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In-vitro Estimation of Phytochemicals & Flavonoids in *Citrus sinensis* & Study their Antimicrobial & Antioxidant Activity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Orange (*C. sinensis*) peel flavonoids have antibacterial, antioxidant, anticancer, anti-inflammatory, and antiviral effects. However, there is a scarcity of information in the literature about orange peels flavonoids. So, we estimated phytochemicals in hydro-methanolic extract of *C. sinensis* peel and flavonoids were separated by Column chromatography and confirmed with TLC and HPLC. After that we evaluated the antimicrobial, antioxidant and nitric oxide scavenging activity. The MIC value of flavonoids against *E. coli, B. subtilis, S. aureus* and *P. aeruginosa* is 19.40, 20.48, 12.45 and 21.44µg respectively. The IC_{50} value of flavonoids for antioxidant and Nitric oxide scavenging activity is 121.4 and 0.88µg respectively. In the current study, *Citrus* peel flavonoids were found to have antibacterial, antioxidant, and nitric oxide scavenging action in methanolic extracts. This study suggests that *C. sinensis* flavonoids could be exploited as an antibacterial and antioxidant source in the future.

Keywords: Citrus sinensis; flavonoids; antimicrobial activity; MIC; antioxidant and nitric oxide scavenging activity.

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1. INTRODUCTION

Around 80-85 percent of the total population relies on Ayurveda for disease treatment, which could be due to its ease of accessibility, low cost, and lack of unfavourable side effects [1]. Ayurveda is very popular among patients and is widely available in developing countries such as India, Pakistan, Bhutan, Srilanka and China [2,3]. India is the world's leading producer of herbal medicine and the birthplace of Ayurveda [4]. *C. sinensis* (Orange) is a juicy fruit, belonging to the *Rutaceae* family and the most widely cultivated plant species in the world. Orange peel is proven to have many medicinal properties as a tonic for the digestive and respiratory system, immune system, skin, antiviral, antibacterial, antioxidant and anticancer [5,6]. Orange peel extract showed antibacterial activity against *Klebsiella pneumonia, Streptococcus mutans, Lactobacilli acidophilus, Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis* [7,6].

The reactive oxidizing and nitrogenous species participate in the oxidation of many biological processes as protective hosts and generate more free radicals such as nitric oxide, O_2. OH' and affect bio-molecules such as carbohydrates, lipids, proteins and nucleic acids [8,9]. It contributes to immune pathologies including cancer, inflammation, diabetes, hypertension, AIDS and aging, etc [10]. Antioxidants have the ability to reduce or inhibit the oxidation of bio-molecules such as lipids, proteins, etc. [11]. Food degradation with lipid peroxidation affects the shelf life of food due to the overproduction of free radicals. During the manufacturing process, the use of antioxidants can extend the shelf life of foods [12]. In recent times, there is a tendency to use natural phytochemicals as sources of antibacterial and antioxidant substances found in all plants and their parts. Many phytochemicals such as phenolic content are used as a source of antioxidants to reduce oxidative degradation in food and maintain food quality in the long term [13,14].

C. sinensis (Orange) is a widely distributed species of plant in the family Rutaceae. It is grown in all regions for its juices, flavour and medicinal importance. Orange peel also has medicinal properties used to strengthen the immune system, digestive system, vitamin deficiencies and skin. Orange peel extracts and flavonoids have shown antibacterial and antioxidant activities. Flavonoids are effective against antibiotic-resistant bacteria, Gram-positive and Gram-negative bacteria, AIDS, Covid-19 and cancer [15,16,17].

On the literature survey, did not find any studies on the flavonoids in orange peels on bacteria, antioxidants and nitric oxide scavenging activity. Therefore, the present work was carried out with the given objectives:

1. Evaluation of phytochemical estimates and isolation and purification of flavonoids from orange peels.
2. Evaluation of antibacterial activity and determination of minimum inhibitory concentration (MIC) of flavonoids
3. For the evaluation of antioxidants, the scavenging activity of nitric oxide and the determination of the IC_{50} value in order to find new drugs against bacteria and free radicals.

