Free radical scavenging potential and total phenolic and flavonoid content of *Ziziphus mauritiana* and *Ziziphus nummularia* fruit extracts

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Background: Nowadays antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in the diseases caused by free radicals. Presently, the active constituents from the natural sources are tested for their free radical scavenging potential. Plants are of enormous importance in the free radical and antioxidant field. **Aim:** The present study was carried out to evaluate the free radical potential, total phenolic and flavonoid content of edible and non-commercial fruits of *Ziziphus mauritiana* and *Ziziphus nummularia* belonging to the family Rhamnaceae. **Materials and Methods:** Free radical scavenging potential of methanolic extracts of the *Ziziphus mauritiana* and *Ziziphus nummularia* fruits (MEZM and MEZN) was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and hydrogen peroxide (H_2O_2) method. The total phenolic content of the methanolic extracts of fruits was determined using gallic acid as a standard. The total flavonoid content was determined using the Aluminium chloride reagent and quercetin as standard. **Statistical analysis:** The results were statistically analysed with the Student's *t-test*. **Results and Conclusions:** The extracts showed significant free radical scavenging activity in a dose dependent manner when compared with ascorbic acid. The highest DPPH radical scavenging activity of MEZM and MEZN was found to be 79.5% and 77.5% respectively at concentration of 250 µg mL^{-1}. The H_2O_2 scavenging effect of MEZM and MEZN was 73.4% and 71% respectively at a concentration of 250 µg mL^{-1}. IC_{50} <1 mg/ ml shows that the fruits possess significant antioxidant activity. The phenolic as well as flavonoid content was found to be higher in MEZM. The fruits can be used as a natural source of antioxidants in combating diseases caused by free radicals.

Key words: Antioxidants, free radicals, Rhamnaceae, *Ziziphus mauritiana*, *Ziziphus nummularia*

INTRODUCTION

The genus *Ziziphus* belongs to the family Rhamnaceae. This genus comprises about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world; of these, 17 are native of India.[1] *Ziziphus* species can grow either as shrublets, shrubs or trees with thorny branches and are used as a hedge to form defensive fences for animals. The genus *Ziziphus* is known for its medicinal properties as a hypoglycaemic, hypotensive, anti-inflammatory, antimicrobial, antioxidant, anti-tumour and liver protective agent and as an immune system stimulant.[2,3] Some species like *Ziziphus mauritiana* (MEZM) and *Ziziphus nummularia* (MEZN) occur on nearly every continent. MEZM and MEZN are found all over the drier tracts, particularly in north-western India and Uttar Pradesh.[4] The fruits of these species are very nutritious and are usually eaten fresh. The fruits are applied on cuts and ulcers. They are also used to treat pulmonary ailments and fevers, to promote the healing of fresh wounds, and for dysentery.[5] The leaves of MEZM are applied as poultices and are helpful in liver troubles, asthma and fever. Plant materials are cheap and significantly contribute to the improvement of human health in terms of cure and prevention of diseases. The MEZN roots and leaves are cooling and used to treat biliousness and headache. The bark is used to treat boils and is effective against diarrhoea. The fruit is believed to purify the blood and helps with digestion.[6]

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Nowadays, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in the diseases caused by free radicals. Oxidative stress occurs when the production of damaging free radicals exceeds the capacity of the body's antioxidant defences to detoxify them. Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. These unpaired electrons are very reactive with adjacent molecules such as lipids, proteins and carbohydrates and can cause cellular damage.[7] The cellular injury caused by oxidative stress has been linked to over 200

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clinical disorders.[8] Traditionally, natural medicines with free radical scavenging properties have been used for various purposes. Presently, the active constituents from the natural sources are tested for their free radical scavenging potential. A number of plants included in *Ayurveda* such as *Emblica officinalis*, *Curcuma longa*, *Momordica charantia*, *Swertia chirata* and *Withania somnifera* have been studied by various researchers for their antioxidant potential. Thus, plants are of enormous importance in the free radical and antioxidant field.[9,10] They supply us with the essential biradical, O_2. Plants expose themselves to high levels of O_2 and so are rich in antioxidant defences and repair systems against oxidative damage.[11]

The present study was carried out to evaluate the free radical potential and total phenolic and total flavonoid content of edible and non-commercial fruits of MEZM and MEZN.

