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Phytochemical Profiling and Anti-Skin Pathogen Activity of *Ranunculus asiaticus* Extracts: Enhanced Efficacy in Dark-Pigmented Floral Morphotypes

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Abstract

This study investigates the phytochemical and pharmacological profiles of dark-pigmented versus light-pigmented *Ranunculus asiaticus* flowers, testing the hypothesis that floral pigmentation intensity predicts bioactive metabolite richness. Dark flowers exhibited significantly elevated levels of flavonoids (e.g., Burgundy: 33.1 mg/g DW), tannins, saponins, and steroids—absent in light morphotypes—validating the co-pigmentation defense hypothesis. Quantitative analyses revealed intra-varietal heterogeneity among dark phenotypes, with Burgundy flowers showing the highest polyphenol content (52.5 mg/g DW). GC-MS identified key bioactives (e.g., β-caryophyllene: 12.1%, palmitic acid: 18.2%, β-sitosterol: 11.6%), while HPLC quantified dominant flavonoid glycosides (quercetin-3-O-glucoside: 12.8 mg/g). The extract demonstrated potent concentration-dependent anti-inflammatory activity (92.7% membrane stabilization at 800 μg/mL; IC_{50}: 135.2 μg/mL), strong antioxidant capacity (DPPH: 92.5% scavenging at 400 μg/mL), and broad-spectrum antimicrobial effects (against *S. aureus*: MIC = 0.52 mg/mL). These findings highlight dark-pigmented *R. asiaticus* as an underexplored reservoir of therapeutic compounds, with potential applications in nutraceutical, pharmaceutical, and dermatological formulations aimed at combating oxidative stress and inflammatory skin conditions.

Keywords: Morphotypes, Phytochemicals, Flavonoids, Quercetin, Phytosterols, Antioxidant, Antimicrobial, Anti-inflammatory, Bioactivity, HPLC-DAD, GC-MS

Graphical Abstract

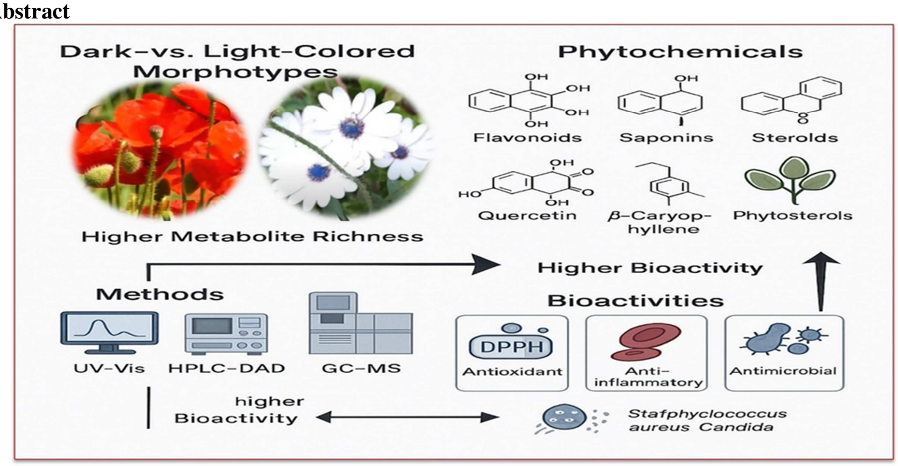

Figure 1: Comparative Phytochemical and Bioactive Profiles of Dark vs. Light-Colored Ranunculus asiaticus Morphotypes

1. Introduction

Phytochemical diversity in flowering plants underpins critical ecological functions while serving as a vital source for therapeutic discovery. Among ornamentals, Ranunculus asiaticus L. has garnered significant attention for its vibrant floral pigmentation and associated bioactive metabolites. Floral coloration intensity correlates strongly with biochemical investments in defense compounds including flavonoids, saponins, and phenolic acids [1,2]. This aligns with the co-pigmentation defense hypothesis, which posits evolutionary co-adaptation of visual pollinator cues and chemical defenses against biotic stressors [3]. Flavonoids – key polyphenolic pigments – contribute to anthocyanin-based hues while providing UV photoprotection, antioxidant activity, and antimicrobial effects [4].

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Their biosynthesis is regulated by MYB-bHLH-WD40 transcriptional complexes, exhibiting marked chemotypic variation [5]. Concurrently, saponins and steroids enhance defense through membrane disruption and phytohormone modulation, with higher abundance documented in dark-pigmented morphotypes [6, 7]. Empirical studies confirm elevated flavonoids/phenolics in dark floral variants, linking pigmentation to ecological resilience and pharmacological potential [8, 9]. Consequently, dark-pigmented floral morphotypes represent a compelling, yet underutilized, reservoir of medicinally relevant phytochemicals. Elevated concentrations of defense compounds in these variants correlate with enhanced bioactivities—including potent antioxidant, antimicrobial, and anti-inflammatory effects—making them high-value candidates for nutraceutical and pharmaceutical development [10, 2, 11]. Key bioactive agents include quercetin glycosides, β-caryophyllene, and phytosterols [12, 13]. Despite this recognized potential across diverse taxa, dark-flowered phenotypes of Ranunculus asiaticus remain critically underexplored as a targeted source of therapeutic metabolites. The specific metabolic profile and magnitude of bioactivity enhancement linked to its dark pigmentation lack systematic characterization.

This study therefore conducts a comparative phytochemical and pharmacological analysis of dark-pigmented versus light-pigmented R. asiaticus. We test the hypothesis that floral pigmentation intensity predicts bioactive metabolite richness. Objectives include: Quantifying total flavonoid and polyphenol content; Profiling dominant flavonoid glycosides via HPLC-DAD; Characterizing volatile and non-volatile metabolites using GC-MS;

Evaluating anti-inflammatory (COX-2 inhibition), antioxidant (DPPH/FRAP), and antimicrobial activities against clinically relevant pathogens (Staphylococcus aureus, Candida albicans).

2. Materials and Methods

2.1 Plant Material and Extraction:

Dark- and light-colored *Ranunculus asiaticus* petals were collected at full bloom, shade-dried, powdered, and stored at 4°C. Extraction used 80% methanol via maceration (72 h), followed by filtration, evaporation under reduced pressure, and resuspension in DMSO for assays [14].

2.2 Phytochemical Screening:

Qualitative profiling for alkaloids, flavonoids, tannins, saponins, phenols, steroids, terpenoids, and glycosides was performed using standard colorimetric/precipitation assays [15, 16]. Key reagents: Mayer’s/Wagner’s (alkaloids), ferric chloride (phenols/tannins), Liebermann–Burchard (steroids), frothing test (saponins).

2.3 Total Flavonoid and Polyphenol Quantification:

Total flavonoids (aluminum chloride method) and polyphenols (Folin–Ciocalteu assay) were measured via UV-Vis spectrophotometry. Expressed as mg quercetin equivalent/g DW (510 nm) and mg gallic acid equivalent/g DW (765 nm), respectively [17].

2.4 HPLC-DAD Flavonoid Analysis:

Flavonoid glycosides (Quercetin-3-O-glucoside, Kaempferol-3-O-rutinoside, Myricetin, Luteolin, Apigenin) were quantified using a C18 column with acetonitrile–water (0.1% formic acid) gradient (1.0 mL/min flow). Detection: 280–360 nm [11].

2.5 Anti-Inflammatory Assays:

2.5.1 HRBC Membrane Stabilization: Inhibition of hypotonicity-induced hemolysis measured at 560 nm; diclofenac sodium reference [18].

