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# Extraction, Purification and Characterization of Antioxidant Fractions from *Zizyphus spina-christi* Fruits

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**Abstract:** In this study, *in vitro* antioxidant activity, total phenolic content and concentration of flavonoids of five different extracts from the *Zizyphus spina- christi* fruits were determined using spectrophotometric methods. Antioxidant activity of extracts were expressed as percentage of DPPH radical inhibition and values were ranged from 31.76% - 90.23% which indicated that zizyphus manifested the strongest capacity for neutralization of DPPH radicals. The total phenolic content ranged from 11.04 - 56.44 mg/g expressed as quercitin equivalent. The concentration of flavonoids in the zizyphus extracts varied from 16.66 - 58.32 mg/g expressed in terms of rutin equivalent (mg of RU/g extract). Methanolic extract of zizyphus showed the highest phenolic and flavonoid concentration and strong antioxidant activity. The significant linear correlation was confirmed between the values for the total phenolic content and antioxidant activity of plant extracts. The high contents of phenolic compounds indicated that these compounds contribute to the antioxidant activity. The zizyphus fruit can be regarded as a promising candidates for natural plant sources of antioxidant with high value.

**Key words:** Antioxidant Activity · Total Phenols · Zizyphus Spina-Christi · Flavonoids

## INTRODUCTION

Modern life style habits cause many people to develop abnormally high levels of oxidative stress which caused mainly by free radicals [1]. Reactive oxygen species (ROS), which consist of free radicals such as hydroxyl ($OH^-$), superoxide ($O_2^-$), nitric oxide (NO), peroxyl ($RO_2^-$), lipid peroxyl ($LOO^-$) radical and non-free radical species such as hydrogen peroxide ($H_2O_2$), singlet oxygen ($O_2^{-1}$), ozon ($O_3$) and lipid peroxide (LOOH) are different forms of activated oxygen [2]. ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. This Ros can generate oxidative stress and produce many pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity [3]. For the protection against free radicals, organisms are endowed with endogenous (antioxidant enzymes) and exogenous defence systems. These systems unable to protect our tissues when the generation of free radicals are significantly increased [4].

In recent years, attention has been paid to the role of diet in human health. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis and cancer [3]. Plant polyphenols are a wide group of secondary metabolites that can range from simple molecules, such as phenolic acids to highly polymerized constituents such as tannins [4,5]. They are compounds with a large number of derivatives in the plant kingdom. All the phenolics, but especially flavonoids, have been reported to have multiple biological effects such as antioxidant activity [6], which can terminate or retard the oxidation process by scavenging free radicals. These antioxidants are considered as possible protection agents for reducing oxidative damage of human body from ROS [7]. Also, they act as anti-inflammatory agents [8], inhibition of platelet

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aggregation [9,10], inhibition of mast cell histamine release [11], antimicrobial activities [12]. Moreover, antioxidant phenolics have been suggested to play a preventive role in the development of cancer and heart diseases.

*Zizyphus spina-christi* is a deciduous shrub which belong to Rhamnaceae family. Fruits are commonly used in folklore medicine for the curing of various diseases [13]. They are wide-spread in the Mediterranean region, Africa, China, India, Australia and tropical America [14]. *Zizyphus spina-christi* has been used in folk medicine as a demulcent, depurative, anodyne, emollient, stomachic, blisters, astringents, bruises and mouth problems [7,10].

The aim of this investigation is to evaluate the free radical scavenging properties in *zizyphus spina-christi* fruit as well as to quantify total phenolic content and concentration of flavonoids in different polarity solvents.

## MATERAL AND METHODS

All chemical used in this study were of analytical grade and purchased from Sigma Co. USA.

Ripe *Zizyphus spina-christi* were collected from local areas in Malaysia (middle area). Washed many times by tap water then by phosphate buffer (pH 7.4), dried using reflected air current in darkness at room temperature (25°C), seeds were removed and then dried again and powdered.

