

Phytochemical Analysis, Antifungal and Antioxidant Activity of Leaf and Fruit of *Zizyphus xylopyrus* (Retz.) Willd. (Rhamnaceae)

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Abstract

In the present study, phytochemical screening, content of total phenolics, antifungal and antioxidant activity of leaf and fruit extract of *Zizyphus xylopyrus* (Retz.) Willd. (Rhamnaceae) was investigated *in vitro*. The leaf and fruit extracts were obtained by maceration process using methanol as solvent. The extracts were screened for phytochemicals by standard tests. Flavonoids, saponins, terpenoids, tannins and phenols were found in both extracts. Antifungal activity of extracts was evaluated by poisoned food technique. Leaf extract suppressed the growth of test fungi to high extent when compared to fruit extract. Antioxidant activity was determined by DPPH radical scavenging, ABTS radical scavenging and Ferric reducing assays. Both extracts exhibited radical scavenging and ferric reducing activity in dose dependent manner with marked activity exhibited by leaf extract. Folin-Ciocalteu reagent method was employed to estimate total phenolic content of extracts. Phenolic content was slightly higher in leaf extract. A positive correlation was observed between phenolic content and antioxidant activity. The plant can be used against phytopathogenic fungi and to prevent and control oxidative damage. Further studies are to be carried out to isolate active principles from the plant and to determine their bioactivities.

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INTRODUCTION

Plants play an important role for the existence and civilization of individuals since prehistoric period. Humans depend on plants for various needs such as medicine, food, spices, fodder, fuel, timber, resin, tannin and dyes (Nandini and Shiddamallayya, 2015). Plants are essential components of ethnomedicine and their use can be rightly called as ethnobotanic medicine. Throughout the world, whole plants or various parts of the plants are used traditionally for treatment of several ailments. The use of herbal medicine is most common in developing and under-developing countries. It is estimated that 80% of world's population rely on traditional medicine for primary health care needs. The dependence of people on herbal medicine is cost effectiveness, health benefits, acceptability and most importantly the accessibility. Plants are extensively used in various systems of medicine such as Ayurveda, Sidda and Unani. The therapeutic potential of plants lies in chemical components called phytochemicals that are present in plants. The most important of them are polyphenolic compounds including flavonoids, alkaloids, glycosides, saponins and terpenoids. These phytochemical are shown to exhibit bioactivities such as antimicrobial, antioxidant, antitumor

and several other pharmacological activities. Plants provide lead compounds for developing new drugs. Plant-derived drugs such as morphine, vinblastine, vincristine, quinine, digoxin, digitoxin, reserpine, taxol and artemisinin have made significant contributions in the field of medicine. Interest in medicinal plants has increased due to various drawbacks that are associated with modern medicine (Fabricant and Farnsworth, 2001; Yineger *et al.*, 2008; Pushpangadan and George, 2010; Kingston, 2011; Mishra *et al.*, 2012; Kulkarni *et al.*, 2014; Ghosh *et al.*, 2014; Alhakmani *et al.*, 2014; Kumar and Shiddamallayya, 2016).

Zizyphus xylopyrus (Retz.) Willd. (Synonym *Rhamnus xylopyrus* Retz.) is a small tree or straggling shrub armed with spines belonging to the family Rhamnaceae. The plant is distributed in India, Pakistan, China and Sri Lanka. Bark is thin and young branches are tomentose. Leaves are simple, up to 6x4.5cm, thin, alternate, elliptic or suborbicular, obtuse at apex. Flowers are in axillary cymes. Fruit is a drupe, 1.5-2.5 cm across and globose (Yoganarasimhan *et al.*, 1981; Modi *et al.*, 2014). Various chemical compounds such as quercetin, quercitrin,