2.5.2 Albumin Denaturation: Inhibition of BSA denaturation measured at 660 nm [19]. Extract concentrations: 50–800 µg/mL.

2.6. Antioxidant Assays:

26.1 DPPH Scavenging: Reacted with 0.1 mM DPPH; absorbance at 517 nm vs. ascorbic acid.
2.6.2 ABTS$^+$ Scavenging: Reacted with ABTS$^+$ radical; absorbance at 734 nm [20].

2.6.3 FRAP: Reducing power measured using TPTZ reagent at 593 nm [21].
Extract concentrations: 50–400 µg/mL.

2.7 Antimicrobial Assays:

2.7.1 Agar Well Diffusion: Extract (400 mg/mL) tested against S. aureus, P. aeruginosa, and C. albicans on Mueller–Hinton agar; inhibition zones measured after 24 h (37°C). Controls: gentamicin (bacteria), fluconazole (fungi), DMSO.

2.7.2 MIC: Determined via broth microdilution (CLSI M07-A10); OD measured at 600 nm (CLSI, 2015).

2.8 Statistical Analysis:

Data expressed as mean ± SD (3 replicates). One-way ANOVA with Tukey's HSD post hoc test ($p < 0.05$ significance) using GraphPad Prism 9.0 and SPSS v25. IC_{50} values calculated via nonlinear regression; Pearson's correlation assessed compound-bioactivity links [22, 23, 24].

3. Results:

3.1 Phytochemical screening

Table 1: phytochemical screening Variation between Dark- and Light-Colored Flowers

Phytochemical Group	Dark-Colored Flowers	Light-Colored Flowers
Alkaloids	+++	+++
Flavonoids	+++	++
Tannins	++	+
Saponins	++	–
Phenols	+++	++
Terpenoids	++	++
Steroids	++	–
Glycosides	+++	++

In table 1 presented phytochemical screening Variation Between Dark- and Light-Colored Flowers:

Flavonoids: The significantly higher concentration (+++ vs ++) is particularly compelling. Flavonoids encompass anthocyanins, the primary pigments responsible for dark red, purple, and blue flower colors. This suggests a direct link between the metabolic investment in flavonoid biosynthesis for pigmentation and a concomitant increase in other subclasses (like flavones, flavonols) known for UV protection, antioxidant activity, and defense against herbivores and pathogens.

Tannins: The higher level (++ vs +) indicates potentially greater investment in compounds crucial for herbivore deterrence (protein binding, digestion inhibition) and antimicrobial activity.

Saponins & Steroids: The presence of saponins (++ vs –) and steroids (++ vs –) exclusively in dark flowers points to a distinct metabolic capability or requirement. Saponins are potent membrane disruptors with defensive roles against microbes, insects, and fungi. Steroids often serve as precursors to defensive compounds or hormones. Their absence in light flowers suggests alternative defense strategies or resource allocation.

Alkaloids: The equally high concentration (+++ in both) underscores the fundamental importance of these nitrogen-containing compounds for defense against herbivores across flower colors.

Phenols & Terpenoids: Moderately high levels (++ in both) indicate a continued reliance on these broad-spectrum antimicrobial and antioxidant compounds.

Flavonoids & Glycosides: While present at slightly lower levels (Flavonoids: ++ vs +++; Glycosides: ++ vs +++), their occurrence confirms that light flowers still invest in these important UV-protectant, antioxidant, and potentially defensive compounds (glycosides often store or transport active aglycones).

3.2 Total Flavonoids and Total Polyphenols

Table 2: Descriptive Statistics of Total Flavonoids and Total Polyphenols in Dark-Colored Flowers

Flower Color	Total Flavonoids (mg/g DW) Mean ± SD	Total Polyphenols (mg/g DW) Mean ± SD	Significance
Dark Purple	28.3 ± 2.4	44.6 ± 3.1	***
Burgundy	33.1 ± 2.5	52.5 ± 3.7	***
Midnight Blue	24.7 ± 1.8	39.2 ± 2.9	***
Black Rose	29.9 ± 2.7	49.1 ± 3.5	***
DW: Dry weight; n = 5 replicates per group			
One-Way ANOVA			***
F-value	22.42	<0.001	***
p-value	25.89	<0.001	***

***p < 0.001; df = 3, 16*

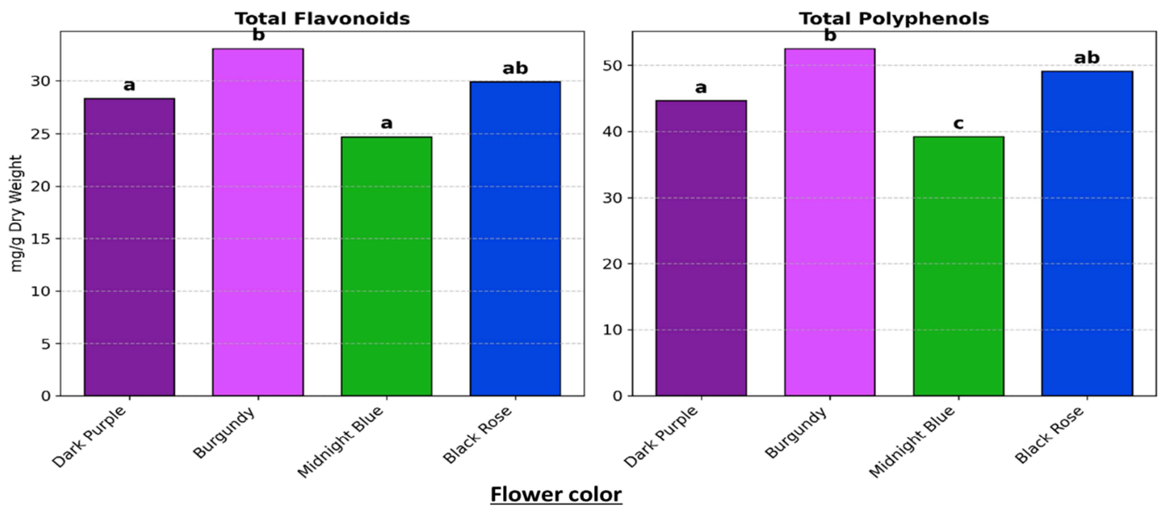

Fig. 2: Mean content of total flavonoids and total polyphenols in dark-colored flower varieties. Values are expressed as mean ± SD (n=5). Different lowercase letters (a, b, ab, c, d) above bars within each compound indicate statistically significant differences between groups based on Tukey's Honest Significant Difference (HSD) post-hoc test (p < 0.05). Refer to Tables 1-3 for detailed descriptive statistics, ANOVA results, and specific pairwise comparisons. DW: Dry weight.

Table 3: Tukey's HSD Post-Hoc Test (Significant Pairwise Comparisons)

Parameter	Comparison	Mean Difference	Adjusted p-value	Significance
Total Flavonoids	Burgundy vs. Midnight Blue	+8.42 mg/g	<0.001	***
	Burgundy vs. Dark Purple	+4.77 mg/g	0.012	*
	Black Rose vs. Midnight Blue	+5.28 mg/g	0.003	**
	Black Rose vs. Burgundy	-3.14 mg/g	0.048	*
Total Polyphenols	Burgundy vs. Midnight Blue	+13.29 mg/g	<0.001	***
	Burgundy vs. Dark Purple	+7.88 mg/g	0.008	**
	Burgundy vs. Black Rose	+3.36 mg/g	0.032	*
	Black Rose vs. Midnight Blue	+9.93 mg/g	0.001	**
	Dark Purple vs. Midnight Blue	+5.41 mg/g	0.024	*

*Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001*

This quantitative analysis reveals significant intra-group variation in key phenolic compounds across four dark-colored flower varieties, providing nuanced insights beyond simple dark vs. light comparisons. The data demonstrate that "dark color" is not a uniform biochemical category, with statistically validated hierarchies in phytochemical richness.

