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Photoprotective properties of *Zanthoxylum rhetsa*: An *in vitro* analysis

Ramesh Kumar Santhanam[1], Syahida Ahmad[2], Faridah Abas[1], Intan Safinar Ismail[1], Yaya Rukayadi[1] and Khozirah Shaari[1*]

[1]*Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia*
[2]*Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia*

ABSTRACT

This research highlights the photoprotective potential of a traditional medicinal plant, Zanthoxylum rhetsa. The photoprotective effect was measured based on the sunscreen protection factor (SPF value) and UV absorption spectrum of various solvent fractions of the plant. The ethyl acetate fraction had the highest SPF value (13.36±0.12) followed by butanol (8.6±0.08), at a test concentration of 100µg/ml. All of the fractions exhibited broad UV spectrum absorption covering both the UVB and UVA regions. The free radical scavenging properties were assessed using the selected antioxidant assays, namely, diphenylpicrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging assays. In both assays, the ethyl acetate exhibited the highest activity followed by the butanol fraction. The DPPH and NO free radical scavenging activity were highly expressed in the ethyl acetate fraction with IC_{50} values of140±1.20 µg/ml and 50 ± 0.35µg/ml, respectively. The total phenolic and flavonoid content were also determined for all the fractions. These results indicate that the bark extract of Z. rhetsa has great potential for use as a natural active ingredient in broad spectrum sunscreen and anti-ageing cosmetic preparations.

Keywords: *Zanthoxylum rhetsa*; Photoprotective; Sunscreen protection factor(SPF); Broad spectrum ;Antioxidant.

INTRODUCTION

Photo ageing generally occurs due to continuous exposure to UV radiation which stimulates the discharge of free radicals in the skin. The free radicals activate the NF-kB and AP-1 pathways and in reverse inhibit the TGF-B pathway, finally promoting the expression of matrix metalloproteinases (MMPs) and inflammatory cytokines [1]. About 95% of the sun's UV-radiation reaching the earth's surface is long wave UV radiation (UVA, 320-400 nm). UVA radiation is able to penetrate the deeper layers of the epidermis and dermis skin, resulting in wrinkle formation and premature ageing [2]. A lower percentage of UV radiation (UVB, 280-320 nm) reaches the earth surface but the radiation is more intense, enough to cause skin reddening and sunburns. Preventing or reducing exposure to UVA and UVB radiation will reduce signs of ageing, deep wrinkles, solar elastosis, coarse textures, telangiectasias and skin cancer [1, 2]. Numerous synthetic organic compounds that absorb UV radiation have been developed to protect skin from the damaging effects of sunlight. These synthetic compounds are either UVA- or UVB-absorbing compounds and hence are used in combination to provide a broad-spectrum UV screen. The necessity to provide high sun protection factor (SPF) and blocking efficiency against both UVA and UVB wavelengths has led to the development of sunscreen formulations with multiple added sunscreen chemicals [3]. Most chemical compounds used in sunscreen products are active in the UVB region while only a few chemicals block the UVA region. It is also a point of growing concern that the safety of many of these compounds has not been established, especially for long-term human use. For example, although broad-spectrum protection is achievable using titanium dioxide, zinc oxides or iron oxides, these are promoted on the basis that they may be less harmful than organic sunscreen absorbers. It should be noted that microfine (nanoparticles) titanium dioxide as a sunscreen product also has no long-term safety data [4]. Hence, there is a need to search for alternative source of effective and safer photoprotective agents that can be utilized in sunscreen products as well as in cosmetic preparations. In general, whole plant extracts have shown

better potential as photoprotective agents due to their complex chemical composition and broad UV absorption spectra as well as their antioxidant power. Although they have not completely replaced the dominance of synthetic materials, the use of these botanical extracts is becoming more common. For example, green tea and black tea have been reported to ameliorate adverse skin reactions following UV exposure, while *Aloe vera* gel assists in cell regeneration [5-7]. Interests in adding natural ingredients in sunscreen formulation are mainly driven by the 'back to nature' movement and the promise of equal or greater efficiency with lesser side effects by using these materials. Furthermore, there is also a growing interest in natural antioxidants present in medicinal plants[8]or herbal extracts that can reduce oxidative damage for use in cosmetic science as beauty products and to maintain the physiological balance of the human skin [9].