vitamin C, carotene, reducing sugars, sterol, fatty acids, kempferol, rutin, xylopyrine, lupeol and betulinic acid are found in different parts of the plant (Modi *et al.*, 2014). The plant is used as wild edible and in folk medicine by various tribal communities. Bark and fruits are employed in making black dye for leather. Various parts of the plant is used in Ayurvedic and traditional medicine for the treatment of several ailments such as obesity, diabetes, snake bite, fever, diarrhoea, insomnia and digestive disorders (Yoganarasimhan *et al.*, 1981; Arinathan *et al.*, 2007; Reddy *et al.*, 2009; Jain *et al.*, 2010; Kulkarni and Adwait, 2011; Modi *et al.*, 2014; Kumar and Shiddamallayya, 2016). *Z. xylopyrus* is shown to exhibit several bioactivities such as antimicrobial (Sameera and Mandakini, 2015), anti-diarrhoeal (Singhal and SenthilKumar, 2011), anti-ulcer (Sharma *et al.*, 2013), antioxidant (Sharma *et al.*, 2013), analgesic (Mishra *et al.*, 2012), anti-inflammatory (Mishra *et al.*, 2012), wound healing (Jain *et al.*, 2015), anti-depressant (Sharma *et al.*, 2009) and antisteroidogenic effect (Dhanapal *et al.*, 2012). In the present study, we screened methanolic extract of leaf and fruit of *Z. xylopyrus* for phytochemicals and determined antifungal and antioxidant activity of leaf and fruit extracts.

MATERIALS AND METHODS

Collection and Identification of Plant

The plant materials were collected during January 2015 in and around Sagara, Shivamogga district, Karnataka. The plant was identified by referring flora (Yoganarasimhan *et al.*, 1981).

Extraction of Leaf and Fruit

The leaves and fruits were separated from the plant materials, washed well using clean water, dried under shade and powdered. Extraction was carried out by maceration process where known quantity of the powdered plant materials (10g) was immersed in 100ml of methanol in clean conical flasks. With occasional stirrings, the flasks were left at room temperature for 48 hours followed by filtering the contents of flasks through muslin cloth followed by Whatmann No. 1. The filtrates were evaporated to dryness at 40°C to get extracts (Kekuda *et al.*, 2015).

Phytochemical Analysis of Leaf and Fruit Extracts

The extracts were screened for detection of phytochemicals namely alkaloids, tannins, flavonoids, steroids, glycosides, saponins, terpenoids and phenols were detected by standard phytochemical tests (Bhandary *et al.*, 2012; Mir *et al.*, 2013; Yusuf *et al.*, 2014).

Antifungal Activity of Leaf and Fruit Extracts

The antifungal effect of leaf and fruit extracts of *Z. xylopyrus* was determined by Poisoned food technique. Test fungi were inoculated aseptically at the centre of control (without extract) and poisoned (0.5mg extract/ml of medium) potato dextrose agar plates followed by incubating the plates at 28±2°C for 72 hours. The diameter of fungal colonies in mutual perpendicular directions was measured using a ruler. Antifungal activity, in terms of inhibition of mycelial growth of test fungi, was determined using the formula:

$$\text{Inhibition of mycelial growth (\%)} = (C - T / C) \times 100$$

where C and T denotes the diameter of colonies in control and poisoned plates respectively (Kekuda *et al.*, 2015).

Antioxidant Activity of Leaf and Fruit Extracts

DPPH Radical Scavenging Activity

Leaf and fruit extracts and ascorbic acid (6.25-200µg/ml) and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical solution (0.004%) were prepared in methanol. 1ml of different concentrations extracts was mixed with 3ml of DPPH radical solution in clean and labeled tubes. The tubes were kept in dark for 30 minutes at room temperature. The absorbance of each tube was measured in spectrophotometer at 517nm. Methanol replacing the extract/ascorbic acid served as control. Scavenging activity (%) of each concentration of extracts/ascorbic acid was calculated using the formula:

$$\text{Scavenging of DPPH radicals (\%)} = (C - T / C) \times 100$$

where 'C' and 'T' denotes to absorbance of DPPH control and absorbance of DPPH in presence of extract/ascorbic acid. The IC₅₀ (Inhibitory Concentration) value was calculated which indicates the concentration of extract/ascorbic acid required to scavenge 50% of free radicals (Kekuda *et al.*, 2015).

