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Flavonoid synergy in antioxidant optimization: a study of *Apium graveolens* and *Orthosipon stamineus*

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ABSTRACT

Oxidative stress, which arises by an imbalance among the formation of free radicals and the body's antioxidant defenses, is a pivotal factor in the pathogenesis of numerous degenerative abnormalities, comprising cardiovascular abnormality. Flavonoids, that are natural chemicals by antioxidant capabilities, have been identified as potential agents for protection against the adverse impacts of oxidative stress. The objective of this study was to ascertain the flavonoid substance of *Apium graveolens* and *Orthosipon stamineus* extracts and their antioxidant activity. The technique comprising qualitative and quantitative phytochemical tests to decide the flavonoid substance of the extracts. In addition, bioactive compounds were screened utilizing LC-HRMS, and antioxidant activity was evaluated utilizing the DPPH technique. The outcomes of this study drawn the presence of flavonoid compounds, alkaloids, tannins, and saponins in the *Apium graveolens* and *Orthosipon stamineus* extracts. The *Orthosipon stamineus* extract was found to contain steroid compounds. The screening of flavonoids compounds utilizing LC-HRMS has drawn the presence of the greatest diversity of flavonoid compounds in the *Apium graveolens* extract. The antioxidant activity assay employed quercetin as the standard, possessing an IC_{50} value of 3.95 µg/mL. *Apium graveolens* extract exhibited an IC_{50} value of 58.86±0.44µg/mL, *Orthosiphon stamineus* extract drawn an IC_{50} value of 61.69±0.21 µg/mL, whereas the combined extract yielded an IC_{50} value of 46.32±0.34 µg/mL. The outcomes indicate that the extract combination shows superior free radical scavenging ability compared to the individual extracts, suggesting its potential to enhance the antioxidant efficacy of bioactive compounds derived by herbal plants.

Keywords: Synergistic impact, Antioxidant activity, flavonoids, *Apium graveolens*, *Orthosiphon stamineus*

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INTRODUCTION

Oxidative stress is a phenomenon associated by the presence of free radicals and reactive metabolites. It happens either by a drop in antioxidant density or in rise in free radical count (Zhang et al., 2015). Free radicals, by their unpaired electrons, are highly reactive and have been related to several major abnormalities, such as cardiovascular abnormality, diabetes, cancer, and arthritis (Jain et al., 2011). These chronic abnormalities represent a huge worldwide health burden, having a substantial impact on mortality and morbidity (Essola et al., 2022). Utilizing natural antioxidants obtained by herbal plants as a free radical scavenging therapy is a successful technique for the prevention of chronic abnormality (Embuscado, 2015).

Herbal medicine is classified as complementary and alternative medicine (CAM) and is used for the prevention and therapy of various abnormalities due to its impactiveness, accessibility, and availability (Yuan et al., 2016). Several plants, such as *Apium graveolens*, *Centella asiatica*, *Orthosiphon stamineus, Panax ginseng* and *Camellia sinensis* have demonstrated the capability to neutralize free radicals, inhibit lipid oxidation, and function as immunomodulators. The combination of these impacts like lowering free radical activity, decreasing lipid oxidation, and changing the immune response, can greatly lessen cell damage that haves improve overall health protection (Banjarnahor & Artanti, 2014; Jain et al., 2011; Wulandari, 2021).

Herbal medications contain phytochemicals such as flavonoids, alkaloids, polyphenols, terpenoids, saponins, phytosterols, and fiber, that have been shown to have therapeutic impacts in the therapy of a variety of abnormality (Hassen et al., 2022; Minarno, 2015). Flavonoids are natural compounds that have a basic structure made up of two benzene rings (C6-C3-C6). They play an essential role as natural antioxidants, neutralizing free radicals, decreasing oxidative stress, and safeguarding biological cells by oxidative damage that can contribute to chronic abnormality (Tamfu et al, 2022; Panche et al., 2016; Ullah et al., 2020). Flavonoids not only have antioxidant activity, but they have also been demonstrated to possess anti-inflammatory, anticancer, and neuroprotective impacts (Shamsudin et al., 2022; Ullah et al., 2020).

