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Evaluation of *in-vitro* antioxidant property and total phenolic content of *Zanthoxylum rhetsa* fruit extracts

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Abstract
Zanthoxylum rhetsa (Roxb.) DC of family Rutaceae is used in many traditional medicines as a therapy for diabetes, spasmodic, diuretic and inflammatory complications. Many chemical constituents like terpenoids, xanthyletin, sesamin, alkaloids, flavonoids and sabinene that are present in this plant attribute to various medicinal activities. Different parts of fruit of *Zanthoxylum rhetsa* were analyzed for their antioxidant property and total phenolic content. The antioxidant property was estimated using DPPH (Diphenyl picryl hydrazyl) radical scavenging activities of various extracts of fruit, seed, and seed coat. The antioxidant property of the fruit, seed and the seed coat were compared with the standard antioxidant chemical constituents like Propyl gallate (PG) and butylated hydroxytoluene (BHT). The total phenolic content in *Zanthoxylum rhetsa* fruits was determined using Folin- Ciocalteau reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/ml Gallic acid equivalent (GAE) using the standard curve equation: y=0.061x-0.002: R^2=0.998. The total phenolic content varied from 0.027±0.0019 to 0.0392±0.011 mg/mL GAE in various parts of fruit extracts. The study reveals that fruits of *Zanthoxylum rhetsa* could be used as a good source of natural antioxidants in pharmaceutical industry.

Keywords: *Zanthoxylum rhetsa*, natural antioxidant, DPPH radical scavenging

Introduction
Zanthoxylum is the largest and most widespread genera and are native to warm temperate and subtropical areas belongs to the family Rutaceae. *Zanthoxylum* is represented by around 12 species in India which include *Z. acanthopodium, Z. armatum, Z. ovalifolium, Z. tomentellum, Z. rhetsa, Z. myriacanthum, Z. pseudoxyphyllum, Z. tetraspermum, Z. burkillianum, Z. nitidum, Z. scandens and Z. oxyphyllum* [8]. *Zanthoxylum rhetsa DC*, commonly called prickly ash, or Satin wood, is a moderate sized deciduous tree that grows up to 20-30 m tall with pale corky bark commonly found in shaded moist localities of tropical regions of India at an altitude of 1,800 mand spreading leafy branches. It is one of the important non wood forest products (NWFP) of Karnataka [6]. *Zanthoxylum rhetsa* fruits are edible and widely used in traditional medicine for their analgesic, anticonvulsant, anti-inflammatory and antitumor properties [16].
Medicinal plants, which form the supportive pillar of traditional medicine, have in the last few decades gained much importance in the pharmaceutical studies. Chemical studies carried out on *Zanthoxylum* species have revealed the occurrence of alkaloids, aliphatic and aromatic amides, lignans, coumarins, sterols, carbohydrate residues etc. Some of these metabolites have reported cytotoxic, molluscicidal, anticonvulsant, anti-sickling, anesthetic, antibacterial, anti-hypertensive and anti-inflammatory properties. The fruits of *Zanthoxylum* and their pericarps are used as a peppery spice in both sweet and savoury preparations and the seeds rich in oil are often used as fertilizer or fuel. The seeds are also used in folk medicine for the treatment of stomach ache, toothache, abdominal pain, ascariosis, diarrhoea and dysentery. It is also used to promote digestion and as a topical antibacterial agent for treatment of infected wounds. Traditional healers have used different species of the *Zanthoxylum* for treatment of a wide range of disorders, including urinary and venereal diseases, rheumatism and lumbago. Being the rich source of minerals and vitamins, the wild edible plants can reduce the risk of diseases like diabetes, cancer, coronary heart disease, neurodegenerative ailment [15]. The alkaloids commonly found in species of the genus *Zanthoxylum* belong to groups of isoquinoline (mainly benzo [c] phenanthridine-type) and quinoline and can be demonstrated as models for anti tumor drugs also.
The world health organization has estimated that 80% of people worldwide rely on herbal medicines for their primary health care needs [26].

During stress, our bodies produce more reactive oxygen species than enzymatic antioxidants which leads to cell death. Generally, the production of these reactive species is slow and is naturally removed by antioxidant enzymes present in the cell [9]. It becomes necessary to use antioxidants as free radical scavengers for removal of ROS to reduce cell damage. Antioxidant molecules have the ability to capture, deactivate and repair the damage caused by free radicals [1]. Antioxidants are widely used in food industries are synthetic materials such as BHT, BHA, Metabisulphite and ethoxyquin. Various studies have shown that usages of synthetic antioxidants are noxious to humans; hence these synthetic antioxidants have to be completely substituted by natural antioxidants of plant derivation [2, 23].

