

COMPARATIVE *IN VITRO* ANTIOXIDANT EVALUATION OF *CITRUS SINENSIS* PEEL EXTRACTS USING DIFFERENT SOLVENTS

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Abstract

The study aimed to evaluate the antioxidant activity of *Citrus sinensis* (orange) peel extracts using different solvents - methanol, ethanol, water, acetone, chloroform, and benzene - highlighting the potential of citrus peel as a valuable agro-waste resource. The highest extraction yield was observed with methanol (16.5%), followed by ethanol (14.8%) and water (12.2%). Antioxidant capacity was assessed using DPPH, ABTS, reducing power, nitric oxide, and hydroxyl radical scavenging assays. The methanolic extract demonstrated the strongest radical scavenging activity across all assays. In the DPPH assay, methanol showed 84.32% inhibition at 300 µg/mL with the lowest IC₅₀ of 52.12 µg/mL, while ethanol and water exhibited 79.45% and 70.23% inhibition, respectively. In the ABTS assay, methanol achieved 78.31% inhibition (IC₅₀: 54.67 µg/mL). The reducing power of methanol extract increased from 0.412 at 25 µg/mL to 1.203 at 300 µg/mL, indicating strong electron-donating ability. In the nitric oxide scavenging assay, methanol extract showed 70.26% inhibition (IC₅₀: 58.34 µg/mL), while in the hydroxyl radical assay, it recorded 66.87% scavenging activity (IC₅₀: 61.78 µg/mL). These results suggest that methanol is the most effective solvent for extracting antioxidant compounds from *Citrus sinensis* peel. The study supports the sustainable utilization of citrus peel waste as a natural antioxidant source and encourages further research into compound identification, *in vivo* validation, and green extraction techniques for industrial application.

Keywords: Antioxidant activity; *Citrus sinensis*; Phytochemicals; Enzymatic and non-Enzymatic; Oxidative stress

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Introduction

Plants are a valuable source of natural compounds that promote human health. Among these phytochemicals found in plant extracts possess strong antioxidant properties, making them promising candidates for therapeutic use. Antioxidants, also known as free radical scavengers, are substances that prevent or delay oxidation, thereby protecting cells from damage caused by free radicals. In recent years, natural antioxidants derived from plants have gained considerable attention due to their therapeutic efficacy and minimal side effects compared to synthetic antioxidants produced industrially. Common natural sources of antioxidants include herbs, vegetables, seeds, spices, and fruits. These plant-based antioxidants are widely studied not only for their biological significance but also for their economic potential (Priyanka *et al.*, 2021). *Citrus sinensis* was chosen for its high content of natural antioxidants like flavonoids, polyphenols, and vitamin C. The peel of *Citrus sinensis* has common agro-waste, low-cost, eco-friendly source for antioxidant extraction, making it ideal for natural health applications. *Citrus sinensis* (sweet orange) is widely consumed, generating large amounts of peel waste during processing. The peel, often discarded, is rich in bioactive compounds like flavonoids and polyphenols with antioxidant potential. Utilizing the abundant agricultural byproduct could reduce environmental waste and offer natural sources for health-promoting agents (Al-Khalifa *et al.*, 2023). Belonging to the Rutaceae family, citrus fruits are cultivated globally across tropical and subtropical regions, with an estimated annual production of around 102 million tons. Beyond their nutritional value as excellent sources of vitamin C, citrus fruits provide essential nutrients such as minerals, vitamins, micronutrients, and dietary fiber, making them an important component of the human diet. The abundance of antioxidants - including phenolic compounds, ascorbic acid, essential oils, and carotenoids - in citrus fruits may provide protection against oxidative cellular damage (Saleem *et al.*, 2023). The study aims to evaluate the antioxidant activity of *Citrus sinensis* peel extracts obtained using various solvents (methanol, benzene, ethanol, chloroform, and acetone). Antioxidant potential was assessed through DPPH, ABTS, hydroxyl radical, reducing power, and nitric oxide scavenging assays. The findings of our present study highlight the potential of citrus peel as a natural alternative to synthetic antioxidants, contributing to both health promotion and environmental sustainability.

