A STUDY OF FREE RADICAL SCAVENGING ACTIVITY AND TOTAL PHENOL CONTENT OF ZANTHOXYLUM RHETSA

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An attempt was made to study the antioxidant activity of the fruit and seed of *Zanthoxylum rhetsa* in terms of its free radical scavenging activity and Total Phenol Content in aqueous and alcoholic medium. In the DPPH assay, the aqueous extract of the fruit showed IC_{50} value of 6.693 ± 1.327mg/mL and the seed showed IC_{50} value of 27.02 ±1.095mg/mL, whereas in alcohol it showed IC_{50} of 16.488 ± 1.825mg/mL and 41.12 ±3.284mg/mL respectively. Total Phenolic content was measured using Folin-Ciocalteu reagent and was found to be 15.53 ± 1.138 mgGAE/g and 1.06 ± 0.567 mgGAE/g for the aqueous extract of fruit and seed, while it was 12.17 ± 0.658 mgGAE/g and 8.81 ± 0.925 mgGAE/g for their ethanolic extract. The free radical scavenging activity of the spice was comparable to that of the standards Butylated Hydroxy Toluene (BHT) and Gallic acid.

INTRODUCTION:

Of the seventeen Sustainable Development Goals, listed by the United Nations, 'Good health and Well-being' is at number 3. Spices contribute vital nutrients essential for good health and form an integral part of Indian food. They are a good source of antioxidants and are often used as home remedies to treat different ailments. India is a well-known country for its spices and traditional medicines, which have a wide selection of physiological and pharmacological properties. (Anupam KR Sachan, 2018) Secondary metabolites of plants, phytochemicals, belonging mainly to the classes such as terpenes and derivatives, phenylpropanoids, isothiocyanates, sulfur compounds, etc., responsible for the food enhancing properties are synthesized by plants to interact with the environment. Several medicinal properties of spices have been recognized for a long time including antioxidant, anti-inflammatory, analgesic, hypoglycemic, etc. (Baiano, 2018)

While oxygen is one of the most essential components for living, an over-production of this reactive species can occur due to oxidative stress brought about by the imbalance of the bodily antioxidant defense system and radical formation. Oxygen is also a highly reactive atom that is capable of becoming part of potentially damaging molecules such as hydroperoxyl radicals, superoxide anions, singlet oxygen, hydrogen peroxide, organic peroxides, nitric oxide, peroxynitrite, and triplet oxygen. (P. Anbudhasan A. Surendraraj, 2014) (Shih Peng Wong, 2006) The generation of free radicals is associated with several normal metabolic processes, in addition to other environmental factors. (Essam Y. Abdul-Hafeez, 2014)

Free radicals are molecules that contain unpaired electron in the outer orbitals and are very reactive within the body by oxidizing (removing an electron from) other atoms, or sometimes reducing (donating their electron to) other atoms. (Almokhtar A Adwas, 2019) These radicals are important parts of groups of molecules called reactive oxygen/nitrogen species (ROS/RNS). ROS has an important role in cell signaling, gene expression, and ion transportation. However, excessive amounts of ROS can have deleterious effects on many molecules like lipid, protein, RNA, and DNA. In order, to stop or reduce the ROS-induced oxidative damage, the living organisms and other organisms have developed an antioxidant defense system. Besides, the intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body. (Jian-Ming Lü, 2010)

Antioxidants are molecules that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical, and thus prevent or reduce the oxidative stress of the physiological system. Based on their sources, antioxidants are classified into natural and synthetic antioxidants. (Mamta Pal, 2014) Natural antioxidants are further divided into two categories, i.e., enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants are uniquely produced in the human body like Superoxide Dismutase, Catalase, glutathione reductase, etc. Whereas, non-enzymatic antioxidants are not found in the body naturally but are required to be supplemented for proper metabolism which includes minerals, vitamins, carotenoids, polyphenols, etc. (Raygani V, 2007) Synthetic antioxidants are artificially produced or synthesized using various techniques. They are basically polyphenolic compounds like butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), etc.