Zanthoxylum rhetsa (Roxb.) DC (Syn. *Zanthoxylumbudrunga*, Fam. Rutaceae) is a spiny, decidious tree that can grow up to 25-30 m tall. The tree is locally known as 'batangberduri' (Malay) or 'tirphal' (Indian), and is native to tropical and subtropical areas including India, Malaysia and other parts of South Asia [10]. Like other species of the same genus, *Z. rhetsa* has thorns or spines on the stem. All parts of the plant are used medicinally. For example, a paste made from the hard spines of *Z. rhetsa* is used for pain relief and to increase lactation in nursing mothers [11]. The bark has been reported to be a remedy for stomach and chest pains, and to treat snake bites. The fruits are a spice, a digestive and an appetizer as well as used to treat urinary diseases and rheumatism [12]. Meanwhile the leaf decoction is used to treat intestinal worm infections [13]. Previous phytochemical investigations on the plant have revealed the presence of amides [14], quinolone and quinazolinealkaloids ,lignans and terpenoids[15, 16, 17, 18]. Scientific investigations on *Z. rhetsa* have shown it to have antimicrobial [14],antinociceptive and anti-diarrhoeal[19], cytotoxic [20, 21]and anti-inflammatory properties.In particular, it was shown that a possible underlying mechanism of the anti-inflammatory property of *Z. rhetsa* bark*via* inhibition ofiNOS and COX-2 in the NF-κB pathway[22]. The seed extract of *Z. rhetsa* was also recently reported to exhibit good UV-protective activity [23] which roused our interest to investigate other parts of the plant for a similar activity. Therefore this study aimed to evaluate the photoprotective potential of the plant through the measurement of its sunscreen protection factor (SPF value) and UV absorption spectrum. In addition, total phenolic, total flavonoid content and the antioxidative effectof the plant was also investigated *via* assessment of its free radical scavenging properties.

EXPERIMENTAL SECTION

Chemicals and Reagents

All chemicals used were of analytical grade; 1, 1-diphenyl-2-picrylhydrazyl, epigallocatechingallate (EGCG), Ascorbic Acid, n-(1-naphthyl) ethylenediaminedihydrochloride,quercetin, gallic acid, FollinCiocalteu's phenol reagent were obtained from Sigma -Aldrich (USA). Sodium nitroprussidedihydrate was purchased from Fluka (USA). Sulphanilamide, aluminium chloride from Friedemann Schmidt (UK), sodium chloride was purchased from Kollin Chemicals. Sodium carbonate was purchased from NacalaiTesque. Solvents used for extraction were all prepurified by distillation.

Plant Material

Bark material of *Z. rhetsa* was collected from Pangkor Island, Malaysia. A voucher specimen (No. SK2226/13) was deposited at the Herbarium of Institute of Biosains, Universiti Putra Malaysia.

Extract Preparation

The bark materialwas cutinto small pieces, dried and ground into fine powder (910 g) using a Wiley mill. The powdered material was then extracted with 100% methanol using ultrasound-assisted extraction technique [24]. The extract was filtered and dried under vacuum at 40-50^{o}C yielding 65 g of crude extract. The crude extract was then resuspended in methanol and subjected to liquid–liquid partitioning into organic solvents of varying polarities, starting with hexane, chloroform, ethyl acetate and butanol. The resultant solvent fractions were dried under vacuum and lyophilized to yield 14 g hexane, 17.4 g chloroform, 2 g ethyl acetate and 5.3 g butanol fractions, all of which were stored at -20^{o}C prior to analysis.

Sun Protection Factor (SPF) Measurements

The *in vitro* SPF value was obtained by following the method [25]. Briefly, the absorbance of a methanolic solution (100 µg/ml) of the test sample was determined on a UV-Visible spectrophotometer at 290-320nm. Methanol was used as a blank and measurements were made in triplicates. The SPF value was then calculated by using the formula:

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where: EE (λ) is the erythemal effect spectrum
I (λ) is the solar intensity spectrum
EE (λ) x I(λ) are constants.
Abs (λ) is the absorbance of test sample
CF is the correction factor (= 10)

Measurement of UVA/UVB Absorption Spectrum
The UV absorption spectrum for each test sample (100 µg/ml in methanol) was measured on a UV-Visible spectrophotometer using 1 cm quartz cell, over a wavelength range of 200-400 nm. The absorption spectrum of the test samples was compared to that of EGCG prepared with the same concentration [26].