Materials and Methods

Sample collection:

Fresh *Citrus sinensis* (oranges) peel was collected from local markets in (Coimbatore) India, during the peak harvesting season (December-January). The collected peels were thoroughly washed with distilled water to remove any adhering dirt and impurities. The cleaned peels were then shade-dried at ambient temperature (approximately $30 \pm 2^\circ\text{C}$) for 7 days, followed by oven drying at 40°C for 24 hours to ensure complete removal of moisture. Once fully dried, the peels were ground into a fine powder using a mechanical grinder. The powder was then passed through a 60-mesh sieve ($250 \mu\text{m}$) to achieve uniform particle size. The resulting powdered material was stored in airtight amber-colored glass containers to prevent photodegradation and was kept at 4°C until further use to maintain stability and prevent oxidation.

Chemicals:

Methanol, Ethanol, DPPH (2,2-diphenyl-1-picrylhydrazyl), Quercetin were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India.), Aluminum chloride, potassium acetate, and Folin - Ciocalteu reagent were obtained from Loba Chemie Pvt. Ltd., Mumbai, India. All chemicals and reagents used in the present study of analytical grade. Reagents were used as per the standardized protocols described in antioxidant assays and were stored according to the manufacturer's guidelines to ensure stability and accuracy.

Extraction of *Citrus sinensis* peeled sample:

Citrus sinensis peel was prepared by mixing 10 g of powdered sample with 100 ml of different solvents, (water, ethanol, methanol, acetone, chloroform, and benzene) in a conical flask. The mixture was kept in shaking incubator (SubZero, India) for three days. The extract was filtered through Whatman No.1 filter paper to obtain the final extract of *Citrus sinensis*. The clear filtrate was then transferred to a stoppered glass flask and stored in a refrigerator (4°C) for further experimental use.

Yield calculation

Extraction yield is used to determine the efficiency of a solvent in extracting compounds from plant material. It is calculated using the formula:

$$\text{Yield(\%)} = \frac{\text{Weight of dried extract}}{\text{Initial weight of sample}} \times 100$$

DPPH radical scavenging assay

The different extracts of *Citrus sinensis* were assessed for their free radical scavenging activity against DPPH radicals, using the method outlined by Singh and Pal (2023) with slight modifications. 0.5ml of 1mM DPPH solution in methanol was mixed with 0.5ml of *Citrus sinensis* peel extract solution of various concentration (25, 50, 100, 200 and 300 µg/ml). Methanol without DPPH serves as the blank and L-Ascorbic acid (25-300 µg/ml) was used as reference standard. Methanol in DPPH without samples acts as control. The reaction mixture was incubated for 30 minutes in dark and the absorbance was measured at 520 nm using UV-Visible spectrophotometer (Chemito 2100). The percentage of Inhibition was determined using the following formula.

$$\% \text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

ABTS radical scavenging assay:

The antioxidant activity of different solvent extracts of *Citrus sinensis* was evaluated using the ABTS radical decolorization assay, following the procedure described by Rumpf *et al.* (2023) with minor modification 7 mM ABTS and 2.45 mM ammonium persulfate was mixed to react for 12-16 hours before use in dark at room temperature to produce ABTS radical cation. 0.7 ml of *Citrus sinensis* peel extract solution of various concentrations (25, 50, 100, 200 and 300 µg/ml) was allowed to react with 0.3 ml of ABTS solution. The absorbance was read at 745 nm and the percentage scavenging activity was calculated.

$$\% \text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of sample}} \times 100$$

Hydroxyl radical scavenging assay:-

The hydroxyl radical scavenging activity of *Citrus sinensis* peel extracts was evaluated using the method described by Kopylchuk *et al.* (2023) with slight modifications. Various concentrations (25, 50, 100, 200 and 300 µg/ml) of extracts were mixed with 10ml of iron-EDTA solution, 0.5 ml of 0.018% EDTA solution, and 1.0 ml of 0.85% DMSO in 0.1M phosphate buffer, pH 7.4 sequentially. 0.5 ml of 0.22% ascorbic acid was added to initiate the reaction and the mixture was incubated at 80-90°C for 15 minutes in a boiling water bath. After 15 minutes, the reaction was stopped by adding 1.0ml of 17.5% ice-cold TCA. Then 3 ml of Nash reagent was added and the tubes

were incubated for 15 minutes at room temperature with all these reaction mixtures except the sample serves as the control. Absorbance was measured spectrophotometrically at 412 nm against reagent blank. The percentage of inhibition was determined using the following formula.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of sample} - \text{Absorbance of control}}{\text{Absorbance without sample} - \text{Absorbance of control}} \times 100$$

Reduction power assay:

The reducing ability of various extracts of *Citrus sinensis* was studied as reported Singhal *et al.* (2014) with slight modifications. The various concentrations (25, 50, 100, 200 and 300 µg/ml) of extracts was mixed with 2.5 ml of 0.2M phosphate buffer, pH 6.6 and 2.5 ml of 1% potassium ferricyanide trichloroacetic acid was added to the mixture. Then the mixture was centrifuged at 3000 rpm for 10 minutes. 2.5 ml of supernatant 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl₃ was added. The absorbance was measured at 700 nm. Absorbance is directly proportional to the reducing power of the peel extracts.

$$\text{Increasing reducing power (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of sample}} \times 100$$

Nitric oxide radical scavenging assay:

The assay was carried out as reported by Ibrahim *et al.*, 2024 and Kunlong Yang *et al.* (2024) with minor modification. 5 mM sodium nitroprusside in 0.025 M phosphate buffer saline pH 7.4 was incubated with different concentration of extracts (25, 50, 100, 200 and 300 µg/ml) and the tubes were incubated at 25°C for 5 hours. All the reaction mixture except the sample serves as the control and the buffer alone serves as the blank. After 5 hours of incubation, 0.5 ml of reaction mixture was removed and diluted with 0.5 ml of Griess reagent. The absorbance was read at 546 nm and the percentage scavenging was calculated.

$$\% \text{ of scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

Statistical Analysis

Each assay was performed in triplicates and the values are expressed as mean ± SD. Statistical significance of the data was analyzed using one-way ANOVA by using Microsoft office 2007 and P value < 0.05 are considered significant.

Results and Discussion

Yield calculation of different solvent extracts:

Table 1 summarizes the extraction efficiency of various solvents used to extract phytochemicals from *Citrus sinensis* peel. Among the solvents tested methanol exhibited the highest extraction yield, indicating the most effective solvent for extracting bioactive compounds from the peel. Due to its high polarity and ability to dissolve a wide range of polar phytochemicals.

The concentration ranges used in each assays were 25 µg, 50 µg, 100 µg, 200 µg and 300 µg. A 10 g powdered sample of *Citrus sinensis* peel was extracted using 100 mL of various solvents with different polarities, including water, ethanol, methanol, acetone, chloroform, and benzene. In addition, quercetin was used as a standard antioxidant for all antioxidant assays to compare the activity of the *Citrus sinensis* peel extracts against a well-established reference compound.

DPPH radical scavenging activity:

Figure 1(a) and Figure 1(b) present the results of DPPH assay through bar graph (Figure 1(a)) shows the percentage of radical scavenging activity across concentration (25-300 µg/ml). A widely used method for evaluating antioxidant activity through free radical scavenging. Among the *Citrus sinensis* peel extracts prepared with solvents of varying polarity, the methanol extract exhibited the highest antioxidant potential across all the tested concentrations closely approaching the activity of the standard quercetin (IC_{50} = 17.6 µg/ml). The methanol showed the lowest IC_{50} value (40.94 µg/ml) among the extracts, indicating strong free radical scavenging activity, whereas benzene showed the weakest (IC_{50} = 452.72 µg/ml). A progressive increase in DPPH scavenging activity was observed with rising extract concentration. Consistent with previous studies, methanol proved to be most effective solvent due to its high polarity and ability to extract phenolics, flavonoids and other antioxidants. Shehata *et al.* (2021) and Ashraf *et al.* (2024) all reported that, superior antioxidant activity in methanolic *Citrus sinensis* peel extracts. In our present study, the

methanolic extract of *Citrus sinensis* peel, was an agricultural byproduct showed the highest antioxidant activity (IC_{50} = 40.94 µg/ml) and the greatest extraction yield (16.5%), significantly outperforming other solvents ($p < 0.05$) and approaching the activity of standard quercetin (IC_{50} = 17.6 µg/ml). These findings support the value added use of citrus peel waste as a natural antioxidant source.