The antioxidant activity of flavonoids is attributed not only to their ability to neutralize free radicals but also to the synergistic interactions that occur when multiple flavonoids are combined (Vinholes & Vizzotto, 2017). The synergistic impact occurs when the antioxidant activity of the flavonoid mixture exceeds the cumulative impacts of each individual molecule (Olszowy-Tomczyk & Wianowska, 2023). However, such interactions may also lead to antagonistic outcomes, in that the cumulative activity is less than expected (Skroza et al., 2022). Despite comprehensive research on the antioxidant impacts of flavonoids, the complex interactions inside of flavonoid combinations continue to be a topic of ongoing investigation. Further study is necessary to thoroughly elucidate the processes underlying both synergistic and antagonistic impacts, as well as to enhance the efficacy of flavonoids as antioxidant agents (Hidalgo et al., 2010).

In this study, celery (*Apium graveolens*) and cat's whisker (*Orthosiphon stamineus*) were chosen because of their known antioxidant properties. The main objective was to explore the synergistic impacts of their flavonoid substance. A phytochemical analysis was performed utilizing liquid chromatography-high resolving mass spectrometry (LC-HRMS) to identify and characterize compounds, comprising flavonoids, polyphenols, and alkaloids, that are linked to antioxidant activity (Rafi at. al, 2023). LC-HRMS allows for both qualitative identification and quantitative assessment of the density of these components, facilitating the identification of molecules that contribute to the observed antioxidant impacts (Cakmak, 2024).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a largely used technique due to its simplicity, speed, and the availability of low-cost spectrophotometers. This assay was employed to evaluate the antioxidant activity of the polyherbal combination. It is particularly favored for its straightforward measurement procedure and quick analysis time. However, it is important to note that, while several techniques exist for assessing antioxidant qualities, these are not equivalent. The DPPH radical model, being physically distinct, offers a unique mechanism for evaluating antioxidant

activity, potentially enhancing our comprehending of the connections among antioxidants assessed by different techniques (Platzer et al., 2021).

This study focused on *Apium graveolens* and *Orthosiphon stamineus* due to their previously established antioxidant properties, that have been assessed utilizing the DPPH technique in earlier research. Both plants demonstrate significant antioxidant potential, making them suitable subjects for investigation in a polyherbal formulation. Nevertheless, despite prior research, there remains a gap in comprehending the synergistic antioxidant properties of these two plants. Researchers have examined each plant independently, but they have not sufficiently explored the combined antioxidant efficacy when used together. This research is time sensitive as it aims to address this gap, potentially leading to the enhancement of more impactive polyherbal formulations by enhanced antioxidant characteristics. The work seeks to clarify the synergistic antioxidant properties of *Apium graveolens* and *Orthosiphon stamineus,* thereby improving our comprehending of polyherbal formulations in therapeutic contexts.

MATERIALS AND METHOS

Materials

The leaves of *Apium graveolens* and *Orthosiphon stamineus* were harvested by The Center for Medicinal and Traditional Plant Research in Tawangmangu. All experimental procedures used chemicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl), sourced by Sigma-Aldrich, to assess antioxidant activity. The additional reagents used in the experiments comprising aluminum chloride ($AlCl_3$), Folin-Ciocalteu (FC) reagent, sodium hydroxide, sodium nitrite, Meyer's reagent, sulfanilamide, sodium nitroprusside, anhydrous acetic acid, sulfuric acid, quercetin, ethanol, methanol (purity grade), sodium acetate, and aquabides. All of these were sourced by Merck.

Method

Essence preparation

A total of 50 grams of *Apium graveolens* leaves and 50 grams of *Orthosiphon stamineus* leaves were weighed separately. All plant elements were positioned in a beaker containing 250 mL of water. We then heated the beakers on a hot plate for 15 minutes to bring the infusion to a boil. Subsequent to boiling, the infusions were permitted to rest for an additional 15 minutes prior to filtration. The volume of the filtrate by the filtration procedure was quantified, and water was incorporated till the total volume reached 250 mL. Subsequently, 230 mL of the filtrate was concentrated utilizing a rotary evaporator at a degree range of 40-60ºC for 210 minutes (Peloan & Kaempe1, 2020).