Antioxidant is a molecule that impedes the oxidation of other molecules. Oxidation is the chemical reaction that transfers electron or hydrogen from substances to the oxidizing agent. These oxidation reactions can produce free radicals which in turn flinch chain reactions; it can cause impairment or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit or delay other oxidative reactions.

The antioxidants which are available commercially are mostly synthetic substances that prevent oxidative stress at lower concentrations and they have the drawback of side effects when taken in *vivo*. Secondary metabolites produced by the plants are the rich source of natural antioxidants and they include carotinoids, flavonoids phenolics and ascorbic acids. These chemicals are produced by the plants for their sustenance and will have the capability to delay or prevent the diseases caused by oxidative stress without any side effects. So, the search for plant derived antioxidants from natural source is gaining much attention of many researchers and medical practitioners.

Presently, there has been an intensified concern globally to ascertain antioxidant compounds which are pharmacologically effective. Plants produce substantial amount of antioxidants such as flavonoids, phenolics and Polyphenolics (tannins) to prevent the oxidative stress [7]. Plants have long been a source of exogenous (dietary) antioxidants. It is believed that 66% of the world's plant species have therapeutic significance and all these have amazing antioxidant potential [13]. The enthusiasm in the exogenous plant antioxidants was first evoked by the identification and subsequent isolation of ascorbic acid constituent from plants [22]. Since then, the antioxidant potential of plants has been recognized noteworthy because increased oxidative stress has been identified as a major factor in the development of many life threatening diseases. In addition, supplementation with exogenous antioxidants or boosting of endogenous antioxidant defenses of the body has been observed to be a promising technique to counter the undesirable effects of oxidative stress [11].

Phenols are potent antioxidants and possess one or more aromatic rings with one or more hydroxyl groups. Phenolic compounds are ubiquitous bioactive compounds distributed in the higher plants ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins [18]. Plant phenolics are largely involved in defense against ultraviolet radiation, aggression by pathogens or predators and it also contribute to plant's colours. Polyphenols have been reported to have antioxidant property [25]. Studies have shown that intake of foods containing phenolic compounds can curtail the risk of heart diseases and lessen the progress of atherosclerosis through acting as antioxidants towards LDP [4, 12]. Phenolic compounds have been known to act as antioxidants due to their capability to donate hydrogen atoms from their hydroxyl groups to radicals and form phenoxyl radicals, hence playing an important role in the prevention of oxidation at cellular and physiological level [21].

Antioxidants have diverse biological effects as a result of their antioxidant activity [4]. Total phenolic content is one of the methods for determining the antioxidant property of plant extracts as they are well known radical scavengers and singlet oxygen quenchers [17]. Antioxidant properties can also be evaluated using 1, 1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay. This assay was first described by Blois, 1958 [3] and was later modified by many researchers. It is one of the most extensively used methods for plant samples. Hence, the present study was undertaken to estimate the total phenolic content and antioxidant activity of *Zanthoxylum rhetsa* fruits.

Materials and methods

The fresh and healthy fruit of *Zanthoxylum rhetsa* has been collected from Kolhapur of Maharashtra state in India. These plant materials were dried at room temperature. The dried material was subjected to size reduction to get coarse powder manually. This powder was extracted with methanol, Carbon tetrachloride, Acetone and Hexane in soxhlet extraction. The extracts were concentrated to a small volume and allowed to dry. After drying, the respective extracts were weighted and percentage yield of extractives were determined.

Standard 1, 1-diphenyl-2-picrylhydrazine (DPPH), Propyl gallate (PG) and butylated hydroxytoluene (BHT) were used for Antioxidant assays of fruit extracts of *Zanthoxylum rhetsa by* radical scavenging assay method. Standard reagent like Folin –Ciocalteau reagent and Gallic acid was used for estimation of total phenolic content. Analytical grade chemicals were used in this study.

