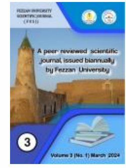
جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/



Comparative Analysis of Antioxidant Properties and Phenolic Contents in Aqueous and Ethanolic Extracts of *Citrus aurantifolia* Using DPPH and ABTS Radical Scavenging Assays

*Ketaam Youness[1] and Shamsi Saad [2]
1 Department of Chemsirty , Faculty of education , Fezzan University , Traghan , Libya
2 Department of Botany , Faculty of science , sebha university , Sebha , Libya

A B S T R A C T

This study investigates the phenolic content and antioxidant properties of Citrus aurantifolia extracts, comparing aqueous and ethanolic variants to ascorbic acid. The total phenolic content was measured using gallic acid equivalents (GAE) and quercetin equivalents (QE) on a dry mass basis. The ethanolic extract displayed a higher phenolic content in GAE terms (109.23 mg GAE/g DM), while the aqueous extract showed higher QE content (80.26 mg QE/g DM). Antioxidant activities were assessed using DPPH and ABTS radical scavenging assays. Both extracts exhibited concentration-dependent antioxidant activities. At 100mg, the aqueous extract demonstrated notable DPPH scavenging activity, though less than ascorbic acid, maintaining superior activity across all concentrations. In the ABTS assay, the ethanolic extract showed a marked increase in scavenging activity with concentration, nearing the efficacy of ascorbic acid at 100mg. The aqueous extract exhibited a progressive but less pronounced increase, indicating moderate antioxidant capacity. Overall, the ethanolic extract of Citrus aurantifolia demonstrated a higher phenolic content and more potent antioxidant activity than the aqueous extract, especially regarding hydrophobic antioxidant compounds. Ascorbic acid remained the benchmark for antioxidant efficacy, consistently exhibiting the highest activity. These findings highlight the impact of extraction solvents on the phenolic content and antioxidant potential of Citrus aurantifolia extracts, offering insights for their potential therapeutic applications.

Keywords: Citrus aurantifolia - Phenolic Content - Antioxidant Activity - DPPH and ABTS Assays - Aqueous and Ethanolic Extracts

تحليل مقارن لخصائص مضادات الأكسدة والمحتويات الفينولية في المستخلصات المائية والإيثانولية لأوراق citrus aurantifolia باستخدام طريقتي ABTS، DPPH

*ختام عبدالقادر يونس صالح[1] ، شمسي عبدالله محمد سعد[2]
[1]قسم الكيمياء، كلية التربية تراغن،جامعة فزان
[2]قسم النبات، كلية العلوم، جامعة سبها .

الملخص

تبحث هذه الدراسة في المحتوى الفينولي وخصائص مضادات الأكسدة لمستخلصات الحمضيات أورانتفوليا، مقارنة المتغيرات المائية والإيثانولية بحمض الأسكوربيك. تم قياس إجمالي محتوى الفينول باستخدام مكافئات حمض الغاليك (GAE) ومكافئات كيرسيتين (QE) على أساس الكتلة الجافة. أظهر المستخلص الإيثانولي محتوى فينوليًا أعلى من حيث

*Corresponding author: E-mail bdalqadrkhtam7@gmail.com Received 5 December 2023 - Received in revised form 9 january 2024 Accepted 12 january 2024