Phytomchemical screening

The phytochemical screening of *Apium graveolens* and *Orthosipon stamineus* extracts comprising the identification of flavonoids utilizing an AlCl3 reagent (Supomo et al., 2021), the detection of alkaloids through the use of Mayer reagents (Supomo et al., 2021), and the determination of tannins through the application of Folin Ciocalteauv (Supomo et al., 2021). The presence of steroids is decided by the Liebermann reaction (Melati & Parbuntari, 2022), while the identification of saponins is based on their ability to form a stable foam (A'yun & Laily, 2015).

Total flavonoid substance assay

The standard curve was done by accurately weighing up to 10 mg of quercetin and transferring it into a 25 mL volumetric flask. We solubilized the quercetin in ethanol, subjected the solving to sonication, and subsequently filled the flask to the designated mark by ethanol. The solving was placed in a cuvette, and its absorbance was measured over the wavelength range of 400–600 nm. Flavonoid substance Assessment: The sample was measured and transferred into a conical flask. Two milliliters of ethanol were introduced, and the mixture was subjected to vortex and sonication for one hour. Subsequently, the sample underwent centrifugation, the supernatant was decanted into a 10 mL graduated flask, and the valid procedure was reiterated to guarantee comprehensive flavonoid concentration (Sulastri et al., 2018).

Liquid Chromatography High-Resolving Mass Spectrometry (LCHRMS) analysis

At the National Research and Innovation Agency (BRIN) in Yogyakarta, the ethyl acetate and water fractions were subjected to LCHRMS examination using an Agilent 6520 Accurate Mass Q-TOF Mass Spectrometer. Separation was achieved with acetonitrile, 5 mM acetate buffer, and water as the solvent system, delivered at 1.5 mL/min. The gradient began with 5% acetonitrile for 0.1 min, ramped to 30% in 10 min, further to 80% within 32 min, and finally returned to the initial condition. The column was maintained at 30 °C, and detection was carried out through Q-TOF HRMS in positive electrospray ionization mode. (Noumi et al., 2020)

Antioxidant activity test by DPPH technique

A 50 ppm DPPH solving was done by dissolving 5 mg of DPPH crystals in 100 mL of methanol (Cruz-Casas et al., 2023). To determine the maximum absorbance wavelength of DPPH, 1 mL of the done 50 ppm solving was combined with 3 mL of methanol in a test tube and mixed till homogeneous. The resulting mixture was subsequently allowed to stand at room temperature for 30 minutes. The optimum wavelength was then identified by measuring absorbance inside of the range of 510 to 525 nm. For sample preparation, 25 mg of each sample namely, *Apium graveolens* extract, *Orthosiphon stamineus* extract, and the combination extract (1:1) were accurately weighed and dissolved in 25 mL of methanol in a volumetric flask to obtain a 100 ppm stock solving. by this stock, a series of diluted solving by density of 10, 15, 20, 25, and 30 ppm were done. A quercetin solving was done to serve as a reference standard. Precisely 5 mg of quercetin was weighed and dissolved in methanol, then transferred to a 50 mL volumetric flask and diluted to volume by methanol to yield a 100 ppm stock solving. by this stock, a series of dilutions were done at density of 1, 2, 3, 4, and 5 ppm. Absorbance measurements were performed utilizing a UV-Vis spectrophotometer. For each test, 1 mL of the sample or standard solving was mixed by 1 mL of 100 ppm DPPH solving and 2 mL of methanol in a test tube, followed by thorough mixing till homogeneous. The mixtures were incubated at room degree for 30 minutes before measuring absorbance at the previously decided optimal wavelength for DPPH. All experiments were employed in triplicate (Pakki et al., 2020).

Calculation of % Inhibition and IC_{50}

Determination of % Inhibition and IC_{50} Value. IC_{50} values were calculated utilizing a linear regression equation of y = bx + a among the density of the test solving (x) by % Inhibition (y).

$$\%\text{inhibition} = \frac{\text{Absorbance Control} - \text{Absorbance Sample}}{\text{Absorbance Control}} \times 100\%$$

Data analysis

The outcomes were statistically analyzed utilizing the analysis of variance (ANOVA) test to see whether there was a significant difference in values among the extracts and the test variables. Following this, the Tukey test was utilized to decide the connection between the density of the test substance and the sample's antioxidant capacity.