2. MATERIALS AND METHODS

Orange peels were collected near to Chandigarh, India and identified by Gaurav Srivastav (Botanist) and dried at room temperature in absence of light after washed. Obtained powder (100 gm) after milling was subjected to extraction with methanol & water in ratio of 60:40 at Soxhlet for 24h. The obtained extract was concentrated in oven at 60^0C and quantify the extracts recovered mass by following formula and store at 4-8^0C for further used.

Y (%) = Mass of extract / mass of sample X 100

2.1 Phytochemical Screening

A minimum amount of extracts were used for phytochemical screening for the compound as total phenol, alkaloids, flavonoids, proteins, amino acid, tannins, saponins, steroids, terpenoids, carbohydrate, reducing sugar and glycosides [11,18]. According to selected method with little modification, 2.0 gm of plant extract was dissolved in 10ml double distilled water and filtered with filter and further used for phytochemical screening:

- **Test for Alkaloid:** Take 3.0 ml filtrate extract solution with addition of few drops Dragendorff's reagent, Appearance of an orange-red colour precipitate indicates the presence of alkaloids.
- **Test for Phenolic Compound:** To 1.0 ml of the extract, 3.0 ml of distilled water

followed by few drops of 10% aqueous Ferric chloride solution was added. The origin of blue or green colour indicates the presence of phenols.

- **Test for Flavonoids:** Take 2.0 ml of test solution and add a small quantity of reduced lead acetate solution (10%). The solution will form yellow colour ppt. In addition to an increasing amount of sodium hydroxide to the residue, Yellow colour of a solution formed which decolourizes after the addition of acids.
- **Test for Carbohydrate:** Take 2.0 ml extract and add few drops of α-naphthol solution in alcohol. Shake and add 2.0 ml conc. H_2SO_4 from the side of test tube. A violet/purple ring at the junction of two liquids appeared.
- **Test for Reducing Sugar:** Add 20 ml of Fehling (A+B) to 5.0 ml of extract in a test tube and boil for 15mins. Then neutralize with 10% sodium hydroxide to pH-7.00. Solution appeared brick red ppt.
- **Test for Glycosides:** Take 2.0 ml extract solution and add 1.0 ml glacial acetic acid and 1.0ml 5% $FeCl_3$ and 1.0 ml conc. H_2SO_4. The reddish-brown colour at the junction of two liquid layers appeared and the upper layer appears bluish-green colour.
- **Test for Protein:** Take 3.0 ml test solution and added 4% NaOH and few drops of 1% $CaSO_4$ solution. The solution appeared violet in colour.
- **Test for Amino Acid:** Take 5.0 ml solution, adds few drops of 40% NaOH and 10% lead acetate solution and then boil it. Black ppt. of lead sulphate formed.
- **Test for Steroid:** To 1.0 ml of extract and added 1.0 ml of chloroform, 2-3 ml of Acetic Anhydride, 1 - 2 drops of conc. H_2SO_4. Dark green colour showed.
- **Terpenoids:** Take 2.0 ml of extract solution, add 2.0ml of chloroform and 2.0 ml of conc. H_2SO_4 and shake well. Chloroform layer (upper) appeared red in colour and acid layer (lower) formed greenish-yellow fluorescence.
- **Tannin:** 1.0 ml of water extract was mixed with 10 ml of distilled water and the filtered. Ferric chloride ($FeCl_3$) reagent 2-4 drops were added to filtrate. A blue-black or green precipitate confirmed the presence of tannins.
- **Saponins:** Take 0.5 ml of extract and 5.0 ml of water and shake well. Foam formed.