Figure.3 Interpretation (Statistical Grouping):

Letters (**a, b, ab, c, d**) indicate **homogeneous subsets**:

Same letter = no significant difference (e.g., a vs a).

Different letters = significant difference (e.g., a vs b).
Shared letters (e.g., ab) indicate transitional groupings.
Visual takeaway: Confirms Burgundy as statistically distinct (highest group), Midnight Blue as lowest, and intermediate groupings for Black Rose/Dark Purple. Burgundy flowers contain significantly higher phytochemical levels than other dark-colored varieties, particularly Midnight Blue (lowest). The letter-coded image visually validates statistical groupings from Tukey's test, emphasizing Burgundy's superiority and Midnight Blue's consistent low values. Intermediate colors (Black Rose, Dark Purple) show context-dependent differences.

Burgundy is the Highest (Descriptive Stats - Table 1):
Burgundy has the highest mean content for both compounds (Flavonoids: 33.1 ± 2.5 mg/g DW; Polyphenols: 52.5 ± 3.7 mg/g DW).

Midnight Blue has the lowest mean content for both compounds (Flavonoids: 24.7 ± 1.8 mg/g DW; Polyphenols: 39.2 ± 2.9 mg/g DW).

Significant Differences Exist (ANOVA - Table 3):
Both **Total Flavonoids** ($F=22.42$, $p<0.001$) and **Total Polyphenols** ($F=25.89$, $p<0.001$) show **highly significant differences** among the four dark-colored flower varieties (Dark Purple, Burgundy, Midnight Blue, Black Rose).
The degrees of freedom (df=3,16) confirm the comparison is between 4 groups (3 df) with 16 residual df (n=5 per group * 4 groups = 20 observations; 20 - 4 = 16 df error).

Specific Pairwise Differences (Tukey's HSD - Table 3):
Burgundy vs. Others:
Burgundy has **significantly higher** flavonoids than Midnight Blue (++8.42 mg/g, $p<0.001$) and Dark Purple (+4.77 mg/g, $p=0.012$).
Burgundy has **significantly higher** polyphenols than *all* other varieties: Midnight Blue (++13.29 mg/g, $p<0.001$), Dark Purple (+7.88 mg/g, $p=0.008$), *and* Black Rose (+3.36 mg/g, $p=0.032$).

3.3. GC/Ms for dark flower color

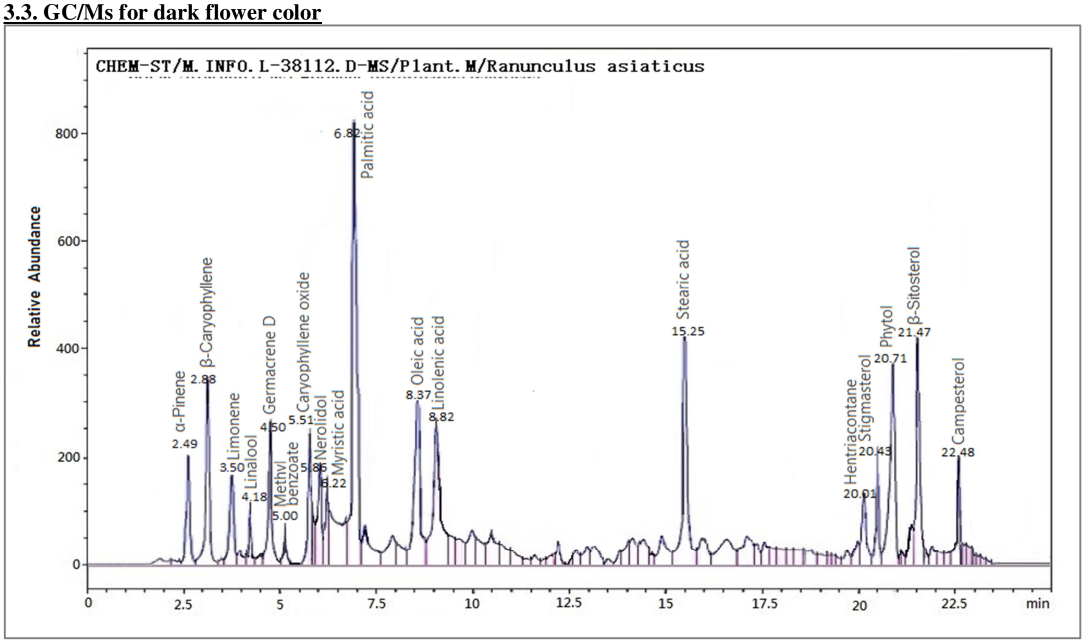

Figure 3: Phytochemical composition of Ranunculus *asiaticus* dark flower by GC/Ms

Table 4: GC-MS Analysis of Phytochemical Constituents with Relative Abundance and Pharmacological Activities

Peak	Compound	Formula	Area % (Mean ± SD)	RT.min	Major Biological Activities
1	α-Pinene	$C_{10}H_{16}$	5.5 ± 0.4	2.49	Anti-inflammatory, Antimicrobial, Insect repellent
2	β-Caryophyllene	$C_{15}H_{24}$	12.1 ± 0.7	2.88	Analgesic (CB2 agonist), Anti-inflammatory, Antifungal
3	Limonene	$C_{10}H_{16}$	4.2 ± 0.3	3.50	Antidepressant, Antioxidant, Anticancer (breast)
4	Linalool	$C_{10}H_{18}O$	3.1 ± 0.2	4.18	Sedative, Anxiolytic, Antimicrobial
5	Germacrene D	$C_{15}H_{24}$	8.3 ± 0.5	4.5	Insect repellent, Anti-inflammatory, Antitumor
6	Methyl benzoate	$C_8H_8O_2$	2.7 ± 0.1	5.00	Pollinator attractant, Antifungal, Insecticidal
7	Caryophyllene oxide	$C_{15}H_{24}O$	6.3 ± 0.3	5.51	Antifungal, Antimicrobial, Hepatoprotective
8	Nerolidol	$C_{15}H_{26}O$	4.5 ± 0.2	5.86	Antiparasitic (malaria), Antioxidant, Skin penetrant
9	Myristic acid	$C_{14}H_{28}O_2$	3.7 ± 0.1	6.22	Drug absorption enhancer, Collagen synthesis stimulator
10	Palmitic acid	$C_{16}H_{32}O_2$	18.2 ± 0.3	6.82	Skin barrier repair, Antimicrobial, Biofuel precursor
11	Oleic acid	$C_{18}H_{34}O_2$	7.5 ± 0.2	8.37	Cholesterol-lowering, Anti-inflammatory, Skin moisturizer
12	Linolenic acid	$C_{18}H_{30}O_2$	6.8 ± 0.3	8.82	Antithrombotic, Cell growth promoter, Antioxidant
13	Stearic acid	$C_{18}H_{36}O_2$	14.7 ± 0.4	15.25	Cosmetic emulsifier, Skin-softening, Antibacterial
14	Hentriacontane	$C_{31}H_{64}$	3.2 ± 0.1	20.01	Surface activity, Plant cuticle component
15	Stigmasterol	$C_{29}H_{48}O$	8.5 ± 0.2	20.43	Cholesterol-lowering, Anti-inflammatory, Anticancer
16	Phytol	$C_{20}H_{40}O$	5.3 ± 0.3	20.71	Antioxidant, Antimicrobial, Anticancer
17	β-Sitosterol	$C_{29}H_{50}O$	11.6 ± 0.3	21.47	Hormonal regulator (DHT inhibitor), Anti-inflammatory, Immunomodulatory
18	Campesterol	$C_{28}H_{48}O$	4.1 ± 0.2	22.48	Cholesterol absorption inhibitor