ABTS Radical Scavenging Activity

Different concentrations of extracts and ascorbic acid (6.25-200µg/ml) were prepared in methanol. 1ml of different concentrations of extracts/ascorbic acid was mixed with 3ml of ABTS (2,2-azinobis 3-ethylbenzothiazoline 6-sulfonate) radical solution (previously generated by mixing ABTS salt and potassium persulfate) in clean and labeled tubes. The tubes were left for 30 minutes at room temperature and the absorbance was measured in a spectrophotometer at 730nm. The radical scavenging activity of extracts/ascorbic acid was calculated using the formula:

$$\text{Scavenging activity (\%)} = (C - T / C) \times 100$$

where 'C' denotes the absorbance of the ABTS solution without extract/ascorbic acid and 'T' represents the absorbance of ABTS solution in the presence of extract/ascorbic acid. The IC₅₀ value was calculated and it denotes the concentration of extract/ascorbic acid required to scavenge 50% of the radicals (Kekuda *et al.*, 2015).

Ferric Reducing Activity of Extracts

The reducing activity of leaf and fruit extracts of *Z. xylopyrus* was determined by Ferric reducing assay (Kekuda *et al.*, 2014). In clean and labeled tubes, different concentrations of extracts/ascorbic acid (6.25-200µg/ml) in 1ml of methanol were mixed with 2.5ml of phosphate buffer (pH 6.6) and 2.5ml of potassium ferricyanide (1%). The tubes were incubated at 50°C for 20 minutes and cooled. To each tube, 2.5ml of trichloroacetic acid (10%) followed by 0.5 ml of ferric chloride (0.1%) was added. The tubes were left at room temperature for 10 minutes and the absorbance was measured at 700nm in spectrophotometer. An increase in the absorbance with increase in concentration of extracts/ascorbic acid indicates reducing power.

Total Phenolic Content of Extracts

To estimate the total phenolic content of leaf and fruit extracts of *Z. xylopyrus*, Folin-Ciocalteu reagent method was used. A dilute concentration of each extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:10) and 2 ml of sodium carbonate (2%) and the tubes were incubated at room temperature for 30 minutes. The absorbance of each tube was measured at 765nm in spectrophotometer.

A standard curve was constructed using different concentrations of Gallic acid (standard, 0-1000µg/ml) and the total phenolic content was represented as mg Gallic Acid Equivalents (Kekuda *et al.*, 2015).

RESULT AND DISCUSSION

Qualitative Phytochemical Analysis of Leaf and Fruit extracts

The medicinal properties of plants are due to the presence of secondary metabolites such as flavonoids, tannins, alkaloids, glycosides, alkaloids, saponins and terpenoids. These chemicals are called phytochemicals. Phytochemistry deals with the study of these phytochemicals. These phytochemicals have profound physiological effects on the health. Therefore, the detection of these chemicals in medicinal plants is important so as to relate the possible therapeutic role with these phytochemicals (Cowan, 1999; Bhandary *et al.*, 2012; Mir *et al.*, 2013; Yusuf *et al.*, 2014). In the present study, we chose methanol as extraction solvent. Among various solvents used for extraction of phytochemicals, methanol seems to be the best solvent for extract as it has been shown that methanol can dissolve many phytochemicals including polyphenolic compounds present in the plants (Cowan, 1999; Gurjar *et al.*, 2012; Naz *et al.*, 2013; Iloki-Assanga *et al.*, 2015). In the present study, maceration process using methanol solvent was performed to obtain extract of leaf and fruit of *Z. xylopyrus*. The phytochemicals that were detected in extracts are shown in Table 1. Flavonoids, saponins, terpenoids, tannins and phenols were detected in both leaf and fruit extracts. Alkaloids were detected in leaf extract only. Glycosides and steroids were not detected in both extracts.