RESULT AND DISCUSSION

Qualitative phytochemical analysis of an extract

Identifying the phytochemical compounds in the extracts of *Apium graveolens,* and *Orthosiphon stamineu*s is a crucial initial step for assessing their potential biological, medicinal, and technical applications (Kandasamy et al., 2023). Each extract was examined for the presence of key phytochemical cohorts, comprising alkaloids, flavonoids, tannins, saponins, steroids, and terpenoids. The qualitative phytochemical analysis outcomes are summarized in Table 1 for the extracts of *Apium graveolens*, and *Orthosiphon stamineus*. The *Apium graveolens* extract fulfills alkaloids, flavonoids, tannins, saponins, and terpenoids, while the extracts by *Orthosiphon stamineus* contain alkaloids, flavonoids, tannins, saponins, steroids, and terpenoids. Emad et al. (2022) indicates that the extract

of *Apium graveolens* leaves has alkaloids, flavonoids, tannins, saponins, and terpenoids. Sivakumar & Jeganathan (2018) report that *Orthosiphon stamineus* extract comprises alkaloids, flavonoids, steroids, terpenoids, tannins, and saponins. Preliminary phytochemical screening is valuable for examining bioactive compounds and may facilitate medication enhancement and discovery (Dubale et al., 2023).

Apium graveolens and *Orthosiphon stamineus* comprising phytochemicals essential for cardiovascular abnormality prevention and antioxidant activity. Flavonoids, the main component, have antioxidant activity through the mechanisms of free radical scavenging and metal ion binding (Babychan et al., 2017). Flavonoids protect cells by oxidative damage, improve endothelial function, reduce inflammation, and inhibit LDL oxidation, that is the first step in the formation of atherosclerosis, a major cause of cardiovascular abnormality (Ullah et al., 2020). The combined use of flavonoids by different plant sources has drawn higher benefits than the use of a single plant, suggesting a synergistic potential in decreasing cardiovascular risk (Speisky et al., 2022).

Table 1. Qualitative Phytochemical analysis of an extract

Essence	Test type	*Reagent/technique*	*The changes caused*	*Outcome*
Apium graveolens	Flavonoid	$AlCl_3$ 1%	Yellow color occurs	+
	Alkaloid	Meyer	Formation of yellow precipitate	+
	Tannins	Folin Ciocalteau	Blue-green color formed	+
	Saponins	Froth	stable foam	+
	Steroids	Libermann Burchard's	brown ring formed	-
Orthosipon stamineus	Flavonoid	$AlCl_3$ 1%	Yellow color occurs	+
	Alkaloid	Meyer	Formation of yellow precipitate	+
	Tannins	Folin Ciocalteau	Blue-green color formed	+
	Saponins	Froth	stable foam	+
	Steroids	Libermann Burchard's	brown ring formed	+

Quantitative flavonoid analysis of extract

The total flavonoid substance in extracts of *Apium graveolens*, and *Orthosipon stamineus* was measured utilizing a colorimetric assay based on the aluminum chloride ($AlCl_3$) technique, by quercetin serving as the reference standard. This technique quantifies flavonoids by detecting the formation of a yellow complex that occurs when $AlCl_3$ reacts by the keto and hydroxyl cohorts found in flavones and flavanols (Sultana et al., 2024), (Syukur et al., 2023). The analysis drawn that the total flavonoid substance in the *Orthosipon stamineus* extract, expressed as quercetin equivalents, was 3.216 mg QE per gram of extract. This means that each gram of the extract fulfills 3.216 milligrams of quercetin. This outcome surpasses the study employed by Hizar et al., (2024); Ho et al., (2014) that noted that the optimal extract of *Orthosiphon stamineus* contained total flavonoids of 74.09 mg/100 g and 45.00 mg/100 g. The flavonoid density in the *Apium graveolens* extract was quantified at 1.850 mg QE per gram. This outcome is inferior to that documented by Kholieqoh et al. (2022), who decided the flavonoid density in *Apium graveolens* to be 13.99 mg QE for the leaf extract and 2.46 mg QE for the stem extract. The observed discrepancies may outcome by various factors, comprising environmental variations, climatic differences, soil composition at the harvest locations, and the enhancement level of the plants at the time of collection (Xu, 2016). Moreover, although analogous fundamental techniques were employed, slight alterations in some parameters, such as incubation degree, may also influence the efficacy of flavonoid concentration (Shi et al., 2022). The total flavonoid density in plant extracts exhibits a robust association by antioxidant action

(Lukman et al., 2024). Flavonoids function as free radical scavengers by donating electrons to neutralize these harmful molecules, thereby decreasing oxidative damage to cells. A higher flavonoid substance is generally associated by higher antioxidant potential, that can have protected the body against oxidative stress and various related abnormalities (Hassanpour & Doroudi, 2023).

