International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 9, Issue 5, 2017

Original Article

QUANTIFICATION OF PHYTOCHEMICAL CONSTITUENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY IN THE LEAVES OF*CITRUS MEDICA*

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Received: 23 May 2017, Revised and Accepted: 22 Jul 2017

ABSTRACT

Objective: In the present investigation, phytochemical assay and *in vitro* antioxidant activity of ethanol (70%), methanol, ethyl acetate and hexane extract of *Citrus medica* leaves were carried out.

Methods: The quantification of total phenolic and alkaloid contents were estimated by taking gallic acid and atropine as standard. *In–vitro* antioxidant activity of extracts was evaluated by using different free radicals (DPPH, superoxide and free radicals).

Results: Ethanol extract of leaves have more phenolic and alkaloid contents than other extracts. The selected plant extract was produced concentration dependent percentage inhibition of different free radicals and produced maximum activity at a concentration of 1000µgm/ml, and there after percentage inhibition was raised gradually to its maximum level with higher concentrations. HPLC analysis revealed the presence of Gallic acid, Catechein, Rutin, Chlorgenic acid, Queracetine and some unknown components which need to be e identified.

Conclusion: Among the four extracts, ethanol extract of *C. medica* showed good antioxidant activity.

Keywords: Alkaloids, Antioxidant, *Citrus medica*, HPLC, Phenolics

DOI: http://dx.doi.org/10.22159/ijcpr.2017v9i5.22153

INTRODUCTION

Since long back human civilization is using plant and plant derived products as a source of medicine. As the plants possess potent activity against several diseases, in the recent scientific developments so many medicinal properties of such plants have been investigated throughout the world. It possesses another advantage like no side effects and economic feasibility. Many plants species are reported to synthesize certain secondary metabolites with important medicinal properties which include antioxidant activity, free radical scavenging abilities, anti-carcinogenic anti-inflammatory, etc. [1]. Hence recently, natural plants are getting ample attention of researchers for sources of biologically active substances like antioxidants. A vast studies is already being carried out on several plants, vegetables and fruits which are rich sources of antioxidants, like vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds and flavonoids which reported to prevent free radical damage, reducing the risk of chronic diseases [2]. During normal metabolic activities in a human cell, various physiological and biochemical reactions generate free radicals and other reactive oxygen species (ROS) as the by-products. Free radicals are chemical species, which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability and thus can cause oxidative damage to important biomolecules like proteins lipids in living cell, ultimately leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans [3]. Thus ROS have been found to play an important role in several diseases such as ageing, atherosclerosis, inflammatory injury, cancer, and cardiovascular disease [4].

Phytochemical assay of crude extracts of several plants reported to bear the antioxidant activity [5, 6]. As a result, some natural products of such plants have been approved as antioxidant drugs. Antioxidant activity of plants mainly results of presence secondary metabolites, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes [7, 8]. In the present study quantification of phytochemical constituents and antioxidant potential of leaves a medicinal plant *Citrus medica* (Lemon), is aimed. Based on the review about the therapeutic values of *Citrus medica,* the present study was attempted to screen the phytochemicals, their quantification and analysis [9, 10].

The *Citrus medica* (Citron) isa plant with fragrant fruit and leaves. It is a prominent member in the genus Citrus belonging to the Rutaceae or Rue family, found in the base region of Himalaya from Gadwall to Sikkim at the height of 4000 feet. It is also seen in Assam, central India and Western Ghats of India and more commonly present in the Mediterranean region and central and southern parts of America [9]. This study represents the first approach for characterization of phytochemicals of *Citrus medica* leaves. The fruit juice of *Citrusmedica* exerts antimutagenicity and anticancer effect [11]. Its aqueous and alcoholic extracts were found to be active as anthelmintic with reference to both the paralysis and death times as compared to the piperazine citrate. The aqueous extract is more active than alcoholic extract. Phytochemical screening states that it contains fixed oils, volatile oils, citric oxide and flavanone glycosides are abundant constituents of citrus leaves and fruits [12].

MATERIALS AND METHODS

Chemicals and drugs

All chemicals and solvents were of analytical grade obtained from Hi-media Mumbai, SD Fine Chemicals Pvt. Ltd., Mumbai, Sigma Chemical Company, U. S. A., Loba chemicals, Mumbai.

Preparation of extracts from *Citrus medica* leaves

The fresh plant material used in the present study was collected from one agro field in Aurangabad district, M. S., India. It is authenticated by the taxonomist of Botany Department of Institute of Science, Aurangabad, M. S., India. Freshly collected plant material was dried under shade and dried material was crushed to obtain a coarse powder. The powdered material was separately extracted in a soxhlet apparatus for 6 h successively with hexane, ethyl acetate, 70 % v/v ethanol and methanol was concentrated to dryness under vacuum by using Rota-vapor.