Table 1: Phytoconstituents in leaf and fruit extracts

Phytochemical	Leaf	Fruit
Alkaloids	+	-
Flavonoids	+	+
Saponins	+	+
Terpenoids	+	+
Glycosides	-	-
Tannins	+	+
Phenols	+	+
Steroids	-	-

Antifungal Activity of Leaf and Fruit Extracts

Humans depend on plants for various needs such as food, medicine, timber and dyes. Due to increase in population, there is an increase in demand for food. Production of crops is constantly threatened by various factors among which pests are one of the important factors. Among various pests which affect crop production, fungi are considered as dominant group of pests as they cause a huge number of disease in agricultural crops. In severe cases, fungal diseases results in >50% of crop loss. One of the widely used approaches to control fungal diseases of crops is the usage of chemical fungicides. These chemicals affect the fungal pathogens and reduce disease incidence. However, the usage of these chemical agents is accompanied with some drawbacks. It results in residual

problem for environment where soil and underground water contamination can possibly occur. Besides, several fungal pathogens have developed resistance against some fungicides. Use of botanicals to control plant diseases seems to be one of the best alternatives for chemical control. Various studies which utilized plants and their components have highlighted the potential of plants to inhibit several fungal pathogens (Gomathi and Kannabiran, 2000; Nduagu *et al.*, 2008; Johnny *et al.*, 2011; Bajpai and Kang, 2012; Ajith *et al.*, 2012; Suprpta, 2012; Bahraminejad *et al.*, 2012; Shweta *et al.*, 2015). In the present study, we screened the antifungal activity of leaf and fruit extract of *Z. xylopyrus* by Poisoned food technique which is one of the widely used methods to evaluate antifungal activity of various kinds of samples including plant extracts. The mycelial growth of test fungi was affected by the extract as evidenced by reduction in colony diameter of test fungi in poisoned plates (when compared with control). Among extracts, leaf extract displayed high inhibitory activity when compared to fruit extract. *A. flavus* was susceptible to high extent than *A. alternata* to extracts.

Table 2: Antifungal activity of leaf and fruit extract

Test fungi	Colony diameter in cm (% inhibition)		
	Control	Leaf extract	Fruit extract
<i>A. alternata</i>	2.8	2.0 (28.57)	2.2 (21.42)
<i>A. flavus</i>	3.4	2.3 (32.35)	2.4 (29.41)

DPPH Radical Scavenging Activity of Leaf and Fruit Extracts

Out of various *in vitro* radical scavenging assays, DPPH assay is simple, rapid, inexpensive and widely used assay. This method uses a stable free radical DPPH and was developed by Blois (1958) in order to determine the antioxidant potential. The assay is based on the measurement of the scavenging capacity of antioxidants. DPPH is a stable, organic, nitrogen centred free radical having an absorption maximum at 517nm in alcoholic solution. In DPPH, the odd electron of nitrogen atom is reduced when it receives a hydrogen atom from antioxidants leading to the formation of corresponding hydrazine (absorption decreases as the electron pairs off). The decolorization is stoichiometric with respect to the number of electrons taken up. DPPH assay is widely used method to measure the antioxidant capacity of plant extracts (Blois, 1958; Wangcharoen and Morasuk, 2007; Krishna and Nair, 2010; Aiyegoro and Okoh, 2010; Kekuda *et al.*, 2015). In the present study, we determined the effect of leaf and fruit extracts of *Z. xylopyrus* to scavenge radicals by DPPH assay. Fading away of color of DPPH radical solution was monitored in presence of varying concentrations of extracts and standard at 517nm. Extracts and ascorbic acid scavenged DPPH radicals in a concentration dependent manner (Figure 1). Among extracts, leaf extract exhibited stronger scavenging potential (IC₅₀ 31.73value µg/ml) when compared to fruit extract (IC₅₀ value 36.79µg/ml). Ascorbic acid exhibited marked scavenging of DPPH radicals (IC₅₀ value 6.17µg/ml). From the results, it is certain that the extracts of *Z. xylopyrus* possess hydrogen donating property and hence the extracts can act as potent free radical scavengers.