DPPH Free Radical Scavenging Assay
The free radical scavenging activity of the extracts on DPPH radical was determined using the method defined in literature [27]. Briefly, a 0.1 mM DPPH in methanol was prepared and each test sample was prepared in methanol at various concentrations (0.02 – 0.1 mg). A reaction mixture made up of 1:1 ratio of DPPH solution to the test sample solution was mixed thoroughly using a vortex mixer and left in the dark at room temperature. After 30 min incubation, the absorbance of the mixture was measured on a spectrophotometer at 517nm. Ascorbic acid was used as positive control and measurements were made in triplicates. The DPPH radical scavenging activity was calculated using the formula:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where:
$Abs_{control}$ is the absorbance of DPPH radical + methanol
Abs_{sample} is the absorbance of DPPH radical + sample extract/standard

Nitric Oxide Free Radical Scavenging Assay
Nitric oxide (NO) radical scavenging activity of the extracts was determined using the method described previously [28]. 60 µl aliquots of the test samples, prepared in various concentrations, were placed into a 96-well flat-bottomed microplate. To each well, 60 µL of 10 mM solution of sodium nitroprusside, prepared in phosphate buffered saline (PBS), were added and the plate was incubated under normal light conditions at room temperature for 150 min. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediaminedihydrochloride, 2.5% H_3PO_4) was added into each well in order to measure the nitrite content. After the chromophore was formed at room temperature over 10 min, the absorbance at 577nm was measured spectrophotometrically. Ascorbic acid was used as positive control and triplicate readings were obtained. The free radical scavenging activity was calculated using the formula:

NO radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where:
$Abs_{control}$ is the absorbance of NO radical + methanol
Abs_{sample} is the absorbance of NO radical + sample extract/standard

Total Phenolic Content
The total phenolic content for all the solvent fractions of *Z. rhetsa* was determined using the Folin-Ciocalteu method [29]. Briefly, 50µl of extract (1 mg/ml) in methanol was mixed with 50 µl distilled water, 50 µl of 10% FollinCiocalteu's phenol reagent and 50µl of 1 M sodium carbonate solution in a 96-well microtitre plate. Methanol was used as blank. Reaction mixtures were incubated for 60 minutes at room temperature and protected from light. The absorbance of the reaction mixture was measured at 750 nm with a microplate reader (SpectraMaxPlus). The total phenolic content was determined using a standard curve created with gallic acid (6.25, 12.5, 25, 50, 100, 200, 300, 400, 500 µg/ml) as standard. Results are expressed as milligram Gallic Acid Equivalents (GAE) per gram of dry plant extract. All tests were replicated in triplicates.

Total Flavonoid Content
The total flavonoid content for all fractions of *Z. rhetsa* bark was determined using spectrophotometric method [30]. 100µl of the plant extract (1 mg/ml) and standard solutions of quercetin(6.25, 12.5, 25, 50, 100, 200, 300, 400, 500 µg/ml) in methanol solution were mixed with 100µlof2% $AlCl_3$solution . Then the reaction mixtures were incubated for an hour at room temperature. The absorbance was measured using SpectraMax Plus microplate reader at λ_{max} 415 nm. Triplicate readings were obtained for all the samples. Total flavonoid contents were expressed as mg Quercetin Equivalent (QE) per gram of dry plant extract.