جامعة فزان العلمية مجلة
Fezzan University scientific Journal

Journal homepage: wwwhttps://fezzanu.edu.ly/

GAE (109.23 مجم GAE / جم DM)، بينما أظهر المستخلص المائي محتوى أعلى من التيسير الكمي (80.26 مجم QE / جم DM). تم تقييم أنشطة مضادات الأكسدة باستخدام فحوصات الكسح الجذري DPPH وABTS. أظهر كلا المستخلصين أنشطة مضادة للأكسدة تعتمد على التركيز. عند 100 ملجم، أظهر المستخلص المائي نشاطًا ملحوظًا في مسح DPPH، على الرغم من أنه أقل من حمض الأسكوربيك، مع الحفاظ على نشاط متفوق عبر جميع التركيزات. في اختبار ABTS، أظهر المستخلص الإيثانولي زيادة ملحوظة في نشاط الكسح مع التركيز، مما يقترب من فعالية حمض الأسكوربيك عند 100 ملغ. أظهر المستخلص المائي زيادة تدريجية ولكن أقل وضوحًا، مما يشير إلى قدرة مضادات الأكسدة المعتدلة. بشكل عام، أظهر المستخلص الإيثانولي لحمضيات أورانتيفوليا محتوى فينولي أعلى ونشاطًا مضادًا للأكسدة أكثر فعالية من المستخلص المائي، خاصة فيما يتعلق بمركبات مضادات الأكسدة الكارهة للماء. ظل حمض الأسكوربيك هو المعيار لفعالية مضادات الأكسدة، حيث أظهر باستمرار أعلى نشاط. تسلط هذه النتائج الضوء على تأثير مذيبات الاستخلاص على المحتوى الفينولي وإمكانات مضادات الأكسدة لمستخلصات الحمضيات أورانتيفوليا، مما يوفر رؤى لتطبيقاتها العلاجية المحتملة.

الكلمات الدالة: المستخلصات المائية، DPPH و ABTSوالإيثانولية، أوراق الحمضيات ، المحتوى الفينولي، نشاط مضادات الأكسدة، فحوصات

Introduction

Citrus aurantifolia, commonly known as the key lime or Mexican lime, is a small, green, spherical fruit belonging to the Rutaceae family. Its cultivation and utilization have a rich history spanning several continents[1], with its origins likely tracing back to Southeast Asia before being introduced to the Mediterranean region and eventually the Americas. Known not only for its distinctive flavor profile, which has found its way into various food delights but also for its array of health benefits, Citrus aurantifolia holds a significant place in culinary and traditional medicine practices[2].

One of the most notable properties of *Citrus aurantifolia* is its potent antioxidant capacity[3]. Antioxidants can prevent or slow damage to cells caused by free radicals, which are unstable molecules the body produces as a reaction to environmental and other pressures[4]. The rich presence of vitamin C, flavonoids, and other bioactive compounds in lime contribute to its antioxidant properties[5], thus making it an essential fruit in pursuing health and wellness. Consuming foods high in antioxidants may reduce the risk of chronic diseases and delay aging. While many studies[6] have explored the general antioxidant properties of citrus fruits, there appears to be a research gap in quantifying and characterizing the specific antioxidant compounds found in *Citrus aurantifolia*.[7] Furthermore, with the proliferation of lime-based products in the market, there is a growing need to establish standardized measures and understand the variations in antioxidant properties across different growth conditions, varieties, and processing methods.

Given this background, the primary aim of this study is to estimate and characterize the antioxidant properties of *Citrus aurantifolia*. Through a systematic approach, we aim to analyze the specific antioxidant capacity present in this fruit, thereby providing a

جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/

comprehensive understanding of its therapeutic potential and guiding future applications in both the food industry and the health sector.

Materials and Methods

Chemicals and Reagents

Folin–Ciocalteu reagent Sodium carbonate (Na2CO3) at 7% (weight/volume) Ethanol1,1-Diphenyl-2-picrylhydrazyl (DPPH) for DPPH Radical Scavenging Assay L-Ascorbic acid as a positive control for DPPH assay ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) for ABTS Radical Reduction Assay

Plant Material

Citrus aurantifolia fruits were obtained from a local market in Sabha City in January 2022. The species was authenticated by the Botany Department at the Faculty of Science, Sebha University

Preparation of Citrus aurantifolia Extract

Following the methods described by [8] The fruits of Citrus aurantifolia were washed, followed by peeling, and subsequently air-dried. The desiccated peels were pulverized into a fine particulate and subjected to maceration using a solution consisting of 95% (v/v) ethanol and distilled water, maintained at ambient temperature for 72 hours. The extraction process was conducted in a repeating manner, with a total of five repetitions. Subsequently, the resultant solution underwent filtration. Subsequently, the solvent was subjected to evaporation under reduced pressure within the temperature range of 40-45°C. The residual substance was subjected to freeze-drying to remove any remaining solvent and, after that, stored at a temperature of -20°C for subsequent analysis.