MATERIALS AND METHODS

Collection of Plant Material

The fruits of MEZM *and MEZN* selected for the present study were collected from the local region in Punjab, India, in October 2011. A voucher specimen (Sample No PH02 and PH03) was deposited in the Department of Pharmacy (Surya group of institutes).

Drugs and Chemicals

Ascorbic acid was obtained from Central Drug House Pvt. Ltd., Mumbai, India. Hydrogen peroxide (H_2O_2) was obtained from E-Merck Ltd., Mumbai, India. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Folin and Ciocalteu reagent, gallic acid and quercetin were obtained from Sigma Chemical Co., St. Louis USA. All other chemical reagents used were of analytical grade, which were procured from different companies (LobaChem, Mumbai, India and Merck Limited, Mumbai, India).

Extraction

Firstly, the matured fruits of MEZM and MEZN were taken and sliced into small pieces. The pieces were dried in the sun and then powdered using a grinder. The fruit powders were extracted by simple maceration process using methanol. The solvent was filtered off using a muslin cloth and residue macerated again with the fresh solvent. Both solvents were combined and filtered using whatman filter paper and concentrated under reduced pressure on a rotary evaporator at 40°C. The crude extracts were used for further investigation.

Phytochemical Screening

The crude extracts were studied for the presence of phytochemicals such as alkaloids, tannins, saponins, flavonoids and carbohydrates using standard procedures.[12]

Qualitative Evaluation of Free Radical Scavenging Activity

DPPH method

The DPPH reagent was used to detect the qualitative free radical scavenging activity of the extract using the method of Oke and Hamburger with some modifications.[13] The crude extract with appropriate dilution was spotted on the silica gel coated plate and developed in a tank containing the mobile phase (hexane:Ethyl acetate, 8:2). The plate was then dried and a methanolic solution of DPPH (0.2%) was sprayed on the plate and the plate was left to dry. The coloration produced on the plate was noted. The colour change of the DPPH spray solution from deep violet to yellow was considered positive.

Quantitative Evaluation of Free Radical Scavenging Activity

DPPH radical scavenging activity

The free radical scavenging activity of the methanolic extracts of MEZM and MEZN fruits was measured by using DPPH method described by Sreejavan.[14] Briefly, a 0.05 mM solution of DPPH in methanol was prepared and 1.5 mL of this solution was added to 0.5 mL of extract solution in methanol at different concentrations (50–250 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1700 Pharma spec Kyoto Japan). A blank without DPPH was used to remove the influence of the colour of the samples. A methanolic solution of DPPH was used as negative control. Ascorbic acid was used as a reference drug, in the same concentrations as used for the extract. All measures were carried out in triplicate. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH radical scavenging activity was calculated using the following equation.

$$\text{Percentage scavenging of DPPH radical} = [(A_o - A_s)/A_o] \times 100$$

where:
A_o is absorbance of the negative control.
A_s is the absorbance of the sample.

H_2O_2 radical scavenging activity

The ability of the extract to scavenge H_2O_2 was determined according to the method of Wettasinghe and Shahidi.[15] The extract solution (1.0 mL) in various concentrations (50–250 µg/mL) was mixed with 2.4 mL of 0.1 M phosphate buffer (pH 7.4), and then 0.6 mL of 43 mM solution of H_2O_2 in the same buffer was added. After 10 min, the absorbance values of the reaction mixtures were recorded against a blank solution containing phosphate buffer without H_2O_2 at 230 nm using a spectrophotometer (Shimadzu UV-1700 PharmaSpec). For each concentration, a separate blank

sample was used for background subtraction. Ascorbic acid was used as a standard and mixture without sample was taken as a control. All tests were done in triplicate. The percentage scavenging of H_2O_2 was calculated as.

