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## Research Article

# Comparative evaluation of phytochemical profiles and antioxidant potential in three citrus leaf varieties (Rutaceae)

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**Abstract**
Citrus species are widely recognised for their bioactive compounds, which contribute to antioxidant and therapeutic properties. Despite extensive research on Citrus fruits, comparative reports on the antioxidant potential of their leaves remain limited. This study aimed to perform a qualitative phytochemical analysis and assess the antioxidant activity of leaf extracts from *Citrus aurantifolia*, *Citrus hystrix*, and *Citrus microcarpa*. Phytochemical screening revealed the presence of alkaloids, triterpenoids, steroids, saponins, flavonoids, reducing sugars, carbohydrates, phenolic compounds, glycosides, and tannins across different solvent extracts. Notably, alkaloids were absent in methanol extracts of *C. aurantifolia* and *C. microcarpa*, while *C. hystrix* tested positive for alkaloids in all extracts. Antioxidant activity was assessed using DPPH radical scavenging, along with non-enzymatic antioxidant analyses for α-tocopherol and carotenoid content. *C. aurantifolia* exhibited the highest total antioxidant activity (10.1 ± 0.04 mg/g fwt), correlating with its phenolic content, whereas *C. microcarpa* recorded the lowest (0.6 ± 0.01 mg/g fwt). Interestingly, DPPH scavenging analysis showed the reverse trend, with *C. microcarpa* achieving the highest scavenging percentage (96.41 ± 1.62%). This apparent discrepancy may result from differences in the antioxidant mechanisms measured by each assay. Additionally, *C. hystrix* contained the highest α-tocopherol concentration (2.30 ± 0.05 µg/g fwt), while *C. microcarpa* showed the greatest carotenoid content (20.40 ± 1.56 mg/g fwt). These variations highlight the diverse phytochemical profiles of Citrus leaves and their potential as natural antioxidant sources. The findings support their future application and potential use in nutraceutical and pharmaceutical development, warranting further studies on pharmacological mechanisms and bioavailability.

**Keywords**: antioxidant, phytochemical profiles, *Citrus aurantifolia*, *Citrus hystrix*, *Citrus microcarpa*

## Introduction

Citrus species, belonging to the Rutaceae family, are widely recognised for their rich phytochemical composition and significant pharmacological properties, including antioxidant, antimicrobial, and anti-inflammatory activities [1]. Among these species, *Citrus aurantifolia* (key lime), *Citrus hystrix* (kaffir lime), and *Citrus microcarpa* (calamansi) (**Figure 1**) have been traditionally used for culinary and medicinal purposes, and are now being increasingly studied due to their diverse bioactive constituents and potential health benefits [2]. Phytochemical investigations of these species have reported flavonoids, alkaloids, tannins, saponins, and terpenoids as major metabolites contributing to their biological activities [3]. For example, *C. aurantifolia* leaf oil is rich in limonene, geranial, and neral, all of which are known for their antioxidant and anti-inflammatory properties [4]. In *C. hystrix*, citronellal predominates have been linked to antimicrobial and antifungal effects [5], while *C. microcarpa* leaf oil contains a diverse profile of sesquiterpenes, such as hedycaryol, β-sesquiphellandrene, and α-eudesmol, which are associated with anti-inflammatory and cytoprotective activities [1].

These metabolites suggest that Citrus leaves may serve as natural sources of antioxidants, which play a crucial role in neutralising free radicals and preventing oxidative stress-related diseases. Oxidative stress has been implicated in the pathogenesis of chronic disorders, including cardiovascular disease,

neurodegeneration, diabetes, and cancer. At the same time, recent global trends demonstrate increasing consumer demand for natural antioxidants and nutraceutical products, driven by concerns over synthetic additives and a preference for sustainable, plant-based bioresources [6,7]. Studies published in 2023–2024 also highlighted the growing nutraceutical market, where plant-derived bioactives, including those from underutilised plant parts, such as peels and leaves, are being prioritised for functional food and pharmaceutical development [8,9].

