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Antioxidant activity of organic extracts of root bark of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew

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Abstract

Extracts of root bark of *ziziphus jujube* were prepared by using methanol, acetone, ethyl acetate, dichloromethane, chloroform and *n*-Hexane through maceration. Their total phenolic contents and total flavonoid contents were determined by using single concentration of all extracts (200µg/mL). Total phenolic contents 207.33, 197.33, 157.33, 97.33, 67.33mg eq GA/g were measured in methanol, dichloromethane, acetone, chloroform, ethyl acetate and *n*-hexan respectively. While total flavonoid contents 162.28, 162.28, 138.59, 133.33, 128.54 and 93,85mg eq rutin/g were measured in acetone, ethyl acetate, chloroform, dichloromethane, n-hexane and methanol extract respectively. Antioxidant activity of organic extracts of root bark of *Ziziphus jujube* was measured by three methods; DPPH, TAC and ABTS assays. Highest antioxidant activity was observed for dichloromethane extract (DPPH 41.26%, ABTS 47.64% and TAC 504.87mg ascorbic acid equivalent/g of extract) and methanol extracts (DPPH 40.14%, ABTS 48.69% and TAC 501.21mg ascorbic acid equivalent/g of extract). The results of antioxidant potential can be clearly related to total phenolic content results as both dichloromethane and methanol extracts were high in total phenolics and have shown high antioxidant potential. A Dose dependent study was conducted by using DPPH and ABTS assays for determining IC_{50} value. Data was analyzed by using one-way ANOVA. Comparison was performed by using LSD test and $p < 0.05$ was considered as significant.

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Introduction

Zizyphus Jujuba (L.) Gaertn. Var. *hysudrica* Edgew is a hybrid of two Zizyphus species namely *Zizyphus mauritiana* and *Zizyphus spina-christi* (Azam-Ali *et al.,* 2006). It grows as medium sized tree possessing leaves which appear glabrous on both surfaces. The plant is rarely seen in wild-form and is usually cultivated to obtain its fruit which is edible. The fruit usually attains maximum of 1-inch length in wild-form in contrast to its cultivated form where it may reach up to the length of 3-inch long and is almost half as wide as its length (Chaudhry, 1969). This variety is distributed in punjab region of Pakistan. *Z. mauritiana* occurs in form of small shrubs to medium sized tree .The natural habitat of plant is warm subtropics and tropics of South Asia.where it exist in its wild form .The cultivated form of plant spreads through Indo-China and Southren China East ward whereas through Malesia it spreads to South East ward. In contrast Z. spina-christi belongs to drier tropical areas of Middle east, Ethiopia, North-East Africa and Eastern Africa. In Iran, Saudi Arabia and farther west Turkey it exist in its wild form. In India, Pakistan, Egypt, Syria, the Mahgreb, Saharan Oases and Zanzibar it exists as minor cultivated plant (Azam-Ali *et al.,* 2006).

The species of Ziziphus are enriched in phytochemicals Vitamin-C, Calcium, Phosphorous, Protein, carotene, Iron, carbohydrates (Bakhshi and Singh, 1974; Singh *et al.,* 1973), Vitamin-B_1 (Thiamine), Vitamin-B_2 (Riboflavin) (Troyan and Kruglyakov, 1972; Kuliev and Guseinova, 1974), Pectin-A (Tomoda *et al.,* 1985), Alkaloids, (Pareek, 2001; Tschesche *et al.,* 1976; Tschesche *et al.,* 1979; Han *et al.,* 1990; Jossang *et al.,* 1996), Flavonoid Glycosides/Spinosins (Woo *et al.,* 1979), Glycoside saponin (Ogihara *et al.,* 1976), Triterpenoic Acids (Lee *et al.,* 2003), Betulinic Acid (Pisha *et al.,* 1995; Kim *et al.,* 1998; Eizhamer and Xu, 2004) properties, Oleanolic acid (Hsu *et al.,* 1997), Phospholipids (Goncharova *et al.,* 1990). Medicinal plants are rich source of bioactive metabolites. These bioactive metabolites vary in amount from plant to plant and in different parts of the same plant. Extraction of these metabolites depend upon two main factors i-e nature of extracting solvent and method adopted for extraction. In this study the extraction of bioactive metabolites was carried out by using six different solvents of varying polarity i-e Methanol, Acetone, Ethyl Acetate, DCM, n-Hexane and Chloroform. Then TPC and TFC was calculated and all the extracts were evaluated for anti-oxidant activity by using DPPH, ABTS and total antioxidant capacity (TAC) was measured byusing Phosphomolybdenum assay.