Table 2. Flavonoid substance of *Apium graveolens* and *Orthosipon stamineus*

Sample	Outcome	Unit
Apium graveolens	1.850±0.046	mg QE/g extract
Orthosipon stamineus	3.216±0.046	mg QE/g extract

Identification of flavonoid substance utilizing LC-HRMS

LC-HRMS is a highly sensitive and exact technology for the detection of chemicals in complex materials, such as plant extracts (Saurina, 2024). The technique integrates liquid chromatography by high-resolving mass spectrometry, enabling the separation, identification, and quantification of compounds. This study employed LC-HRMS to analyze the flavonoid substance in *Apium graveolens* and *Orthosipon stamineus* extracts, utilizing electrospray ionization (ESI) in positive mode. In this mode, protons ($H^{+)}$ are added to the target molecule, forming a pseudo molecular ion [M+H]+, that is then detected (Della Vedova et al., 2022). The positive ionization technique of electrospray ionization is advantageous for its high sensitivity and impactive fragmentation, making it an ideal approach for the detection of flavonoids and enabling detailed structural analysis through controlled fragmentation (Yamamoto et al., 2023).

Table 3 illustrates the flavonoid profile of *Apium graveolens* L. extract, presenting the outcomes of the LC-HRMS analysis. The primary flavonoid detected in the extract was apigenin, a unique compound present in celery, that substantially influences the extract's overall composition, accounting for 44.80% of the total area. The investigation identified multiple flavonoids, comprising apigenin, diosmetin, kaempferol, apigetrin, cynaroside, and kaempferol-3-O-rutinoside. Apigetrin (7.27%) was recognized as a glycosidic derivative of apigenin, displaying a peak area of 2.53E+08. This molecular data verifies that apigetrin is a crucial constituent of the extract, in conjunction by apigenin. Furthermore, the identification of kaempferol 3-O-glycoside (4.14%) and cynaroside (1.30%) enhances our comprehending of the flavonoid composition in the extract. These outcomes align by the detailed flavonoid profile documented for celery, where molecular masses (m/z) and retention times (RT) provide critical information for further research.

Kaempferol-3-O-rutinoside was identified, corroborating the existence of multiple kaempferol derivatives in the extract, contributing 0.60% to the total area. The chemicals were identified through precise m/z values, and the LC-HRMS analysis successfully clarified the composition of these flavonoids. This study's outcomes align by the research of Mohamud Dirie et al. (2025), that identified apigenin-7-glucoside, rutin, and apigetrin in the ethanolic extract of celery leaves via LC-HRMS. Ahmed et al. (2022) identified apigenin, diosmetin, apigetrin, and kaempferol in Apium graveolens extract via LC-HRMS. These outcomes corroborate the substantial presence of flavonoids in celery and emphasize the consistency of outcomes across various studies, highlighting the importance of these compounds in the antioxidant capacity and bioactive potential of celery extract. This study identified flavonoids, specifically apigenin, kaempferol, and apigetrin, linked to various health benefits, comprising antioxidant, anti-inflammatory, and anti-cancer impacts. These bioactive compounds are essential in mitigating oxidative stress, a significant contributor to the onset of chronic abnormalities comprising cardiovascular abnormality, diabetes, and cancer (Siripongvutikorn et al., 2024); (Abdulrasheed-Adeleke et al., 2023).

Table 3. Flavonoid substances were identified in *Apium graveolens L.* and *Orthosipon stamineus* extracts utilizing LC-HRMS by positive ESI ionization mode