RESULTS AND DISCUSSION

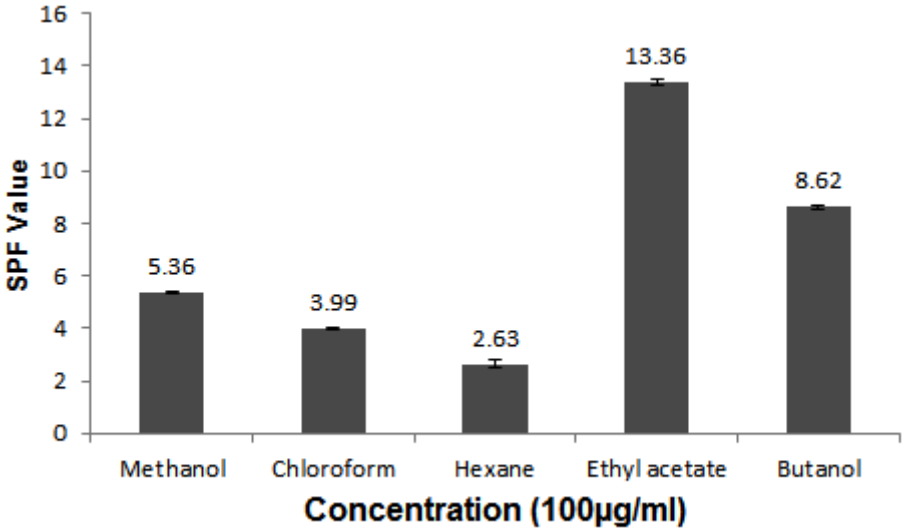
Sun Protection Factor (SPF) Value

The effectiveness of a sunscreen is measured as a function of their SPF value, defined as the ratio of the least amount of ultraviolet energy required to produce minimal erythema or burning on sunscreen protected skin to the amount of energy required to produce the same erythema on unprotected skin [31].Thus the SPF value indicates the ability of a sunscreen product to reduce UV-induced erythema.

The *in vitro* SPF value was determined by a spectrophotometric method using the UVB region which is considered to be the region of greatest incidence during the day in which people are exposed longer to the sun's radiation. From Figure 1, it can be seen that the SPF value of ethyl acetate (13.36±0.12) and butanol (8.62±0.08) fractions showed higher SPF values than the rest of the solvent fractions, at a test concentration of 100µg/ml. The other plant extract which are commonly used as the main ingredient in several sunscreen products available now in the market shows less SPF value, especially the extract of *Camellia sinensis* has an SPF value of 18.10±0.05 and Aloe vera extract has an SPF value of 1 at a test concentration of 200µg/ml [32]. Thus it was clearly visible that the SPF value of the ethyl acetate and butanol fractions of *Z. rhetsa*bark has appreciably enhanced sun-blocking properties. The chloroform and hexane fractions of *Z. rhetsa* however showed lesser ability in sun-blocking, with lower SPF values of 3.98±0.06 and 2.63±0.16, respectively. Earlier, it was also reported [23] that a formulation containing seed extract of *Z. rhetsa* has an SPF value of 1.09 with an ultra-boot star rating 2 which approaches towards sunscreen activity.

Figure 1. Sunscreen Protection Factor value of various *Z.rhetsa* bark solvent fractions

Data are expressed as mean± standard deviation. (n=3)