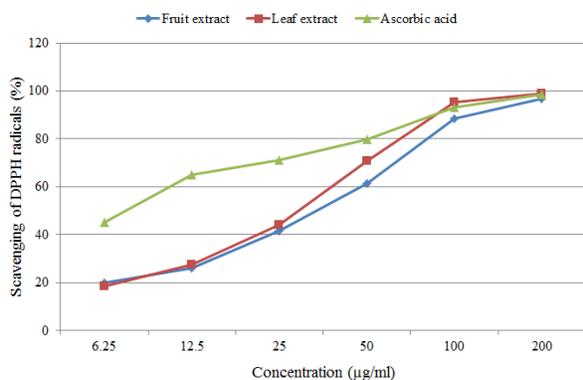


Figure 1: DPPH radical scavenging activity of leaf and fruit extracts

ABTS Radical Scavenging Activity of Leaf and Fruit Extracts

The assay involving scavenging of ABTS radical cation is another commonly used *in vitro* radical scavenging assay. Unlike DPPH method, scavenging of ABTS radical cation requires generation of radical cation. This can be done by reacting ABTS stock solution (7 mM) with potassium persulphate (2.45 mM) and incubating the mixture in dark condition at room temperature for 12-16 hours. The radical solution was diluted with water to an absorbance of 0.70 at 734 nm. Scavenging of ABTS radical cations is useful in determining the antioxidant capacity of both lipophilic and hydrophilic antioxidants. This method has been used to evaluate radical scavenging potential of variety of samples including plant extracts. A compound having the property of donation of electrons will reduce the blue-green ABTS radical solution to colorless neutral form which is indicated by the suppression of characteristic long wavelength absorption spectrum of radical (Re *et al.*, 1999; Wangcharoen and Morasuk, 2007; Camacho-Luis *et al.*, 2008; Krishna and Nair, 2010; Aiyegoro and Okoh, 2010; Kekuda *et al.*, 2015). In the present study, we evaluated the potential of leaf and fruit extracts of *Z. xylopyrus* to scavenge ABTS radical cations and the result is shown in Figure 2. Both extracts were able to scavenge ABTS radicals in a concentration dependent manner. Leaf extract displayed marked scavenging activity (IC_{50} value 4.61 µg/ml) when compared to fruit extract (IC_{50} value 8.37 µg/ml). Ascorbic acid exhibited stronger scavenging activity (IC_{50} value 3.05 µg/ml) when compared to extracts. From the results, it is confirmed that the extracts of *Z. xylopyrus* possess electron donating property and hence the extracts can act as potent free radical scavengers.