No	Metabolite Name	Formula	RT (min)	Calcu-lated Mass	% Area
1	Apigenin	$C_{15}H_{10}O_5$	8.52	270.05	44.80
2	Diosmetin	$C_{16}H_{12}O_6$	8.69	300.06	29.29
3	Kaempferol	$C_{15}H_{10}O_6$	7.72	286.05	12.21
4	Apigetrin	$C_{21}H_{20}O_{10}$	6.53	432. 10	7.27
5	Kaemferol 3-O-glikosida	$C_{22}H_{22}O_{11}$	6.70	462.11	4.14
6	Cynaroside	$C_{21}H_{20}O_{11}$	5.99	448.10	1.30
7	Kaempferol-3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	6.54	594.16	0.60
8	3-hydroxyflavanone	$C_{15}H_{10}O_3$	9.17	240.10	0.40
1	1-(5.8-Dimethoxy-2.2-dimethyl-2H-chrome-6-yl) ethanone	$C_{15}H_{18}O_4$	11.37	262.12	64.29
2	Tangeritin	$C_{20}H_{20}O_7$	9.99	372.12	26.15
3	(5.7-Dihydroxy-3'.4'.6-trimethoxyflavone)	$C_{18}H_{16}O_7$	9.94	344.10	9.56

The extract of *Orthosiphon stamineus* comprised several notable flavonoids, comprising tangeritin, 1-(5,8-dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone, and 5,7-dihydroxy-3',4',6-trimethoxyflavone, as drawn by the LC-HRMS analysis. A synthesis of reference comparisons, TraceFinder software, and standard-specific identification definitively confirmed the presence of these substances and outlined their bioactive components. The extract comprises several significant flavonoids, by 1-(5,8-Dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone identified as the principal constituent. This chemical exhibited the most substantial contribution to the extract's composition, by a peak area of 64.29%. Characterized by a retention time of 11.37 minutes and a molecular weight of 262.12, 1-(5,8-Dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone demonstrates considerable potential as a notable bioactive component in the extract. This molecule highlights the importance of polymethoxylated flavonoids in the profile due to its recognized antioxidant properties. Tangeritin, a polymethoxylated flavonoid, exhibited a peak area of 26.15%, signifying its substantial contribution to the flavonoid composition of the extract. This molecule exhibited a retention time of 9.99 minutes and a molecular mass of 372.12, by its fragmentation pattern indicating the neutral loss of a methoxy cohort, consistent by recognized flavonoid fragmentation patterns.

Tangeritin, an additional polymethoxylated flavonoid, was identified by a peak area of 26.15%, that indicates its substantial contribution to the extract's flavonoid profile. This molecule demonstrated a retention time of 9.99 minutes and a molecular mass of 372.12, by its fragmentation pattern revealing the neutral loss of a methoxy cohort, aligned by established flavonoid fragmentation patterns. Tangeretin has been thoroughly investigated for its notable antioxidant and anticancer characteristics, highlighting its importance in therapeutic applications by botanical extracts. Furthermore, 5,7-dihydroxy-3',4',6-trimethoxyflavone was detected, exhibiting a peak area of 9.56%. This molecule, by a retention time of 9.94 minutes and a molecular mass of 344.10, displayed a unique fragmentation pattern, thereby validating its identification. This molecule augments the

flavonoid profile of the extract and enriches our comprehension of its bioactive characteristics. Comprehending its bioactive characteristics is important when creating novel therapeutic techniques.

Tangeritin and 1-(5,8-dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone are bioactive compounds known for their significant antioxidant capabilities. 1-(5,8-dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone protects cells by oxidative stress, a significant factor in the advancement of cardiovascular abnormality, by preserving endothelial function and inhibiting LDL oxidation. Tangeritin, a polymethoxylated flavonoid, reduces free radicals, preventing oxidative cellular damage, and also inhibits inflammatory pathways by blocking NF-κB and COX-2. Moreover, tangeritin induces apoptosis in cancer cells by obstructing the PI3K/AKT and MAPK/ERK signaling pathways. The bioactive properties of these compounds make them promising candidates for therapeutic applications in preventing and treating abnormality related to oxidative stress and cancer (Yan et al., 2023), (Raza et al., 2020).