Total Phenolic Contents

The overall phenolic content in the extract was determined using the Folin–Ciocalteu assay[9], with some adjustments to the procedure. A solution comprising 130 μL of deionized water, 10 μL of extract, and 10 μL of Folin–Ciocalteu reagent was produced. Following a duration of 5 minutes, a volume of 100 μL of a sodium carbonate solution with a concentration of 7% (w/v) was introduced. The solution was subjected to incubation in a light-restricted environment for a duration of 30 minutes. Subsequently, the optical density of the solution was measured at a wavelength of 734 nm using a Jenway 7405 Spectrophotometer.

Antioxidant Assays

DPPH Radical Scavenging Assay

Free radical scavenging activity was assessed [9] using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) test with minor adjustments. Different quantities of the extract were made in ethanol solutions and subsequently combined with a 0.2 mM DPPH solution. L-Ascorbic acid was utilized as a positive control in the experiment. The measurement of absorbance was conducted at a wavelength of 517 nm following a 30-minute incubation period at room temperature under dark conditions.

ABTS Radical Reduction Assay

The ABTS assay was performed following a previously established protocol[10], with slight modifications. A solution of ABTS•+ was produced and subsequently diluted to obtain an

جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/

absorbance value of 0.70 ± 0.02 at a wavelength of 750 nm. The extract was combined with the solution above, and the resultant mixture's absorbance was measured at a wavelength of 734 nm following a 10-minute incubation period at ambient temperature in the absence of light.

Result

Total Phenolic Contents

Plant extract	Total phenol content (mg GAE/g DM)	Total phenol content (mg QE/g DM)
Aqueous extract *Citrus aurantifolia*	58.25	80.26
Ethanolic extract *Citrus aurantifolia*	109.23	43.37

Table 1: Total phenolic content of aqueous and ethanolic extracts of *Citrus aurantifolia* expressed in mg GAE/g DM and mg QE/g DM.

The total phenolic content in different extracts of *Citrus aurantifolia* was quantified using two standards: gallic acid equivalents (GAE) and quercetin equivalents (QE) on a dry mass basis.

For the aqueous extract of *Citrus aurantifolia*, the total phenolic content was determined to be 58.25 mg GAE/g DM and 80.26 mg QE/g DM. On the other hand, the ethanolic extract of *Citrus aurantifolia* exhibited a total phenolic content of 109.23 mg GAE/g DM and 43.37 mg QE/g DM. These results suggest a variation in the phenolic content based on the extraction solvent utilized, with the ethanolic extract demonstrating a notably higher phenolic content when assessed with GAE, while the aqueous extract showed a higher phenolic content when assessed with QE

DPPH Radical Scavenging Results

The presented Figure(1) describes the antioxidant activity of aqueous and ethanolic extracts of *Citrus aurantifolia* compared to ascorbic acid, employed as a control, at varying concentrations (100mg, 75mg, 50mg, and 25mg). The activity is expressed as a percentage of inhibition.

Observations indicate that both extracts of *Citrus aurantifolia* and ascorbic acid exhibit dose-dependent antioxidant activities. The aqueous extract at 100mg concentration presents a notable antioxidant activity, which appears to decrease stepwise with the reduction in concentration. Similarly, the ethanolic extract displays significant inhibition at the highest concentration (100mg), with a marked decrement as the concentration decreases, suggesting that the antioxidant potency is concentration-dependent.

Comparatively, ascorbic acid, which serves as a benchmark for antioxidant efficacy, exhibits superior inhibition at the 100mg concentration, asserting its robust antioxidant properties. At reduced concentrations (75mg, 50mg, and 25mg), ascorbic acid consistently maintains higher antioxidant activity than the *Citrus aurantifolia* extracts.

جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/

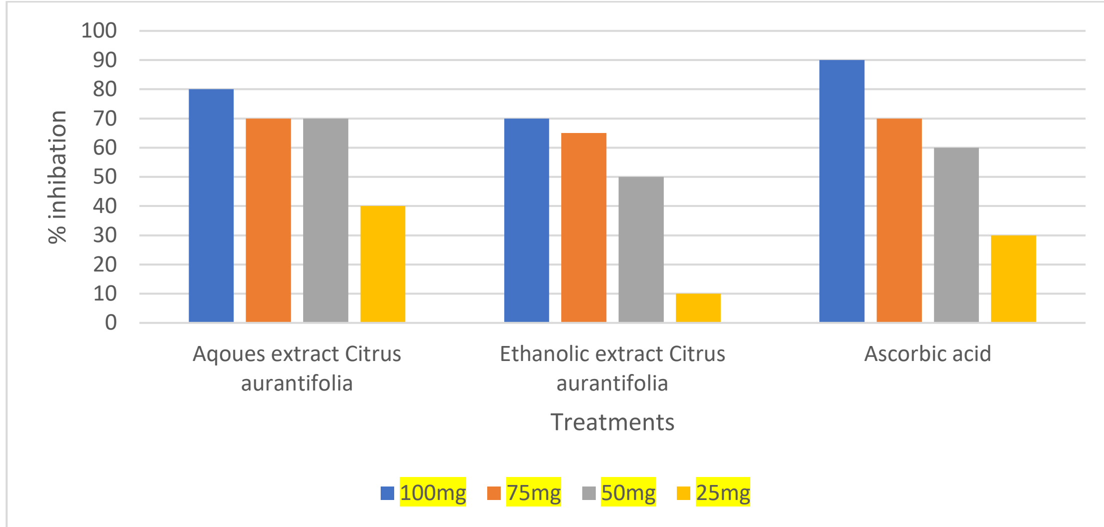


Figure 1 illustrates the percentage inhibition of free radicals by aqueous and ethanolic extracts of Citrus aurantifolia, as well as ascorbic acid, at four concentrations: 100 mg, 75mg, 50mg, and 25mg

ABTS Radical Reduction result

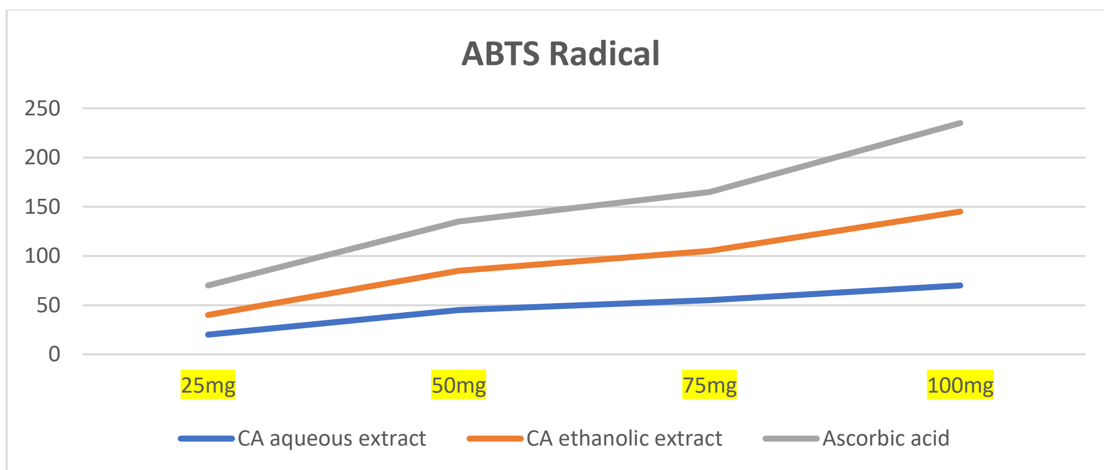


Figure 2 displays the scavenging activity on the ABTS radical by Citrus aurantifolia aqueous extract, Citrus aurantifolia ethanolic extract, and ascorbic acid over a range of concentrations (25 mg to 100 mg)

The antioxidant activity of *Citrus aurantifolia* (CA) extracts was assessed using the ABTS radical scavenging assay, and the results were compared to the known antioxidant ascorbic acid. The assay results are quantified as trolox equivalents and are presented in terms of their capacity to scavenge ABTS radicals at various concentrations (25mg, 50mg, 75mg, and 100mg).