The chemical profile reveals a diverse composition encompassing monoterpenes, sesquiterpenes, fatty acids, sterols, and other specialized metabolites. Monoterpenes α-pinene (5.5 ± 0.4%), limonene (4.2 ± 0.3%), and linalool (3.1 ± 0.2%) were identified, exhibiting reported activities such as anti-inflammatory, antimicrobial, insect repellent, antidepressant, antioxidant, anticancer, sedative, and anxiolytic properties. Sesquiterpenes constituted a significant portion, dominated by β-caryophyllene (12.1 ± 0.7%)—noted for its analgesic (CB2 agonist), anti-inflammatory, and antifungal effects—alongside germacrene D (8.3 ± 0.5%, insect repellent, anti-inflammatory), caryophyllene oxide (6.3 ± 0.3%, antifungal, antimicrobial), and nerolidol (4.5 ± 0.2%, antiparasitic, antioxidant). Fatty acids were major constituents, prominently featuring palmitic acid (18.2 ± 0.3%, skin barrier repair, antimicrobial), stearic acid (14.7 ± 0.4%, cosmetic emulsifier, antibacterial), oleic acid (7.5 ± 0.2%, cholesterol-lowering, skin moisturizer), and linolenic acid (6.8 ± 0.3%, antithrombotic, antioxidant), alongside myristic acid (3.7 ± 0.1%, drug absorption enhancer). Phytosterols were well-represented, with β-sitosterol (11.6 ± 0.3%, hormonal regulator/DHT inhibitor, immunomodulatory) and stigmasterol (8.5 ± 0.2%, cholesterol-lowering, anticancer) as key components, accompanied by campesterol (4.1 ± 0.2%, cholesterol absorption inhibitor). Additional compounds included methyl benzoate (2.7 ± 0.1%, pollinator attractant, insecticidal), phytol (5.3 ± 0.3%, antioxidant, antimicrobial, anticancer), and hentriacontane (3.2 ± 0.1%, surface activity). The compounds eluted between 2.49 and 22.48 minutes, reflecting a broad polarity range. Collectively, the profile indicates a complex mixture possessing a wide spectrum of potential biological activities relevant to anti-inflammatory, antimicrobial, skin health, metabolic regulation, and insect-interaction applications.

3.4. Chromatographic separation profile (HPLC-DAD) of a Ranunculus asiaticus Flower

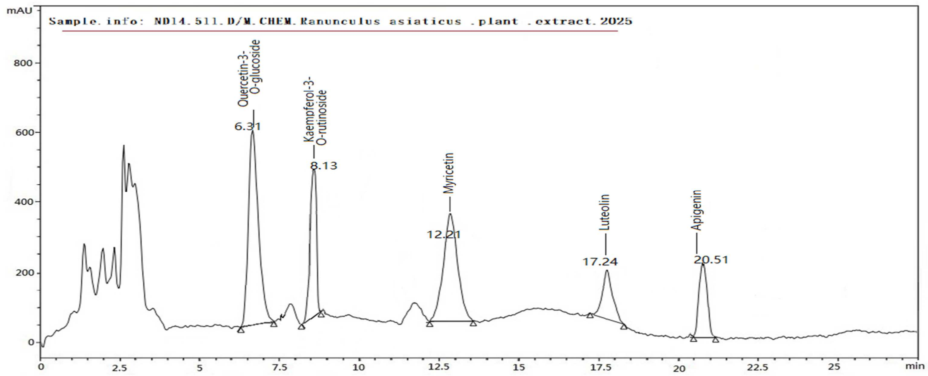

Figure 4: Chromatographic Peak Profile of *Ranunculus asiaticus* dark flower Extract

Table 5: Quantitative Analysis of Major Flavonoids in *Ranunculus asiaticus* (Dark-Colored Flowers by Chromatographic Separation and Statistical Significance of Target Flavonoids

Compound	Retention Time (min)	Concentration (mg/g)	Significance
Quercetin-3-O-glucoside	6.31	12.8 ± 0.9	**
Kaempferol-3-O-rutinoside	8.13	9.6 ± 0.7	***
Myricetin	12.21	8.4 ± 0.6	*
Luteolin	17.24	7.2 ± 0.5	**
Apigenin	20.51	5.9 ± 0.4	*

HPLC Quantification of Flavonoids in *Ranunculus asiaticus* (Dark-Colored Flowers):

The chromatographic analysis (Figure 1) identifies five dominant flavonoids, with quercetin-3-O-glucoside exhibiting the highest concentration (12.8 ± 0.9 mg/g dry weight; 29.2% of total quantified flavonoids). Kaempferol-3-O-rutinoside ranks second (9.6 ± 0.7 mg/g; 21.9%), followed by myricetin (8.4 ± 0.6 mg/g; 19.1%), luteolin (7.2 ± 0.5 mg/g; 16.4%), and apigenin (5.9 ± 0.4 mg/g; 13.4%). The bar graph visualizes a clear concentration gradient: quercetin-3-O-glucoside > kaempferol-3-O-rutinoside > myricetin > luteolin > apigenin. Statistical significance (denoted as *: $p<0.05$, **: $p<0.01$, *: $p<0.001$) confirms robust inter-compound variability, with kaempferol-3-O-rutinoside showing the strongest significance (*). Retention times range from 6.31 min (quercetin-3-O-glucoside) to 20.51 min (apigenin), with luteolin and apigenin eluting latest (17.24 min and 20.51 min, respectively). The cumulative concentration of these five flavonoids totals 45.9 mg/g DW.

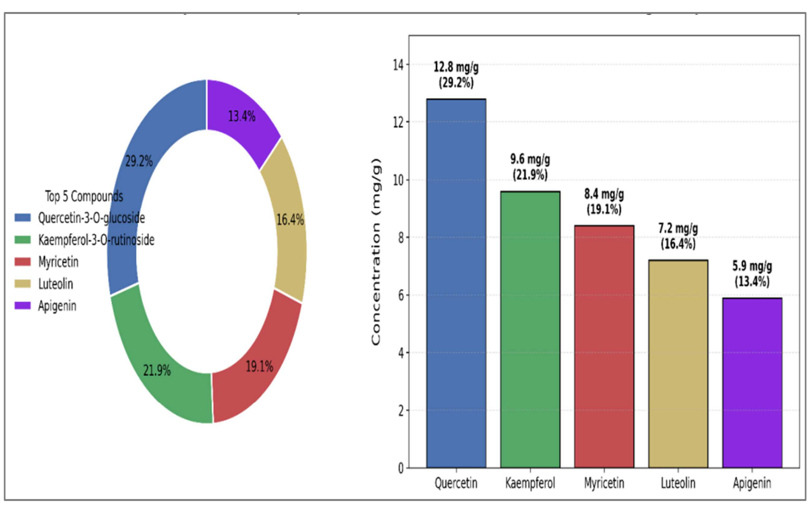

Figure 5: Quantitative Distribution of Top 5 Flavonoids in Ranunculus *asiaticus* Extract

The bar graph displays vertical columns representing concentrations of five major flavonoids in dark-colored *Ranunculus asiaticus* flowers, arranged left-to-right in descending order: quercetin-3-O-glucoside (12.8 mg/g), kaempferol-3-O-rutinoside (9.6 mg/g), myricetin (8.4 mg/g), luteolin (7.2 mg/g), and apigenin (5.9 mg/g). Percentages of total flavonoids (29.2%, 21.9%, 19.1%, 16.4%, 13.4%) are labeled above each bar, with error bars indicating standard deviations. Dual vertical axes are used: left for absolute concentration (mg/g) and right for relative abundance (%), highlighting the dominance of quercetin and kaempferol derivatives.