Although the antioxidant activity of various Citrus species has been reported, most research focused on fruits, peels, or essential oils. Comparative studies on Citrus leaves remain scarce, despite their traditional use and availability as agricultural by-products. Furthermore, variations in antioxidant activity across species and solvent extracts, as well as the differences in mechanisms measured by distinct antioxidant assays, have not been comprehensively addressed. The novelty of this study lies in conducting a comparative phytochemical and antioxidant assessment of *C. aurantifolia*, *C. hystrix*, and *C. microcarpa* leaves, thereby providing new insights into their potential as sustainable natural antioxidant sources for nutraceutical and pharmaceutical applications.

## Materials and Methods

### Plant collection and extraction

The fresh leaves of *C. aurantifolia*, *C. hystrix*, and *C. microcarpa* (250 g each) were collected from Mersing, Johor, Malaysia. The species was identified based on morphological characteristics using standard references (Flora of Peninsular Malaysia, Flora of Java), and the identification was verified by botanists at Universiti Malaysia Terengganu (UMT). The leaves were washed, air-dried at room temperature for seven days, and ground into powder. Extraction was performed by soaking 250 g of powdered leaves in 500 mL of analytical grade hexane (Cat. No. 270504), ethyl acetate (Cat. No. 270989), and methanol (Cat. No. 34860) (Sigma-Aldrich, USA) for 72 h at room temperature. Extracts were concentrated using a rotary evaporator (Büchi Rotavap R-200CH-9230, Switzerland) under reduced pressure at 35 °C – 40 °C.

### Preliminary phytochemical screening

Qualitative phytochemical screening of the extracts was conducted to identify alkaloids, saponins, and tannins (Braymer's test) [10], steroids [11], flavonoids ($H_2SO_4$ test), phlobatannins [12], reducing sugars (Fehling's test) [13], carbohydrates (iodine test) [13], and phenolics (ferric chloride test) [14]. All assays were performed in triplicate to confirm consistency of the results.

### Scavenging effect on DPPH radical

The DPPH radical scavenging activity was determined following a previous method [15]. Briefly, 0.1 mL of extract was mixed with 0.25 mL of 0.2 mM DPPH in methanol, shaken, and left at room temperature for 30 min. Absorbance was recorded at 517 nm, with butylated hydroxytoluene (BHT, 20 mM in methanol) as the positive control. All assays were carried out in triplicate. The scavenging effect was calculated as follows:

$$\text{Scavenging Activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad \text{(Eq. 1)}$$

### Determination of α-tocopherol Content

Fresh leaves (0.5 g) were ground with 1.5 mL acetone and a small amount of sand at 0 °C – 4 °C, followed by extraction with 0.5 mL hexane. After vortexing and centrifugation (10,000 rpm, 10 min), the supernatant was collected and re-extracted twice. The assay was performed as described in [16]. Hexane extracts were mixed with 0.4 mL of 0.1% PDT (in ethanol) and 0.4 mL of 0.1% ferric chloride (in ethanol), adjusted to 3.0 mL with ethanol, and allowed to react for 4 min. After adding 0.2 mL orthophosphoric acid, the mixture was incubated at room temperature for 30 min, and absorbance was measured at 554 nm. A standard curve of α-tocopherol (Sigma Type V, Cat. No. T3634) was used for quantification. Analyses were performed in triplicate.

(a) (b) (c)

**Figure 1.** (a) *Citrus aurantifolia* (key lime), (b) *Citrus hystrix* (kaffir lime) and (c) *Citrus microcarpa* (calamansi)

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**Determination of carotenoid content**

Carotenoids were extracted following a reported method [17]. Leaf samples (0.5 g) were homogenised in 3.0 mL of 80% acetone and centrifuged (10,000 rpm, 10 min). Absorbance of the supernatant was measured at 663.2, 646.8, and 470 nm. Carotenoid content was calculated as:

$$C_a = 12.25A_{663.2} - 2.79A_{646.8} \quad \text{(Eq. 2)}$$

$$C_b = 21.50A_{646.8} - 5.10A_{663.2} \quad \text{(Eq. 3)}$$

$$C_{x+c} = \frac{1000A_{470} - 1.82C_a - 85.02C_b}{198} \quad \text{(Eq. 4)}$$

Where, $C_a$ = chlorophyll a (mg/L), $C_b$ = chlorophyll b (mg/L), $C_{x+c}$ = carotenoids (mg/L). All analyses were performed in triplicate.