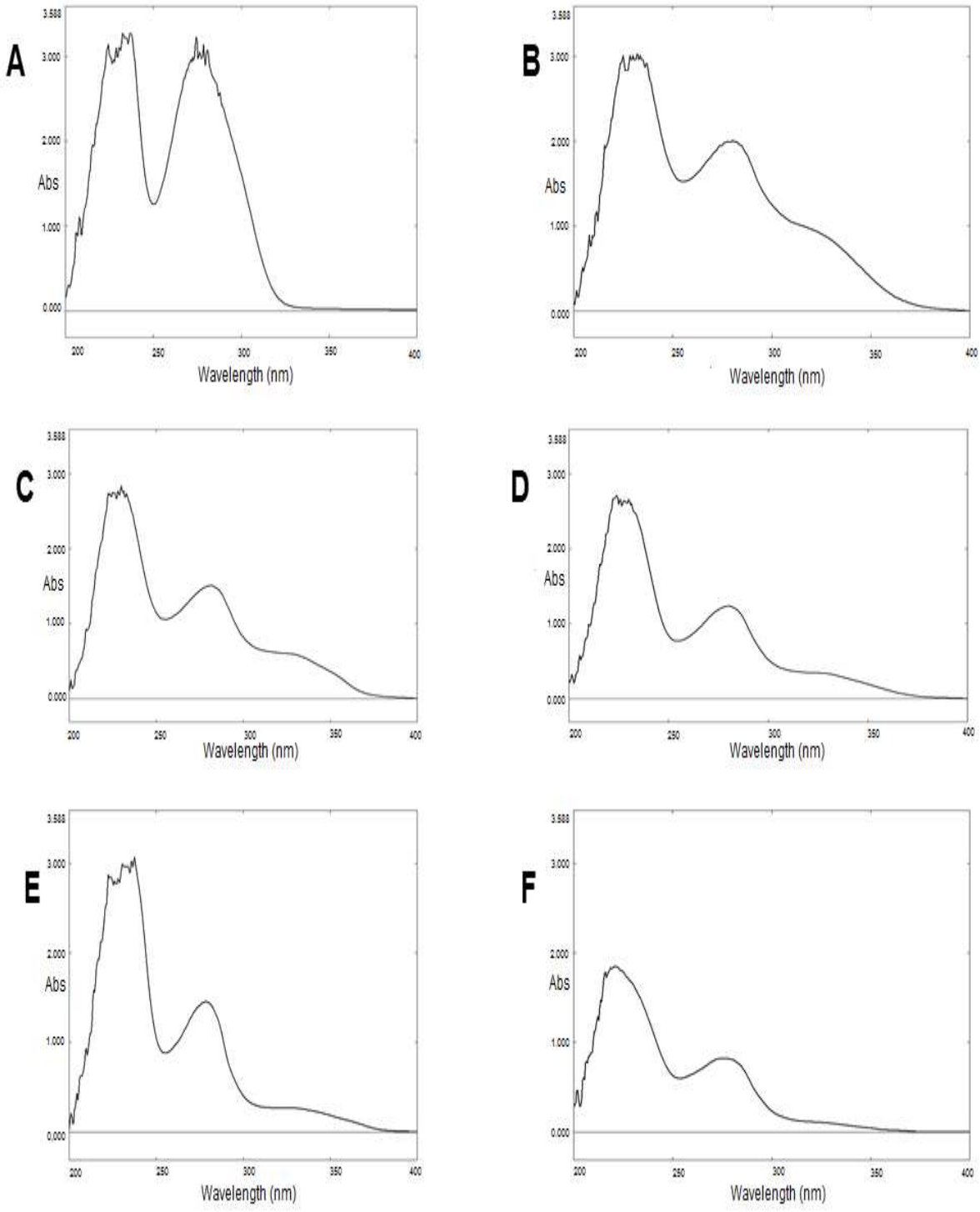
UVAbsorption Spectra

In order to protect the skin from harmful UV radiation, a sunscreen product should have high SPF value and a wide range of absorbance within the UV region of 290 to 400nm.Figure 2 shows the UV absorption capacity of all the fractions in comparison to Epigallocatechingallate (EGCG), measured at a test concentration of 100µg/ml over a wavelength range of 200-400nm. The results revealed that, while it did show high UVB (280 – 320nm) absorption, EGCG exhibited only low UVA(320 -400nm) absorption. In contrast, the ethyl acetate and butanol fractions of *Z. rhetsa* exhibited good UVB absorption and moderate UVA absorption capacities. This indicated that *Z. rhetsa* may be a potential sun-blocking ingredient to be incorporated in broad UV spectrum sunscreen products.

DPPH Free Radical Scavenging Activity

Assays based on the scavenging effect of DPPH have been widely used to measure the antioxidant potential of a substance. The DPPH free radical scavenging capacity of the solvent fractions were compared with Ascorbic acid is shown in Figure 3, while Table 1 lists their IC_{50} values. The data showed that the ethyl acetate and butanol fractions exhibited the highest free radical scavenging activity when compared with all other *Z. rhetsa* test fractions with IC_{50} values of 140 ± 1.20 and 168 ± 0.76µg/ml, respectively.

Figure 2. UV Absorption spectraof various *Z.rhetsa* bark solvent fractions at 100µg/ml. EGCG(A) Ethyl acetate (B), Butanol(C) , Methanol(D), Chloroform(E) and Hexane (F) fractions



Nitric Oxide Free Radical Scavenging Activity

Continuous exposure of the skin to UV radiation leads to the liberation of NO free radicals, an important reactive oxgen species (ROS) that plays a vital role in instigating inflammation, melanogenesis, photoageing, immunosuppression and erythema. Various parts of *Z. rhetsa* have been reported to be used as a remedy for inflammation in traditional medicine. In fact, the effectiveness of the essential oils obtained from the seeds of *Z. rhetsa* as a topical application for inflammatory dermatosis has been confirmed in a clinical trial study [33]. In the current study, the NO free radical scavenging property of the test fractions of *Z. rhetsa* bark was analyzed and the results compared to that of ascorbic acid The percentage inhibition and IC_{50} values are shown in Figure 4 and Table 1, respectively. From Figure 4 it was clearly observed that all the fractions showed more than 50% NO free radical

scavenging property at concentrations exceeding 200µg/ml. These findings supported previous literature reports on the high antioxidant and anti-inflammatory properties of this species.