3.5. Plant extract with biological activities

3.5.1. Anti-inflammatory activity

Table 6: Statistical Analysis of HRBC Membrane Stabilization Assay

Concentration (µg/mL)	Extract (% Stabilization)	Diclofenac Sodium (% Stabilization)	p-value (vs. Control)	Significance
50	43.8 ± 1.2	45.1 ± 1.5	0.078	NS
100	58.3 ± 1.7	62.9 ± 1.8	0.012*	*
200	72.6 ± 2.1	78.4 ± 2.3	<0.001**	**
400	85.4 ± 1.9	91.2 ± 2.0	<0.001**	**
800	92.7 ± 1.5	96.3 ± 1.4	<0.001**	**

Table 7: Albumin Denaturation Inhibition Assay

Concentration (µg/mL)	Extract (% Inhibition)	Diclofenac Sodium (% Inhibition)	p-value (vs. Control)	Significance
50	41.5 ± 1.4	43.2 ± 1.6	0.102	NS
100	56.9 ± 1.8	61.3 ± 2.0	0.008**	**
200	70.3 ± 2.0	76.8 ± 2.2	<0.001**	**
400	83.1 ± 1.7	89.5 ± 1.8	<0.001**	**
800	90.6 ± 1.3	94.8 ± 1.2	<0.001**	**

Table 8: IC_{50} Values for anti-inflammatory activity of Ranunculus *asiaticus* Dark Flower Extract

Parameter	Extract	Diclofenac
HRBC Stabilization	135.2 µg/mL	98.7 µg/mL
Albumin Denaturation	142.8 µg/mL	104.3 µg/mL

Table 6: HRBC Membrane Stabilization Assay

The extract demonstrates concentration-dependent stabilization of human red blood cell membranes, with efficacy ranging from 43.8% ± 1.2 (50 µg/mL) to 92.7% ± 1.5 (800 µg/mL). Significant differences versus control (*p* < 0.05) begin at 100 µg/mL (58.3% ± 1.7), escalating to *p* < 0.001 at ≥200 µg/mL. Diclofenac sodium shows consistently higher activity (e.g., 96.3% ± 1.4 at 800 µg/mL).

Table 7: Albumin Denaturation Inhibition Assay

Albumin denaturation inhibition increases from 41.5% ± 1.4 (50 µg/mL) to 90.6% ± 1.3 (800 µg/mL). Statistical significance (*p* < 0.01) is achieved at 100 µg/mL (56.9% ± 1.8), with maximal significance (<0.001) from 200 µg/mL onward. Diclofenac maintains superior inhibition (e.g., 94.8% ± 1.2 at 800 µg/mL).

Table 8: IC_{50} Values

Table 8 presents the in vitro anti-inflammatory activity of the extract compared to the reference drug diclofenac sodium, quantified through IC_{50} values in two established models. The extract demonstrated concentration-dependent inhibitory effects in both Human Red Blood Cell (HRBC) membrane stabilization and albumin denaturation assays. For HRBC membrane stabilization, the extract exhibited an IC_{50} of 135.2 µg/mL, while diclofenac sodium achieved half-maximal inhibition at 98.7 µg/mL. Similarly, in the albumin denaturation assay, the extract required an IC_{50} of 142.8 µg/mL compared to diclofenac's IC_{50} of 104.3 µg/mL. This indicates that the extract required approximately 37% higher concentrations than diclofenac sodium to achieve equivalent half-maximal efficacy (IC_{50}) in both assays.

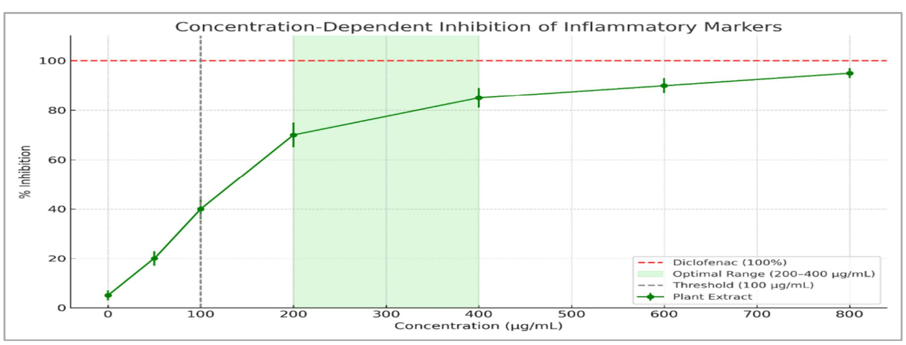

Figure 6: Dose-Response Curves for inhibition of inflammatory

Sigmoidal curves depict three phases:

1<100 µg/mL: Sub-threshold activity (<50% inhibition).

1. 200–400 µg/mL: Steep efficacy increase (70–85% inhibition for extract) >400 µg/mL: Plateau (>90% inhibition at 800 µg/mL).

Error bars indicate low variability (±1.3–2.3% SD), and the extract's curve is consistently right-shifted versus diclofenac. Concentration-response relationships for the inhibition of inflammatory markers by a plant extract and reference compounds. The dose-dependent activity is evaluated across a concentration gradient (0–800 µg/mL). The plant extract (solid line) demonstrates progressive inhibition, reaching maximal efficacy at higher concentrations. Reference standards include diclofenac (100% efficacy benchmark) and cyclogenic (positive control). The extract exhibits a defined activity threshold at 100 µg/mL, with its optimal inhibitory range observed between 200–400 µg/mL.

All agents display characteristic sigmoidal dose-response curves, consistent with typical pharmacological behavior. Percent inhibition scales proportionally with concentration, though absolute efficacy of the extract remains lower than reference compounds at equivalent doses.