Phytochemical studies

Phytochemical studies were carried out for hexane, ethyl acetate, hydro alcoholic (70% v/v ethanol) and methanol extracts of *C. medica* to detect the presence of different phytochemical constituents like alkaloids, steroids, terpenoids, tannins, flavonoids, saponins, glycosides, amino acids etc. by using standard procedure [13-15].

Quantification of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteau reagent [16]. Singleton *et al.* Folin-Ciocalteau colorimetry is based on a chemical reduction of the reagent having a blue absorption with a maximum at 765 nm. The intensity of light absorption at that wave length is proportional to the concentration of phenols. By using standard gallic acid calibration curve, measure the concentration of phenolic content in gallic acid total equivalents using unit's mg/g (GAE).

Quantification of total alkaloid content

Total alkaloid content was determined by *Fazel et al.* [17] method. The plant extract (1 mg/ml) was dissolved in 2N HCl and then filtered. One ml of this solution was transferred to a separating funnel and then 5 ml of BCG (bromocresol green) solution along with 5 ml of phosphate bufferof pH 7 were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of complex in chloroform was measured at 470 nm. All experiments were performed thrice; the results were averaged and reported in the form of mean±SEM

***In vitro* antioxidant activity**

For the assessment of free radical scavenging activity, the hexane, ethyl acetate, Ethanol (70% v/v) and methanol extracts were dissolved and 5 % dimethyl sulphoxide (DMSO) respectively.

Superoxide radical scavenging activity

Superoxide scavenging activity of the plant extract was determined by McCord and Fridivich method [18], which depends on light induced superoxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity is frequently used to evaluate the free radical scavenging effectiveness of various antioxidant substances [2]. It was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the $Fe^{2+}/EDTA/H_2O_2$ system. The hydroxide radical's attacks deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances.

DPPH radical scavenging activity

The scavenging activity for DPPH free radicals was measured according to the procedure described by [19]. This method is based on the reduction of alcoholic DPPH solution (dark blue in color) in the presence of a hydrogen donating antioxidant converted to the nonradical form of yellow colored diphenyl-picrylhydrazine. Lower the absorbance higher the free radical scavenging activity [20].

HPLC analysis

Separation and quantification of the various components in the ethanolic (70% v/v) extract was carried out using a reversed-phase high performance liquid Chromatographic system with the UV detector. *(Younglin (S. K) isocratic System UV Detector* i.e. having constant wavelength for detection) consisting of UV Detector and*Autochro-3000* software. The size of column 4.6 × 250 mm consisting of silica particles of diameter 5 µm and modified with alkyl chain having 18 Carbons (C_{18})(Grace) as the stationary phase. In pair formation agent (0.1 % trifluoroacetic acid) enhances the retention of highly charged molecules due to charge compensation. The column was thermo stated at ambient temperature. Samplessize of 20 µl were injected with flow rate 0.5 ml/min. The initial mobile phase was a dilute aqueous solution consist of a mixture of water/acetic acid (98:2, v/v) was used (solvent A). Aqueous acetonitrile (50:50, v/v) with 0.5 % (vol.) acetic acid additive was used as solvent B. Following gradient was used for elution: 10 % of B at 0 min, 55 % of B at 50 min, 100 % f B at 60 min and 10 % of B at 65 min. A wavelength of 280 nm was used for the detection of gallic acid, chlorgenic acid, catechin, rutin, quercetin etc. as standard compounds. The identification of each compound was carried out comparing the retention time and UV-Vis spectra of the peaks with those previously obtained by the injection of standards. Each compound was quantified as mg/100g of dry sample material by using the peak area because peak area is proportional to the quantity of that compound in the sample [21].

RESULTS AND DISCUSSION

Quantification of phytochemical constituents

Quantified phenolic contents of *Citrus medica* leaves extracts were ranging from 42.45±0.48 to 11.32±0.45 mg/g. The ethanolic extracts (70%) have found more phenolic content (42.45±0.4845 mg/g) than the other extracts. Alkaloid content was ranging from 47.65±0.46 to 19.65±0.75 mg/g. Here also ethanol extract (70%) has more alkaloidal content (47.65±0.46 mg/g) than other extracts (table 1).

Table 1: Total phenolic and alkaloid content (mg/g) *of Citrus medica* leaves extract

Name of the extract	Total phenolic content (mg/g)	Total alkaloid content (mg/g)
Hexane	11.32±0.45	19.65±0.75
Ethyl acetate	23.5±0.24	34.89±0.67
Methanol	19.45±0.34	27.67±0.54
Ethanol (70% v/v)	42.45±0.48	47.65±0.46

***In vitro* antioxidant activity**

The results of the present research revealed that, the hexane, ethyl acetate, methanol and ethanol (70% v/v), extracts of *Citrus medica* leaves were found to possess concentration dependant scavenging activity on DPPH radicals (fig. 1). The mean IC_{50} values for DPPH radical of hexane, ethyl acetate, methanol and ethanol (70% v/v), extracts of *Citrus medica* leaves were found to be 118 µg, 134 µg, 248 µg, 345 µg, 45 µg respectively. The mean IC_{50} value for ascorbic acid was found to be 45 µg. (table 2).