Figure 3.Percentage of DPPH free radical scavenging activity of various *Z.rhetsa* bark solvent fractionsin comparison to ascorbic acid (positive control)

Data are expressed as mean ± standard deviation. (n =3)

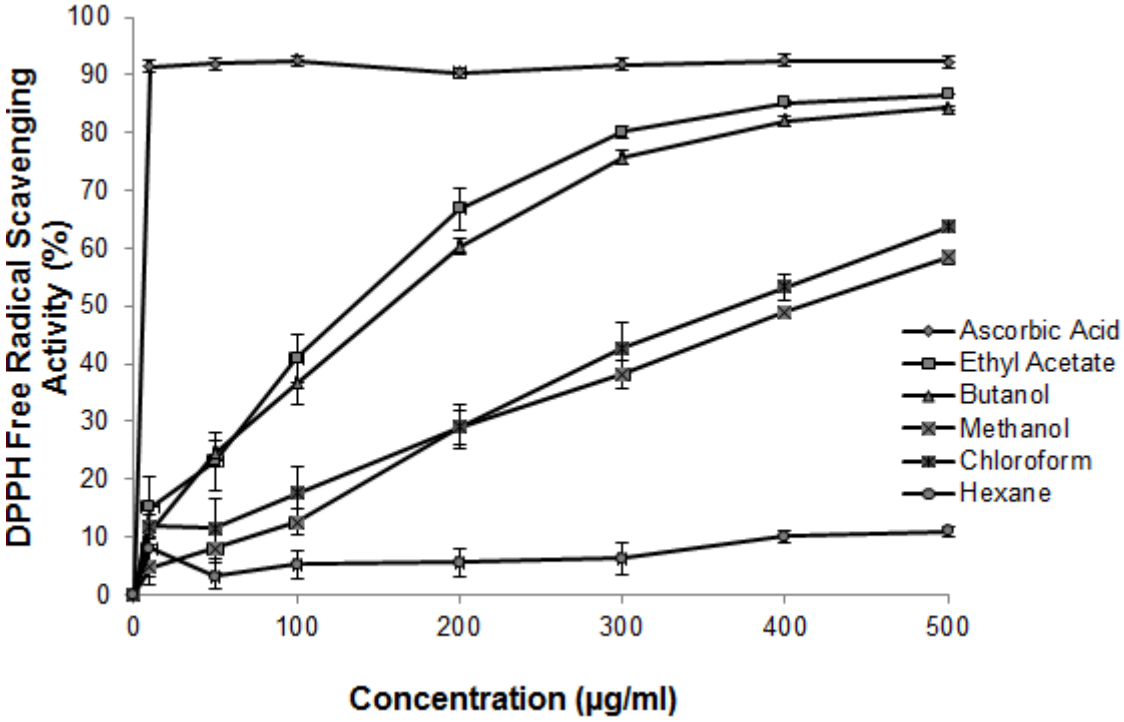


Figure 4.Percentage of NO Free radical scavenging activity of various *Z.rhetsa* bark solvent fractions in comparison to ascorbic acid (positive control)

Data are expressed as mean ± standard deviation. (n = 3)

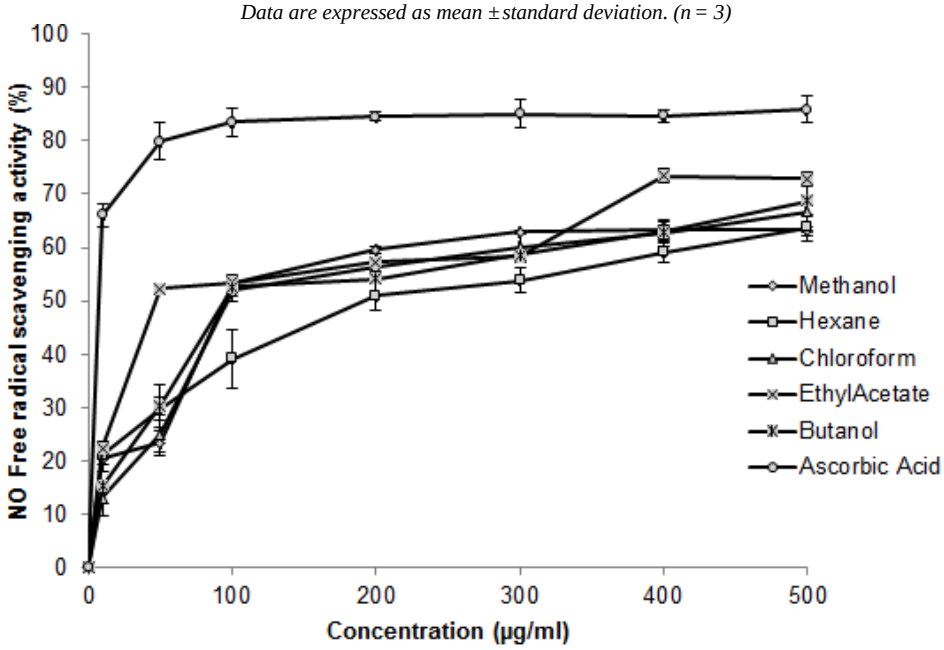


Table 1. IC_{50} values for DPPH and NO free fradical scavenging activity for various *Z. rhetsa* bark solvent fractions