Preparation of the crude extract:

The seed, seed coat and the entire fruit were homogenized separately into fine powder. The powder of each samples were taken in a clean, round bottomed flask and soaked in hexane, carbon tetrachloride, acetone and methanol. Then these coarse powders were subjected to Soxhlet extraction in 4 different solvents as mentioned for its extraction as per standard procedure according to their polarity. The filtrate was kept in an open space to evaporate the solvent thus crude extract was obtained.

Evaluation of Antioxidant property

The free radical scavenging effect of different parts like seed, seed coat and the entire fruit extracts were assayed *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) according to the method described by Cunndet *et al.*, 1997 [5], reported by Kapoor IP *et al*., 2009 [10]. 3.0 mL of methanolic solution of different parts of fruit (0.5-2.0 mg/mL) was mixed thoroughly with 1 mL of 0.1 mM solution of DPPH (in methanol). The reaction mixture was shaken well and incubated at room temperature for 30 minutes in dark. The absorbance of the mixed solution was measured at 517 nm on double beam Shimatzu UV- spectrophotometer against methanol as blank Control (without any additive) and standards BHT and PG (in place of extracts) were also analyzed. The free radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{AC - AT}{AC} \times 100$$

Ac = Absorbance of control sample
At = Absorbance of test sample

Estimation of Total Phenolic Content (TPC)

TPC were measured using Folin–Ciocalteau method described by Singleton and Rossi [19], reported by Maurya *et al*., 2010 [20]. Gallic acid stock solution (0.05 mg/ml) was prepared by dissolving 0.005g of Gallic acid in 100 ml of distilled water. Various dilutions of Gallic acid were prepared from this stock solution. Calibration curve was plotted by mixing 1ml of aliquots of 0.00125 to 0.1 mg/ml of Gallic acid solution with 2.5 ml of FC reagent (diluted tenfold) and 2 ml of sodium carbonate (7.5%). The absorbance was measured after 30 minutes at room temperature at 765 nm.

Concentration of 1 mg/mL of extracts was prepared in methanol and 1 mL of sample was used for the determination of total phenolic content. For each sample, duplicate assays were performed. Total phenolic content was calculated as Gallic acid Equivalent by the following equation;

$$T = C * M/V$$

Where T is the total phenolic content in mg/mL of extracts as GAE, C is the concentration of gallic acid established from calibration curve in mg/mL, V is the volume of extract solution in mL and M is the weight of extract in grams.

Statistical analysis: All experiments were carried out in duplicate and the results were expressed as mean ± SD. The correlation coefficient between DPPH assay and total phenolic content by Folin–Ciocalteau method was done.

Results and Discussion

Percentage yield of extracts (as shown in table 1 and Figure 1)

Seed extracts: The percentage yield of seed extracts of *Zanthoxylum rhetsa* in different solvents are 7.442, 7.114, 6.914 and 5.694 for hexane, carbon tetrachloride, acetone and methanol respectively.

Seed coat or pericarp extracts: The percentage yield of pericarp extracts of *Zanthoxylum rhetsa* in different solvents are 0.234, 0.914, 2.188 and 11.038 for hexane, carbon tetrachloride, acetone and methanol respectively.

Whole fruit extracts: The percentage yield of entire fruit extracts of *Zanthoxylum rhetsa* in different solvents are 3.69, 3.406, 7.1 and 11.32 for hexane, carbon tetrachloride, acetone and methanol respectively.

Table 1: Percentage yield of *Zanthoxylum rhetsa* fruit extracts

Solvents	Fruit	Pericarp	Seed
Methanol	11.32	11.038	5.694
Acetone	7.1	2.188	6.914
Carbon tetrachloride	3.406	0.914	7.114
Hexane	3.69	0.234	7.442