2.2 Determination of Total Phenolic Component

The total amount of phenolic contents was determined using the method by Folin Ciocalteu's phenol reagent with slight modification. 1.0 ml of the sample was mixed with 2.0 ml of Folin Ciocalteu's phenol reagent (10%). After 3.0min, 1.0 ml of saturated Na_2CO_3 (~35%) was added to the mixture and made up to 10 ml by adding distilled water. The reaction was kept in the dark for 90 min, observed under UV-Vis spectrophotometer at 760 nm absorbance. Tannic acid was used as a standard with varied concentrations from 50 ppm to 200 ppm. The results were expressed as mg of Tannic acid equivalents/g of extract and the same procedure was done with extract and calculated as mean value ± SD (n = 3) [11,18].

2.3 Determination of Flavonoid Content

Flavonoids content of the isolated crude extract was determined by method with slight modification. Take 0.5 ml of the sample (Extract) containing 1.25 ml of distilled water. Then added 0.075 ml of 5% sodium nitrite solution and allowed to stand for 5 min. Added 0.15 ml of 10% aluminium chloride, after 6 min 0.5 ml of 1.0 M sodium hydroxide was added and the mixture was diluted with another 0.275 ml of distilled water. The absorbance of the mixture at 510 nm was measured immediately. The flavonoid content was expressed as mg of Quercetin equivalents/g sample and the same procedure was done with Extract of peel and calculated as mean value ± SD (n=3) [11,18].

2.4 Separation & Purification of Flavonoids from Crude Extract of Peels

Combined solvent (methanol: water) was used as mobile phase in silica gel column chromatography (200 g, 60-200 mesh). 10gm extracts were loaded in a glass column with silica gel and a solvent system (methanol: water) was used for running the sample. For confirmation of separation, thin layer chromatography was performed by taking sample with mobile phase (Glacial acetic acid: Methanol: water) using 10µl of sample and fractions were further screened for flavonoids [19,11].

For determination of wavelength, spectrophotometry was considered on UV/Visible

spectrophotometer with spectra (200-500 nm) using Quercetin dihydrate as standard. 1ml of fraction, 1 ml pure water, 5 ml acetate buffer (pH-3.8±0.5), and 3ml $AlCl_3$ (0.1M) solution added and after shaking kept for 30minuts [20]. Absorbance was measured and considered wavelength and then, reversed-phase high-performance chromatography (HPLC) was performed for the determination of flavonoids. The 20 µl of column chromatography purified liquid extracts was injected into HPLC (reversed-phase, C18, 250 mm x 416 mm, Shimadzu) at 350 nm for 16min at 1.0 ml/min with HPLC grade Methanol: Water (60:40) as mobile phase [19,11,20].

2.5 Antibacterial Activity and MIC Determination

Four pathogenic bacteria namely *Escherichia coli* (MTCC 25922), *Bacillus subtilis* (MTCC 3256), *Pseudomonas aeuroginosa* (MTCC 1688) and *Staphylococcus aureus (*MTCC 6810) were collected from MTCC, Chandigarh. After subculture, Agar well diffusion was used for antimicrobial activity with slight manipulation. 0.5 ml (<1.0 x 10^5 CFU/ml) of 48 h old cultures of test organisms were inoculated into different sterile Petri-plates and 15-20 ml sterile Muller Hilton agar media was poured into each Petri-plate (90 x 90 mm). The Petri-plates were gently shaken for proper mixing and allowed to solidify. Thereafter, four wells were punched of 5mm diameter with a sterilized cork borer. For each well 50µl (25 – 100 µg/ml) of flavonoids were added. The plates were incubated at 37^0C for 24h and then the zone of inhibition was measured in mm (Kumar et al, 2019). The experiment was carried out in triplicates and calculated as mean value ± SD (n = 3) and manually calculated minimum inhibitory concentration (MIC) [20,21].

