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ESTIMATION OF BIOACTIVE CONSTITUENT OF HYDROALCOHOLIC EXTRACT OF *ZIZIPHUS MAURITIANA* USING RP-HPLC

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ABSTRACT

Ziziphus mauritiana is one of the underutilized herbs having potential to heal various ailments. It is reported in the ancient literature that whole plant as fruits, leaves, seed and root possess pharmacological activity. Several polyphenols have been tested in *Ziziphus mauritiana*. The flavonoid content is important because of the pharmacological properties of these compounds, whereas quercetin has been proved to be an antioxidant, antiinflammatory and hepatoprotective compound. A simple reverse phase high-performance liquid chromatography (RP-HPLC) method for the separation and quantitative determination of bio active constituent of hydroalcoholic extract of *Ziziphus mauritiana* has been developed and validated. The use of aC-18 column with mobile phase enabled the efficient separation of bio active constituent of hydroalcoholic extract within a 30 min analysis. The RP-HPLC method was validated as per ICH guidelines. The proposed RP-HPLC method was found to be simple, precise and accurate.

Key words:*Ziziphus mauritiana*, Hydroalcoholic extract, Quercitin, RP-HPLC Method

INTRODUCTION:

Plants contains various active phytocompounds which includes vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites which are well to do in antioxidant activity (Zheng and Wang, 2001; Cai et al., 2003).Numerous crude extracts and pure natural compounds from plants are reported to have antioxidant and radical-scavenging activities and intensive research has been carried out, either to characterize the antioxidant properties of extracts and/or to isolate and identify the compounds responsible for those activities seeking the development of natural antioxidant formulations in the areas of food, medicine and cosmetics (Boligon et al., 2009; Su et al., 2009; Kintzios et al., 2010).

Ziziphus mauritiana commonly known as Indian Jujube and 'Ber' is a tropical fruit

found in many parts of the world especially in Pakistan, India and Africa (Gupta et al., 2012; Azam-Ali, 2006). *Ziziphus mauritiana* Lam. belongs to the family Ramnaceae. It possesses anti-diabetic, anti-inflammatory, anti-plasmodial, and anti-microbial, as well as haemolytic, sedative, anxiolytic, diuretic, analgesic and antioxidant properties (Goyal et al., 2012). The leaves of *Ziziphus mauritiana* are eaten with catechu as an astringent. They are considered as diaphoretic and are prescribed for typhoid in children. They are also used as poultices. A decoction of the bark of *Ziziphus mauritiana* is used for the treatment of diarrhoea and dysentery. The bark is also used as an astringent in gingivitis. *Ziziphus mauritiana* root is used as bitter and cooling, cures headache. Decoction of roots is used in fever, and as powder applied to old wounds and ulcer.

High-performance liquid chromatography (HPLC) has been successfully applied to analyze plant extracts and products (Baratto et al., 2012;Fonseca et al., 2010). This method has several advantages, such as efficient separation, identification and quantification of similar compounds. However, to the best of our knowledge, no RP-HPLCmethod has been reported to evaluate extracts and products from *Ziziphus mauritiana*.In this context, the aim of the present work was to develop and validate a new RP-HPLC method for the determination of bioactive constituent that could be present in hydroalcoholic extract of *Ziziphus mauritiana*.

Materials and methods

Chemical and reagents

All the chemicals and reagents used were of analytical grade.

Plant material

The plant *Ziziphusmauritiana*was collected from local area of Bhopal (M.P.) in the month of January 2018.

Extraction procedure

Powdered plant material of *Ziziphus mauritiana* was shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place. Defatted dried powdered of *Ziziphus mauritiana* was extracted with hydroalcoholic solvent using maceration process for 48 hrs, filtered and dried using vaccum evaporator at 40 ºC.

Qualitative phytochemical tests

The extracts obtained by solvent extraction were subjected to various qualitative tests to detect the presence of plant constituents [Khandelwal, 2005;Kokate, 1994].

Identification of bioactive compound by TLC

Identification of bioactive compound was performed by thin layer chromatography (TLC) on silica plates (60F254, aluminium backed, 200 µm layer thickness, 10.0 x 5.0 cm). The presence of flavonoids, phenyl propanoids, alkaloids, terpenes, steroids, coumarins, quinones and proanthocyanidins were investigated using adequate development systems and revealers (Harborne, 1973; Roberts EAH &Cartwright , 1957; Wagner &Bladt, 1996). After development, the plates were air dried and sprayed with the revealers in a fume hood.

Total flavonoid content estimation

Determination of total flavonoids content was based on aluminium chloride method (Olajuyigbe & Afolayan, 2011). 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.10 mg of dried extract was dissolved in 10 ml methanol and filtered. Three ml (1mg/ml) of this extract was for the estimation of flavonoid.1 ml of 2% $AlCl_3$methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

High-performance liquid chromatography (HPLC)

Identification and quantification of bioactive constituents from the hydroalcoholic extract of *Ziziphus mauritiana*

Standard stock preparation: Standard stock of quercitin was prepared by dissolving 10 mg of dry extracts in 10 ml milique water.