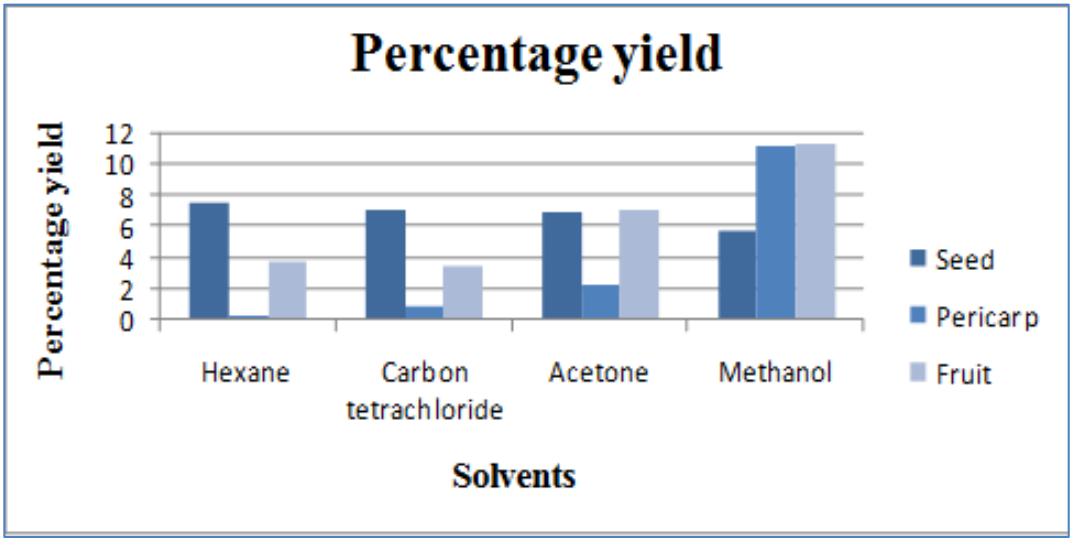

Fig 1: Percentage yield of crude extracts from various parts of fruits of *Zanthoxylum rhetsa*.

Antioxidant property

DPPH radical scavenging activities of methanolic extracts of fruit (seed, seed coat and the whole fruit) were measured as percentage inhibition of free radical. The antioxidant activity was estimated at different concentrations of extract namely 0.5mg/mL, 1mg/mL, 1.5mg/mL and 2 mg/mL. The free radical scavenging activity of the extracts ranged from 85 to 95% as shown in the Table 2. Different parts of *Zanthoxylum rhetsa* fruit showed DPPH scavenging effect. The scavenging effect increased, as the concentration increased as shown in figure 2. The free radical scavenging activity of the extracts was compared with the synthetic antioxidants like BHT and PG. The methanolic extract of fruit and seed coat showed strong Antioxidant activity in comparison with the standard antioxidant, butylated hydroxytoluene (BHT), but lower than that of Propyl gallate (PG).

Table 2: Percentage inhibition of free radical scavenging activities of different parts of fruit of *Zanthoxylum rhetsa*

Concentration mg/mL	PG	BHT	Seed	Seed coat	Whole fruit
0.5	97.45	90.30	84.19	95.72	85.63
1.0	97.81	91.24	85.40	95.81	94.15
1.5	97.90	91.44	86.08	96.24	95.06
2.0	98.04	92.35	86.93	96.40	95.26

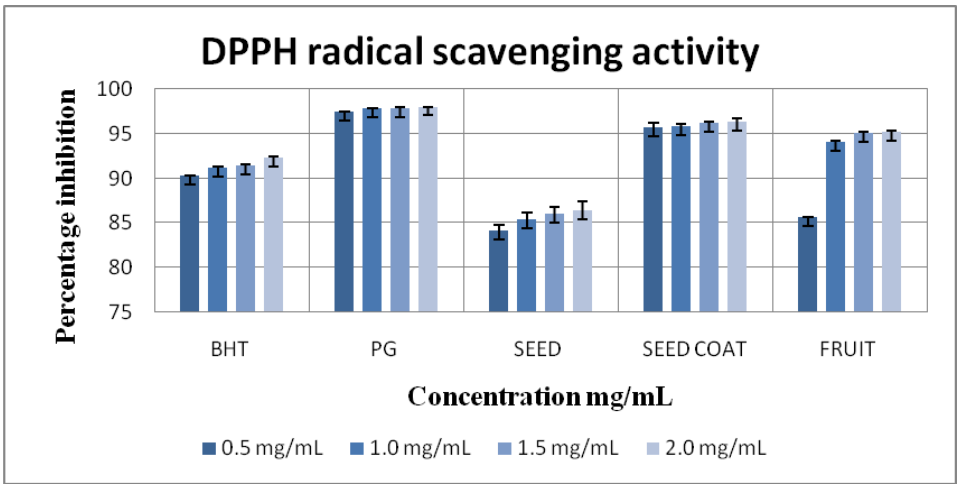

Fig 2: DPPH radical scavenging activity of various parts of *Zanthoxylum rhetsa* fruit. Values are the means of duplicate determination ± standard deviation.