2.6 Antioxidant Activity

The antioxidant activity of the flavonoids and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH-0.002% in methanol) method [20]. The diluted working solution of the standard (Ascorbic acid) and flavonoids (10 mg/ml) was prepared using the respective solvents. In 3ml of the total reaction solution, 2 ml of flavonoids/standard solution (200-1000 µg/ml) and 1.0 ml of DPPH were mixed and allowed to react at 37^0C for 30 min. afterward; absorbance values were measured at 520 nm and converted into percent antioxidant activity. The percentage antioxidant activity was calculated by the following formula and calculated as mean value ± SD (n = 3) [22,23].

Percent (%) inhibition of DPPH activity = (A – B) / (A *100)

Where

A = Absorbance of the blank
B = Absorbance of the sample

2.7 Nitric Oxide Radical Scavenging Activity

Scavengers of nitric oxide are complete with oxygen, leading to reduced production of nitrite ions. The standard (Ascorbic acid) and *Citrus* peel flavonoid were dissolved in distilled water. 2 ml Sodium Nitroprusside (5 mM) in phosphate buffer saline (0.025 mM, pH-7.4) was incubated with 0.5 ml of different concentrations (200-1000 µg/ml) of standard and flavonoids were incubated at 30^0C for 3 h. After 3 h incubation, samples were diluted with 1ml of Griess reagents (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% Naphthylethylene diamine dihydrochloride in water) and incubated for 30 min [11]. The absorbance of the colour developed during diazotization of nitrite with sulphanilamide and its subsequent coupling with Napthylethylene diamine hydro-chloride was observed at 550nm on a spectrophotometer. Calculated the % inhibition by formula and plot graph in compared to standard and calculated as mean value ± SD (n = 3) [24,25].

Nitric Oxide Scavenging (%) = $(\mathbf{A_{control} - A_{test}}) / (\mathbf{A_{control} X\ 100})$

Where,

$A_{control}$ = Absorbance of control reaction
A_{test} = Absorbance in the presence of the samples of extract

3. RESULTS AND DISCUSSION

Herbs contain a variety of ingredients that can be separated using chromatography techniques. There are numerous references available for describing the various components and their functions. Flavonoids, a phenolic compound, have been shown in recent studies to be

antimicrobial agents, antioxidants, and nitric oxide scavengers. Flavonoids, which are secondary metabolites of plants, have antibacterial, antioxidant, cleansing, carcinogenic, and other disease-fighting properties [26,27].

The presence of total phenol, alkaloids, flavonoids, proteins, amino acids, tannins, saponins, steroids, terpenoids, carbohydrate, reducing sugar, and glycosides were observed in a hydro-methanolic extract of *C. sinensis*. During the screening, we discovered that alkaloids, phenolic compounds, steroids, terpenoids, flavonoids, tannins, saponins, and reducing sugar seem to have been present, but glycosides, carbohydrates, proteins, and amino acids have not been (Table 2). The peels' phenolic content, flavonoids, terpenoids, and alkaloids may contribute to antimicrobial, antioxidant, anti-inflammatory, and anti-cancer activity. We estimated the content of phenolic and flavonoids using a spectrophotometer after acknowledging their existence in the peels. 26.5 mg hydro-methanolic extract was found in 300 gm of orange peels. The total phenolic content and flavonoids in hydro-methanolic extract powder were 32.4 µg Tannic Acid equivalent/gm and 62.59 µg Quercetin equivalent/gm, respectively, using the standard curve (tannic acid-y=0.015x + 1.004, R2 =0.994 and Quercetin-y=0.001x + 0.019, R2 = 0.998) (Table 1). The solvent system (Methanol + Water) was used for column chromatography, spectrum and HPLC and total of five fractions were collected. After qualitative confirmation of flavonoids, the entire fraction were mixed and further screened for re-confirmation. The yield is 62.59 µg and had percentage of 0.21%. By spectrum scanning of wavelength of flavonoids sample, the corresponding wavelength of most absorption peak had been identified in Orange – 287nm and 332 nm although HPLC was performed at 350 nm due to common similarity with standard spectrum (Fig. 1).