Powdered fruits were homogenized in phosphate buffer (pH 7.4) using ultra homogenizer, then transferred to dark-colored 5 flasks (25 g in each flask) then mixed with 200 ml of solvents with different polarities (water, methanol, ethyl-acetate, acetone, petroleum ether) respectively and stored at 25°C. After 24 h, infusions were filtered through Whatman No.1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Each sample was extracted using Soxhlet extractor for eight hours as reported before [15]. The reflexed samples were dried *in vacuo,* the extractive value were calculated on dry weight basis from the formula given below :

$$\%\ \text{extractive value (yield\%)} = \frac{\text{weight of dry extract}}{\text{weight taken for extraction}} \times 100$$

**Dpph Based Free Radical Scavenging Activity:** The free radical scavenging activities were assayed using stable DPPH following standard method [16]. The reaction mixture contained 1.8 ml of 0.1mM DPPH and o.2 ml of each serial dilution (0.5-2) of the methanol extracts. Simultaneously, a control was prepared without sample extracts. The reaction mixture was allowed to incubate for 5 min at room temperature in the dark and scavenging activity of each fraction were quantified by decolourization at 515nm. Percentage of free radicals scavenging activity was expressed as percent inhibition from the given formula :

$$\%\ \text{Inhibition of DPPH radical} = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

**Determination of Total Phenolic Contents in the Extract:** The reaction mixture was prepared by mixing 0.5 ml methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was determined spectrophotometrically at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

**Determination of Flavonoid Concentrations in Zizyphus Extracts:** The content of flavonoids in the extracts were determined according Quettier method [16]. The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$, solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

**Evaluation of Antioxidant Activity:** The ability of the extract to scavenge DPPH free radicals was assessed by the standard method [17]. The stock solution of extracts were prepared in methanol to achieve the concentration of

1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 µg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of methanolic solution of DPPH in concentration of 1 mg/ml. After 30 min incubation in darkness at room temperature (23°C), the absorbance was recorded at 517nm.

Control sample contained all the reagents except the extract. Percentage inhibition was calculated using equation 1, whilst $LC_{50}$ values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values± standard deviation (n = 3).

**Statistical Analysis:** Data were expressed as mean ± standard errors using computerized SPSS program (version 15). Differences between means in different groups were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan s multiple range test . The differences were considered significant at level $P \leq 0.05$.

## RESULTS

Aqueous, methanol, ethyl acetate, acetone and petroleum ether extract were prepared to examine the biochemical characters of bioactive fractions of the zizyphus spina-christi. We examined the total phenolic contents, flavonoid concentration and antioxidant activity. The yield of extract obtained from 100 g of dried zizyphus fruits was determined for each extract with the Folin-Ciocalteu reagent as in Table 1. Our results showed a very high contents in methanolic extract. Similarly, the flavonoid contents were markedly higher in the methanolic extract with a value of 8.12 mg rutin equivalent/g DW compared to the other extracts as in Table 2.

The total phenolic contents in the examined zizyphus extracts using the Folin-Ciocalteu s reagent is expressed in terms of quercitine equivalent. The standard curve equation is :

$y = 9.118 x - 1.041, r^2 = 1.022$

The values obtained for the concentration of total phenols are expressed as mg of QE/ g of extract (Table 2).

The total phenolic contents in the examined extracts ranged from 11.04 to 56.44 mg QE/g. The highest concentration of phenols was measured in methanolic, acetone and water extracts. Ethyl acetate and petroleum ether extracts contain considerably smaller concentration of phenols. It is clear from these results that the total phenolic contents in zizyphus extract depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained.

The concentration of flavonoids in various extracts of zizyphus was determined. The content of flavonoids was expressed in terms of rutin equivalent. The standard curve equation :

Table 1: The yields of solid residue after extraction and evaporation from 100 g of dried zizyphus fruit.

| Extract | Yields (g) |
|---|---|
| Methanol | 27.88± 0.122 |
| Water | 18.62±0.108 |
| Ethylacetate | 2.70± 0.089 |
| Acetone | 1.99± 0.080 |
| Petrolium ether | 0.54± 0.36 |

Each value is the average of three measurements ± standard deviation.