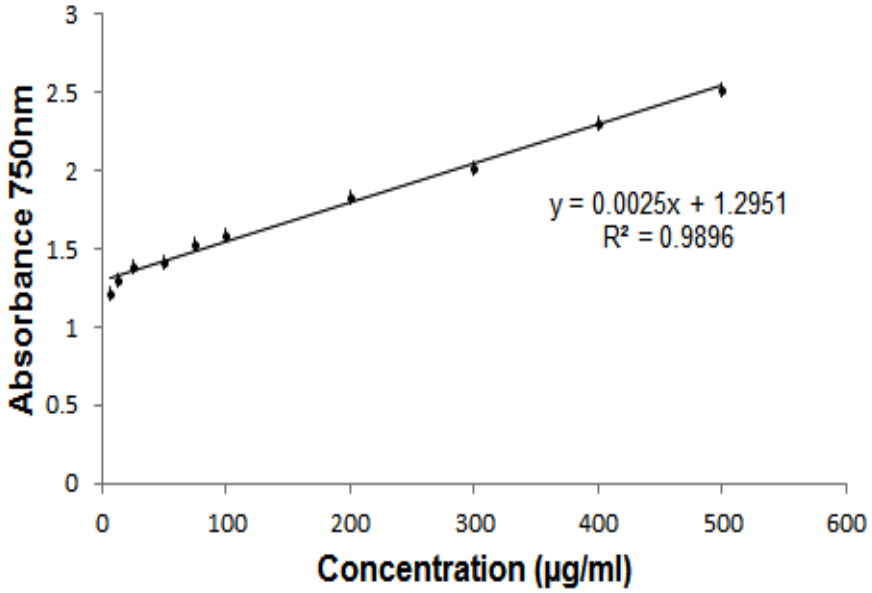
Calculations were done using GraphPad Prism software version 6.02

	IC_{50}(µg/ml)					
Free radical Scavenging Assay	**Ascorbic Acid**	**Ethyl Acetate**	**Butanol**	**Methanol**	**Chloroform**	**Hexane**
DPPH	<5	140 ±1.20	168±0.76	505± 1.54	434 ±1.08	>600
NO	23 ±0.97	50 ±0.35	69±0.74	79±0.52	70±1.34	197±1.11

Total Phenolic Content

The total phenolic content of all the solvent fractions of *Z. rhetsa* was examined using FolinCiocalteu's method and expressed in terms of mg Gallic Acid Equivalent (GAE)/g of the dry plant extract using gallic acid standard calibration curve shown in Figure 5. The phenolic compounds of the fractions reduced the phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids present in the Folin's reagent to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) under alkaline condition in the presence of sodium carbonate. The existence of blue oxides depicts the quantity of phenolic compounds. The total phenolic content obtained for the solvent fractions are tabulated in Table 2. Previous research reported that the total phenolic content of the fruits of *Z. rhetsa*was 0.061±0.29g/100g FW [34]. In the present study, the ethyl acetate and butanol fractions of *Z. rhetsa* bark showed highest total phenolic content of 20.47±0.09 and 14.14±0.185 mg GAE/g of the dry weight of extract, respectively. Lower amounts were detected in the chloroform (7.95±0.27mg GAE/g extract) and hexane (5.38±0.21mgGAE/g extract) fractions. These results suggested that the rich phenolic content of the ethyl acetate and butanol fractions are possibly responsible for the photoprotective property of *Z. rhetsa*