The Ethanol (70% v/v), methanolic, ethyl acetate and hexane extracts *C. Indica* leaves were found to possess concentration dependent scavenging activity on superoxide generated by photo reduction of riboflavin.(fig. 2). The mean IC_{50} values for superoxide radicals of hexane, ethyl acetate, methanol, ethanol (70% v/v) and ascorbic acid, extracts of *Citrus medica* leaves were found to be 245 µg, 123 µg,154 µg, 296 µg, 23 µg respectively. The mean IC_{50}value for ascorbic acid was found to be 23 µg (table 2).

The Ethanol (70% v/v), methanolic, ethyl acetate and hexane extracts *M. Ferrea* leaves were found to possess concentration dependent scavenging activity on hydroxyl radicals and the results were given (fig. 3). The mean IC_{50} values for hydroxyl radicals of hexane, ethyl acetate, methanol, ethanol (70% v/v) and ascorbic acid, extracts of *Citrus medica* leaves were found to be 189 µg, 256 µg, 287 µg, 335 µg and 64 µg respectively (table 2). The mean IC_{50} value of ascorbic acid was found to be 59.3µg (table 2).

HPLC analysis

In order to identify the certain active component in the plant extracts sample, HPLC analysis was performed. As ethanolic extract was found to give more concentration dependent scavenging activity on DPPH radicals, superoxide radicals and hydroxyl radicals, it was subjected for HPLC analysis. The result of the above analysis showed the presence of compounds like Gallic acid, Catechein, Rutin, Chlorgenic acid, Queracetine and some unknown components which need to be identified.

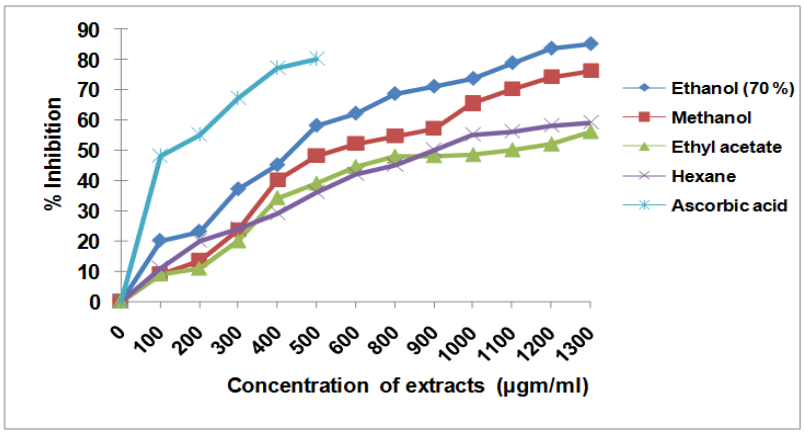


Fig. 1: Concentration dependent percent inhibition of DPPH radical by extracts of Citrus medica leaves and ascorbic acid

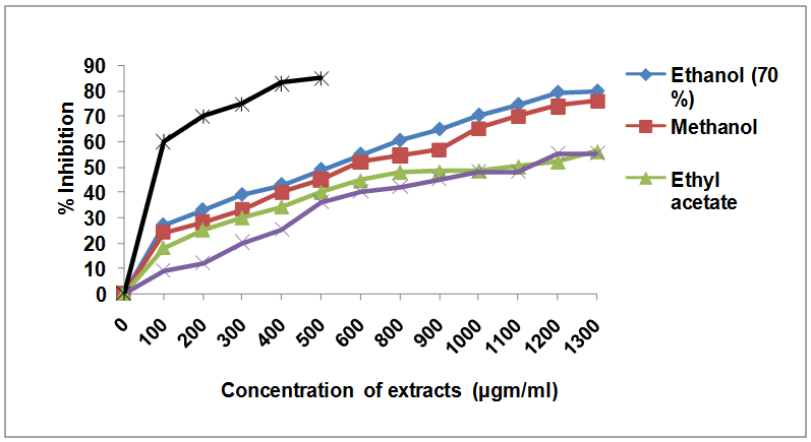


Fig. 2: Concentration dependent percent inhibition of superoxide radical by different extracts of Citrus medica leaves and ascorbic acid