Total phenolic content of *Zanthoxylum rhetsa*

The total phenolic content was determined using Folin-Ciocalteau reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/ml gallic acid equivalent. The calibration curve showed linearity for gallic acid in the range of 0.00125 to 0.1 mg/ml with a correlation coefficient (R^2) of 0.998 (Figure 3). Concentration of 1 mg/mL of extracts was used for the determination of total phenolic content. The absorbance of various fruit extracts of *Zanthoxylum rhetsa* were shown in Table 3. Table 4 shows the concentration of phenols in seed, seed coat and the whole fruit. Maximum phenolic content was found in whole fruit extract of *Zanthoxylum rhetsa* followed by its seed coat and seed alone.

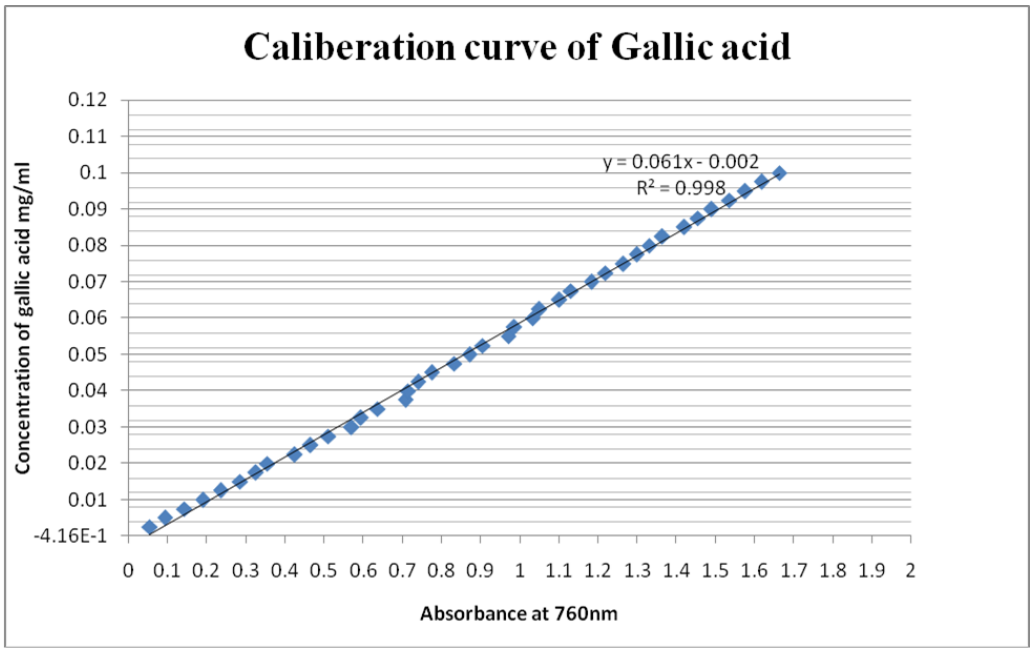

Fig 3: Standard curve obtained for Gallic acid

Table 3: Absorbance of *Zanthoxylum* fruit extracts

Concentration (mg/ml)	1 mg/ml
Seed	0.481
Seed coat	0.579
Fruit	0.677

Table 4: Total phenolic content in various fruit extracts of *Zanthoxylum rhetsa*

Concentration mg/ml	1 mg/ml
Seed	0.027±0.0019
Seed coat	0.033±0.005
Fruit	0.0392±0.011