The research identified 5.7-Dihydroxy-3',4',6-trimethoxyflavone, by the chemical formula $C_{18}H_{16}O_7$. The molecule, identified at m/z 345.09570, demonstrated fragmentation at m/z 345.09574, suggesting a minor loss of a methoxy cohort. The fragmentation pattern of this flavonoid corresponds by the characteristics of other flavone derivatives, suggesting that this molecule may augment the antioxidant properties of the extract. The outcomes align by Ouyang et al. (2024) research, that identified 20 flavonoids in *Orthosiphon stamineus*, of that five—rutin, isoquercitrin, astragalin, sinensetin, and tangeritin—were specifically distinguished via LC-HRMS analysis. The presence of notable flavonoids, comprising tangeritin and 1-(5,8-dimethoxy-2,2-dimethyl-2H-chromen-6-yl) ethanone, highlights the importance of these compounds in enhancing the antioxidant and anti-inflammatory attributes of *Orthosiphon stamineus*. The LC-HRMS outcomes offer an extensive examination of the flavonoid composition, emphasizing their possible therapeutic uses, especially in the therapy of oxidative stress-related situations (Muscolo et al., 2024). The findings suggest that further exploration of the underlying mechanisms by which these flavonoids act could support the enhancement of novel therapeutic approaches.

Antioxidant activity

The DPPH assay represents a largely applied technique for assessing antioxidant potential, largely because it is straightforward and economically efficient. In this procedure, antioxidant molecules quench free radicals by transferring hydrogen atoms to DPPH, which results in a detectable shift in color from purple to yellow. The extent of this color shift reflects the compound's ability to reduce DPPH, thereby indicating its antioxidant potential (Mohammed et al., 2016). Table 4 and Figure 1 display the outcomes of antioxidant testing, specifically the percentage of inhibition and IC_{50} values, for both the extracts and the nano polyherbal formulation. In assessing antioxidant activity, the maximum absorption wavelength (λ max) of DPPH identified in this study was 517 nm, that aligns well by the established range of 515 to 520 nm noted by Mohammed et al. (2016). This wavelength was subsequently used to decide the appropriate incubation period and to analyze both the reference solving and the extracts. Previous research indicates that a 30-minute incubation period is optimal for DPPH analysis; therefore, our test solving's were incubated for 30 minutes under dark situations. The use of a dark environment is critical to prevent the generation of free radicals other than DPPH, that could interfere by the outcomes (Rosidi, 2020).

As the density of the sample solving increases, the measured absorbance value correspondingly decreases. This trend occurs because a higher number of antioxidant molecules are present to neutralize the DPPH radicals (Baliyan et al., 2022). The reduction in absorbance is therefore used to quantify the capacity of antioxidant compounds to scavenge DPPH radicals. By applying linear regression analysis, the connection among density and inhibition can be characterized, by the IC_{50} value serving as an indicator of antioxidant potency—a lower IC_{50} reflects stronger antioxidant activity. An antioxidant's capacity is generally drawn by a lower IC_{50} value. To calculate this value, we use a linear regression model that plots sample density on the x-axis against percentage inhibition on the y-axis. The IC_{50} value is a common and reliable measure for assessing

a sample's antioxidant capacity (Itam et al., 2021). Specifically, we decide the IC_{50} by inputting the percentage inhibition into the regression equation as the dependent variable. A smaller IC_{50} corresponds to stronger antioxidant activity, whereas a larger value suggests weaker activity (Rivero-Cruz et al., 2020).

In this research, quercetin, a flavonoid recognized for its potent free radical scavenging capacity and diverse biological effects, served as the primary standard in evaluating antioxidant activity. Antioxidant activity is classified by IC_{50} values: $IC_{50} < 50$ µg/mL indicates high activity; 50 µg/mL $< IC_{50} <$ 100 µg/mL denotes substantial activity; 100 µg/mL $< IC_{50} <$ 150 µg/mL suggests moderate activity; 150 µg/mL $< IC_{50} <$ 200 µg/mL represents low activity; and $IC_{50} >$ 200 µg/mL signifies minimal activity.

Table 4. Antioxidant Activity of celery extract, cat's whisker + celery DPPH technique

Sample	IC_{50} (µg/mL)	*P-value*
Quercetin	3.95±0.11	
Apium graveolens	58.86±0.44	
Orthosipon stamineus	61.69±0.21	0.016
Apium graveolens + *Orthosipon stamineus*	46.32±0.34	