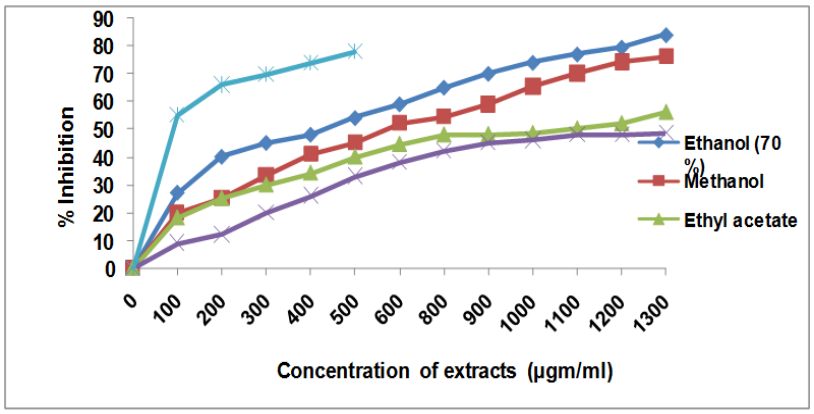
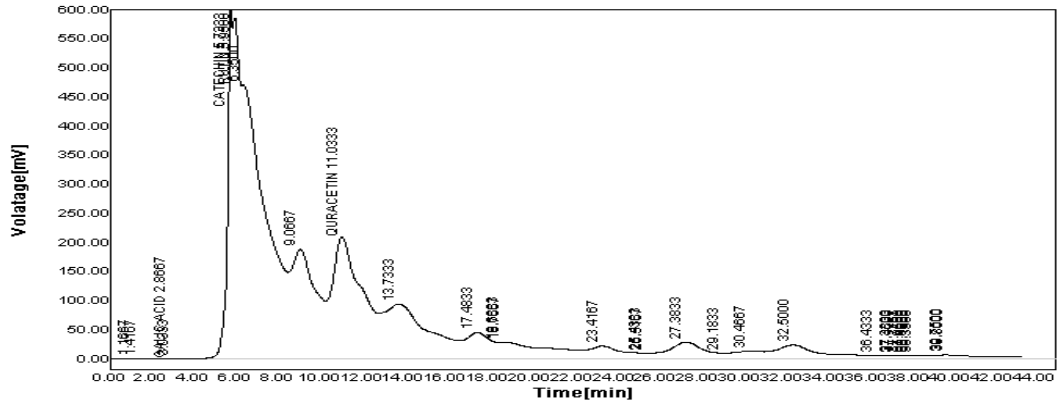


Fig. 3: Concentration dependent percent inhibition of hydroxyl radical by different extracts of Citrus medica leaves and ascorbic acid

Table 2: *In vitro* 50% inhibition concentration (IC_{50}) of different extracts of *Citrus medica* leaves on DPPH, superoxide and hydroxyl free radicals

Name of the extract	IC_{50} value (µ g)		
	DPPH radical	Superoxide radical	Hydroxyl radical
Hexane	118	245	189
Ethyl acetate	134	123	256
Methanol	248	154	287
Ethanol (70% v/v)	345	296	335



S. No.	Name	RT [min]	Area[mV*s]	Area %
1		1.1667	6.3090	0.00
2		1.4167	2.9671	0.00
3	GALIC ACID	2.8667	2.8812	0.02
4		3.0333	2.8170	0.00
5	CATECHIN	5.7333	9374.9990	7.42
6	RUTIN	5.9500	13927.8164	11.02
7		6.3500	37288.0859	29.50
8		9.0667	15577.4395	12.33
9	QURACETIN	11.0333	20228.5137	16.01
10		13.7333	13922.0586	11.02

Fig. 4: HPLC profile report of ethanolic extract of *Citrus medica*

CONCLUSION

Various Chemical compounds *viz*. alkaloids, steroids, phenols, glycosides from *Citrus medic* plant are known to be useful in the treatment of many diseases [22]. Thus, leaves containing these compounds may serve as a potential source of bioactive compounds in the treatment of diseases.

The plant extracts prepared from leaves in various solvents showed presence very important compounds especially phenolic and alkaloids which have their medicinal uses in recent years. Alkaloids are related with medicinal uses for centuries including their role in cancer treatment [23]. The phenolic compounds in this plant may contribute to its antioxidant properties and thus the usefulness in herbal treatment [3]. Hence during the preparation of some antimicrobial and antioxidant compounds Phenols is found to use.

The results of phytochemical and HPLC suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for Radical's reactivity. The plant extracts were capable of scavenging super oxide, hydroxyl and DPPH in a concentration dependent manner. The data clearly indicated that the extracts ethanol, methanol (70%), hexane, ethyl acetate and of *C. medica* showed good antioxidant activity. Among the all the ethanol (70%) extract showed better activity.

CONFLICT OF INTERESTS

Declare none

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How to cite this article

- Mayavatis Patil. Quantification of phytochemical constituents and *in vitro* antioxidant activity in the leaves of *Citrus medica*. Int J Curr Pharm Res 2017;9(5):119-123.