

# In Vitro Evaluation of the Antioxidant Activities in the Seeds Extract from *Citrus Medica* L.

Shikha Rani<sup>1</sup>, Dr. N. S. Gill<sup>2</sup>

Department of Pharmaceutical Chemistry, Rayat Institute of Pharmacy, Railmajra

Email: shikhavishalshakur[at]gmail.com

**Abstract:** From classical times, plants are wealthy source of effective and safe medicines. Herbal medicines have been the main source of primary health maintenance in many nations. The benefits of natural products with medicinal properties is as ancient as human advancement and, for a long time, mineral, plant and animal products were the main origins of drugs. The reasons for this were that pure compounds were easily obtained, structural moderations to manufacture potentially more active and safer drugs could be easily executed and the commercial power of the pharmaceutical companies was increasing. Citrus fruits are surrounded by the most extensive horticultural products enjoying universally as item of diet. They are rich in vitamin C, minerals and carries distinct flavors. *Citrus medica* L., Generally called as a Citron in English and Bijapura in Ayurvedic literature is member of Rutaceae family. Several parts of Citron are generally used in Indian traditional system of medicine. Leaves are useful to induce sleep. In earliest literature citron was observed as an antidote of each kind of poison. *Citrus medica* L. leaves carries anthelmintic and estrogenic activities; fruit has analgesic, anticancer and antiulcer activities; peel carries many characteristics including hypoglycemic, anti - cholinesterase, hypocholesterolemic, hypo - lipidemic, antimicrobial and anthelmintic properties; seed has anti - diabetic, hypocholesterolemic, hypolipidemic and estrogenic activities. Apart from medicinal uses, the plant has high scale value because of its edible and nutritive fruit, useful wood, latex and bark and provides generous occupations support to local citizens.

**Keywords:** Citron, *Citrus Medica* L., Rutaceae, Phytochemistry, Pharmacological activities

## 1. Introduction

Natural products have been the origin of most of the active elements of medicines (Alan. L. Harvey, 2008). They (secondary metabolites) have been the most fruitful origin of unrealized drug leads (Mishra B. B, et al., 2011). Natural products imply huge and different secondary metabolites with an entire choice of organic actions those have confirmed with their various methods, especially in human and veterinary and also in agriculture. Natural products are derived from microorganisms, marine animals, and also from plant sources. Natural products that are sold as herbal and/or food additives for medications cover - up half of the main 50 drugs sold in European pharmacies. Ayurveda, the traditional Indian medicine remains the most earliest, with sound rational root. The use of Natural products as health - care administration system might be showed a great objection in early human development (Adhikari P. P, et al., 2017).

Plants provide rich natural antioxidants. Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts, grains and some meats, poultry and fish (Kebede Muluken, et al., 2019). Antioxidants are the substances that obstruct oxidation. Oxidation is a chemical reaction that can manufacture free radicals, thereby leading to chain reactions that may harm the cells of organisms. Antioxidants such as thiols or ascorbic acid (vitamin C) restrict these chain reactions (Dabelstein W, et al., 2007). Antioxidants have important preventive roles, not only on undesirable changes in the flavor and nutritional quality of food, but also on tissue damage in various human diseases. They are effective in prevention of degenerative illnesses, such as different types of cancers, cardiovascular and neurological diseases, cataracts and oxidative stress dysfunctions (Sharma. S. K, et al., 2013).

*Citrus* is a genus of flowering trees and bushes in the rue family, Rutaceae. Plants in the genus produce **citrus fruits**, like Citrus Sinensis (Sweet Orange), Citrus Limon (Lemon), Citrus Aurantium (Bitter Orange), Citrus Paradisi (Grape fruit), Citrus Medica (Citron) (Wu, Guohong Albert, 2017). The Citrus genus is consisting of various species and each species has many varieties. Taxonomic description is very difficult and the accurate number of species is still not clear because there are many spontaneous and commercial hybrids. According to the Swingle system, 16 species were identified and the Tanaka system identifies 156 species in the genus (AC Matheyambath, 2016). All species of citrus have traditional medicinal value. Citrus preparations, in case for prepared or fresh fruit products, from the biggest producing countries are an important materials for universal trade and of fabulous economic value and impact. In addition to the use as a food or drink source, citrus products from some of the wild species not grown commercially are also of value as agents of traditional medicinal and healthy utilization, In spite of the diversity of fruit types, however, nearly 70% of the world's citrus production is sweet orange (Manuel T, et al, 2008).