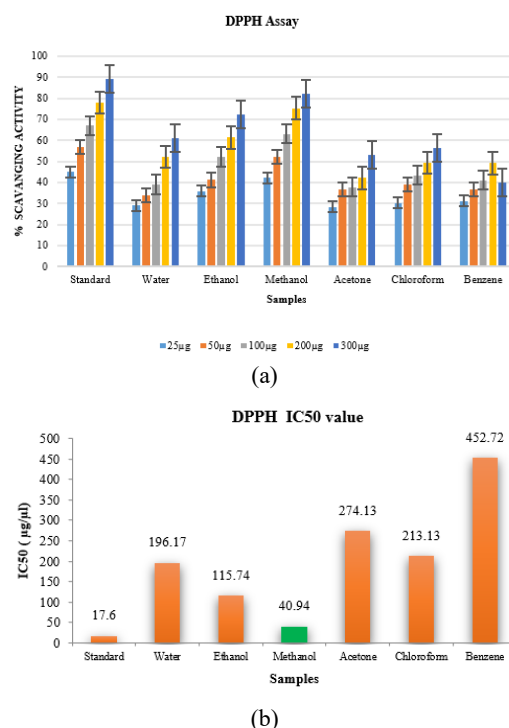


Figure 1. (a) DPPH Radical Scavenging Activity of *Citrus sinensis* Peel Extracts The bar graph represents % scavenging activity of extracts (25-300 µg/mL) in DPPH assay. Figure (b) DPPH radical scavenging activity (IC_{50}) of *Citrus sinensis* peel extracts in various solvents. Values are mean $\pm$ SD (n = 3). Statistical significance compared to standard: $p < 0.05$ (*)

Table 1. Extraction Yield of *Citrus sinensis* Peel in Different Solvents (10 g in 100 mL)

S.No	Solvent	Initial Peel Weight (g)	Volume of Solvent (mL)	Dried Extract Weight (g)	Extraction yield (%)
1.	Methanol	10	100	1.65	16.5%
2.	Ethanol	10	100	1.48	14.8%
3.	Water	10	100	1.22	12.2%
4.	Benzene	10	100	0.57	5.7%
5.	Chloroform	10	100	0.89	8.9%
6.	Acetone	10	100	1.33	13.3%

ABTS radical scavenging activity:

Figure 2(a) and Figure 2(b) shows that, the ABTS assay with bar graph (Figure 2(a)) represents the percentage of radical scavenging activity across concentration (25-300 µg/ml) which confirmed that methanol extract of *Citrus sinensis* peel exhibited the lowest IC₅₀ value (46.6 µg/ml) and the highest extraction yield (16.5%) indicating superior antioxidant activity compared to other solvents. These differences were statistically significant ($p < 0.05$). This aligns with Chen *et al.* (2011), who reported the strong antioxidant potential in methanol extracts of orange peels. Dongre *et al.* (2024) found methanol to be most effective solvent among various extracts of *Citrus sinensis*, while Sarkar *et al.* (2024) highlighted the abundance of phenolic and dietary fibers in citrus peel supporting their use in nutraceutical and bioethanol production. Therefore, the findings of our study reveal that, *Citrus sinensis* peel was a fruit processing waste, demonstrates excellent potential as a sustainable source of natural antioxidants, particularly when extracted with methanol.

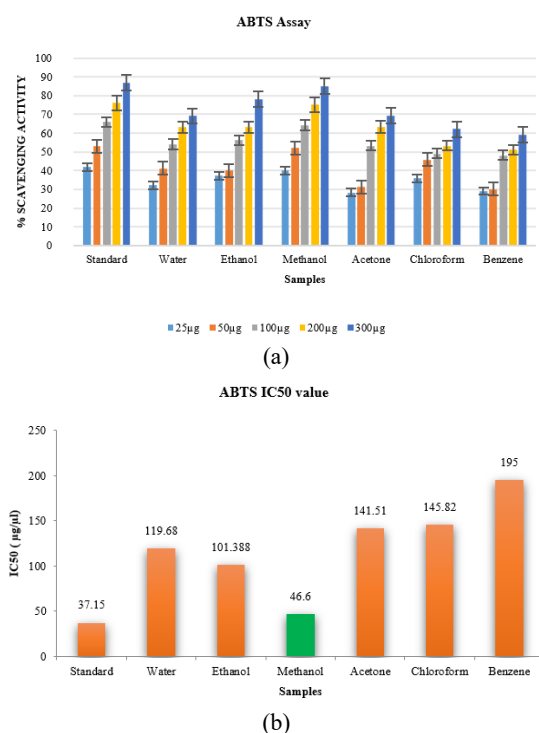


Figure 2. (a) ABTS Radical Scavenging Activity of *Citrus sinensis* Peel Extracts. The bar graph represents % scavenging activity of extracts (25–300 µg/mL) in ABTS assay. 2(b) ABTS radical scavenging activity (IC₅₀) of *Citrus sinensis* peel extracts in various solvents. Values are mean $\pm$ SD ($n = 3$). Statistical significance compared to standard $p < 0.05$ (*)

Hydroxyl radical scavenging activity:

Figure 3(a) and Figure 3(b) shows the hydroxyl radical scavenging assay with bar graph represents (Figure 3a) the percentage of radical scavenging across concentration (25-300 µg/ml) which revealed that methanolic extract of *Citrus sinensis* peel exhibited the lowest IC₅₀ value (39.31 µg/ml), indicating the highest antioxidant potency among all the tested solvents and significantly ($p < 0.05$) more effective compared to ethanol (IC₅₀=47.65 µg/ml, water (109.29 µg/ml), chloroform (109.49 µg/ml) and benzene (182.21 µg/ml). Additionally the methanol extract yielded the highest extraction efficiency followed by ethanol and water. The results align with studies by Pavithra and Vadivukkarasi (2015) and Sontakket *et al.* (2020), confirming that methanolic extracts are rich in phenolic compounds and acts as potent natural antioxidants. Therefore, the findings of our present study reveal that, *Citrus sinensis* peel was an agro-waste rich source of natural antioxidants and the potential of methanolic extracts for developing functional foods, natural health products or phyto-pharmaceuticals through the isolation of bioactive compounds.

Reducing power assay:

The reducing power assay demonstrated that the methanol extract of *Citrus sinensis* peel had the lowest IC₅₀ value (43.61 µg/ml) among all the solvents extracts, indicating the highest antioxidant reducing activity. This activity was statistically significant ($p < 0.05$) when compared to other solvent extracts. The concentration dependent percentage graph (Figure 4a) methanol extract showed a progressive increased in reducing power from 25-300 µg/ml. These findings are in line with previous studies by Liu and Tsai (2012) and Oikeh *et al.* (2014), reported that enhanced reducing ability in methanolic citrus extracts due to phenolic content and efficient Fe^{3+} to Fe^{2+} conversion. This supports the conclusion of our study that, the *Citrus sinensis* peel, particularly when extracted with methanol is a potent source of natural antioxidants, suitable for incorporation into functional foods and therapeutic formulations.

Nitric oxide radical scavenging activity:

The nitric oxide (NO) scavenging assay results revealed that *Citrus sinensis* peel extracts exhibited a concentration dependent increase in antioxidant activity across all solvent types (Figure 5a). Among them, the methanolic extract demonstrated the highest scavenging potential, achieving a maximum inhibition of ~ 85% at 300 µg/ml and showing a significant lower IC₅₀ value of 37.37 µg/ml, compared with other solvent extracts. The

extraction yield data further supports with methanol yielding 16.5% which was the highest among all tested solvents. Statistical analysis ($p < 0.05$) confirms the significance of these findings particularly for methanol extract when compared to the standard. The findings are supported by Biswas *et al.* (2010), Jayachandra *et al.* (2012) and Parul *et al.* (2013), reported that, the antioxidant potential of methanolic plant extracts is linked to the phytoconstituents cable of scavenging NO radicals. These findings supports our current studies, that antioxidant potential of *Citrus sinensis* peel, suggesting its promising role in development of natural therapeutics for oxidative stress related disorders.

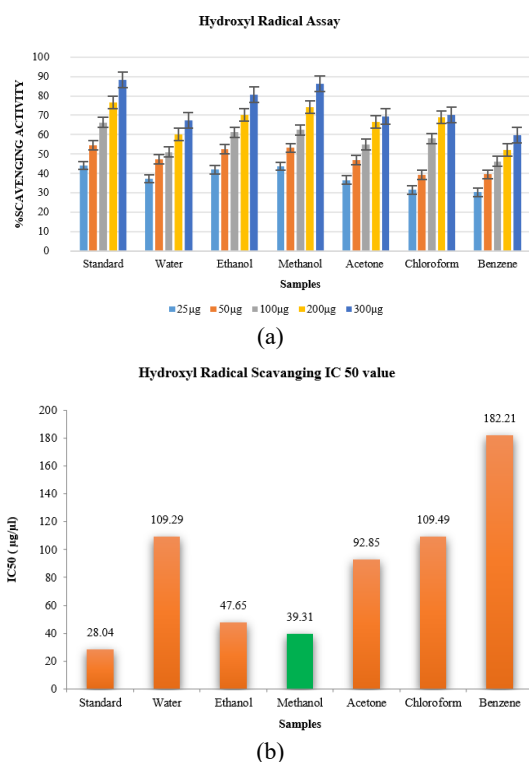


Figure 3. (a) Hydroxyl Radical Scavenging Activity of *Citrus sinensis* Peel Extracts. The bar graph represents % scavenging activity of extracts (25-300 µg/mL) in hydroxyl radical assay. 3(b) Hydroxyl radical scavenging activity (IC₅₀) of *Citrus sinensis* peel extracts in various solvents. Values are mean $\pm$ SD (n = 3). Statistical significance compared to standard < 0.05 (*)