The use of fruits, vegetables, herbs, and spices as natural antioxidants has received great interest due to the negative health effects developed by the use of synthetic antioxidants. At elevated temperatures, synthetic antioxidants are highly volatile and unstable. Studies show that synthetic antioxidants are noxious for human health. Thus, substitution by natural antioxidants is required. (P. Anbudhasan A. Surendraraj, 2014) (Raygani V, 2007)

Zanthoxylum rhetsa is commonly used as a spice in cooking in many regions of Maharashtra as well as Goa. Both the seed as well as the fruit are used. *Zanthoxylum rhetsa* is one of the important Non-Wood Forest Product (NWFP) of Western Ghats of Karnataka, India, but little or no attention is given to this species in regards to its domestication, conservation, and utilization of genetic diversity. Thus, this study aids the evaluation of the phenolic content and antioxidant activity of *Zanthoxylum rhetsa* (fruit and seed), commonly known as Teppal. It is mainly found in shaded moist localities of tropical regions of India at an altitude of 1,800m. It is a deciduous tree, 20m high with 15-20 mm thick bark, brown, mottled with white, armed with conical prickles. (Resat Apak, 2016) The fruits of Zanthoxylum and their pericarps are used as a peppery spice in both sweet and savory preparations and the seeds rich in oil are often used as fertilizer or fuel. (Temin payum, 2013) Various parts of this plant have been predominantly employed by Indian tribes for the treatment of various disorders like diabetes, inflammation, rheumatism, toothache, microbial infections, and diarrhea. (Hajra PK, 1997)

MATERIAL AND METHOD:

Collection of sample:

The spice Z*anthoxylum rhetsa* was collected from a local spice shop in Mahim, Mumbai. Both, the fruit and seed, were used for analysis.

Chemicals:

Ethanol, DPPH˙ [2, 2-diphenyl-1-picrylydrazyl], butylated hydroxytoluene (BHT), Folin–Ciocalteu reagent, sodium carbonate, Gallic acid were all acquired from S.D. Fine Chemicals. All were of analytical grade.

Preparation of extract:

The fruit and seed of the spice were separated, washed with distilled water and dried at room temperature. The dried spices were then ground using a mechanical grinder and stored in an air-tight container for further study. 0.5g of the spice powder was weighed and mixed with 10cm^3 water and was extracted by keeping it on a rotatory shaker for an hour. Whatman filter paper no. 41was used to filter the solution and the filtrate was diluted up to 25cm^3 using the solvent. The same procedure was carried out using ethanol as a solvent. These solutions were then used for the study.

Preparation of reagents:

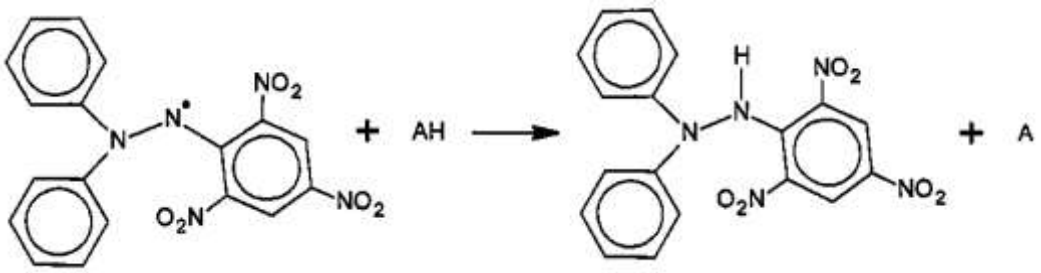
1) DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution was prepared by dissolving 48mg of DPPH in 250mL of ethanol.
2) BHT (Butylated hydroxytoluene) solution was prepared by dissolving 100mg of BHT in 25mL of ethanol.
3) Gallic acid solution was prepared by dissolving 100mg of gallic acid in 25mL of ethanol.
4) 100 cm^3 of 1M Na_2CO_3 solution was prepared by dissolving 10.59g of sodium carbonate with distilled water in a 100 cm^3 standard flask.
5) 10 cm^3 of FC reagent was prepared by mixing 1 cm^3 of the reagent with 9 cm^3 of distilled water.