Percentage scavenging of H_2O_2=$[(A_o-A_1)/A_o]\times100$

where:
A_o is the absorbance of the control
A_1 is the absorbance of the extract/standard.

Determination of IC_{50} Value

IC_{50} values were calculated from the linear regression of the % antioxidant activity versus extracts concentrations.

Determination of Total Phenolic and Total Flavonoid Content

The total phenolic content of each fruit extract was determined by the Folin-Ciocalteu method.[16] The aqueous solution of each extract (10% v/v, 0.5 mL) was added to 2.5 mL of 0.2 N Folin-Ciocalteu reagent and placed at room temperature for 5 min. Then, 2 mL aqueous solution of sodium carbonate (75 g/L) was added. The solution was incubated for 2 h and the absorbance was measured at 760 nm against a blank (water). A standard calibration curve was plotted using gallic acid (0–250 mg/L). The results were expressed as mg of gallic acid equivalents (GAE)/100 g of fruit weight.

The total flavonoid content was determined according to the Dowd method.[17] Five mL methanolic solution of each fruit extract (0.4 mg/mL) was mixed with 2 mL methanolic solution of aluminium trichloride ($AlCl_3$) (2%). The absorbance was read at 415 nm after 10 min against a blank sample consisting of a methanol (2 mL) and plant extract (2 mL) without $AlCl_3$. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as mg of quercetin equivalents (QE)/100 g of fruit weight.

Statistical Analysis

Data are reported as mean of three determinations. The results obtained were statistically analysed with the Student's *t*-test using a significance level of $P<0.05$. Microcal origin (version 6.0) was used for graph plotting.

RESULTS

The percentage w/w yield of the methanolic extract of MEZM and MEZN fruits is given in Table 1.

Preliminary phytochemical screening of the methanolic extract of MEZM and MEZN fruits revealed the presence of chemical constituents such as carbohydrates, saponins and tannins [Table 2]. The flavonoids were found to be present in maximum amount.

The free radical scavenging activity was evaluated by DPPH and H_2O_2 method. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptivity of the DPPH radical reduces when the odd electron of DPPH radical becomes paired with hydrogen atom from a free radical scavenging antioxidant to form the reduced DPPH-H (1,1-diphenyl-2-picrylhydrazine). The structure of DPPH and its reduction by an antioxidant are shown in Figure 1.[18] TLC based DPPH spray method was used for evaluation of qualitative method antioxidant activity. The change in coloration from purple to yellow showed that the methanolic extract of fruits possess free radical scavenging activity.

MEZM and MEZN showed concentration-dependent DPPH radical scavenging activity. The highest radical scavenging activity was found to be 79.5% (MEZM) and 77.5% (MEZN) at concentration of 250 µg/mL, which was comparable with

Table 1: Percentage yield

Plant	Weight of fruits (g)	Weight of extract (g)	Yield w/w (%)
Ziziphus mauritiana	150	19.50	13
Ziziphus nummularia	150	17.60	11.7

Table 2: Phytochemical chemical screening of MEZM and MEZN

Chemical constituents	MEZM	MEZN
Alkaloids	–	–
Flavonoids	++	++
Saponin	+	–
Cardiac glycosides	+	–
Carbohydrates	+	+
Phytosterols	+	+
Tannins	+	+

MEZM – *Ziziphus mauritiana*; MEZN – *Ziziphus nummularia* +: presence of chemical constituent. –: absence of chemical constituent. ++: presence of chemical constituent in maximum amount

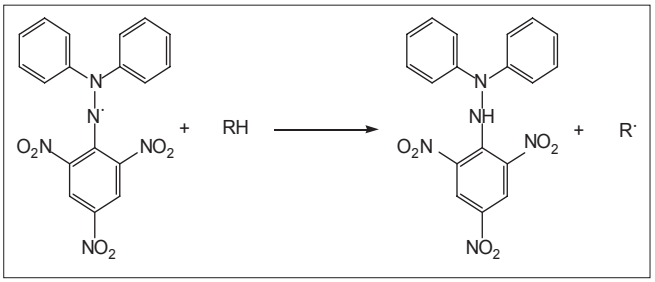

Figure 1: Structure of DPPH and its reduction by an antioxidant

the scavenging effect of ascorbic acid. The results are shown in Table 3 and graphically represented in Figure 2.