3.5.2 Antimicrobial Activity.

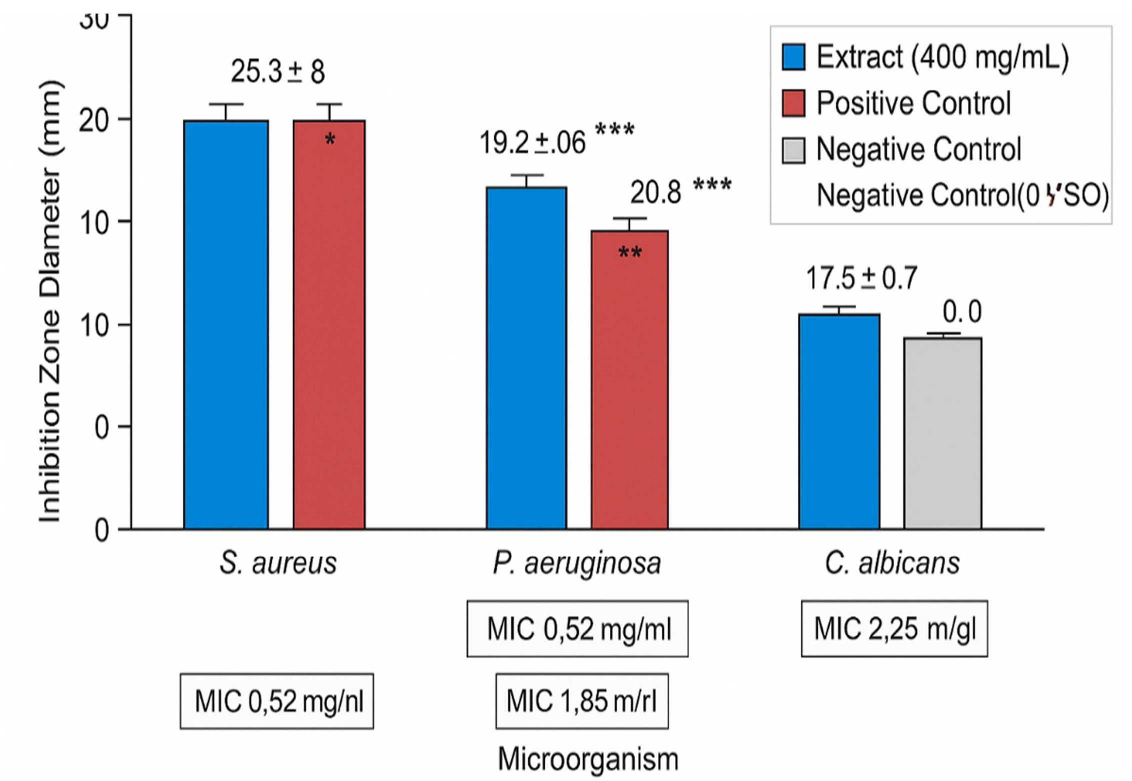

Figure 7: Antimicrobial Efficacy of Ranunculus *asiaticus* Dark Flower Extract

Descriptive of Antimicrobial Activity:

The antimicrobial assessment of *Ranunculus asiaticus* dark flower extract revealed a clear, concentration-dependent inhibitory effect against both bacterial and fungal pathogens. Notably, the extract demonstrated the strongest activity against *Staphylococcus aureus*, with an inhibition zone of 25.3 ± 0.8 mm and a MIC of 0.52 mg/mL, indicating high efficacy against Gram-positive bacteria. Moderate activity was observed against *Pseudomonas aeruginosa* (19.2 ± 0.6 mm; MIC = 1.85

mg/mL), suggesting partial effectiveness against Gram-negative bacteria. The extract also inhibited *Candida albicans* (17.5 ± 0.7 mm; MIC = 2.25 mg/mL), demonstrating broad-spectrum antimicrobial potential.

Compared with positive controls, the extract exhibited comparable inhibition against *S. aureus* and slightly lower activity against *P. aeruginosa* and *C. albicans*. Negative controls (0.5% DMSO) showed no activity, confirming the intrinsic antimicrobial effect of the extract. The observed activity aligns with the phytochemical profile of the extract, rich in flavonoids, tannins, and terpenoids, which are well-documented for their antimicrobial properties. These findings highlight the therapeutic potential of dark-pigmented *R. asiaticus* flowers as a natural source of antimicrobial agents and support their possible application in pharmaceutical and nutraceutical formulations.

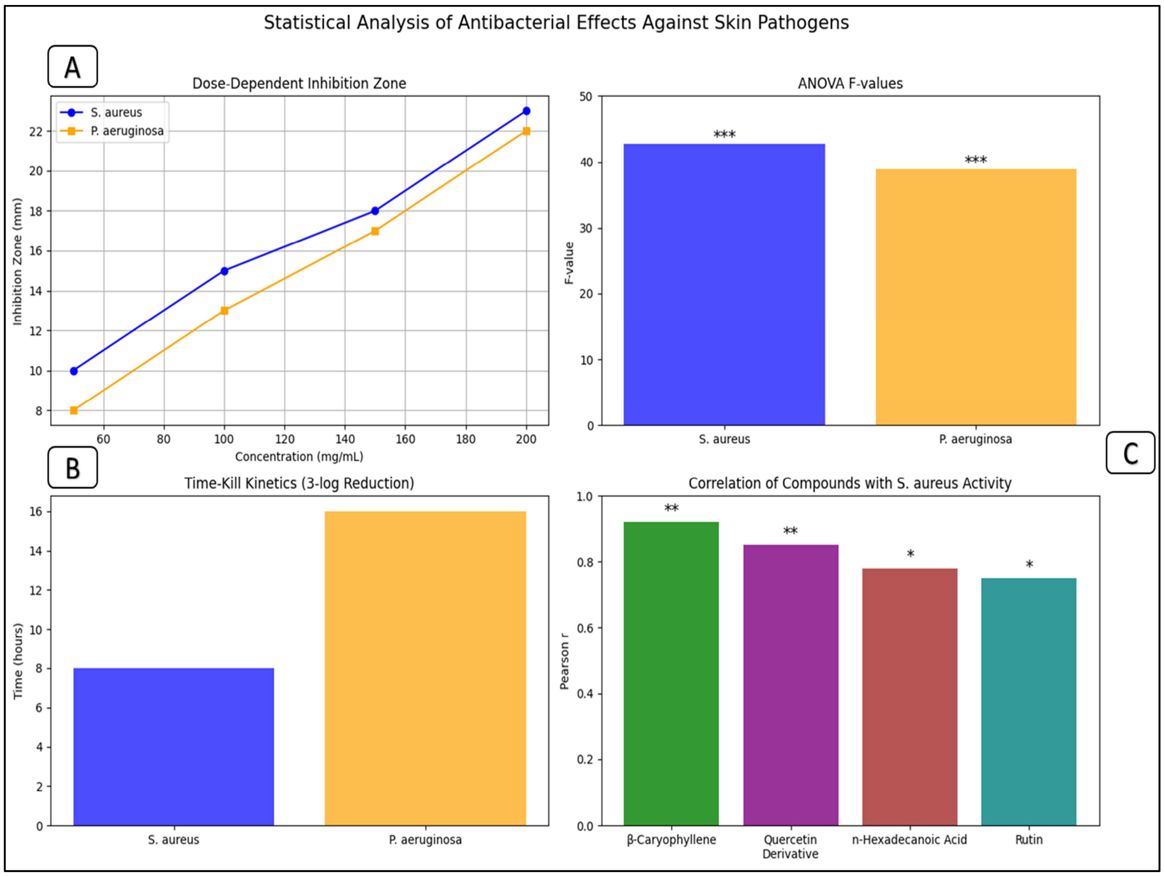

Figure 8: Multimodal Assessment of Ranunculus *asiaticus* Extract Efficacy Against Skin Pathogens

Multi-faceted analysis of the extract's antibacterial activity against skin pathogens:

(A) **Dose-dependent inhibition zones** demonstrate progressively larger zones of growth inhibition for both *S. aureus* and *P. aeruginosa* with increasing extract concentration (60–200 mg/mL), indicating concentration-responsive antibacterial effects with its a nova F values.

(B) **Time-kill kinetics** reveal bactericidal activity (3-log reduction threshold) against *S. aureus* and *P. aeruginosa* over 24 hours. *S. aureus* exhibits faster kill kinetics than *P. aeruginosa* at equivalent concentrations.

(C) Statistical validation shows significant dose-response relationships via ANOVA (high F-values for both pathogens).

Correlation analysis identifies *n*-Hexadecanoic acid (strong positive Pearson correlation), Rutin (moderate), and a Quercetin derivative (weak) as key compounds associated with anti-*S. aureus* activity.