Plant extract preparation: Hydroalcoholic extract of *Ziziphus mauritiana* was prepared by dissolving 10 mg of dry extract in 10 ml milique water.

Chromatographic conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTcauto sampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. Inertsil ODS-C18 (150 mm length × 4.6 mm inner diameter, 5µ particle diameter) analytical column from Phenomenex Inc. (Torrance, CA, USA) was used. Column oven temperature was 30°C, UV detector was used, separation mode was isocratic, mobile phase was acetonitrile-methanol (50:50 v/v) and flow rate was 1 ml/min. Total chromatographic run time was 30 min and injection volume was 20 µl. Plant extract and quercitin standard were separately run on chromatographic column. Based on the retention period of quercitin standard and its

corresponding peak in the crude extract chromatogram identification and quantification of the same was achieved. Spiking the standard quercitin solution with the hydroalcoholic extract of *Ziziphus mauritiana* was done to confirm presence of quercitin in the extract.

Results and discussion

Phytochemicals are the core of phytomedicines; their therapeutic efficiency directly correlates with the presence of various phytochemicals. Table 1shows the presence of alkaloids, flavonoids, glycosides, diterpenes**,** protiensand amino acid sin hydroalcoholic extract of *Ziziphus mauritiana.*

Table 1: Phytochemical screening of hydroalcoholic extract of *Ziziphusmauritiana*

S. No.	Test	***Ziziphusmauritiana***
1.	Detection of alkaloids: a) Hager's test: b) Dragendroff'stest:	 -ve -ve
2.	Detection of carbohydrates: a) Fehling's test:	 -ve
3.	Detection of glycosides: a) Legal'stest:	 +ve
4.	Detection of saponins a) Froth test:	 -ve
5.	Detection of phenols a) Ferric chloride test:	 -ve
6.	Detection of flavonoids a) Alkaline reagent test: b) Lead acetate test:	 +ve -ve
7.	Detection of proteins and aminoacids a) Xanthoproteictest:	 +ve
8.	Detection of diterpenes a) Copper acetate test:	 +ve

The content of total flavanoid compounds (TFC) content was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: Y = 0.06X+0.019, R^2= 0.999**,** where X is the quercetin equivalent (QE) and Y is the absorbance.

Table 2: Preparation of calibration curve of quercetin

S. No.	Concentration	Absorbance
0	0	0
1	5	0.352
2	10	0.61
3	15	0.917
4	20	1.215
5	25	1.521

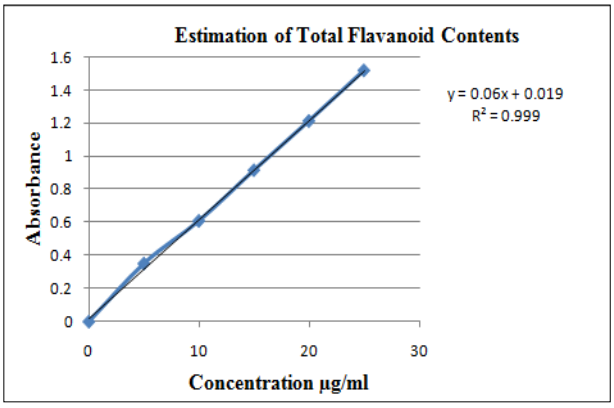

Figure 1: Estimation of total flavonoid content

Table 3: Total flavonoid content

S. No.	Extracts	Total flavonoid (mg/100mg)
1.	*Hydroalcoholic extract*	0.816

Quantification of flavonoid content showed that hydroalcoholic extract of *Ziziphus mauritiana* contained 0.816mg (QEmg/100mg) of extract (Table 3).

Table 4: TLC of hydroalcoholic extract

S.No.	Extract	Toluene: Ethyl acetate: Formic acid (5:4:1)
		Quercetin
1.	Hydro alcoholic	0.864

Identification of bioactive compound was performed by thin layer chromatography (TLC) using Toluene: Ethyl acetate: Formic acid (5:4:1) as solvent system. Development of TLC plate confirmed the presence of quercitin (Table 4).

Reverse phase C-18 column equilibrated with mobile phase methanol: acetonitrile (50:50, v/v) was used. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 256 nm. Each of the standard drug solutions were injected 3 times and the

mean peak area of drug was calculated and plotted against the concentration of the drug. The sample solution was chromatographed and a concentration of quercetin in extract sample was found out using regression equation.