**Statistical analysis**

All experiments were conducted in triplicate (n = 3). Results were expressed as mean ± standard deviation and analysed using one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

**Results and Discussion**

**Qualitative phytochemical screening test**

Phytochemical screening was carried out to identify secondary metabolites in the leaves of *C. aurantifolia*, *C. hystrix*, and *C. microcarpa*. Each sample was extracted with solvents of different polarities (hexane, ethyl acetate, and methanol), resulting in nine crude extracts. The phytochemical assays tested for alkaloids, triterpenoids, steroids, saponins, flavonoids, phlobatannins, reducing sugars, carbohydrates, phenolic compounds, glycosides, and tannins. The summarised results are presented in **Table 1**.

Alkaloids were detected in all crude extracts, except in the methanol extracts of *C. aurantifolia* and *C. microcarpa*, while *C. hystrix* consistently showed positive results across all solvents. These findings align with a previous report, where the methanol extract of *C. aurantifolia* lacked alkaloids, contrasting with *C. hystrix* [18]. Triterpenoids were present in the hexane and ethyl acetate extracts of *C. microcarpa* and in the ethyl acetate extract of *C. aurantifolia*, but were absent in all methanol extracts. Steroids were consistently present in *C. hystrix* across all solvents. Saponins were confirmed in all hexane extracts but were absent in methanol extracts. Among the ethyl acetate extracts, only *C. hystrix* tested negative.

Flavonoids were found in all *C. microcarpa* extracts, as confirmed by the colour change with $H_2SO_4$. Nevertheless, they were absent in the ethyl acetate extract of *C. hystrix* and the methanol extract of *C. aurantifolia*. This observation agrees with some studies but differs from others that reported flavonoids in *C. hystrix* [1]. Phlobatannins were absent in all extracts, which could be attributed either to the inherent genetic traits of Citrus species or to solvent limitations in extracting such compounds. While reducing sugars were present in all *C. hystrix* extracts, they were present only in the hexane and ethyl acetate extracts of *C. aurantifolia* and *C. microcarpa*. Similarly, carbohydrates were present in all *C. hystrix* extracts, but were detected only in the hexane and ethyl acetate extracts of *C. aurantifolia* and *C. microcarpa*. Phenolic compounds were absent in *C. hystrix*, but present in the hexane and ethyl acetate extracts of *C. microcarpa* and in the ethyl acetate extract of *C. aurantifolia*. Glycosides were found in all *C. microcarpa* extracts, while tannins were observed in the ethyl acetate and methanol extracts of *C. microcarpa* and the ethyl acetate extract of *C. aurantifolia*.

**Table 1**. The phytochemical screening test of *C. aurantifolia* (CA), *C. hystrix* (CH), and *C. microcarpa* (CM)

| Phytochemical Test | Hexane Extract | | | Ethyl Acetate Extract | | | Methanol extract | | |
|---|---|---|---|---|---|---|---|---|---|
| | CA | CH | CM | CA | CH | CM | CA | CH | CM |
| Alkaloid | + | + | + | + | + | + | - | + | - |
| Triterpenoids | - | - | + | + | - | + | - | - | - |
| Steroids | + | + | - | - | + | - | - | + | - |
| Saponins | + | + | + | + | - | + | - | - | - |
| Flavonoids | + | + | + | - | - | + | - | + | + |
| Phlobatannins | - | - | - | - | - | - | - | - | - |
| Reducing Sugar | + | + | + | + | + | + | - | + | - |
| Carbohydrates | + | + | + | + | + | + | - | + | - |
| Phenolic | - | - | + | + | - | + | - | - | - |
| Glycosides | + | + | + | - | - | + | - | + | + |
| Tannins | - | - | + | + | - | + | - | - | - |

+: Positive result; -: Negative result

These compounds are widely recognised for their pharmacological roles in modulating oxidative stress and inflammation [19]. The observed solvent-dependent variations underscore the role of polarity in metabolite extraction using non-polar solvents, such as hexane preferentially extracted triterpenoids and steroids, while polar solvents like methanol were more effective in recovering phenolics and flavonoids. Interestingly, phlobatannins were absent in all extracts, which could reflect a genetic trait of Citrus species or limited solubility in the solvents employed [20]. The absence of phlobatannins aligns with other reports showing their rarity in Rutaceae members compared to woody plants, such as Acacia [21]. This selective phytochemical profile highlights the unique metabolomic fingerprint of each Citrus species and provides a biochemical rationale for the variability observed in antioxidant assays.