The aqueous extract of CA demonstrated a progressive increase in ABTS radical scavenging activity with increasing concentration, indicating a dose-dependent response. Starting at a

جامعة فزان العلمية مجلة
Fezzan University scientific Journal

Journal homepage: wwwhttps://fezzanu.edu.ly/

lower antioxidant activity at 25mg, the aqueous extract exhibited a less steep activity increase than the ethanolic extract and ascorbic acid. At 100mg, the aqueous extract reached an activity level significantly lower than ascorbic acid, suggesting a moderate antioxidant capacity.

The ethanolic extract of CA showed a more pronounced increase in activity with concentration. At 25mg, the ethanolic extract's ability to quench ABTS radicals was more effective than the aqueous extract but still lower than ascorbic acid. However, its scavenging activity at 100mg was closer to that of ascorbic acid, indicating a high level of antioxidant potential, possibly due to the higher extraction of hydrophobic antioxidant compounds in ethanol.

Ascorbic acid, used as a control, exhibited the highest antioxidant activity across all concentrations, which is consistent with its established efficacy as a powerful antioxidant. It started with the highest activity at the 25mg concentration and maintained its superior position throughout the range of concentrations tested. The increase in activity with concentration was steeper than both CA extracts, suggesting that ascorbic acid has a more robust and consistent radical scavenging activity.

Discussion

The comparative analysis of the antioxidant activities and phenolic content of *Citrus aurantifolia* extracts provides insightful revelations about the influence of extraction solvents on the phytochemical profile of plant-based extracts. The phenolic content, a pivotal component associated with antioxidant activity, varied notably between the aqueous and ethanolic extracts when benchmarked against two standards: gallic acid equivalents (GAE) and quercetin equivalents (QE).

The aqueous extract displayed a higher phenolic content using the QE standard,this comes in agreement with previous reports[11, 12] while the ethanolic extract exhibited greater values with the GAE standard. This discrepancy could be attributed to the polarity of the solvents influencing the solubility of different phenolic compounds[13]. Ethanol, being a less polar solvent compared to water, is more effective in extracting non-polar compounds[14], which may include phenolics that have a greater resemblance to gallic acid's structure, hence the higher GAE values. Conversely, water may preferentially extract a different profile of phenolic compounds, potentially those structurally similar to quercetin, aligning with the higher QE values observed[15].moreover Research on Citrus aurantifolia aqueous water extract has shown that it possesses strong antioxidant properties, as demonstrated by its radical scavenging and reducing abilities [3] These properties are attributed to the presence of phenolic and flavonoid compounds in the extract , The extract also exhibits anti-inflammatory and anti-cancer [16]

The DPPH radical scavenging assay further supports the findings from the phenolic content analysis. It reveals a concentration-dependent increase in antioxidant activity for both extracts of *Citrus aurantifolia*. However, despite the higher phenolic content reported for the ethanolic extract with the GAE, this did not uniformly translate to a proportionately higher antioxidant activity in the DPPH assay at lower concentrations[17]. This could imply that not

جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/

all phenolic compounds exert the same level of radical scavenging activity, and that the extract's ability to donate electrons or hydrogen to stabilize the DPPH radical can vary[18]. Ascorbic acid, with its high antioxidant activity across the concentration spectrum, is a potent control. Its consistent performance across both DPPH and ABTS assays underscores its established role as a benchmark for antioxidant efficacy. The consistency of ascorbic acid's antioxidant response serves as a valuable reference point for assessing the *Citrus aurantifolia* extracts[19].

In the ABTS radical scavenging assay, the pattern of increased activity with rising concentrations, observed for both the aqueous and ethanolic extracts, points to a dose-dependent antioxidant activity which agrees with [20]. However, the steeper gradient of increase in activity for the ethanolic extract over the aqueous one implies that the ethanol-soluble compounds within *Citrus aurantifolia* may have a stronger ability to neutralize the ABTS radical. This is particularly evident at the 100mg concentration, where the ethanolic extract approached the antioxidant activity levels of ascorbic acid[21].