As a result of normal cellular metabolism and various environmental factors like pollutants and smoke Reactive Oxygen Species (ROS) are being produced by living organisms. The highly reactive nature of ROS can cause very serious damage to various components of cell structure like carbohydrates, lipids, proteins and nucleic acids. By binding to these components' ROS can also alter their functions (Layras *et al.,* 2016; Marnett *et al.,* 1999; Sayre *et al.,* 2001). Oxidative stress is the term used to describe the state where balance between oxidants and anti-oxidants is shifted to oxidants. A very critical factor for maintenance of cell viability, activation and proliferation of various cell functions and performance of various organ functions is the continuous regulation of reducing and oxidizing (redox) processes within body. An integrated anti-oxidant system comprised up of various enzymatic and non-enzymatic anti-oxidants possessed by all aerobic organisms is very effective because it can block all harmful effects caused by ROS. Antioxidant system got over whelmed giving rise to various pathological conditions. Various lung diseases like Acute respiratory distress syndrome, Idiopathic pulmonary fibrosis, Chronic obstructive pulmonary disease, asthama (Andreadis *et al.,* 2003), cancer (Valko *et al.,* 2006), diabetes, neurological disorders(Jenner,2003 ; Kasparova *et al.,* 2005; Sayer *et al.,* 2001), hypertension (Kerr *et al.,* 1999), atherosclerosis, ischemia/perfusion (Kasparova *et al.,* 2005) are all among various pathological conditions/diseases arise from oxidative stress.

Hence deal with Oxidative stress and problems related to it antioxidants from external source are required. Ascorbic acid, gallic acid, butylated hydroxytoluene

(BHT), butylated hydroxyanisole (BHA) and tributyl hydroquinone (TBHQ) are all among synthetic antioxidants. They help body to cope with oxidative stress and also used in food industry as an additive to inhibit oxygen induced lipid peroxidation. But in recent years it came into notice that these are not safe chemicals. Ascorbic acid and gallic acid possess pro-oxidant properties (Abarikwu *et al.,* 2015; Podmore *et al.,* 1998). BHA, BHT and TBHQ are reported to cause dermatitis, Urticaria, asthama, behavioral changes and cancer (Gharavi and El-Kadi, 2004). Functional and histological changes occur in lungs, liver, kidney and thyroid upon long term exposure to BHT. So it is need of hour to discover antioxidants having non-toxic nature and natural origin.

Materials and method

The experiment was designed for the estimation of total phenolic content, total flavonoid content and determination of antioxidant potential of different organic extracts from root bark of *Ziziphus jujube* Gaertn (L) variety *hysudrica* Edgew

Collection of Plant Material

Ziziphus jujube Gaertn (L) variety *hysudrica* Edgew was collected from Lahore, Pakistan. The bark of the root was separated from the inner root by physical means. Which was then shade dried for 10 days and ground, sieved and got properly stored in dessicator.

Chemicals

All the material and reagents used to conduct this study were taken from PCSIR Labs complex and University of the Punjab Lahore. All the chemicals were of AR grade and used as such without further purification.

Preparation of extracts

Hundred grams of each finely ground root bark material of *Zizyphus Jujuba* (L.) Gaertn. Var. *hysudrica* Edgew was poured into six different flasks and extracted against various solvent having different polarities like: methanol, acetone, ethyl acetate, dichloromethane, *n*-Hexane and chloroform. Flasks were allowed to continuously stir for 72 hours the material was then filtered and the resulting filtrates were air dried.

Estimation of total phenolic content

Folin-Ciocalteu reagent method was used to determine total phenolic content of all extracts. Single dilution of 200µg/mL for each dried extract was made in methanol. 200µL of dilution was then mixed with 400 µL of Folin-Ciocalteu reagent in a volumetric flask. The solution was then mixed with 0.2mL of 7% Na_2CO_3 solution, after heating it at 25°C for 5-10 mins. The final dilution was done by using deionized distilled water and final volume was made upto 10ml in volumetric flask. The mixture was held at 25°C for 2 hours before taking absorbance at 765nm (McDonald *et al.,* 2001). Gallic acid was used as a standard for which calibration curve was plotted. Equivalent of permg gallic acid (GAE) per gram of dried sample (mg/g) were calculated to determine the total phenolics.