Flavonoids were tested for antimicrobial activity against four microorganisms (*Escherichia coli, Bacillus subtilis, Pseudomonas aeuroginosa,* and *Staphylococcus aureus*) using the agar plate diffusion method. Ciprofloxacin was used as the standard, and the zone of inhibition was measured in millimetre. For each pathogen, the flavonoids concentrations are 25, 50, 75, and 100 µg/ml. Flavonoids demonstrated concentration dependent antimicrobial activity against pathogens, with the highest zone of inhibition at 100 µg/ml for *E. coli* and *B. subtilis*. As shown in Fig. 2, the zone of inhibition for standard (Ciprofloxacin-30 µg/ml) is 28, 30, 27, 32mm for selected pathogens. In comparison to *S. aureus* and *P. aeruginosa, E. coli* and *B. subtilis* are more susceptible to flavonoids. Despite the reality that pathogen antimicrobial susceptibility is closer to standard than sample. However, when we calculated the minimum inhibitory concentration (MIC), we discovered that *P. aeruginosa* (21.45±2.21 µg/ml) outperformed *E. coli* (19.41±2.75 µg/ml), *B. subtilis* (20.49±4.32 µg/ml), and *S. aureus* (12.46±1.41 µg/ml).

The antioxidant properties of plants or phytochemicals are tested using various methods such as DPPH, hydrogen peroxide, and nitric oxide. The antioxidant activity of flavonoids found in orange peel was studied in comparison to Ascorbic acid (standard). The DPPH scavenging activity was determined using different concentrations of ascorbic acid and flavonoids, which were 100, 400, 600, 800, and 1000 µg/ml, respectively (Fig. 3). The interpretation of the data revealed that as the concentration of flavonoids increased, so did the scavenging activity. Similarly, the IC_{50} was calculated using regression analysis of the graph and was found to be 10.06±2.19 µg for ascorbic acid and 121.41±0.21 µg. By scanning the wavelength of five flavonoids sample, the corresponding wavelength of most absorption peak had been identified in Orange – 287 nm, 332 nm g for orange flavonoids. Free radicals start chain reactions, which can cause cell damage or death. Flavonoids act as antioxidants by reducing oxidation reactions or removing free radicals through neutralization with peroxide, lipid peroxyl, and/or hydroperoxide.

Flavonoids from a hydro-methanolic extract of orange peels were evaluated for nitric oxide scavenging activity. The nitric oxide scavenging activity of flavonoids was tested at concentrations ranging from 200 to 1000 µg/ml (Fig. 4). The IC_{50} value of flavonoids was 0.88±0.21 µg, calculated using the standard curve (Ascorbic acid = y=0.070x and r2=-42.6). The flavonoids demonstrated free radicals scavenging by participating in scavenge of nitrite radicals generated by sodium nitroprusside at neutral pH. The antioxidant activity increased with increasing flavonoid concentration, which is concentration dependent [28,29].

Table 1. Crude extracts, total phenolic & flavonoids content of *Citrus sinensis* peels

Plant Name	Plant Part	Quantity (gm)	Extract (mg)	Total Phenolic Content (µg of Tannic acid equivalent/serving)	Flavonoids (µg of Quercetin /serving)
Citrus sinensis	Peel	300	26.5	32.43	62.59

Table 2. Qualitative phytochemical analysis

Parameter	Alkaloids	Glycosides	Reducing sugar	Phenolic compound	Steroids	Terpenoids
Citrus peel	+	-	+	+	+	+
Parameter	**Carbohydrates**	**Protein**	**Amino acids**	**Flavonoids**	**Tannins**	**Saponins**
Citrus peel	-	-	-	+	+	+