The moderate increase in ABTS radical scavenging activity by the aqueous extract, even at the highest concentration, underscores the potential limits of water-extracted phytochemicals in neutralizing this particular radical, or it may indicate a lower presence of compounds that react optimally in the ABTS assay conditions.

The disparities in antioxidant activities and phenolic contents between the extracts highlight the complexity of antioxidant assays and the influence of extraction methods on the perceived antioxidant capacity[22, 23]. Moreover, these variations suggest that the antioxidant profile of *Citrus aurantifolia* cannot be fully characterized by a single extraction method or antioxidant assay.

The findings emphasize the need for a multi-faceted approach when evaluating plant extracts' antioxidant capacity. Future work should explore the specific phenolic compounds responsible for the activity espically the phenolic coumpunds which have polarity in water, using further testing such as HPLC and LC-MS and consider a broader array of antioxidant assays like FRAP to elucidate the therapeutic potential of Citrus aurantifolia fully.

Conclusion

This study on Citrus aurantifolia extracts reveals important distinctions in phenolic content and antioxidant properties between aqueous and ethanolic extracts, with the ethanolic variant showing higher efficacy, especially regarding hydrophobic antioxidant compounds. While employing rigorous methods like DPPH and ABTS assays to establish these findings, the study is limited by its focus on only two types of extracts and its heavy reliance on ascorbic acid as a comparative benchmark. Despite these limitations, the insights gained are significant for various sectors, including nutraceutical development, therapeutic research, and the food industry, where these extracts could be used for health supplements, disease treatment research, and natural food preservation.

جامعة فزان العلمية مجلة
Fezzan University scientific Journal

Journal homepage: wwwhttps://fezzanu.edu.ly/

Recommendations and suggestions for further studies

- explore the use of additional solvents with varying polarities to potentially uncover a broader range of phenolic compounds and antioxidant activities in Citrus aurantifolia.
- Utilize advanced chromatographic techniques such as High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) to identify and quantify the specific phenolic compounds responsible for the observed antioxidant activities.
- Conduct comparative studies with other citrus species to understand the unique or superior properties of Citrus aurantifolia in terms of antioxidant activities and phenolic contents.
- Further research into the specific anti-inflammatory and anti-cancer properties of the extracts, given their potential therapeutic significance as highlighted in previous studies.
- Investigate the long-term stability and shelf-life of these extracts, which is crucial for their potential application in food preservation and pharmaceuticals.

Acknowledgment

We want to express our thanks to Libyan Medical research center staff for providing the reagent used in the study.

References

1. Rivera-Cabrera, F., et al., *Acid limes. A review.* Fresh produce, 2010. **4**(1): p. 116-122.
2. Karthikeyan, V. and J. Karthikeyan, *Citrus aurantium (bitter orange): A review of its traditional uses, phytochemistry and pharmacology.* International journal of drug discovery and herbal research, 2014. 4(4): p. 766-772.
3. Loizzo, M.R., et al., *Evaluation of Citrus aurantifolia peel and leaves extracts for their chemical composition, antioxidant and anti-cholinesterase activities.* Journal of the Science of Food and Agriculture, 2012. 92(15): p. 2960-2967.
4. Zulaikhah, S.T., *The role of antioxidant to prevent free radicals in the body.* Sains Medika Journal of Medical & Health, 2017. 8(1).
5. Patil, B.S., G.K. Jayaprakasha, and K.N.C. Murthy, *Beyond vitamin C: the diverse, complex health-promoting properties of citrus fruits.* Citrus Research & Technology, 2017. 38(1): p. 107-121.
6. Park, Y.-S., et al., *Quantitative assessment of the main antioxidant compounds, antioxidant activities and FTIR spectra from commonly consumed fruits, compared to standard kiwi fruit.* LWT-Food Science and Technology, 2015. 63(1): p. 346-352.
7. Marzouk, B., *Characterization of bioactive compounds in Tunisian bitter orange (Citrus aurantium L.) peel and juice and determination of their antioxidant activities.* BioMed research international, 2013. 2013.
8. Orji, J.O., *Studies on the Antimicrobial, Nutritional and other Potential Uses of Canarium Schweinfurthii (Ube Okpoko).* 2010, UNIZIK.
9. Asadujjaman, M., M.A. Hossain, and U.K. Karmakar, *Assessment of DPPH free radical scavenging activity of some medicinal plants.* Pharmacology online, 2013. **1**: p. 161-165.