Figure 5.Gallic Acid Standard Calibration Curve



Total Flavonoid Content

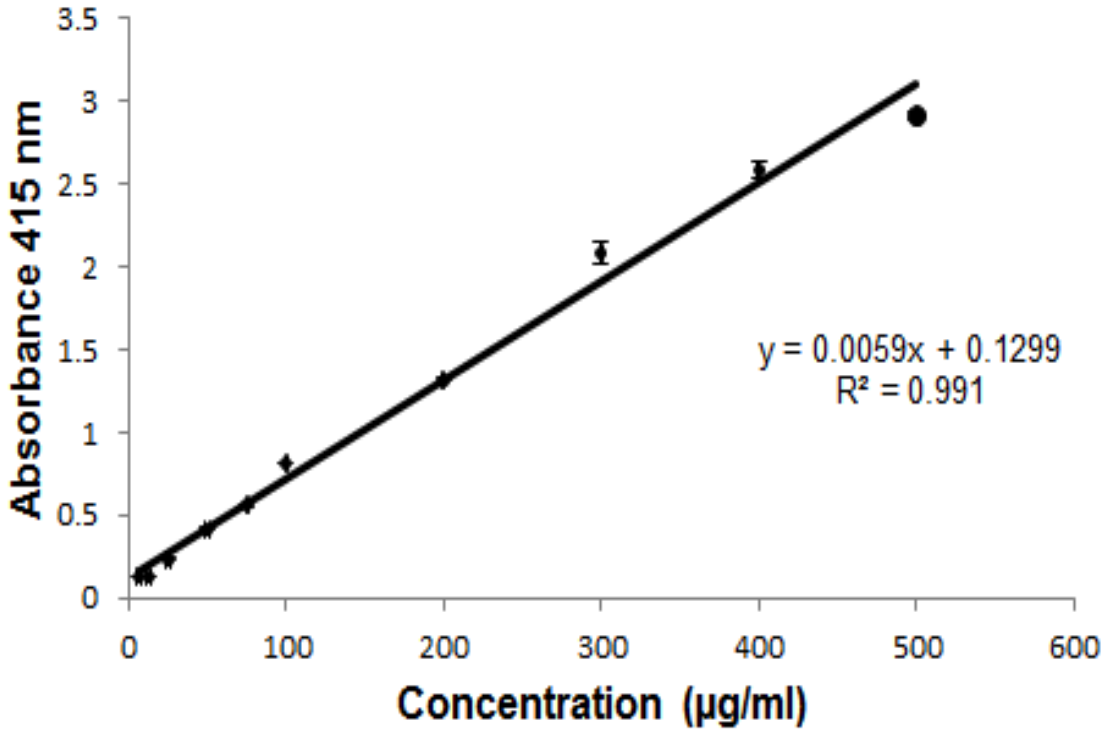
The total flavanoid content of all the solvent fractions of *Z. rhetsa* bark was analysed using aluminium chloride method and their values were expressed in terms of mg Quercetin Equivalent (QE)/g of the dry plant extract using the quercetin standard calibration curve shown in Figure 6. Flavonoids present in the plant extract will form stable acid complexes with aluminium chloride by binding it with their C-4 keto group and either the C-3 or C-5 hydroxyl group. In addition, aluminium chloride also forms acid labile complexes with the ortho dihydroxyl groups in the flavonoid A- or B-ring. In the present study, the total flavonoid content (Table 2) of the butanol fraction of *Z. rhetsa* bark was calculated to be 3.07±0.24 mgQE/g dry weight of the extract while the content in the ethyl acetate fraction was calculated to be 1.59±0.12mgQE/g dry weight of the extract. The total flavonoid contents in chloroform and hexane fractions were insignificant

Table 2. Total phenolic and total flavonoid contents of *Z. rhetsa*bark extracts expressed in terms of mg of GAE/g of plant extract and mg of quercetin/g of plant extract

Respectively Data are expressed as mean±standard deviation

Sample	mg of GAE /g of Plant Extract	mg of QE/g of Plant Extract
Ethyl Acetate	20.47±0.09	1.59±0.12
Butanol	14.14±0.18	3.07±0.24
Methanol	9.45±0.29	1.52±0.04
Chloroform	7.95±0.27	-
Hexane	5.38±0.21	-

Figure 6.Quercetin Standard Calibration Curve



CONCLUSION

Antioxidants from the natural source play a major role in the photoaging pathway by inhibiting the oxidative stressand retain stability by scavenging free radicals triggered by the UV rays. Researchers reported that the plant extract which possesspotential antioxidant activity and UV absorption capacity can prevent photo-aging and skin cancer [35]. The present study showed that the *Z. rhetsa* has significant antioxidant properties and UV absorption capacity, especially the ethyl acetate and butanol fractions.The high level of phenolic compounds and flavonoids present in the active solvent fractions could be responsible for theirphoto-protective effect. These results indicate that the bark extract of *Z. rhetsa* can be utilized as a natural active ingredient in broad spectrum sunscreens and anti-ageing cosmetic preparations.

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