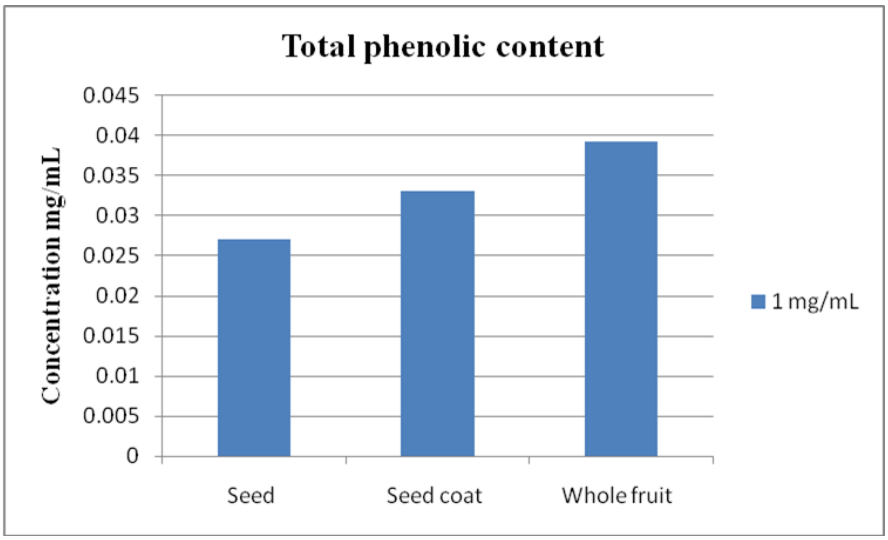

Fig 4: Total phenolic content of *Zanthoxylum rhetsa*

Polyphenols forms a large group of phytochemicals with excellent antioxidant properties and play a major role as free radical scavengers required in the maintenance 'redox homeostasis' responsible for various degenerative diseases. Phenols are potential antioxidants. Estimation of total phenolics is essential in the study of antioxidant properties of plants. Total phenolic content was assessed as equivalents of Gallic acid. TPC were measured using Folin–Ciocalteau method described by Singleton and Rossi [19]. Among the different parts of fruit of *Zanthoxylum rhetsa* studied, maximum phenolic content was found in whole fruit extract of *Zanthoxylum rhetsa* (0.0392±0.011 mg/mL GAE) followed by its seed coat (0.033±0.005 mg/mL GAE) and seed (0.027±0.0019 mg/mL GAE).

The antioxidant activity using DPPH was first described by Blois, 1958. DPPH (Diphenyl picryl hydrazyl) is commercially available stable free radical which is purple in color. Antioxidant molecules when incubated with DPPH neutralize it to Diphenyl hydrazine. The completion of neutralization is determined by the change in DPPH radical color to either colorless to pale yellow. The degree of neutralization of four different concentrations of fruit extracts were measured at 517 nm, which gave the scavenging potential of antioxidants in tested extracts.

DPPH radical scavenging activities of different parts of fruit of Zanthoxylum *rhetsa* varied from 80-95%. The seed coat showed highest antioxidant property (96% of DPPH inhibition) followed by whole fruit (95% of DPPH inhibition) and seed (86% of DPPH inhibition).

The results of antioxidant capabilities by DPPH assay was correlated to total phenolic content estimation by Folin-Ciocalteau method. The present study indicates a moderate positive linear relationship between two assays. Antioxidant ability of plant extracts to scavenge free radicals not only depends on extract composition but also depend on the conditions of the test used. The presence of different antioxidant components in plant extracts makes it relatively difficult to measure each antioxidant component separately. Therefore Single method is not sufficient to determine the antioxidant property of plant extract to understand various modes of action of antioxidants. Various workers have reported antioxidant property of Zanthoxylum species by DPPH method. Narayanaswamy *et al*, 2012 [14] reported that stem bark of *Zanthoxylum tetraspermum* can be a potential natural antioxidant.

It should be emphasized that there is a great difference between antiradical and antioxidant activity. The antiradical activity characterizes the ability of compounds to react with free radical while antioxidant activity represents the ability to inhibit the process of oxidation. Consequently, the tested system using a stable free radical gives information on radical scavenging or antioxidant activity. In order to obtain the information about the real antioxidant activity with respect to lipids or food stabilization, it is necessary to carry out on real products.

Conclusion

Zanthoxylum has proven to be a very valuable *genus* to the discovery and utilization of medicinal and agrochemical natural products. This is a difficult *genus* with many different, similar and not well-researched species. So, there is a need of research to develop its economic value, its regeneration potentiality and for conservation strategies. The present study revealed that extract of different parts of fruits of *Zanthoxylum rhetsa*, which contain highest amount of phenolic content, also exhibited the greatest antioxidant activity. The seed coat alone and the whole fruit showed highest phenolic content (0.033±0.005 mg/mL GAE and 0.0392±0.011 mg/mL GAE) also showed highest antioxidant activity (96% of DPPH inhibition and 95% of DPPH inhibition). The free scavenging activity of *Zanthoxylum rhetsa* may be due to hydroxyl groups existing in the phenolic groups. Hence, the fruit of *Zanthoxylum rhetsa* could be a good source of antioxidant due to its free radical scavenging activity. But further studies are required to clarify *invivo* potential of this plant.

Abbreviation: mm – millimetre; % - percent; mg- milligram; nm – nanometre;

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