جامعة فزان العلمية مجلة
Fezzan University scientific Journal
Journal homepage: wwwhttps://fezzanu.edu.ly/

10. Nenadis, N., et al., *Estimation of scavenging activity of phenolic compounds using the ABTS•+ assay.* Journal of agricultural and food chemistry, 2004. **52**(15): p. 4669-4674.
11. Al Namani, J., et al., *Phytochemical screening, phenolic content and antioxidant activity of Citrus aurantifolia L. Leaves grown in two regions of oman.* Iranian Journal of Pharmaceutical Sciences, 2018: p. 27-34.
12. Kusumawati, I.G.A.W., I.M.W.A. Putra, and I.B.A. Yogeswara, *In vitro ACE inhibitory activity and bioactive compounds of aqueous extract of Citrus amblycarpa.* Majalah Obat Tradisional, 2021. **26**(2): p. 117-122.
13. Babbar, N., et al., *Influence of different solvents in extraction of phenolic compounds from vegetable residues and their evaluation as natural sources of antioxidants.* Journal of food science and technology, 2014. **51**: p. 2568-2575.
14. Kua, Y.L., et al., *Ethyl lactate as a potential green solvent to extract hydrophilic (polar) and lipophilic (non-polar) phytonutrients simultaneously from fruit and vegetable by-products.* Sustainable Chemistry and Pharmacy, 2016. **4**: p. 21-31.
15. Clímaco, G.N., R. Vardanega, and L.H. Fasolin, *Hibiscus sabdariffa L. leaves as an alternative source of bioactive compounds obtained through high pressure technologies.* The Journal of Supercritical Fluids, 2023. **200**: p. 105968.
16. Karimi, E., et al., *Phenolic compounds characterization and biological activities of Citrus aurantium bloom.* Molecules, 2012. **17**(2): p. 1203-1218.
17. Bechis, G., et al. *Fast chiral GC recognition of citrus essential oils evaluated with greenness metrics: is this analysis sustainable in industrial quality control?* in *53rd INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS Book of Abstract*. 2023.
18. Canadanovic-Brunet, J.M., et al., *Free-radical scavenging activity of wormwood (Artemisia absinthium L) extracts.* Journal of the Science of Food and Agriculture, 2005. **85**(2): p. 265-272.
19. Kumari, S., N. Sarmah, and A. Handique, *Antioxidant activities of the unripen and ripen Citrus aurantifolia of Assam.* International Journal of Innovative Research in Science, Engineering and Technology, 2013. **2**(9): p. 4811-4816.
20. Gomathi, D., et al., *In vitro free radical scavenging activity of ethanolic extract of the whole plant of Evolvulus alsinoides (L.) L.* Chinese journal of integrative medicine, 2015. **21**: p. 453-458.
21. Loizzo, M.R., et al., *The Influence of ultrafiltration of Citrus limon L. burm. cv femminello comune juice on its chemical composition and antioxidant and hypoglycemic properties.* Antioxidants, 2019. **8**(1): p. 23.
22. Zhao, H., et al., *Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (Hordeum vulgare L.).* Journal of agricultural and food chemistry, 2006. **54**(19): p. 7277-7286.
23. Kalia, K., et al., *Effects of extraction methods on phenolic contents and antioxidant activity in aerial parts of Potentilla atrosanguinea Lodd. and quantification of its phenolic constituents by RP-HPLC.* Journal of Agricultural and Food Chemistry, 2008. **56**(21): p. 10129-10134.