Methodology/ Antioxidant assays:

1) DPPH˙ free radical scavenging assay:

The free radical scavenging capacity was evaluated using 2, 2-diphenyl-1-picrylydrazyl (DDPH) according to the procedure described by (Blois MS, 1958)

DPPH˙ is a stable compound, commercially available as organic nitrogen radical with a deep purple color. On accepting hydrogen from the corresponding antioxidant, DPPH˙ loses its original purple color and turns yellow in color. Following reaction shows the free radical scavenging activity of an antioxidant:



2,2-diphenyl-1-picrylydrazyl 2,2-diphenyl-1-picrylydrazine

1cm^3 of DPPH$^{\cdot}$ reagent was added to a different amount of spice extracts (200-1000µL) and the total volume was made up to 4 cm^3 using distilled water. The solutions were then kept in dark for the duration of 30minutes and the absorbance was measured at 530nm using a UV-Visible spectrophotometer. The same procedure was repeated for the preparation of control, where no extract was added. Gallic acid and BHT were used as standards. Percentage scavenging capacity was calculated using the following formula:

$$\% \text{ scavenging} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Further, the corresponding IC_{50} values were calculated. Results were obtained in triplicates.

2) Total phenolic content by Folin-Ciocalteu method:

The total phenolic content in the sample was evaluated using Folin-Ciocalteu method as described by (Singleton VL, 1965)

FCR is believed to contain hetero-polyphosphotungstates-molybdates and works by oxidation-reduction mechanism. (Aruna P Jadhav, 2012) (Chen, 2015)The reaction takes place only in basic conditions. Under acidic conditions, the mixture of FCR and phenolic compounds is stable but is unstable in alkaline solution. (Sushil Chandra Sati, 2010) Also, FCR is non-specific for phenolic compounds as it can be reduced by non-phenolic compounds (vitamin C). (Aruna P Jadhav, 2012) Therefore, sodium carbonate, is used to provide an alkaline environment. Phenolate ion is formed on dissociation of phenolic compounds, in alkaline conditions, which further reduces the FCR and renders the solution blue.

Electron transfer reaction occurs between reductants and Mo (VI), as it is believed that molybdenum is easier to reduce in the complex:

$$\text{Mo (VI)} + e^{-} \rightarrow \text{Mo (V)}$$

There is no coordination complex formed as the blue compound formed between phenolate ion and FCR are independent of the structure of phenolic compounds. (Aruna P Jadhav, 2012) The colour intensity of the blue chromogen thus formed can be measured by taking its absorbance at 550nm.

RESULT:

1) DPPH˙ free radical scavenging assay:

DPPH assay is used to determine the free radical scavenging capacity, and is related to electron donation capacity of an antioxidant. As antioxidants react with DPPH˙, it is reduced to DPPH-H, and as a result, the absorbance is reduced. Hence, as the concentration of the spice increased its scavenging activity also increased. (Figure 1-4) The scavenging capacity of *Z. rhetsa* was comparable to that of the standards. (Figure 5-6)

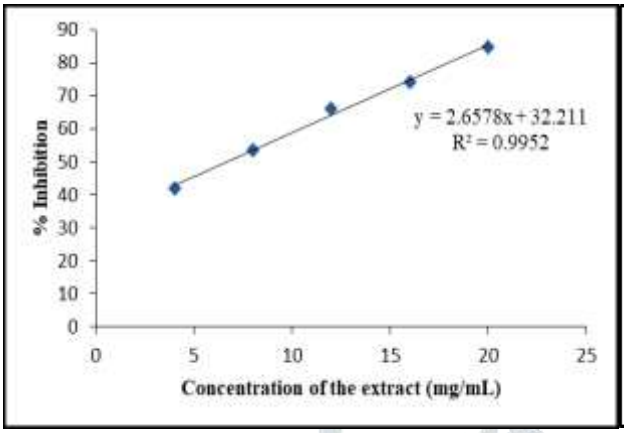


Figure 1

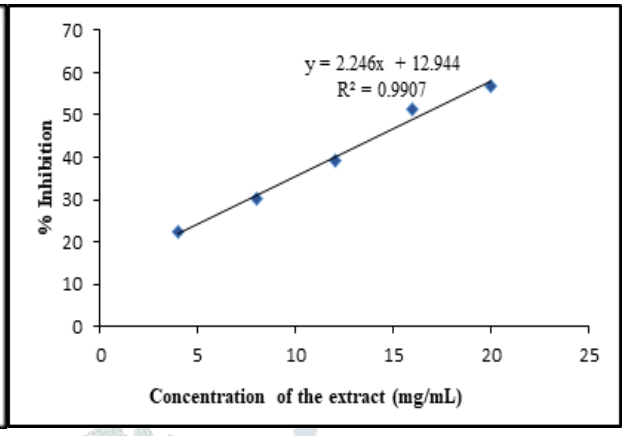


Figure 2

Percentage scavenging capacity of aqueous and ethanolic extract of the fruit of *zanthoxylum rhetsa* respectively.