**Antioxidant activity and free radical scavenging assays**

Antioxidants are essential in reducing oxidative stress by neutralising free radicals that damage DNA, proteins, and lipids, leading to diseases such as cancer and neurodegenerative disorders [22,23]. Secondary metabolites, such as alkaloids, flavonoids, saponins, and glycosides, have been reported as effective free radical scavengers [24]. The antioxidant activity assay (expressed as mg/g.fwt) revealed that *C. aurantifolia* exhibited the highest activity (10.1 ± 0.042 mg/g.fwt), correlating with its positive phenolic content. *C. hystrix* displayed moderate activity (7.1 ± 0.035 mg/g.fwt), whereas *C. microcarpa* recorded the lowest (0.6 ± 0.005 mg/g.fwt) (**Figure 2**). These findings are consistent with reports linking phenolic compounds to strong antioxidant activity [25].

Interestingly, the DPPH assay showed a contrasting trend. *C. microcarpa* exhibited the highest radical scavenging capacity (96.41 ± 1.62%), followed by *C. hystrix* (79.01 ± 1.57%) and *C. aurantifolia* (75.68 ± 1.09%). The positive control, BHT, displayed a scavenging activity of 98.62 ± 1.11% (Figure 3). This apparent contradiction highlights methodological differences: DPPH % inhibition reflects the efficacy of free radical neutralisation, while mg/g.fwt indicates the concentration of total antioxidants. Therefore, *C. microcarpa* may contain compounds with strong radical-scavenging efficiency even if its total antioxidant load is lower. Such discrepancies have also been documented across Citrus species [26-28]. Overall, the results emphasise that both assays provide complementary insights as one measures capacity per weight unit, while the other measures scavenging efficiency, underlining the need to interpret antioxidant data from multiple perspectives.

**Non-enzymatic Antioxidant Assays**

The non-enzymatic antioxidant assays revealed clear interspecific variations in α-tocopherol and carotenoid content (Figures 4 and 5). *C. hystrix* exhibited the highest α-tocopherol concentration (2.30 ± 0.05 µg/g.fwt), suggesting a strong vitamin E–mediated defence mechanism [30]. In contrast, *C. aurantifolia* showed the lowest α-tocopherol content (0.51 ± 0.04 µg/g.fwt). On the other hand, *C. microcarpa* had the highest carotenoid level (20.40 ± 1.56 mg/g.fwt), significantly exceeding the other two species. Carotenoids are known for their photoprotective and antioxidant roles [29].