The MEZM and MEZN showed concentration dependent H_2O_2 radical scavenging activity. The maximum H_2O_2 scavenging effect of was 73.4% (MEZM) and 71.0 (MEZN) at a concentration of 250 µg/mL, which was comparable with the scavenging effect of ascorbic acid. The results are shown in Table 4 and graphically represented in Figure 3.

The free radical scavenging potential is inversely proportional to IC_{50} value, which was calculated from the linear regression of the % antioxidant activity versus extracts concentrations. MEZM and MEZN possess IC_{50} values of 0.133 and 0.142 g/mL, respectively, for DPPH radical scavenging activity and IC_{50} values of 0.136 and 0.144 g/mL, respectively, for H_2O_2 radical scavenging activity. The results are given in Table 5. IC_{50}<1 mg/mL shows that the fruits possess significant antioxidant activity.[19]

The total phenolic content of the methanolic extracts of fruits was determined using a standard curve of gallic acid (R^2=0.9996). The total flavonoid content was determined using the $AlCl_3$ reagent and quercetin as standard (R^2=0.9993). The results are given in Table 6. The results show that the phenolic as well as flavonoid content was found to be higher in the fruits of MEZM.

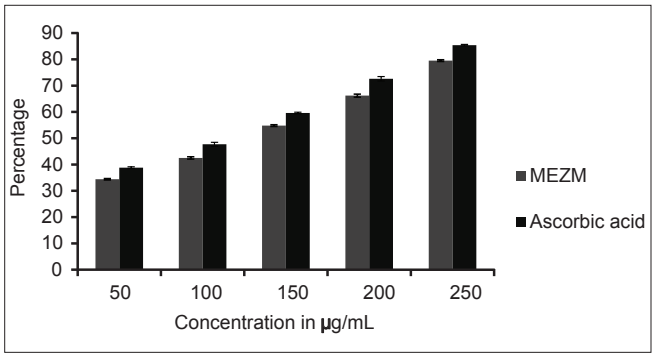

Figure 2: Graphical representation of percentage scavenging of DPPH radical by MEZM and MEZN. (Values are the average of triplicate experiments and represented as mean±S.E.M.)

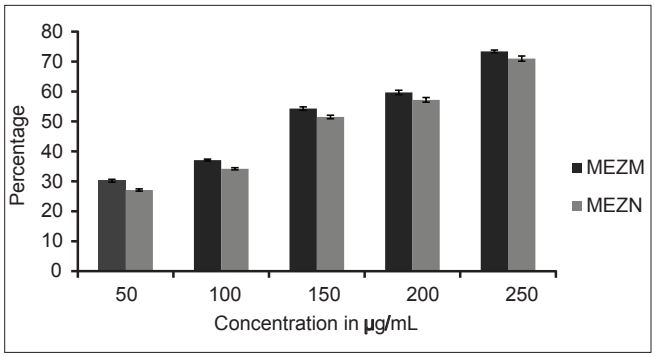

Figure 3: Graphical representation of percentage scavenging of H_2O_2 by MEZM and MEZN. (Values are the average of triplicate experiments and represented as mean±S.E.M.)

DISCUSSION

DPPH scavenging effect of MEZM and MEZN may be attributed due to the phytoconstituents with radical scavenging potential such as flavonoids. Scavenging of H_2O_2 by may be attributed to the compounds, which can donate electrons to H_2O_2, thus neutralizing it to water.[20] Hydrogen peroxide is the second intermediate produced during the stepwise electron reduction of molecular oxygen. It may also be generated directly during a two-electron reduction of molecular oxygen. It can act as both oxidizing and reducing agent. Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to highly