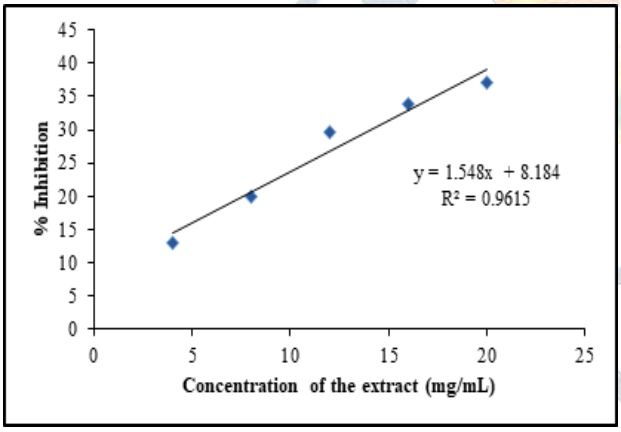


Figure 3

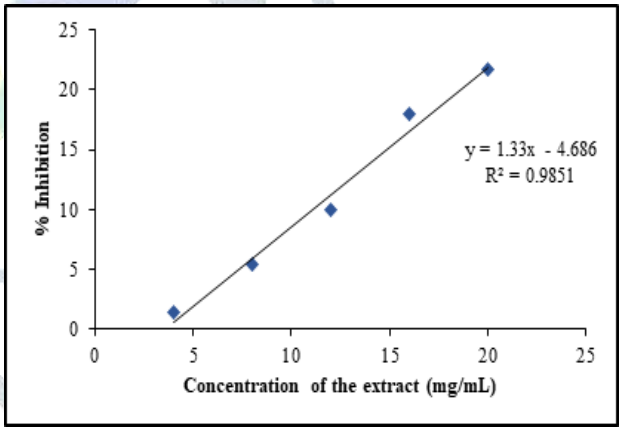


Figure 4

Percentage scavenging capacity of aqueous and ethanolic extract of the seed of *zanthoxylum rhetsa*

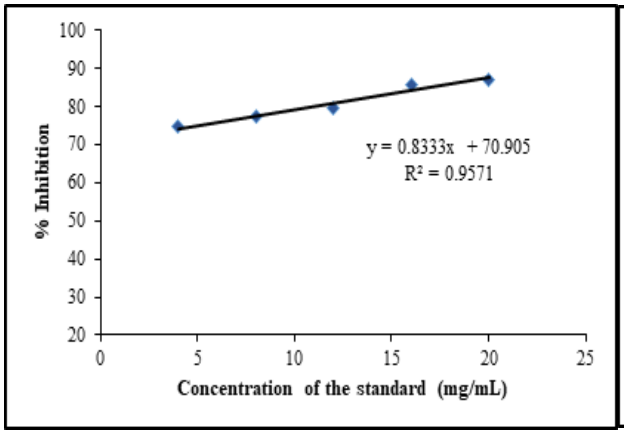


Figure 5

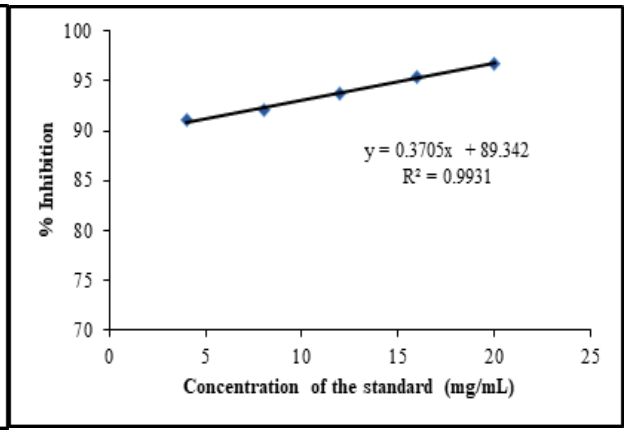


Figure 6

Percentage scavenging capacity of standards, BHT and gallic acid, respectively.