Estimation of total flavonoid content

Initially 2mL of methanol was used to dissolve 100µL of 200µg/mL of crude extract which was then diluted by using 4ml distilled water. Then 0.6ml of each 10% $AlCl_3$ and 5% $NaNO_2$ were added in above mixture which was then allowed to stay at ambient conditions for 10 mins. Final reaction mixture was made by adding 4 mL of 1M NaOH and final volume of 20mL was made up by using distilled water. The absorbance was then measured at 510nm after mixture was allowed to stand for 25 mins (Chang *et al.,* 2002). Rutin was used as a standard for which calibration curve was plotted. Equivalent of mg Rutin per g of dried sample (mg/g) were calculated to determine total flavonoid.

Estimation of antioxidant potential

The anti-oxidant potential of root bark extracts in different solvents was estimated by using different protocols which are described as follows

DPPH assay for radical scavenging activity

Stock solution was prepared by dissolving DPPH (2.4mg) in 100 mL methanol and got stored at 20°C. The working stock solution was prepared by dilution of stock solution with methanol. 500µl of samples having concentration of 200µg/mL were mixed with 500µL of working stock solution. The absorbance was

then measured at 517nm after mixture was incubated for 15 minutes in dark (Manzocco *et al.,* 1998). The following equation was used to determine DPPH scavenging activity of various solvent extracts.

Percentage inhibition (%) = [(Abs of control – Abs of sample)] / Abs of control × 100

Ascorbic acid was used as a standard. 50% inhibition of DPPH radical was determined by using IC_{50} values.

Phosphomolybdate Assay (Total Anti-oxidant Capacity)

1ml of reagent solution (0.6 M sulfuric acid, 28nm Sodium phosphate and 4mM ammonium molybdate) was shaken with 0.1ml of 200µg/mL dilution. The test tubes got covered after which incubation was done at 95°C for 90 minutes by using water bath. The absorbance of mixture was measured at 695nm after samples got cooled (Prieto *et al.,* 1999). Ascorbic acid was used as a standard. Total anti-oxidant capacity was estimated asmg Ascorbic acid equivalent per g of dried extract.

ABTS radical scavenging activity

In this method disappearance of the ABTS radical cation was calculated to determine the activity. The stock solution was prepared by mixing ABTS (7mM) and potassium persulfate (2.4mM). The solution was then placed in dark at room temperature for 12-16 hours. The working stock solution was prepared by diluting stock solution with 1ml of $ABTS^+$ solution and 60% methanol. $ABTS^+$ solution was made fresh for each assay. 1ml of 200µg/mL dilution of different extracts was mixed with 1ml of working stock solution in order to react extracts with $ABTS^+$. The absorbance was then measured at 734nm (Seram *et al.,* 2006). BHT was used as standard. The following formula was used to determine percentage inhibition.

Percentage inhibition Activity = [1-(Abs of sample / Abs of control)]*100

IC_{50} was used designate anti-oxidant capacity of test samples which is a concentration necessary for a 50% reduction of ABTS.

Results and discussion

Organic extracts of root bark of *Ziziphus jujube* Gaerten (L) Var. *hysudrica* were prepared by using six solvents (methanol, acetone, ethyl acetate, dichloromethane ,chloroform and *n*-hexane) having varying polarities through maceration. These extracts were concentrated by removing solvent under reduced pressure and their concentrated form was kept at 4°C in fridege for further studies.

Total phenolic content and Total flavonoid content

The results of TPC in different types of extracts of root bark of *Ziziphus jujube* Gaerten (L) Var. *hysudrica* were given in fig. 1. These results showed that among various extracts ethylacetate and n-hexane differ significantly from each other in term of TPC.. Methanol and dichloromethane extracts have shown high concentration of TPC as compared to other extracts. The results of TFC in different extracts of *Ziziphus jujube* Geartn (L) var. *hysudrica* were given in following fig. 2. These results have shown that all the extracts were significantly different from each other except acetone and ethyl acetate extracts ($p < 0.05$). Acetone and Ethyl acetate extracts have shown high concenteration of TFC as compared to other extracts. The high concenteration of phenolic and flavonoid content in methanol and acetone respectively reflect polar nature of these compounds. But not all phenolics and flavonoids are polar these compounds cover range from polar to intermediate polar to nonpolar compounds (tocopherol is a nonpolar phenolic) that's why high concenteration of phenolics and flavonoids were observed with dichloromethane and ethyl acetate extracts. It may be happen due to presence of polar and intermediate polar phenolics and flavonoids in abundance in plant as secondary metabolites.