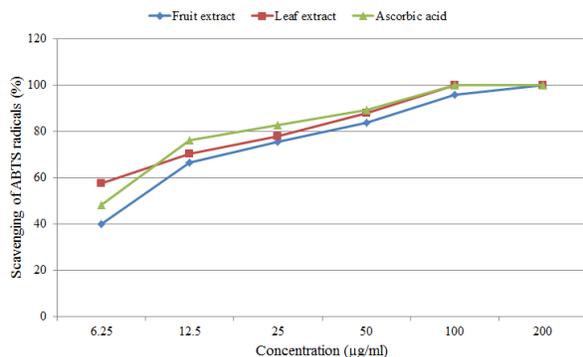


Figure 2: ABTS radical scavenging activity of leaf and fruit extracts

Ferric Reducing Activity of Leaf and Fruit Extracts

Reduction of Fe (III) is often used as an indicator of electron donating property of samples and is an important mechanism of antioxidant action (Ebrahimzadeh *et al.*, 2010). The reducing ability is an indication of total capacity of host to withstand adverse effect of stress induced by free radicals. Reducing ability reflects the electron donating property which is associated with antioxidant activity. The presence of reductants (antioxidants) in extracts would result in the reduction of ferric complex to ferrous form and this can be determined by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Upon addition of free Fe^{3+} to the reduced product, formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN)_6]_3$, having a strong absorbance at 700nm is observed. An increase in absorbance with an increase in concentration of compound indicates reducing capacity of compound (Ebrahimzadeh *et al.*, 2010; Gulcin *et al.*, 2011; Meir *et al.*, 1995; Chung *et al.*, 2006; Aiyegoro and Okoh, 2010; Kekuda *et al.*, 2011). In the present study, we screened the leaf and fruit extracts of *Z. xylopyrus* for ferric reducing activity and the result is shown in Figure 3. On increasing the concentration of extract, a corresponding increase in the absorbance of reaction mixtures was observed which indicated reducing power of the extracts. Among extracts, marked reducing potential was observed in case of leaf extract. However, ascorbic acid displayed higher reducing potential. It is evident from this study that the extracts of *Z. xylopyrus* exhibit reductive ability and hence, the extracts could serve as electron donors, terminating the radical chain reactions (Chung *et al.*, 2006).

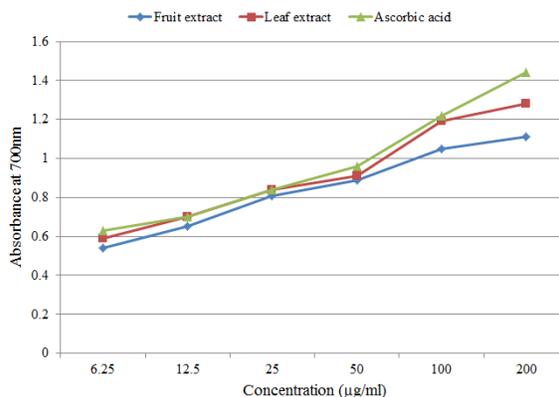


Figure 3: Ferric reducing activity of leaf and fruit extracts

Total Phenolic Content of Leaf and Fruit Extracts

In recent years, there has been immense interest in antioxidants from natural origin especially from plants. Polyphenols including flavonoids appears to be promising antioxidant chemical species in plants. The presence of phenolic compounds in plants is related to several health benefits including antioxidant activity. FCR method is a simple, oldest and rapid method used for estimating the total phenolic content in variety of samples including plant extracts. Phenolic compounds react with FCR under basic conditions resulting in the formation of a blue colored complex with absorption maxima near 750nm (Boonyuen *et al.*, 2009; Ebrahimzadeh *et al.*, 2010; Aiyegoro and Okoh, 2010; Kekuda *et al.*, 2015). In the present study, we estimated the phenolic content of leaf and fruit extracts by FCR method. There was no much variation in the phenolic content of both the extracts. Leaf extract was found to contain slightly high phenolic content (68.16mg GAE/g) when compared to fruit extract (62.62mg GAE/g).

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In this study, a positive correlation between antioxidant activity and phenolic content was observed i.e., leaf extract exhibited higher antioxidant activity when compared to fruit extract.

CONCLUSIONS

Leaf and fruit extract of *Z. xylopyrus* displayed antifungal and antioxidant activity. The observed bioactivities could be attributed to the presence of phytochemicals in the extracts. Bioactivities shown were higher in case of leaf extract when compared to fruit extract. The plant can be used against phytopathogenic fungi and oxidative damage. Further studies are to be carried out to isolate and characterize active components from extracts and to determine their biological activities.

Conflict of Interest

Conflict of interest none declared.

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