The corresponding IC50 values of the fruit of *zanthoxylum rhetsa* calculated from mean scavenging capacity (Figure 7) for aqueous extract and ethanolic extract was found to be 6.69 ± 1.326 mg/mL and 16.49 ± 1.825 mg/mL respectively. While, for the seed it was found to be 27.01 ± 1.09 mg/mL and 41.12 ± 3.284 mg/mL respectively. (Figure 8)

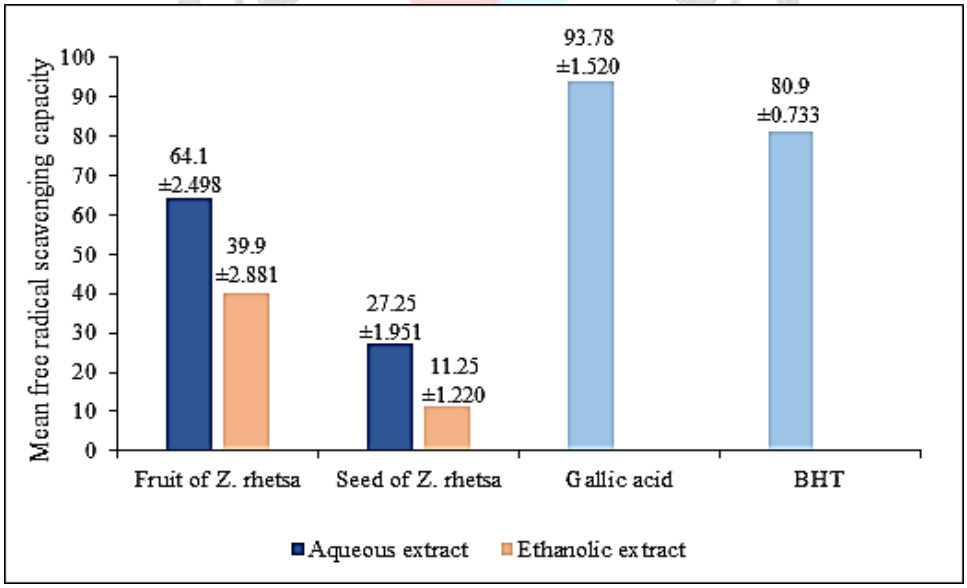


Figure 7: Mean percentage Scavenging activity of fruit and seed of *Z. rhetsa* and the standards

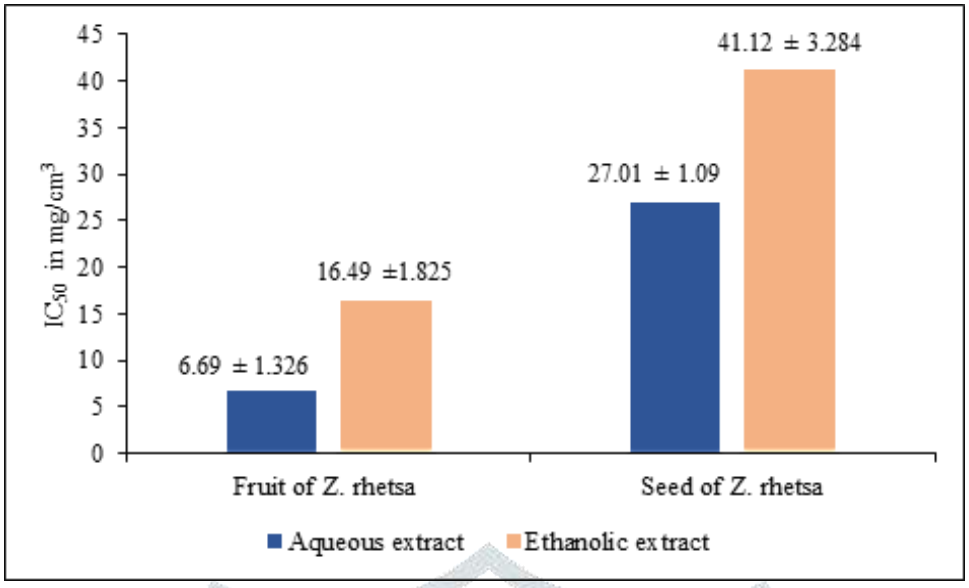


Figure 8: IC_{50} values of fruit and seed of *Z. rhetsa*

2) Total phenol content by Folin-Ciocalteu method:

Folin-Ciocalteu reagent was employed for the determination of total phenolic content of *Z. rhetsa*. The absorbance values of different concentrations of standard gallic acid solution were obtained at 550nm.(Figure 9) The total phenolic content of the extracts were determined using the calibration curve of gallic acid. (Table 1)

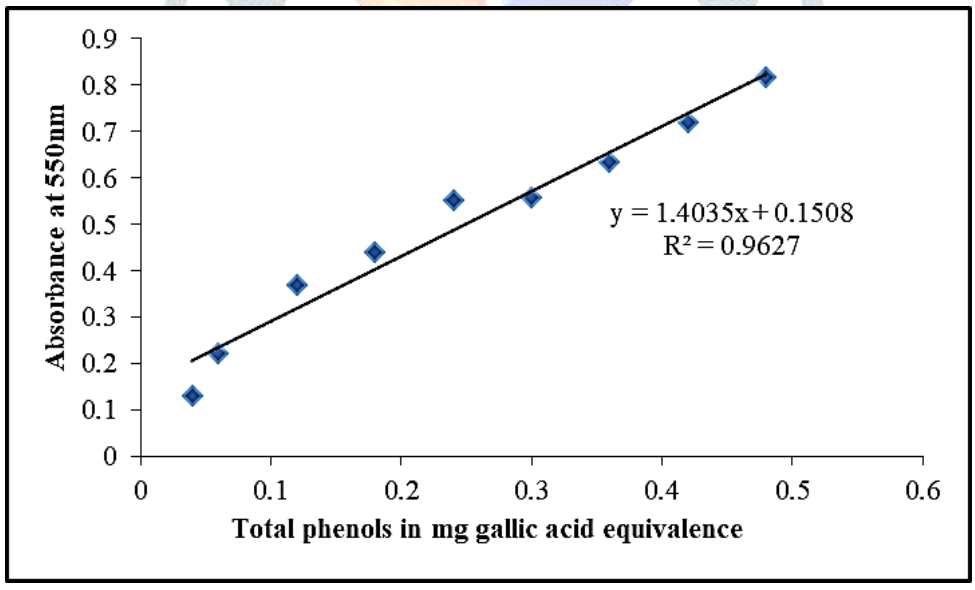


Figure 9: Total phenolic content of Gallic acid.

	Z. rhetsa fruit		*Z. rhetsa* seed	
	Aqueous extract	Alcoholic extract	Aqueous extract	Alcoholic extract
Absorbance	0.364	0.315	0.153	0.266
Concentration	0.1553	0.1217	0.0106	0.0881
Gallic acid equivalent (mg GAE/g of the dried powder)	15.53 ± 1.138	12.17 ± 0.657	1.06 ± 0.566	8.81 ± 0.925

Table 1: Total phenolic content of fruit and seed of *Z. rhetsa*

It was expressed in mg GAE/g of dried spice powder. It was evaluated to be15.53 ± 1.138 mgGAE/g and 1.06 ± 0.566 mgGAE/g for the aqueous extract of fruit and seed respectively, while it was 12.17 ± 0.657 mgGAE/g and 8.81 ± 0.925 mgGAE/g for the ethanolic extract. (Figure 10)

