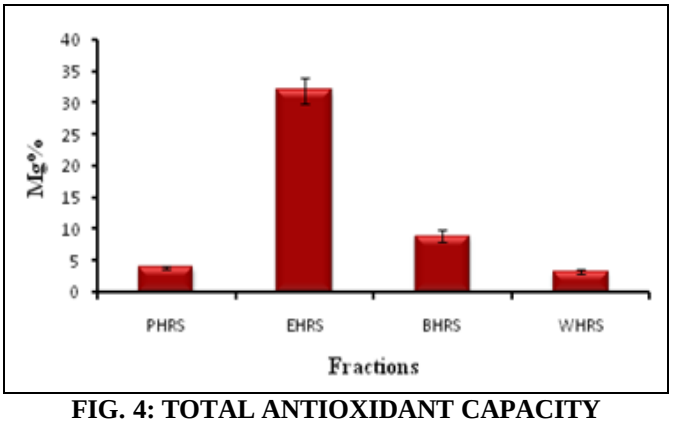

FIG. 4: TOTAL ANTIOXIDANT CAPACITY

Among the different fractions, EHRS showed higher activity (32 mg %). The other fractions registered moderate phosphor molybdenum reduction. Ascorbic acid is used as a standard.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [34]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes so that they can act as primary and secondary antioxidants [35]. The results obtained are given in **Fig. 5**. From these results, it is demonstrated that all the tested fractions showed a non-linear dose-dependent activity. The results were compared with standard ascorbic acid. The more reducing power was exhibited by EHRS.

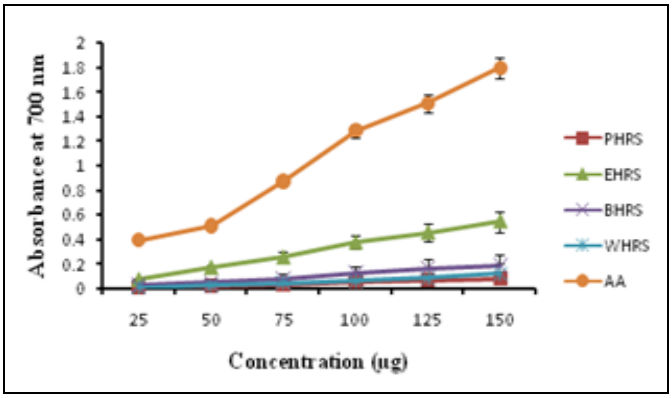

FIG. 5: TOTAL REDUCING POWER

CONCLUSION: Thus, from the results of present *in-vitro* antioxidant study, it is clear that ethyl acetate fraction of ethanolic extract of *Hibiscus*

rosa sinensis possesses a significant antioxidant potential, which is comparable with standard antioxidant molecules. The fraction possesses a good radical scavenging activity, which would be promising for the development as ingredients of functional foods and nutraceuticals. The strong antioxidant activity exhibited by EHRS could be attributed to high phenols, flavonoids, etc. So it can be considered as a potential source of natural antioxidants for therapeutic or industrial purpose and as an alternative for synthetic products, which are known for their multiple disadvantages. Further investigation is currently underway to figure out the mode of action and to identify specific phytochemicals responsible for their antioxidant activities

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CONFLICT OF INTEREST: Nil

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