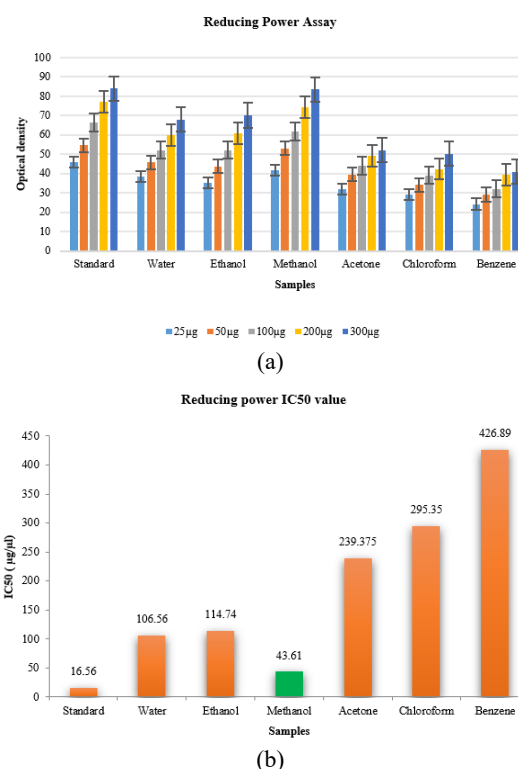


Figure 4. (a) Reducing power Radical Scavenging Activity of *Citrus sinensis* Peel Extracts. The bar graph represents % scavenging activity of extracts (25-300 µg/mL) in reducing power radical assay. (b) reducing power radical scavenging activity (IC₅₀) of *Citrus sinensis* peel extracts in various solvents. Values are mean $\pm$ SD (n = 3). Statistical significance compared to standard $p < 0.05$ (*)

Conclusions

The present study confirms that *Citrus sinensis* (orange) peel, a commonly discarded agricultural waste, is a rich source of natural antioxidants. Among the tested extracts, the methanol extract exhibited the highest yield and strongest antioxidant potential across various assays, indicating its efficiency in recovering bioactive phytochemicals. These findings highlight the potential of citrus peel waste as a sustainable and low-cost source of natural antioxidants for food, nutraceutical, and pharmaceutical applications. Future research would focus on detailed compound profiling (LC-MS/MS

or HPLC), *in vivo* validation, toxicity evaluation, and the adoption of green extraction technologies to enhance both sustainability and industrial relevance.

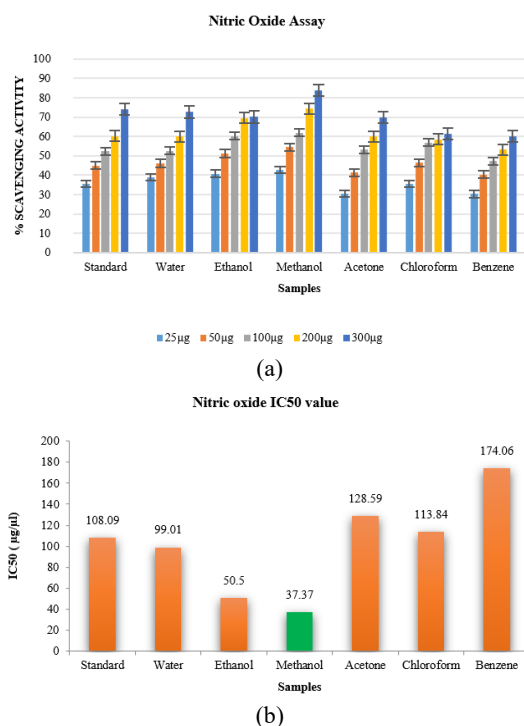


Figure 5. (a) Nitric oxide Radical Scavenging Activity of *Citrus sinensis* Peel Extracts. The bar graph represents % scavenging activity of extracts (25-300 µg/mL) in nitric oxide radical assay. (b) nitric oxide radical scavenging activity (IC₅₀) of *Citrus sinensis* peel extracts in various solvents. Values are mean ± SD (n = 3). Statistical significance compared to standard p < 0.05 (*)

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