The citron is a fragrant fruit with the botanical name *Citrus medica* L., which spreads to both the Swingle & Tanaka system. It is an enlarged member in the genus Citrus, belonging to Rutaceae or Rue family, sub - family Aurantioideae (Meena A. K, et al., 2011). The family Rutaceae consist of aromatic & medicinal plants, which are used in traditional medicine. Citron developed in Assam, central India & Western Ghats of India (Kalpesh Panara, et al., 2012). It is more often present in the Mediterranean region, Central & Southern part of America. It is also available in Japan, China, Bangladesh, Arabia, Australia, tropical & subtropical areas, in the month October to January (Beatriz AA, et al., 2005). Citron has long been known to carries many essential nutritional ingredients such

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as phenolics, flavanones, ascorbic acid (vitamin C), and pectin, which are known as potent antioxidants. It is a potent antiscorbutic and used for evacuating poison and correcting noxious breath. Thus, we can use some fruits like citron (*Citrus medica* L.) in several formulations and can promote its health potential in lowering the prospect of various common diseases (Navnidhi Chhikara, et al., 2018).

## 2. Material and Method

### 2.1 Collection of plant

The fruits of *Citrus Medica* L. Were collected in the month of November and December from the village Joh of district Una, Himachal Pradesh (India). After that, seeds were separated from the fruits by removing their pulp. The healthy seeds were selected from them for future procedure.

### 2.2 Chemicals

Ethanol, pet ether, Ascorbic acid, Sodium nitroprusside, Sulphuric acid, Conc. HCl, N - (1 - Naphthyl) ethylene diamine, DPPH, Nitric acid, Chloroform and sodium hydroxide.

### 2.3 Preparation of the Seed's extract:

The seeds were separated from fruits, washed, cleaned and after that seeds were shade dried and powdered using kitchen grinder at the temperature below 20°C. The powder of seeds was extracted with ethanol for 72 hours at room temperature by cold maceration process. And the extract was decanted and filtered through Whatman No.1 filter paper. Resulting filtrate was defatted with Petroleum ether so as to remove vegetable fat. Defatted extract was concentrated to remove the excessive solvent. After filtration, the filtrate served for phytochemical and antioxidant studies (Aqil & Ahmad, 2003). The extraction procedure is given below:

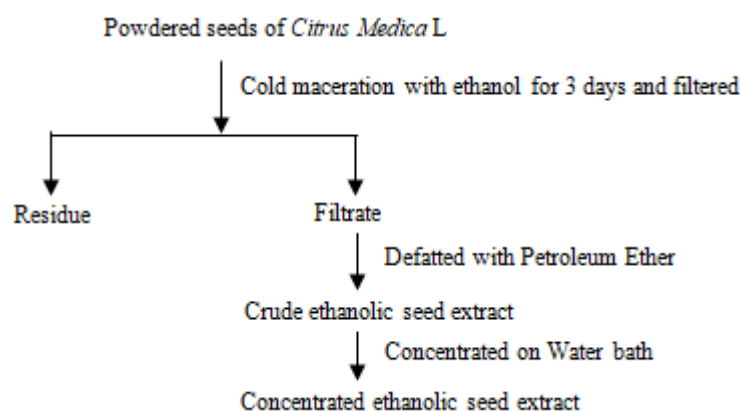


Figure 2.1: Flow chart for seed's extract

### 2.4 Preliminary phytochemical screening of the extract

Phytochemical tests were carried out for various constituents like Flavonoids, Tannins, Alkaloids, Steroids, Sterols, Carbohydrates, Glycosides (Anthraquinone Glycosides, Saponin glycosides, Cardiac glycosides), Amino acids, Proteins and volatile oil.

#### 2.4.1 Test for Alkaloids

- **Dragendroff's test:** To 1 mL of test filtrate, two drops of Dragendroff's reagent (Potassium bismuth iodide solution) were added and observed for the formation of prominent reddish - brown precipitates.
- **Hager's Reagent test:** Hager's reagent (picric acid solution) was added to the extract, yellow coloured precipitates were observed.
- **Mayer's Reagent test:** Mayer's reagent (potassium iodide solution) was added to the extract, cream coloured precipitates show the presence of alkaloids.
- **Wagner's test:** To 2 - 3 mL the test solution, few drops of Wagner's reagent was added, reddish brown coloured precipitates show the presence of alkaloids.

#### 2.4.2 Test for Steroids

- **Salkowski test:** To this extract, 2mL of Chloroform and 2mL of concentrated sulphuric acid were added, shaken well and observed the coloration of chloroform and acid

layers. Chloroform layer as red in color and acid layer as greenish yellow fluorescence.