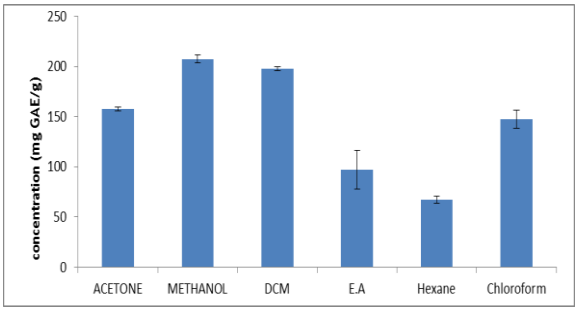

Fig. 1. Determination of Total Phenolic Content (TPC) of different extracts of *Ziziphus jujube* Gaertn(L) var. *hysudrica* Edgew.

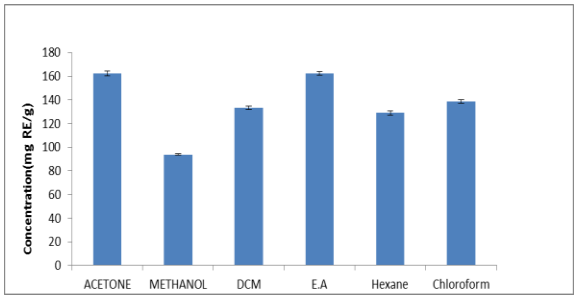

Fig. 2. Total Flavonoid Content (TFC) of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew.

The results of DPPH Assay in different types of extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew are given in Fig. 3. It is shown by graph that all extracts were significantly different from each other except acetone and chloroform extracts ($p < 0.05$). Methanol and dichloromethane extracts have shown high DPPH activity as compared to other extracts.

Results of Total anti-oxidant power assay (*Phosphomolybdenum assay*) of different extracts of *Ziziphus jujube* Geartn (L) var. *hysudrica* Edgew were given in fig. 4. It is shown by graph that all extracts were significantly different from each other except methanol, dichloromethane, Chloroform and hexane extracts ($p < 0.05$). Methanol and dichloromethane extracts have shown high TAC (Total anti-oxidant capacity) as compared to other extracts.

Results of ABTS assay of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew are shown in fig. 5. It is shown by graph that all extracts were significantly different from each other ($p < 0.05$). Methanol and dichloromethane extracts have shown high ABTS activity as compared to other extracts.

Results of antioxidant activity in terms of% inhibition were for DPPH assay 41.26%, 40.14%, 32.08%, 29.47%, 28.65% and 27.16% for dichloromethane, methanol, ethyl acetate, acetone, chloroform and *n*-hexane extracts. Whereas for ABTS assay readings were 48.69%, 47.64%, 39.54%, 39.01%, 35.94%, 32.67% for methanol, dichloromethane, ethyl acetate, acetone, *n*- hexane and chloroform extracts.

Anti-oxidant activity performed by all three methods (DPPH assay, ABTS assay and Phosphomolybdate assay) highest activity was observed for dichloromethane and methanol extracts. The results of antioxidant potential can clearly related to TPC results both dichloromethane and methanol extracts were high in total phenolics and have shown high antioxidant potential.

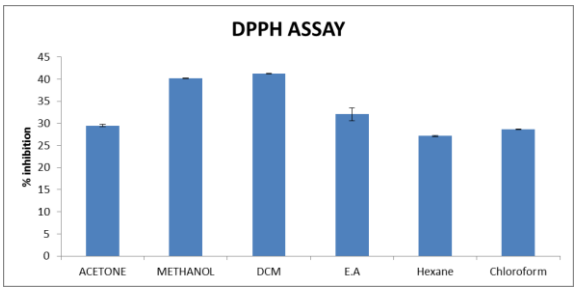

Fig. 3. DPPH Assay of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew.