Table 3: Percentage scavenging of DPPH radical by MEZM and MEZN

Conc. of extract (µg/mL)	Percentage scavenging of DPPH radical		
	MEZM	MEZN	Ascorbic acid
50	34.4±0.27	32.1±0.79	38.8±0.32
100	42.5±0.44	41.2±0.64	47.7±0.75
150	54.8±0.34	52.3±0.67	59.6±0.29
200	66.2±0.58	64.2±0.22	72.6±0.85
250	79.5±0.83	77.5±0.64	85.4±0.27

Values are the average of triplicate experiments and represented as mean ± S.E.M; DPPH — 2,2-diphenylpicryl hydrazyl; MEZM – *Ziziphus mauritiana*; MEZN – *Ziziphus nummularia*

Table 4: Percentage scavenging of H_2O_2 by MEZM and MEZN

Conc. of extract (µg/mL)	Percentage scavenging of H_2O_2		
	MEZM	MEZN	Ascorbic acid
50	30.2±0.46	27.1±0.37	34.1±0.27
100	37.1±0.32	34.2±0.36	42.2±0.56
150	54.3±0.57	51.5±0.57	57.5±0.34
200	59.7±0.73	57.2±0.78	64.0±0.67
250	73.4±0.45	71.0±0.86	77.4±0.55

Values are the average of triplicate experiments and represented as mean ± S.E.M; MEZM – *Ziziphus mauritiana*; MEZN – *Ziziphus nummularia*

Table 5: IC_{50} values of MEZM and MEZN

Extract	DPPH radical scavenging (g/mL)	H_2O_2 radical scavenging (g/mL)
MEZM	0.133	0.136
MEZN	0.142	0.144

DPPH — 1-diphenyl-2-picryl-hydrazyl; MEZM – *Ziziphus mauritiana*; MEZN – *Ziziphus nummularia*

Table 6: Total phenolic and total flavonoid content of MEZM and MEZN

Extract	Phenolic content (mg GAE/100 g of fruit)	Flavonoid content (mg QE/100 g of fruit)
MEZM	2870±3.57	2528±6.55
MEZN	58±4.84	54±5.97

Values are the average of triplicate experiments and represented as mean ± S.E.M; MEZM – *Ziziphus mauritiana*; MEZN – *Ziziphus nummularia*

reactive hydroxyl radicals in the cell. Thus, removing H_2O_2 from biological systems is very important.[21] The ability of plant extract to scavenge H_2O_2 could also reflect its ability to inhibit the formation of hydroxyl radical *in vivo*.[22] H_2O_2 scavenging effect of extract may be attributed due to the phytoconstituents such as flavonoids.

Flavonoids can prevent injury caused by free radicals in various ways. Through *in vitro* experiments, it has been found that flavonoids possess anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties.[23] Flavonoids have been found to be prominent inhibitors of cycloxygenase and lipoxygenase Flavonoids prevent synthesis ofprostaglandins that suppress T-cells. The immune cells communicate with chemical signals called cytokines, which are controlled by flavonoids.[24] Flavonoids are potent bioactive molecules that possess anticarcinogenic effects because they can interfere with the initiation, development and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis.[25] Flavonoids, Kaempferol 3-O-β-isorhamninoside and rhamnocitrin 3-O-β-isorhamninoside, isolated from Rhamnus alaternus L leaves possess anti cancer and antioxidant potential.[26] Flavonoids such as Swertish and spinosin were isolated from *Ziziphus jujube*.[27] Flavonoids with antioxidant potential have been isolated from the leaves of *Phyllostachys pubescens*.[28]

Flavonoids directly scavenge the free radicals. Free radicals oxidize the flavonoids to produce a more stable and less reactive radical.[29] Radicals are rendered inactive by flavonoids according to the following equation:
Flavonoid(OH)+R•Flavonoid(O•)+RH
Where: R• is a free radical.

CONCLUSION

The results of this study showed that the fruits of MEZM and MEZN possess significant free radical scavenging potential. The fruits of MEAM possess higher radical scavenging potential, total flavonoid and total phenolic content. The correlations indicate that phenolic compounds and flavonoids are the major contributors to the free radical scavenging potential of these fruits.

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