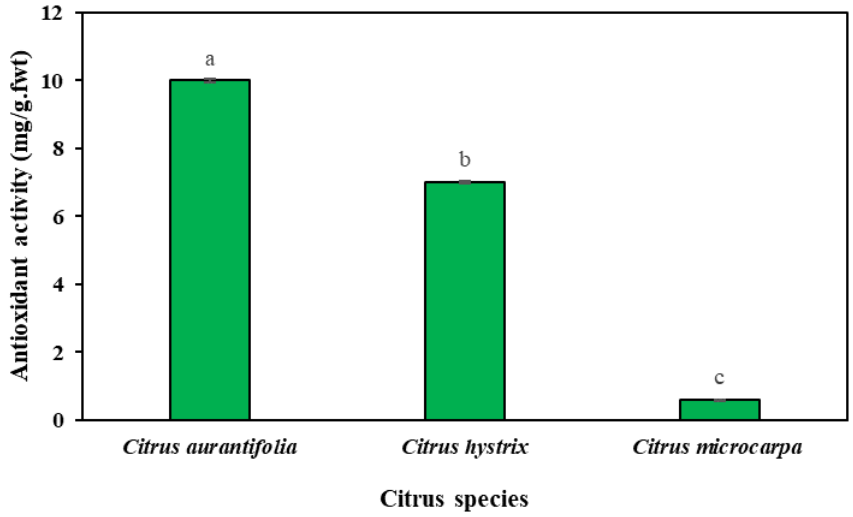


**Figure 2**. Antioxidant Activity (mg/g.fwt) of methanolic crude extract of *Citrus aurantifolia, Citrus hystrix*, and *Citrus macrocarpa.* Values are means ± SD, n=3. Notes where different letters (a-c) indicate significant differences at $p < 0.05$

The variation in these antioxidant compounds may be attributed to genetic differences, environmental factors, and species-specific metabolic pathways influencing secondary metabolite biosynthesis [27]. Collectively, the results highlighted the potential of *C. hystrix* as a source of vitamin E and *C. microcarpa* as a carotenoid-rich species, both of which have promising applications in nutraceuticals and functional foods.

Non-enzymatic antioxidants (α-tocopherol and carotenoids) provide another perspective on the antioxidant defence system of Citrus leaves. *C. hystrix* showed the highest α-tocopherol concentration, reinforcing its role in preventing lipid peroxidation and stabilising cell membranes [31]. Meanwhile, *C. microcarpa* was distinguished by its exceptionally high carotenoid levels, playing dual roles in scavenging free radicals and providing photoprotection against oxidative stress [32]. Carotenoids also act synergistically with phenolics, enhancing antioxidant stability and prolonging bioactivity [33]. These interspecies variations may be attributed to differential expression of biosynthetic genes and environmental influences on metabolite accumulation [34]. Importantly, the complementary profiles observed high α-tocopherol in *C. hystrix* and elevated carotenoids in *C. macrocarpa*, highlighting species-specific strengths that can be strategically harnessed for nutraceutical development.

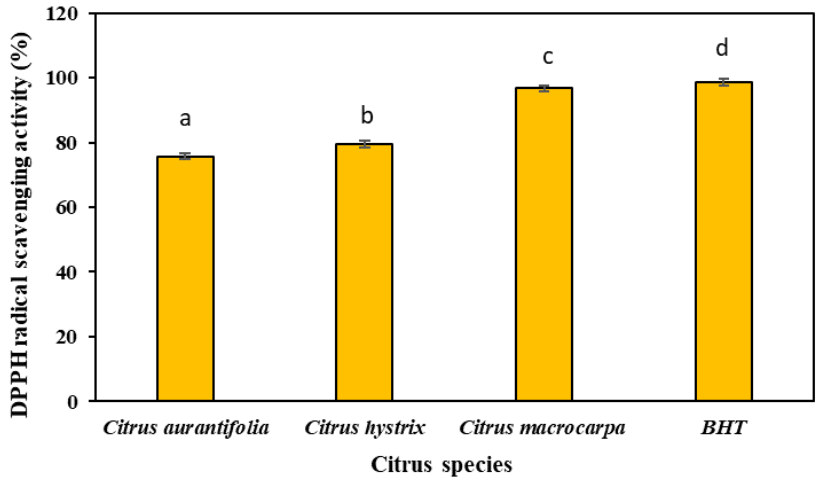


**Figure 3**. DPPH scavenging activities (%) in the methanolic crude extract of *Citrus aurantifolia, Citrus hystrix* and *Citrus macrocarpa* compared to butylated hydroxytoluene (BHT, 20 mM) as the positive control. Values are means ± SD, n=3. Notes where different letters (a-d) indicate significant differences at $p < 0.05$