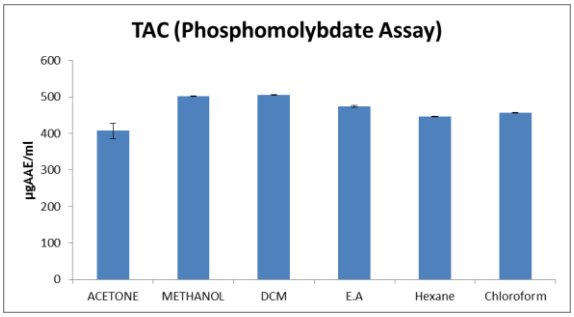

Fig. 4. TAC (Phosphomolybdate Assay) of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew.

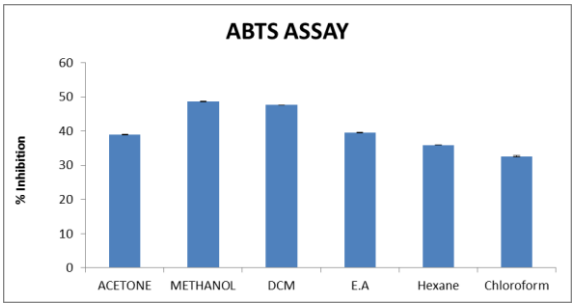

Fig. 5. ABTS Assay of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew.

A Dose dependent study was conducted by using DPPH and ABTS assays for determining IC_{50} value of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew. Results in term of IC_{50} value by using DPPH assay were given in fig. 6. IC_{50} value of different extracts was found to be Acetone 600,

Methanol 400, Dichloromethane 400, Ethylacetate 600, Hexane 800 and Chloroform 600. The respective $1/IC_{50}$ values were calculated for all the extracts and were found to be Acetone 0.00166, Methanol 0.0025, Dichloromethane 0.0025, Ethylacetate 0.00166, Hexane 0.0013 and chloroform 0.00166. As antioxidant activity has inverse relation with IC_{50} and direct relation with $1/IC_{50}$. So results were interpreted as methanol and dichloromethane (IC_{50} 400, $1/IC_{50}$ 0.0025) extracts have shown high activity followed by acetone, ethylacetate and chloroform extracts (IC_{50} 600, $1/IC_{50}$ 0.00166). Whereas *n*-hexane extracts has shown least (IC_{50} 800, $1/IC_{50}$ 0.0013) antioxidant activity by DPPH assay.