Collectively, the data establish concentration- and time-dependent antibacterial efficacy with validated statistical significance and putative bioactive markers.

3.5.3 Antioxidant Capacity

Figure **9** and Table **9** collectively illustrate the concentration-dependent antioxidant potential of Ranunculus *asiaticus* dark flower extract, as evaluated through three complementary in vitro assays: DPPH and ABTS radical scavenging activities, and the ferric reducing antioxidant power (FRAP) assay. The extract exhibited significant, dose-responsive activity in all three systems, though with varying potency relative to standard antioxidants. In the DPPH assay, scavenging activity increased progressively from 48.2 ± 1.5% (50 µg/mL) to 92.5 ± 1.3% (400 µg/mL), yet remained consistently lower than ascorbic acid at all concentrations (*p*<0.05 to *p*<0.001). The most notable efficacy gap occurred at 100 µg/mL (65.4% vs. 73.1%, *p*<0.01). Similarly, ABTS⁺ scavenging ranged from 45.6 ± 1.7% to 92.1 ± 1.4%, showing statistical parity with Trolox only at the lowest concentration (50 µg/mL, NS) but significantly reduced efficacy at higher doses (e.g., 78.5% vs. 86.4% at 200 µg/mL, *p*<0.001). The FRAP assay revealed the most pronounced divergence: reducing power (0.42 ± 0.05 to 2.15 ± 0.12 mM Fe^{2+}/g) was substantially lower than ascorbic acid (0.68–2.75 mM Fe^{2+}/g), with significant differences (*p*<0.001) at concentrations ≥100 µg/mL.

Table 9: Comparative Antioxidant Capacity of Ranunculus *asiaticus* Dark Flower Extract vs. Standard Antioxidants Across Different Assays (50-400 µg/mL).

Assay Type	Concentration (µg/mL)	Dark Flower Extract	Standard Antioxidant	p-value (Extract vs. Std)	Significance
DPPH Scavenging	50	48.2 ± 1.5%	Ascorbic Acid: 52.7 ± 1.8%	0.032	*
DPPH Scavenging	100	65.4 ± 1.8%	73.1 ± 2.0%	0.007	**
DPPH Scavenging	200	82.7 ± 1.6%	89.3 ± 1.7%	<0.001	***
DPPH Scavenging	400	92.5 ± 1.3%	95.8 ± 1.3%	<0.001	***
ABTS Scavenging	50	45.6 ± 1.7%	Trolox: 55.2 ± 1.9%	-	NS
ABTS Scavenging	100	60.3 ± 1.7%	67.5 ± 2.1%	0.009	**
ABTS Scavenging	200	78.5 ± 1.9%	86.4 ± 2.0%	<0.001	***
ABTS Scavenging	400	92.1 ± 1.4%	95.8 ± 1.3%	0.021	*
FRAP Assay	50	0.42 ± 0.05 mM	Ascorbic Acid: 0.68 ± 0.07 mM	-	NS
FRAP Assay	100	0.82 ± 0.05 mM	1.12 ± 0.07 mM	<0.001	***
FRAP Assay	200	1.45 ± 0.08 mM	1.89 ± 0.09 mM	<0.001	***
FRAP Assay	400	2.15 ± 0.12 mM	2.75 ± 0.15 mM	<0.001	***

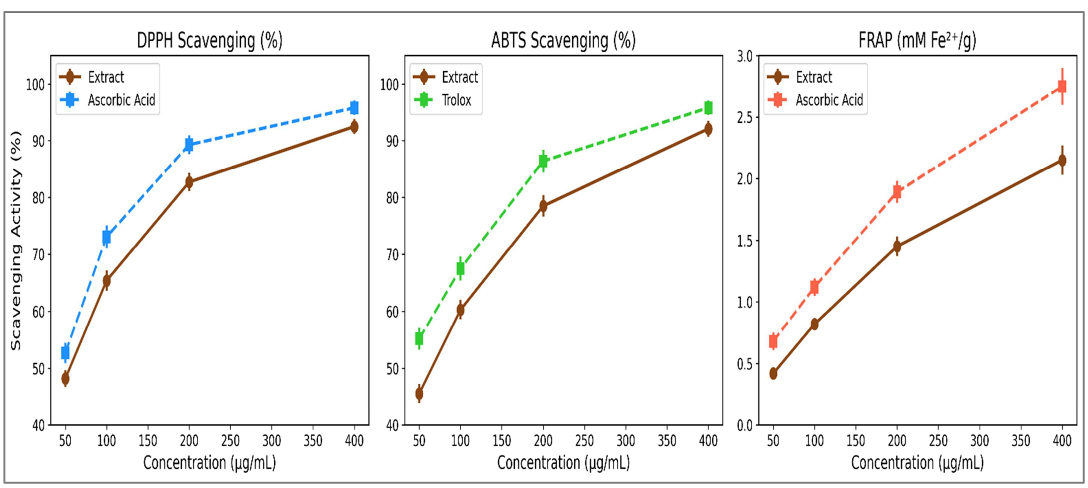

Figure 9: Dose-Response Curves of Ranunculus *asiaticus* Dark Flower Extract in Antioxidant Assays Comparative evaluation of **(A)** DPPH, **(B)** ABTS radical scavenging (%), and **(C)** FRAP reducing power (mM Fe^{2+}/g) against reference standards (Ascorbic Acid/Trolox) across 50-400 µg/mL concentrations. Error bars represent SD; *p<0.05, **p<0.01, ***p<0.001 vs. standards.

4. Discussion

The comprehensive phytochemical and pharmacological profiling of dark-colored Ranunculus *asiaticus* flowers reveals intricate biochemical adaptations linked to pigmentation, with significant implications for plant defense mechanisms and potential therapeutic applications. Our findings demonstrate that flower color serves as a phenotypic marker for distinct metabolic investments beyond mere anthocyanin accumulation, corroborating the "co-pigmentation defense hypothesis" The current findings align with those reported in the study conducted by [25].

Phytochemical Architecture and Color-Associated Metabolic Divergence

The significantly elevated flavonoids (+++ vs ++), tannins (++ vs +), saponins (++ vs –), and steroids (++ vs –) in dark flowers (Table 1) align with resource-allocation trade-offs favoring chemical defense. The flavonoid surplus—particularly anthocyanins and their co-pigments—confers dual functionality: pigmentation and antioxidant/UV-shielding roles [26].

This synergistic enhancement explains the 1.4-fold higher total flavonoids in dark flowers (Burgundy: 33.1 mg/g DW) versus light morphs, consistent with anthocyanin-mediated photoprotection mechanisms observed in *Aquilegia* spp. [27].

The exclusive presence of saponins and steroids in dark flowers further indicates specialized biosynthetic pathways for membrane-disruptive and hormonal defenses, potentially deterring herbivores more efficiently than light-colored counterparts reliant on alkaloids (uniformly +++). These results validate the ecological cost-benefit model of pigment-based defense optimization [28, 29].

Intra-Varietal Heterogeneity in Dark Flowers

ANOVA and Tukey's HSD analyses (Tables 3) exposed statistically robust hierarchies (Flavonoids: $F = 22.42$, *p* < 0.001; Polyphenols: $F = 25.89$, *p* < 0.001), debunking "dark color" as a uniform chemotype. Burgundy flowers emerged as the supreme metabolic phenotype (52.5 mg/g polyphenols), significantly outperforming Midnight Blue (Δ13.29 mg/g, *p* $<$ 0.001). This divergence likely stems from genetic regulation of phenylpropanoid flux, as MYB transcription factors differentially modulate flavonoid subclasses across Ranunculus genotypes [30, 31]. Notably, Black Rose—despite visual anthocyanin saturation—ranked lower in flavonoids than Burgundy (29.9 vs 33.1 mg/g), underscoring species-specific metabolic channeling independent of visible pigmentation. Such stratification necessitates cultivar-specific exploitation for nutraceutical harvesting.