- **Sulfur test:** Small amount of sulfur powder was added to the extract solution, it sinks at the bottom.

#### 2.4.3 Test for Flavonoids

- **Shinoda test:** To the test solution, few magnesium turnings and concentrated hydrochloric acid was added dropwise, pink scarlet or crimson red colour observed.
- **Alkaline Reagent test:** To the extract solution, few drops of sodium hydroxide solution was added, intense yellow colour was formed which turns to colourless on addition of few drops of dilute acetic acid indicate presence of flavonoids.
- **Zinc Hydrochloride test:** To the extract solution, mixture of zinc dust and conc. Hydrochloric acid was added. It gives red colour after few minutes.

#### 2.4.4 Test for Amino Acid

- **Millon's test:** To the extract, about 2mL of Millon's reagent was added. White precipitates indicate the presence of amino acids.
- **Ninhydrine test:** To the extract, Ninhydrine solution was added & boiled. Violet colour indicates the presence of amino acids.

#### 2.4.5 Test of Glycosides

About 50mg of the extract was hydrolyzed with concentrated hydrochloric acid for 2 hours on a water bath, filtered. The filtrate was subjected to the following tests.

##### a) Test for Cardiac glycosides

**Baljet's test:** The test solution was treated with picric acid or sodium picrate, orange colour was formed.

##### b) Test of Saponin Glycosides

**Foam test:** Filtrate was taken and 20mL of distilled water was added and shaken for 15 min in a graduated cylinder and observed for formation of a layer of stable foam.

##### c) Test for Anthraquinone Glycosides

- **Brontragger's Test:** Dilute sulphuric acid was added to 3 mL extract, boiled for 10 min and filtered, to the cold filtrate chloroform was added and shaken well, organic layer was separated which indicates the presence of Anthraquinone glycoside.
- **Nitric Acid Test:** To the test solution, concentrated nitric acid was added, orange brown colour formed which indicates the presence of Anthraquinone glycoside.

#### 2.4.6 Test of Carbohydrates

**Molisch test:** 1mL of test solution was taken and two drops of alcoholic solution of  $\alpha$  - naphthol (Molisch's reagent) was added. The mixture was shaken and 1mL of conc.  $H_2SO_4$  was added slowly from the sides of the test tube. The test tube was cooled in ice water and allowed to stand. Then the test tube was observed for violet - purple ring formation at the junction.

**Fehling solution Test:** 1 ml of Fehling's A and 1 ml of Fehling's B solution were mixed, then boiled for 1 min. Equal volume of test solution was added and heated in boiling water bath for 5 - 10 min. Appearance of first yellow and then brick red precipitate indicates the presence of reducing sugars.

#### 2.4.7 Test of Proteins

**Biuret test:** To 2 mL test solution, about 2mL Biuret reagent was added, Violet colour indicates the presence of proteins.

**Xanthoproteic test:** To 5mL of test solution, 1 mL of concentrated nitric acid was added and boiled, yellow precipitates formed. After cooling it, 40% sodium hydroxide solution was added, orange colour was formed.

$$\% \text{ Radical scavenging activity} = \frac{\text{Absorbance of Control} - \text{Absorbance of test sample}}{\text{Absorbance of Control}} \times 100$$

#### 2.4.2 Quantitative Evaluation of NO Free Radical Scavenging Activity

##### Reagents

10 mM sodium nitroprusside  
Phosphate buffer saline pH 7.4

##### Griess Reagent:

30 ml of 0.1% (1mg/mL) solution of N - (1 - naphthyl) ethylenediamine dihydrochloride (Component A).  
25mL of 1% (10mg/mL) solution in 5% O - phosphoric acid (Component B)

#### 2.4.8 Test of Sterols

**Molischott test:** A few mg of the extract was heated with 1mL mixture of sulphuric acid and water (5: 1), red violet colored confirmed the presence of sterols

#### 2.4.9 Detection of tannins

**Ferric chloride test:** A few mg of extract was added to the distilled water, and then filtered it. To the filtrate few drops of ferric chloride was added, bluish green or brownish green colour appears which indicates the presence of Tannins.

#### 2.4.10 Detection of Volatile Oils

**Sudan III test:** To the small amount of test sample, SudanIII solution was added. Red colour obtained by globules indicates the presence of volatile oil (Anonymous, 2001; Evans, 2002; Kokate, 2006; Khandelwal, 2010).