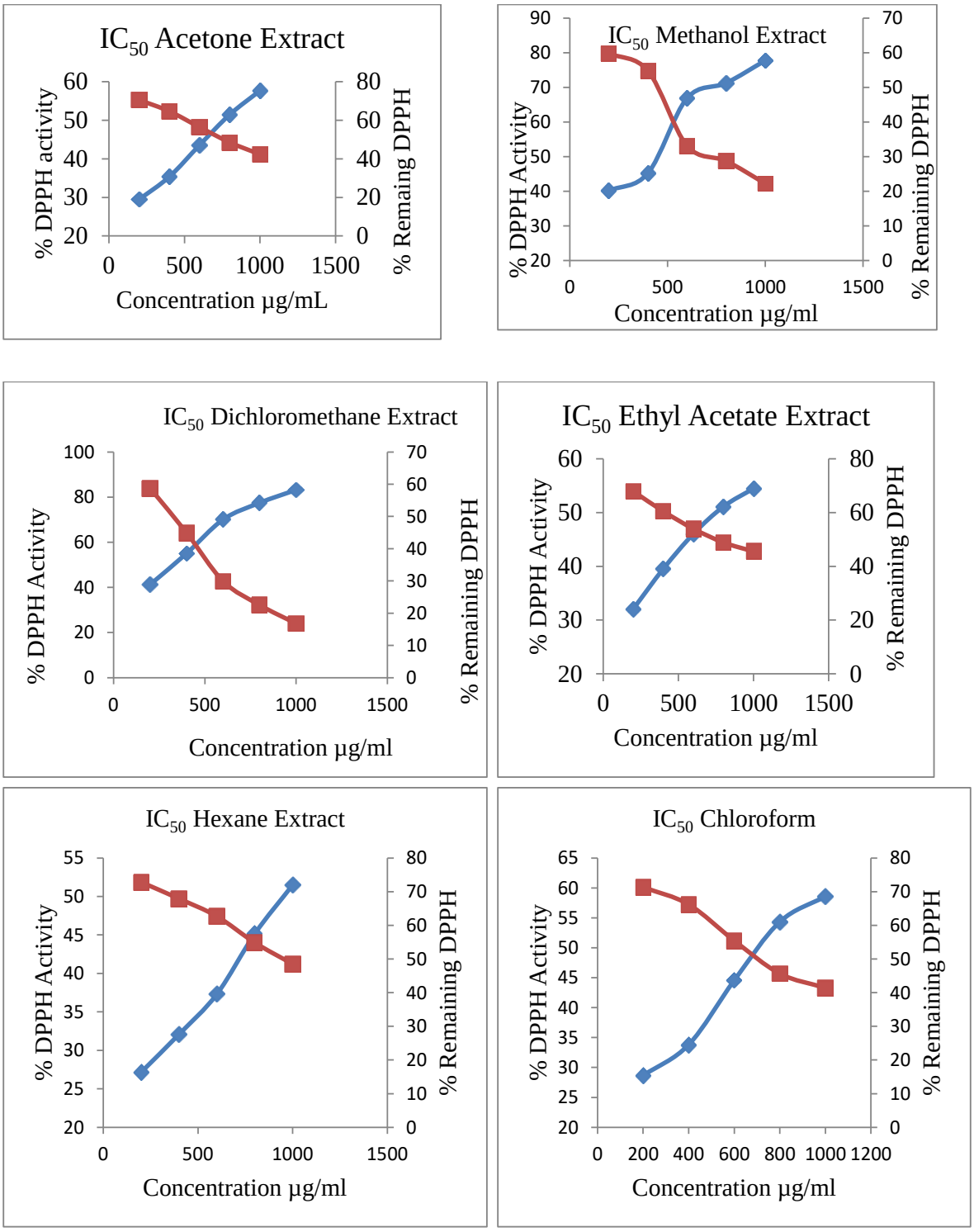

Fig. 6. Determination of IC_{50} (DPPH Assay) of different extracts of *Ziziphus jujube* Gaertn (L) var. hysudrica *Edgew.*

Results in term of IC_{50} value by using ABTS assay were given in fig. 7. IC_{50} values of different extracts were found to be acetone 400, methanol 400, dichloromethane 400, ethyl acetate 400, *n*-hexane 600 and chloroform 600. The respective $1/IC_{50}$ values were calculated for all the extracts and were found to be acetone 0.0025, methanol 0.0025, dichloromethane 0.0025, ethyl acetate 0.0025, *n*-hexane 0.00166 and chloroform 0.00166. According to IC_{50} study by using ABTS model it comes out to be that acetone, methanol, dichloromethane and ethyl acetate extracts have shown high activity (IC_{50} 400, $1/IC_{50}$ 0.0025)as compared to *n*-hexane and chloroform extracts (IC_{50} 600, $1/IC_{50}$ 0.00166).