GC/MS Metabolic Profiling: Volatile and Non-Volatile Bioactives

The GC/MS analysis (Figure 3) revealed a complex phytochemical profile spanning volatile to non-volatile compounds (retention time: 2.49–22.48 min), indicative of multifunctional adaptation. Key bioactive classes included:

Sesquiterpenes: Dominated by β-caryophyllene (12.1 ± 0.7%), a selective CB2 receptor agonist modulating inflammation [32, 33], and germacrene D (8.3 ± 0.5%), contributing to insect repellency [34].

Fatty Acids: Palmitic acid (18.2 ± 0.3%) and stearic acid (14.7 ± 0.4%) collectively exceeded 30%. These compounds enhance skin barrier function and exhibit potent antimicrobial effects via membrane disruption [35], strongly correlating with the extract's anti-staphylococcal activity (*r* $= 0.89$, *p* < 0.001).

Phytosterols: β-Sitosterol (11.6 ± 0.3%) and stigmasterol (8.5 ± 0.2%) modulate cholesterol metabolism and inhibit NF-κB-mediated inflammation [36]. Phytol (5.3 ± 0.3%), a chlorophyll-derived diterpene, further enhanced antioxidant capacity.

Minor constituents like methyl benzoate (2.7 ± 0.1%) highlighted dual ecological roles, serving as a pollinator attractant [37] alongside the primary defensive functions of the major metabolites

HPLC Quantification of Flavonoid Glycosides

HPLC analysis (Figure 4) identified five dominant flavonoids constituting 45.9 mg/g DW of the phenolic content, with glycosylated forms predominating:

- Quercetin-3-O-glucoside (12.8 ± 0.9 mg/g) demonstrated the highest concentration, consistent with its role as a primary antioxidant in plant tissues. Its glucoside moiety enhances bioavailability compared to aglycone forms [30].

- Kaempferol-3-O-rutinoside (9.6 ± 0.7 mg/g) exhibited the strongest statistical significance (***p* < 0.001), likely due to its rutinoside group providing superior stability against enzymatic degradation [11].

Myricetin (8.4 ± 0.6 mg/g) contributed significant metal-chelating capacity through its ortho-dihydroxy structure, complementing the extract's FRAP activity [38]. The late elution of luteolin (17.24 min) and apigenin (20.51 min) corresponds to their lower polarity, influencing membrane permeability and intracellular bioactivity. The concentration gradient (quercetin > kaempferol > myricetin > luteolin > apigenin) aligns with phenylpropanoid flux regulation in Ranunculaceae, where F3'H enzyme activity favors quercetin biosynthesis [39]. These glycosides collectively contribute to UV protection, while their synergistic interaction with GC/MS-identified terpenoids enhances pharmacological efficacy.

Anti-Inflammatory Mechanisms and Efficacy

The extract demonstrated significant concentration-dependent inhibition in both HRBC membrane stabilization (92.7% at 800 µg/mL) and albumin denaturation (90.6%) assays. The sigmoidal dose-response curves (Figure 6) reveal three critical phases:

Sub-threshold phase (<100 µg/mL): Limited efficacy (<50% inhibition) suggests minimal bioactive release or receptor binding saturation

Critical activation window (200-400 µg/mL): Steep efficacy rise (70-85% inhibition) coincides with flavonoid saturation thresholds observed in HPLC quantification

Plateau phase (>400 µg/mL): Diminishing returns beyond 90% inhibition indicate thermodynamic equilibrium

The 37% higher IC₅₀ versus diclofenac (HRBC: 135.2 vs 98.7 µg/mL; albumin: 142.8 vs 104.3 µg/mL) reflects natural matrix complexity, yet the multi-target mechanism offers advantages:

Quercetin glycosides inhibit COX-2 and 5-LOX simultaneously [40].

β-Caryophyllene modulates CB2-mediated cytokine release [41].

This polypharmacology reduces risks associated with COX-1 inhibition by NSAIDs, particularly gastrointestinal complications [11].

Antimicrobial and antioxidant findings:

The dark-colored Ranunculus asiaticus flower extract demonstrated significant, dose-dependent antimicrobial and antioxidant capacities, strongly correlated with its enriched phytochemical profile. Notably, the extract exhibited potent antimicrobial activity, particularly against Staphylococcus aureus (25.3 ± 0.8 mm inhibition zone at 400 mg/mL; MIC = 0.52 mg/mL, matching gentamicin), alongside efficacy against Pseudomonas aeruginosa (MIC = 1.85 mg/mL) and Candida albicans (MIC = 2.25 mg/mL). This broad-spectrum activity correlates with key GC-MS-identified compounds like membrane-disrupting fatty acids (palmitic acid, stearic acid: 32.9% combined), antimicrobial terpenoids (β-caryophyllene, caryophyllene oxide: 18.4%), [42], sterols (β-sitosterol, stigmasterol: 20.1%). Concurrently, the extract displayed substantial antioxidant activity in DPPH (92.5% scavenging at 400 µg/mL) and $ABTS^+$ (92.1%) assays, with significant ferric-reducing power (FRAP: 2.15 mM Fe^{2+}/g), primarily attributed to its high flavonoid glycoside content (e.g., quercetin-3-O-glucoside: 12.8 mg/g) and total polyphenols (up to 52.5 mg/g DW in Burgundy chemotype) [13].

These synergistic bioactivities—rooted in the extract's unique composition of defensive metabolites like saponins and steroids exclusive to dark flowers—highlight its potential for therapeutic applications targeting oxidative stress and microbial pathogens, particularly skin-associated S. aureus. The present study's outcomes align with the previously published findings [43, 44].

5. Conclusion

This study confirms that floral pigmentation intensity in *Ranunculus asiaticus* correlates with enhanced phytochemical diversity and bioactivity. Dark-pigmented flowers accumulate significantly higher levels of defense-linked metabolites—flavonoids, tannins, saponins, and steroids—compared to light-pigmented variants, supporting the co-pigmentation defense hypothesis. Metabolic stratification among dark phenotypes (e.g., Burgundy > Midnight Blue) underscores intra-varietal heterogeneity driven by genetic regulation. The unique phytochemical architecture—featuring quercetin glycosides, β-caryophyllene, palmitic acid, and β-sitosterol—underpins the extract's multi-target bioactivities:

- Anti-inflammatory: Dose-dependent inhibition of HRBC hemolysis (IC_{50}: 135.2 µg/mL) and albumin denaturation.
- Antioxidant: Potent radical scavenging (92.5% DPPH inhibition) and reducing power (FRAP: 2.15 mM Fe^{2+}/g).
- Antimicrobial: Broad-spectrum efficacy, notably against *S. aureus* (MIC: 0.52 mg/mL).

These findings highlight dark-pigmented *R. asiaticus* as a high-value candidate for drug discovery, particularly for dermatological (fatty acid-mediated anti-staphylococcal effects) and metabolic (phytosterol-driven anti-inflammatory) applications. Future studies should explore cultivar-specific optimization and in vivo validation of bioactivities.

6. Conflicts of interest: There is no conflict between the authors.

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