#### 2.4 Antioxidant Activity

In vitro evaluation of free radical scavenging activities is done by following 2 methods:

- 1) DPPH Method
- 2) NO Method

#### 2.4.1 Quantitative Evaluation of DPPH Free Radical Scavenging Activity

##### Reagents

0.1Mm DPPH Solution

Phosphate buffer saline pH 7.4

The free radical scavenging activity of extracts was measured by 1, 1 - diphenyl - 2 - picryl hydrazyl (DPPH) method. Various concentrations (25, 50, 75, 100 $\mu$ g/ml) of extract and ascorbic acid were prepared in ethanol in different test tubes. Volume of the samples and ascorbic acid were adjusted to 3 ml by adding ethanol. Methanolic solution of DPPH (100  $\mu$ M or 0.1 mM) was prepared and 1 ml of this solution was added to each test tube, shaken vigorously and were allowed to stand at 27°C for 30 min. A control was prepared as described above without samples or standard. Absorbance was measured at 517 nm. All the tests were performed in triplicate and the results averaged (Kurian et al., 2008).

Radical scavenging activity was expressed as the inhibition percentage and was calculated by using the formula.

##### Procedure

To 1ml of sodium nitroprusside, 2.5 ml, phosphate buffer saline pH 7.4 was added and mixed with 1 ml of extract at various concentrations (25, 50, 75, 100 $\mu$ g/ml), then the mixture was incubated at 25° C for 180 minutes. The extract was mixed with 3ml of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples and the absorbance was measured at 546 nm (Sreejayan & Rao, 1997). Ascorbic acid was used as a standard. All the tests were performed in triplicate and the results averaged.

The percentage inhibition of nitric oxide radical generated was calculated by using the following formula:

$$\% \text{ Radical scavenging activity} = \frac{\text{Absorbance of Control} - \text{Absorbance of test sample}}{\text{Absorbance of Control}} \times 100$$

### 3. Results

#### 3.1 Phytochemical Screening

Preliminary phytochemical screening of ethanolic extract of *Citrus Medica* L. seeds was done. The ethanolic extract of seeds indicated the presence of Carbohydrates, steroids, sterols, volatile oil, alkaloids and Anthraquinone glycosides.

**Table 3.1:** Phytochemical screening of ethanolic extract

S. No.	Phytoconstituents	Intensity
1.	Alkaloids	+++
2.	Glycosides (G)	
	• Cardiac G.	-
	• Anthraquinone G	++
	• Saponin G.	-
3.	Carbohydrates	+++
4.	Steroids	++
5.	Sterols	+
6.	Tannins	+
7.	Flavonoids	-
8.	Amino acids	-
9.	Proteins	-
10	Volatile Oils	+

Here (-) indicates absence of chemical constituents/ (+) indicates presence of chemical constituents / (+++) indicates higher content of chemical constituents.

#### 3.2 In Vitro Antioxidant Activity

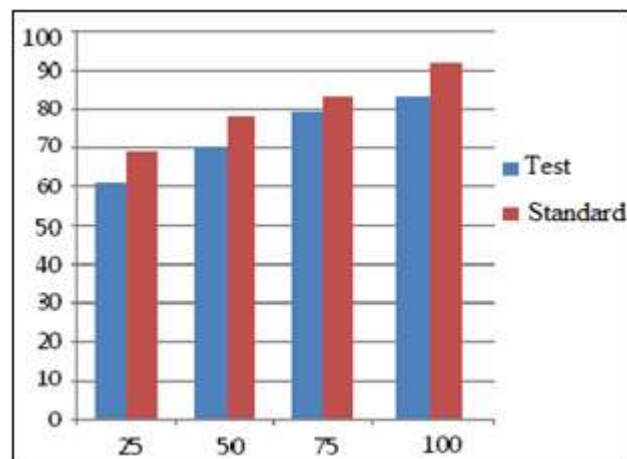
##### 3.2.1 Quantitative Estimation of Antioxidant Activity Using DPPH Method

DPPH is a free radical compound that has been widely used to determine the free radical - scavenging ability of various samples. DPPH decreases significantly upon exposure to proton radical scavengers (Yamaguchi, Takamura, Matoba, & Terao, 1998). The free radical scavenging potentials of extracts from citron and ascorbic acid at different concentrations were tested by DPPH method and the results are shown in Figures 5.2. Antioxidants react with DPPH, which is a nitrogen - centered radical with a characteristic absorption at 517 nm and convert to 1, 1 - diphenyl - 2 - picryl hydrazine (Jayaprakasha et al., 2004).