The tests for antioxidant activity utilizing the DPPH technique drawn that quercetin had a very low IC_{50} value of 3.95 ± 0.11 µg/mL, which means it is very impactive as an antioxidant. The extract by *Apium graveolens* drawn strong antioxidant activity by an IC_{50} value of 58.86 ± 0.44 µg/mL, while *Orthosiphon stamineus* had a slightly higher IC_{50} value of 61.69 ± 0.21 µg/mL, which means both extracts are impactive antioxidants, but not as strong as quercetin. The amalgamation of *Apium graveolens* and *Orthosiphon stamineus* extracts exhibits an enhancement in antioxidant activity, as drawn by the IC_{50} value of 46.32±0.34 µg/mL. This value indicates that the cumulative impact surpasses the aggregate of the individual impacts. The significant (*P-value* < 0,05) drop in IC_{50} by this combination shows that the active ingredients by both extracts work together to boost the overall antioxidant activity. The various flavonoids in both extracts may collaboratively enhance the overall radical scavenging action (Yuhernita & Juniarti, 2011). This outcome aligns by the concept that amalgamating extracts might amplify the various modes of action of their components, outcoming in an enhancement of total antioxidant capacity (Tristantini, Ismawati, Pradana, 2016).

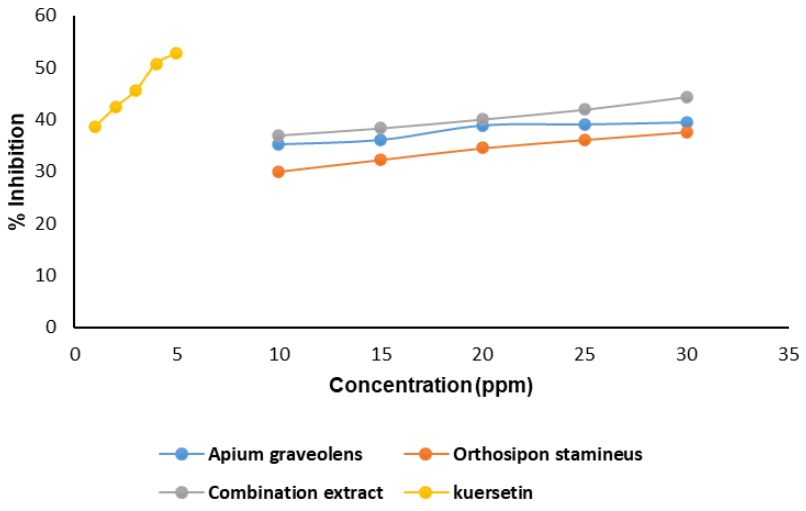

Figure 1. Antioxidant activity profiles of *Apium graveolens* extract, *Orthosiphon stamineus* extract, and their combined extract

The antioxidant properties of flavonoids are mostly decided by their chemical structure (Qin et al., 2018). Flavonoids have a basic structure made up of two ring-shaped parts (A and B) connected by another ring (C), and the presence of hydroxyl (-OH) cohorts plays a big role in their ability to act as antioxidants. The way hydroxyl cohorts in combined flavonoids connect through hydrogen bonding can increase their overall ability to neutralize free radicals (Naróg & Sobkowiak, 2023). The three-dimensional structure of flavonoids considerably enhances their synergistic impacts. The arrangement of hydroxyl and methoxy (-OCH3) cohorts on flavonoid rings can influence how well they donate electrons and how impactive they are at neutralizing free radicals (Huynh et al., 2024). Moreover, flavonoids can establish persistent complexes by transition metals, thereby diminishing their availability for involvement in oxidative processes. This complicated shape, together with the capacity of flavonoids to synergistically augment the activity of antioxidant enzymes, comprising superoxide dismutase (SOD), catalase, and glutathione peroxidase, highlights their function in strengthening cellular defense mechanisms against oxidative stress.

CONCLUSION

The outcomes of this study indicate that the combination of *Orthosiphon stamineus* and *Apium graveolens* extracts possesses an impressive antioxidant capacity. This activity is attributable to the presence of various antioxidant agents, comprising phenolic, alkaloid, and flavonoid compounds such as apigenin, apigetrin, diosmetin, kaempferol, kaempferol 3-O-glucoside, cynaroside, kaempferol-3-O-rutinoside, 3-hydroxyflavanone, 1-(5.8-Dimethoxy-2.2-dimethyl-2H-chrome-6-yl)-ethanone, tangeritin, and (5.7-Dihydroxy-3',4',6-trimethoxyflavone). These outcomes offer substantial evidence for the possible use of herbal combinations as a significant source of bioactive chemicals, underscoring their importance for the advancement of natural antioxidant therapy. This potential indicates that there should be further research into the synergistic impacts of these compounds when used in combination.

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