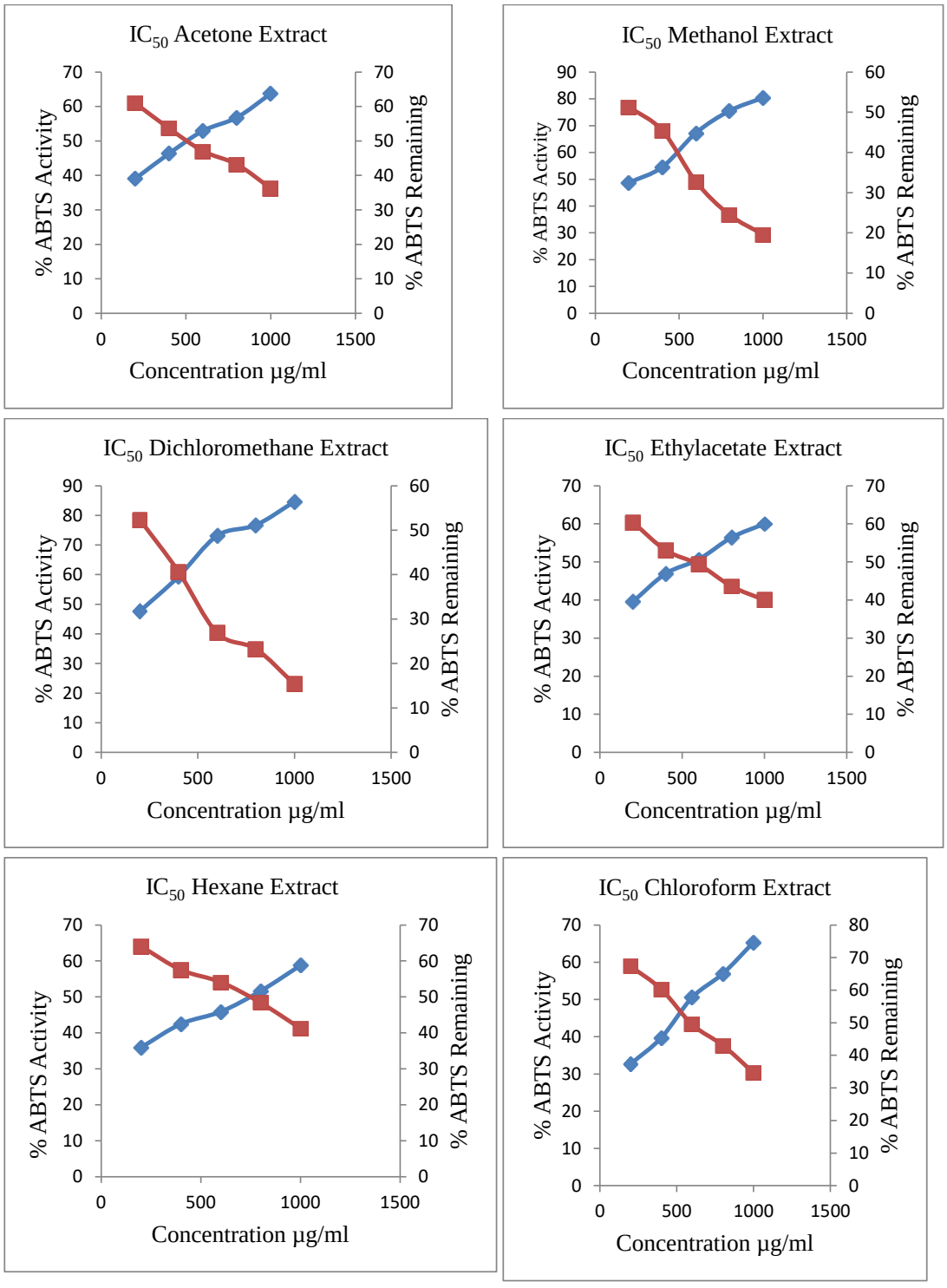

Fig. 7. Determination of IC_{50} (ABTS) of different extracts of *Ziziphus jujube* Gaertn (L) var. *hysudrica* Edgew.

The strong antioxidant activity was exhibited by various ziziphus species which was strongly related to the total phenolic contents present in the plant (Na *et al.,* 2001, Ziping *et al.,* 2009, Leila *et al.,* 2019, Salima *et al.,* 2018, Abalka *et al.,* 2011, Olufunmiso *et al.,* 2011). Ziziphus species contain phenolics and flavonoids as secondary metabolites (Rizwan *et al.,* 2017). Most common phenolics which occur in various ziziphus species includes caffeic acid, p-Coumaric acid, p-hydroxybenzoic acid, ferulic acid and vanillic acid (Gretchen *et al.,* 2005). Phenolic compounds have tendency to act as free radical scavengers (Riedel *et al.,* 2007). Inactivation of lipid free radicals and inhibition of decomposition of hydrogen peroxide into free radicals are two possible mechanism of action through which phenolics exert their antioxidant action (Maisuthisakul *et al.,* 2007)

Conclusion

Extraction of root bark of *Ziziphus jujube* Gaertn(L) var. *hysudrica* Edgew was done by using six different organic solvents of varying polarities(methanol, acetone, ethyl acetate, dichloromethane, chloroform and n-hexane). TPC and TFC was estimated for all of the extracts. Methanol and dichloromethane extracts have shown high content of total phenolics. Antioxidant potential of all extracts was measured by using three different in-vitro models (DPPH assay, ABTS assay and Phosphomolybdate assay).

According to all three models methanol and dichloromethane extracts have shown high antioxidant potential as compared to the other extracts. A dose dependant study was also conducted to calculate IC_{50} and $1/IC_{50}$ values by using DPPH and ABTS assay. According to DPPH assay methanol and dichloromethane extracts have shown high antioxidant activity. Whereas according to ABTS assay methanol, dichloromethane, ethyl acetate and acetone extracts have shown high activity as compared to chloroform and *n*-hexane extracts. So it was interpreted from study conducted that antioxidant activity was directly related to total phenolic content present in the extract.

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