**Table 3.2:** DPPH Scavenging activity of *Citrus Medica* L. seeds

S. No.	Concentration	Mean Absorbance (Test)	%age (Test)	Mean Absorbance (Standard)	%age (Standard)
1.	25	0.593	61.01	0.472	68.97
2.	50	0.458	69.89	0.336	77.91
3.	75	0.317	79.16	0.258	83 - 04

4.	100	0.256	83.17	0.124	91.84
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**Figure 3.1:** Graphical representation of NO scavenging activity

Ethanolic extract of *Citrus Medica* L. seeds exhibited DPPH scavenging activity. With the increase in concentration of seed extract, the antioxidant activity increased proportionally with the maximum activity of 83.17% at 100µg/mL. The absorption decreased proportionally with the increase in the concentration of extract. The maximum absorption was found to be of control sample excluding drug.

##### 3.2.2 Quantitative Estimation of Antioxidant Activity Using Nitric Oxide method

Nitric oxide scavenging activity was determined according to the method reported by Green et al., 1982. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. The nitrite ions produced diazotizes sulphanilamide and then the diazonium salt reacts with N, N - Naphthyl ethylene diamine dihydrochloride to give a pink colour chromophore which has a maximum absorption at 546 nm.

**Table 3.3:** NO Scavenging activity of *Citrus Medica* L. seeds

S. No.	Concentration	Mean Absorbance (Test)	%age Inhibition (Test)	Mean Absorbance (Standard)	%age Inhibition (Standard)
1.	25	0.061	66.14	0.057	68.33
2.	50	0.052	71.11	0.043	76.12
3.	75	0.046	74.45	0.035	80.56
4.	100	0.038	78.88	0.028	84.44

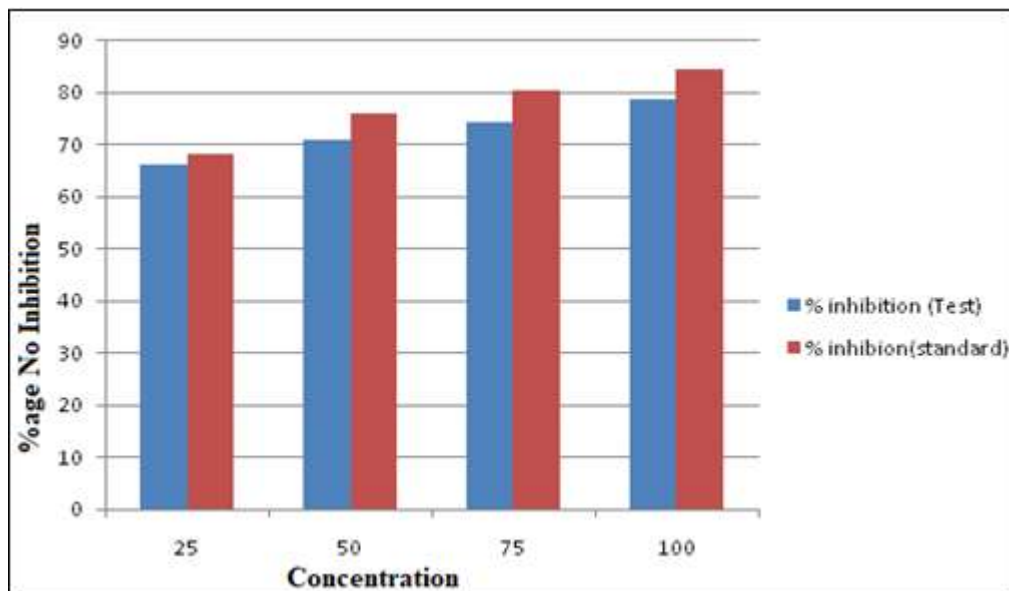


Figure 3.2: Graphical representation of NO scavenging activity

Ethanol extract of *Citrus medica* L. seeds exhibited NO scavenging activity. With the increase in concentration of seed extract, the antioxidant activity increased proportionally with the maximum activity of 78.8% at 100 $\mu$ g/mL. The absorption decreased proportionally with the increase in the concentration of extract. The maximum absorption was found to be of control sample excluding drug.

#### 4. Conclusion

*Citrus medica* L. is the most ancient wild crop of citrus family known to have various pharmacological and nutraceutical properties. The presence of phytochemicals in different parts of plant is responsible for showing various activities. This study suggests that *Citrus medica* L. may be used to discover natural bioactive products which might lead to the development of new drugs with antioxidant properties in the field of medicine.

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