Table 2: Total phenolic contents in zizyphus extracts expressed in terms of quercitin equivalent (mg of QE/g of extract)

| Extract | Mg of QE/g of extract |
|---|---|
| Methanol | 56.44± 1.028 |
| Water | 52.77± 1.062 |
| Ethyl acetate | 40.90± 0.881 |
| Acetone | 48.88± 0.331 |
| Petroleum ether | 11.04± 0.125 |

Each value is the average of three analyses ± standard deviation

Table 3: Concentrations of flavonoids in the zizyphus extracts expressed in terms of rutin equivalent (mg of RU/g extract).

| Extract | Mg of RU/g of extract |
|---|---|
| Methanol | 58.32± 1.033 |
| Water | 16.66±0.411 |
| Ethyl acetate | 50.71± 1.224 |
| Acetone | 57.88± 1.051 |
| Petrolium ether | 21.02± 0.308 |

Each value is the average of three analyses ± standard deviation.

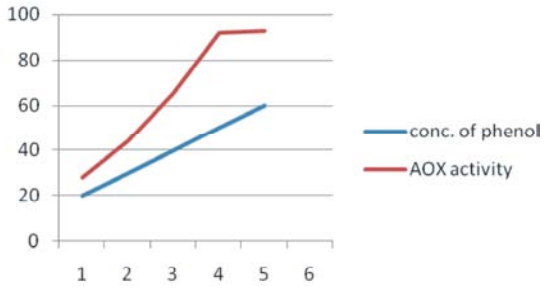


Fig 1: Methanol extract shows the highest antioxidant activity according to the concentration of standard phenols.

Y = 23.44x - o.o77, r̂ = 1. 371 mg of RU/g of extract (4)

The concentration of flavonoids in the zizyphus extract ranged from 21.02 to 58.32 mg/g. Methanolic, acetone and ethyl acetate extracts contains the highest flavonoid concentration.

The concentration of flavonoids in methanol extract was 58.32 mg RU/g, while it was 57.34 mg RU/g in acetone extract. The lowest flavonoid concentration was measured in petroleum ether and water extract as shown in (Table 3). The concentration of flavonoids in zizyphus extracts depend on the polarity of solvents used in the extract preparation.

The antioxidant activity of these five zizyphus extracts showed different values. The obtained values varied from 31.76% to 90.23%. The largest capacity to neutralize DPPH radicals was found for methanolic extract, which neutralize 52% of free radicals at the concentration of 198.72 µg/ml. A moderate activity was found for acetone, aqueous and ethyl acetate extracts. Due to low activity of petroleum ether extract, $IC_{50}$ are not calculated for it. In comparison to $IC_{50}$ values of BHA and chlorogenic acid, methanolic extract from zizyphus manifested the strongest capacity for neutralization of DPPH radicals.

## DISCUSSION

Plant polyphenols are a wide group of secondary metabolites that can range from simple molecules, such as phenolic acids to highly polymerized constituents such as tannins. Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups, therefore, the phenolic content of plants may contribute directly to their antioxidant action [18,19]. Therefore, the phenolic content of plants may contribute directly to their antioxidant action [20]. A significant linear correlation was found between the values for the concentration of phenolic compounds as shown in Table 2 and the antioxidant activity of extracts from zizyphus. Numerous investigations of the antioxidant activity of plant extracts have confirmed a high linear correlation between the values of phenol concentration and antioxidant activity [21-24].

Flavonoids are class of secondary plant metabolites with significant antioxidant and chelating properties. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups [25-28]. Methanolic and acetone extract from zizyphus have high concentration of total phenols as in Table 2 and flavonoids in Table 3, which is in correlation with intense antioxidant activity of these extracts. The concentration of flavonoids in various zizyphus extracts was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of rutin equivalent according to the equation 4 and it is the highest in the methanolic extracts because of the polarity of methanol [29,30]. The activity was determined using a methanol solution of DPPH reagent. DPPH is very stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. Usually a freshly prepared DPPH solution exhibits deep purple color which fades when anti molecules quench DPPH free radical by providing H atoms or by electron donation and convert them into a colorless branched product (i.e. 2,2 diphenyl-1-hydrazine) [32-35].

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities [36,37], their separation still remains a big challenge for the process of identification and characterization of bioactive compounds.

The antioxidant nature of zizyphus is defined mainly by the presence of a β-ring chatechol group (dihydroxylated β-ring) capable of readily donating hydrogen electron to stabilize a radical species [38]. The presence of 2,3 unsaturation in conjugation with a 4-oxo-function in the C-ring and the presence of functional groups capable of binding transition metal ions, such as iron also responsible for the antioxidant nature of zizyphus [39-41].

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