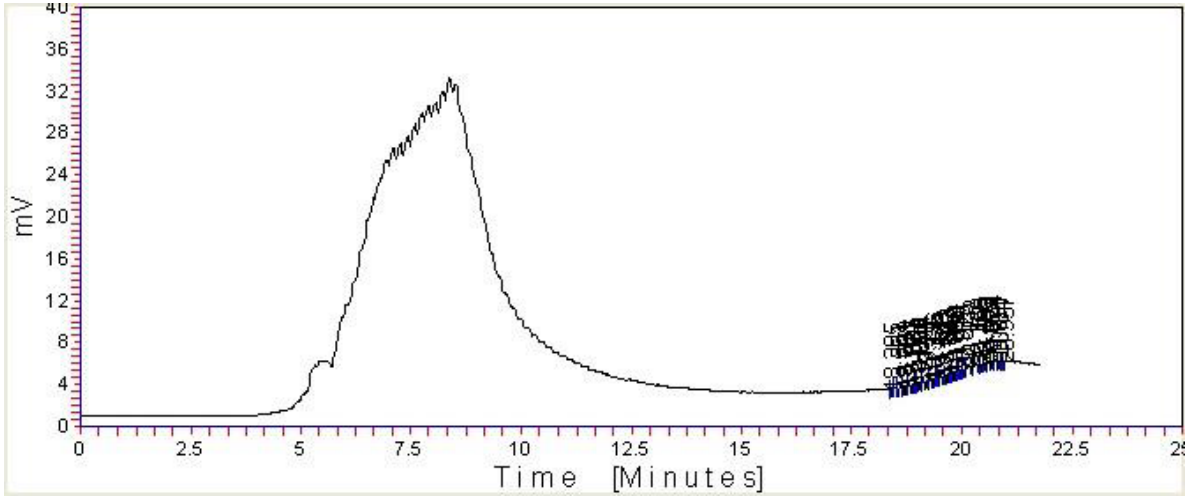

Fig. 1. HPLC graph of orange peels flavonoids

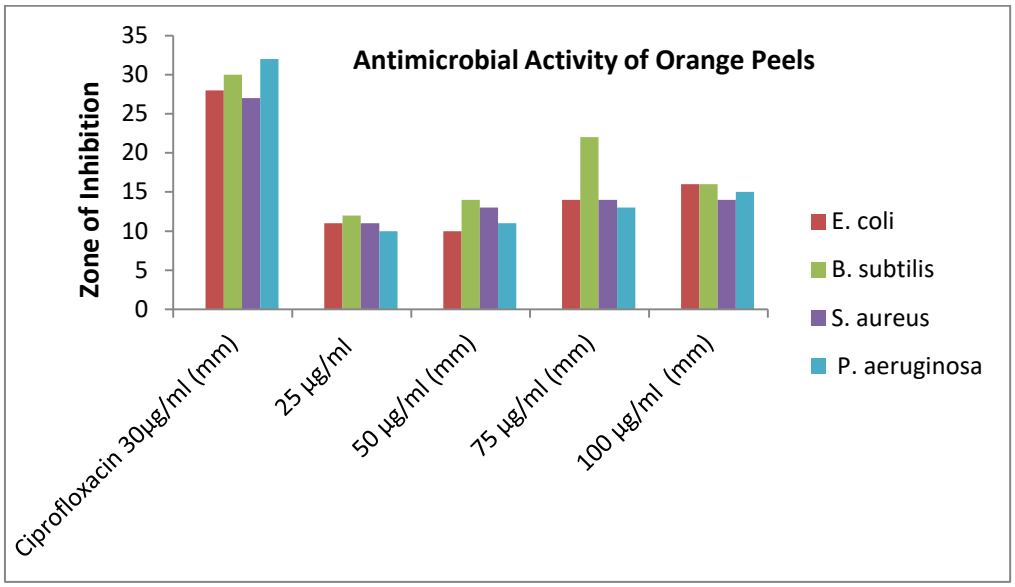

Fig. 2. Antimicrobial activity of *Citrus sinensis* peels flavonoids

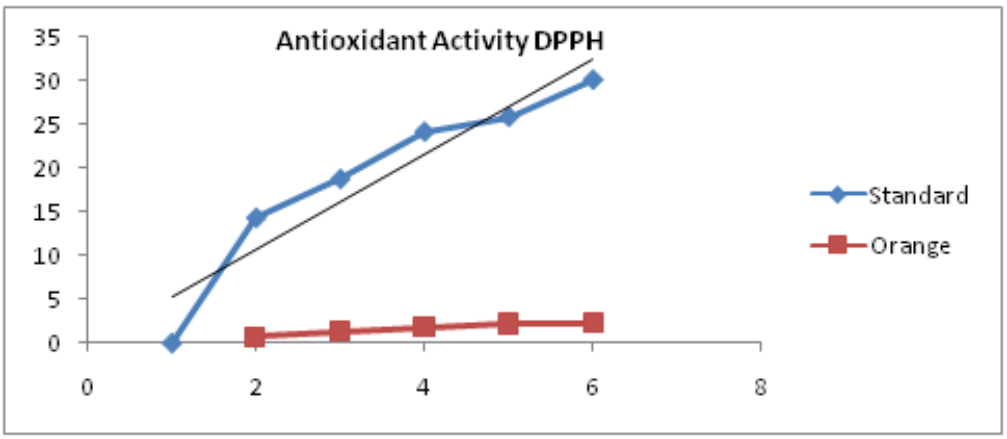

Fig. 3. Antioxidant activity of *Citrus sinensis* peels flavonoids

Free radical scavenging activity of flavonoids against DPPH and nitric oxide is assessing that higher scavenging activity; more anti-oxidizing agent might be possessing anticancer properties.

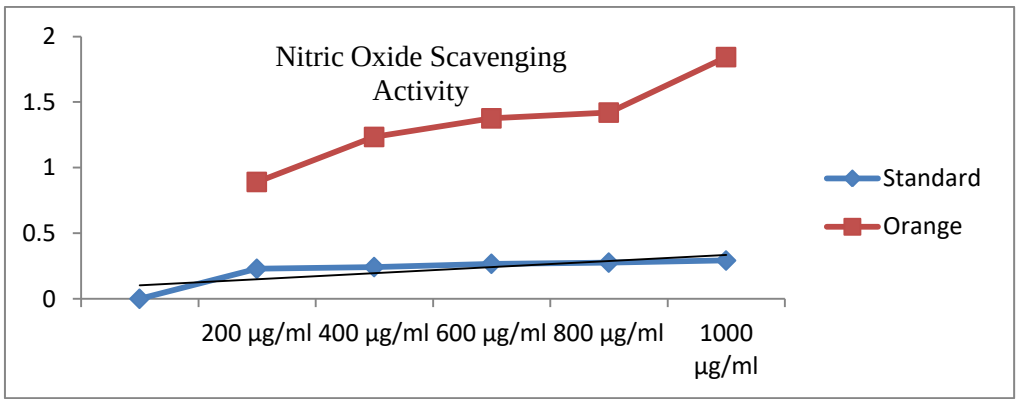

Fig. 4. Nitrite scavenging activity of *Citrus sinensis* peels flavonoids

4. CONCLUSION

The presence of active compounds such as phenolic content, alkaloids, carbohydrates, terpenoids, and flavonoids was observed in the phytochemical analysis of orange peels. It contains a high concentration of phytochemicals that have biological activity. Flavonoids were also found to have antimicrobial and antioxidant properties. As a result of these findings, plant extracts and their flavonoids with significant biological activity should be chosen. Further research into pure flavonoids may open the way for the discovery of a core constituent of *Citrus sinensis* peels as a biological activity of a pure or mixed compound. The study highlights the efficacy of "Ayurved" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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