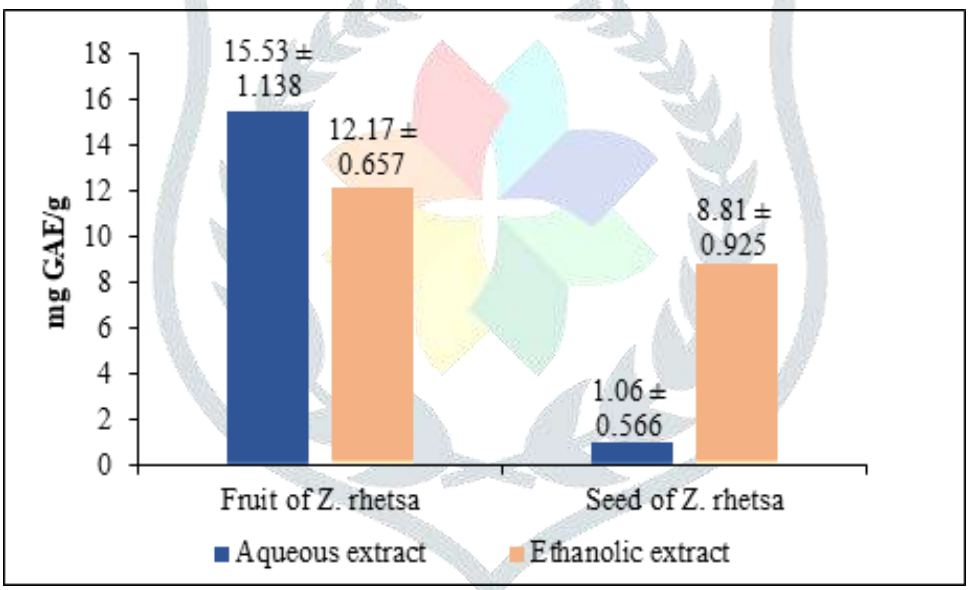


Figure 10: Total phenolic content fruit and seed of *Z. rhetsa*

DISCUSSION:

1) DPPH· free radical scavenging assay:

DPPH assay was used to determine the free radical scavenging capacity, and is related to electron donation capacity of an antioxidant. BHT and Gallic acid were used as standards, which gave a linear plot with the equation y = 0.8333x + 70.905, R^2 = 0.9571 (Figure no. 5) and y = 0.3705x + 89.342, R^2 = 0.9931(Figure no. 6) respectively.

It was observed that the aqueous extracts of both, fruit and seed of *Z. rhetsa* showed greater free radical scavenging activity i.e. 64.1±2.489% and 27.25±1.951% respectively. While their ethanolic extracts showed lesser scavenging activity i.e. 39.90±2.881% and 11.25±1.220% respectively. Thus, the corresponding IC_{50} values for the same were less as compared to that of their ethanolic extracts.

2) Total phenol content by Folin-Ciocalteu method:

Folin-Ciocalteu reagent was employed for the determination of total phenolic content. Gallic acid was used as a standard compound and the total phenolic content was expressed as mg Gallic acid equivalence per gram.

The calibration curve showed linearity for gallic acid in the range 0.04 to 0.48mg/mL with a correlation coefficient (R^2) of 0.9627. (Figure 9) Extract of 20mg/mL concentration was used for the determination of total phenolic content.

Fruit of *Z. rhetsa* showed the higher total phenolic content, 15.53 ± 1.138 mgGAE/g, in the aqueous medium, whereas the seed showed the higher, 12.17 ± 0.657 mgGAE/g, in the ethanolic medium.

CONCLUSION:

The study showed that the spice *Z. rhetsa* showed significant antioxidant property which was comparable to the standards, BHT and Gallic acid. There was a linear relationship between the concentration and the free radical scavenging activity of the spice. The fruit of *Z. rhetsa* showed higher amount of percentage scavenging capacity than the seed. The radical scavenging capacity of the aqueous extracts of both, the fruit and the seed, was greater than that of their ethanolic extracts.

The concentration of the extracts and their total phenolic content showed a linear co-relationship. There is also a linear co-relation between antioxidant capacity and total phenolic content of the fruit extracts but not in the case of the seed extracts. Hence, it can be inferred that the radical scavenging capacity of the fruit of spice, *Z. rhetsa,* is majorly due to the phenolic content. Whereas, in the case of seed the radical scavenging capacity is not only due to phenolic content but also due to some other probable phytochemicals.

Based on the results, it is possible to conclude that the spice shows higher antioxidant capacity in the aqueous medium. The obtained results might be considered sufficient for the further studies of the isolation and identification of the phytochemicals.

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