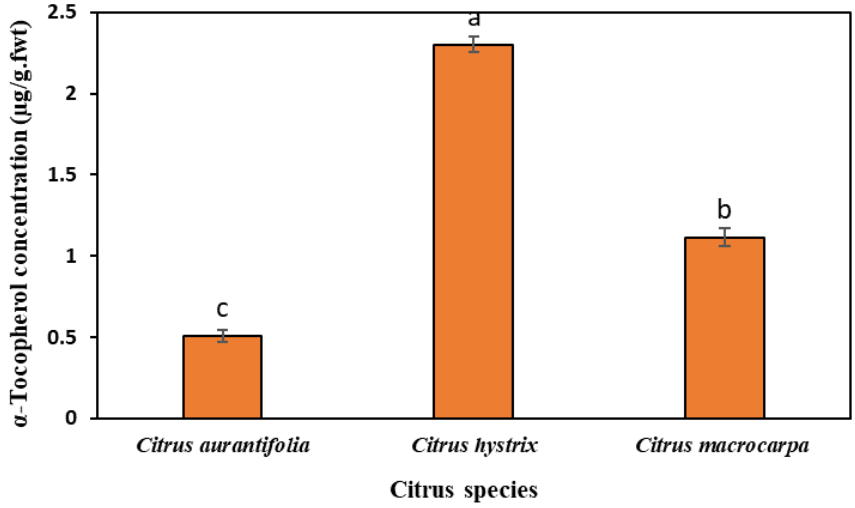


**Figure 4**. α-Tocopherol concentration (ug/g.fwt) of *Citrus aurantifolia, Citrus hystrix*, and *Citrus macrocarpa*. Values are means ± SD, n=3. Notes where different letters (a-c) indicate significant differences at $p < 0.05$

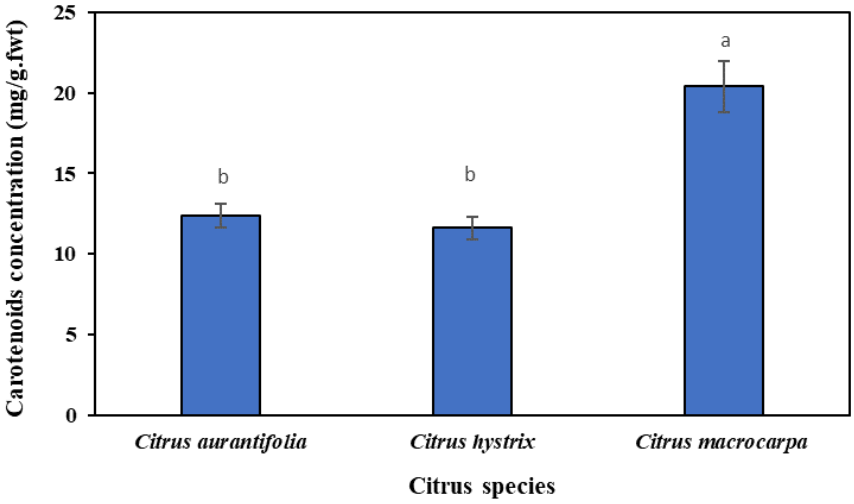


**Figure 5**. Carotenoid concentrations (mg/g.fwt) of *Citrus aurantifolia, Citrus hystrix*, and *Citrus macrocarpa*. Values are means ± SD, n=3. Notes where different letters (a-c) indicate significant differences at $p < 0.05$

## Conclusion

This study provides a comprehensive assessment of the phytochemical composition and antioxidant potential of *C. aurantifolia*, *C. hystrix*, and *C. microcarpa* leaf extracts. The detection of diverse secondary metabolites, including alkaloids, flavonoids, triterpenoids, steroids, saponins, and phenolic compounds, highlighting the rich phytochemical profiles of these species. Notably, *C. aurantifolia* exhibited the strongest antioxidant activity (mg/g.fwt) in association with its phenolic content, while *C. microcarpa* displayed the highest DPPH radical scavenging efficiency. In addition, the elevated α-tocopherol concentration in *C. hystrix* and the abundant carotenoid levels in *C. microcarpa* further underscore their value as sources of natural antioxidants. The observed interspecific variations emphasise the influence of solvent polarity on metabolite recovery and antioxidant activity.

Collectively, these findings support the potential of Citrus leaf extracts as natural antioxidants for mitigating oxidative stress-related diseases. Future studies may focus on bioavailability, mechanisms of action, and therapeutic efficacy to facilitate their application in nutraceuticals and pharmaceutical formulations. By advancing the understanding of their pharmacological benefits, these Citrus species may contribute to the development of novel antioxidant-based health products.

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