Table 5: Preparation of calibration curve

S. No.	Conc.	Mean AUC
1.	0	0
2.	5	410.235
3.	10	892.541
4.	15	1387.324
5.	20	1889.548
6.	25	2317.181

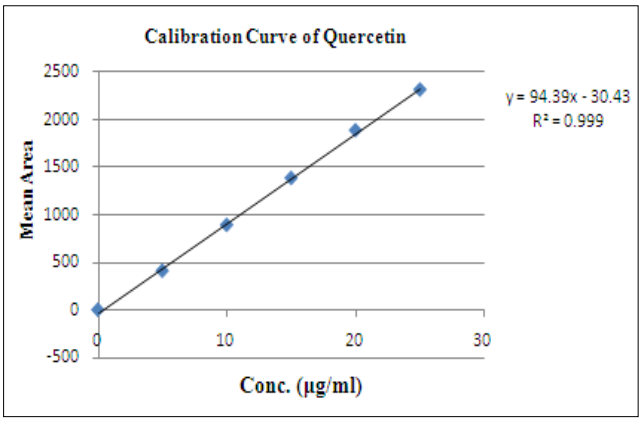

Figure 2: Calibration curve of the quercetin

Table 6: Characteristics of the analytical method derived from the standard calibration curve

Compound	Linearity range µg/ml	Correlation co-efficient	Slope	Intercept
Quercetin	5-25	0.999	94.39	-30.43

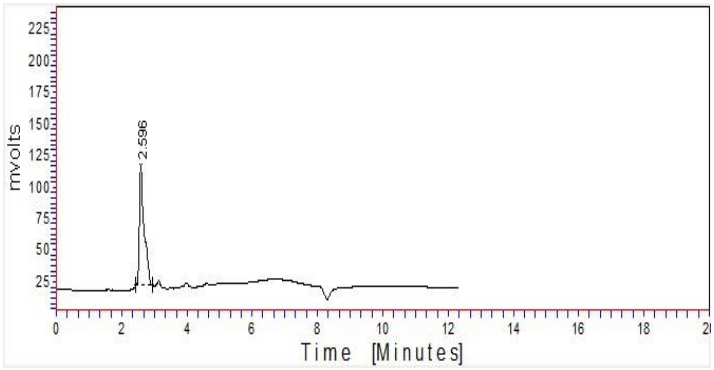

Figure 3: Chromatogram of standard Quercetin

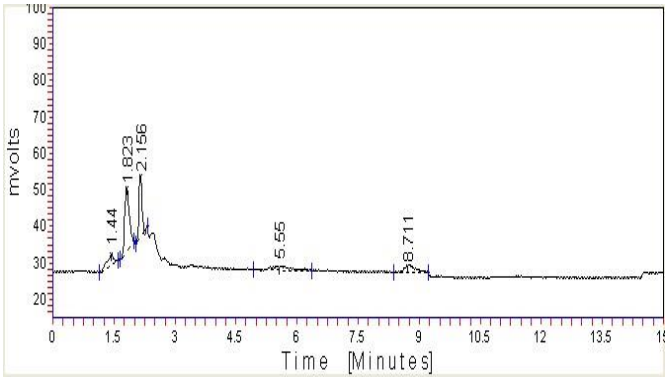

Figure 4: Chromatogram of hydroalcoholic of *Ziziphus mauritiana*

Table 7: Quantitative estimation of Quercetin in Hydro alcoholicextract

S. No.	Extract	RT	Area	% Assay
1.	*Ziziphus mauritiana*	2.156	70.173	0.106

Standardization of the plant extract was done by quantifying the major active components of the plant extract. The major active component quercitin is present in plant and

the hydroalcoholic extract of the plant *Ziziphus mauritiana* was separated from other constituents by reverse phase HPLC analysis. Under the optimized chromatographic conditions, the reaction time and peaks for standard quercitin and hydroalcoholic extract is shown in Figure 3 and 4.

In the present study qualitative assessment of phytochemical constituents of *Ziziphus mauritiana* plant extract showed presence of alkaloids, flavonoids, glycosides, diterpenes**,** protiens and amino acids. The quantitative analysis revealed presence of flavonoid content in *Ziziphus mauritiana* extract. The developed RP-HPLC method will assist in the standardization of *Ziziphus mauritiana* using biologically active chemical markers. The proposed HPLC methods for simultaneous estimation of quercitin from *Ziziphus mauritiana* seems to be accurate, precise, reproducible and repeatable. *Ziziphus mauritiana* also contained a number of other constitute, which are currently the subject of further investigation, apart from those standards studied. Our results provide a fully validated RP-HPLC method for quality control of plant extracts and phytopharmaceuticals containing *Ziziphus mauritiana* using quercitin as a chemical marker. Validation of the method as per ICH guidelines showed that the method is in compliance with the current guideline. The method was found to be robust.

CONCLUSION

In conclusion, besides being simple and specific, this method showed reasonable accuracy, precision and linearity. It did not involve laborious time-consuming sample preparations and can therefore be considered suitable for the routine quantitative analysis of quercitin in formulations containing *Ziziphus mauritiana*, as well as in its extracts. Considering that natural substances can be responsible for the protective effect against the risk of many disease processes, the results described in this paper suggest that *Ziziphus mauritiana* may serve as a stimulus for further study to assess the antioxidant activity of compounds isolated from the different parts of this species.

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