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Comparative Phytochemical Analysis And Antioxidant Activity Of Marigold, Pink Periwinkle, And Butterfly Pea Flowers Using Methanol Solvent

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Abstract:
*This study focused on a comparative phytochemical analysis of butterfly pea (*Clitoria ternatea*), pink periwinkle (*Catharanthus roseus*), and marigold (Tegetes erecta), flowers using methanol as the solvent. To identify and quantify secondary metabolites we employed liquid chromatography-mass spectrometry (LC-MS). Also treated with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) for the antioxidant activity assay and the antimicrobial properties tested by the disk diffusion tests. Our findings revealed diverse phytochemical profiles for each flower species. Marigolds showed high levels of alkaloids, carotenoids, and flavonoids. Pink periwinkles enclosed alkaloids, proteins, and phenolic compounds. Butterfly peas displayed alkaloids, terpenoids, and flavonoids. Moreover, the DPPH assay revealed potent antioxidant activity in pink periwinkle and marigolds methanolic extracts. The results highlight the potential health benefits of flowers extract and provide valuable insights for further research on their therapeutic applications. The study targeted to compare the phytochemical composition, antioxidant potential, and antimicrobial activity of methanol extracts from marigold, pink periwinkle, and butterfly pea flowers. Liquid chromatography-mass spectrometry analysis revealed the presence of various phenolic acids in the flower extracts. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay demonstrated antioxidant capacity. The results showed that the flower extracts have significant antioxidant properties. The butterfly pea flower extracts representing the strongest activities. These conclusions suggest that the flowers have the potential to be natural sources of bioactive compounds with pharmaceutical and nutraceutical applications.*

Keywords: *alkaloids, Catharanthus roseus, Clitoria ternatea, methanol, phenols, phytochemicals, Tegetes erecta linn., antioxidant activity.*

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I. Introduction

Plants produce primary and secondary metabolites, which are known commonly as phytochemicals. These organic chemicals show various characters in plant survival and protection. Plant growth and development depends on primary metabolites, whereas secondary metabolites produced during the stationary phase. Secondary metabolites help plants to adjust to environmental changes, for biotic or abiotic challenges, additional heat, and critical temperature changes. The color, taste and smell, of plants are due to secondary metabolites. Secondary metabolites are used in pesticides, coloring agents, flavoring agents, antimicrobial products, and pharmaceuticals (Randive et al., 2023). For primary healthcare, many developing countries still depend on medicinal plants. The estimation of World Health Organization (WHO) is that over 80% of the population in developing countries uses medicinal plants as traditional medicines. These remedies are cause of development of modern Phytomedicine integrating traditional and modern medical practices. Alkaloids, terpenoids, cardiac glycosides, phytosteroids, phenols, flavonoids, saponins, and tannins are plant produced secondary metabolites. These metabolites show various biological properties. Pharmaceutical companies find these areas valuable for the development of new drugs. (K.Sahira et al., 2015).

In this study inspection of phytochemical compounds were done in methanol extracts of three medicinal flowers: Tegetes erecta Linn (marigold), Clitoria ternatea (pink periwinkle), and Catharanthus roseus (butterfly pea). The phytochemicals in these selected flowers are used to treat various diseases.

Cordofan pea, bluebell vine, Asian pigeon wings, or butterfly pea are different names of Clitoria Ternatea. It is native to tropical regions of Asia and eastern Africa. The secondary metabolites of clitoria

ternatea treat various ailments, such as skin and eye diseases, inflammation and fever. (Karel et al., 2018, & Oguis et al., 2019)

Old maid, Cape periwinkle, Bright eyes, Madagascar periwinkle, are other names of Pink periwinkle. It is native to Madagascar. It has both ornamental and medicinal properties. Many studies revealed that C. roseus extracts have medicinal properties. The secondary metabolites of marigold treat vaginal discharge, diarrhea, chest and intestinal pain, throat ailments, toothache, high blood pressure, sore throat, edema, and swelling. Furthermore, it can purify blood, heal wounds, and boost immunity. (Varunesh et al., 2022)

Tegetes Erecta Linn. is a vibrant and versatile ornamental plant. It belongs to kingdom Plantae. Marigolds are popular for their ornamental beauty, and widely used for different cultural and religious rituals. Additionally, the marigold's petals are significant source of carotenoids. Marigolds used for their medicinal properties in Ayurvedic and Unani traditions. The flowers and leaves of marigold have anti-inflammatory, antiepileptic, antipyretic, and antimicrobial effects (Md. T. R et al., 2020). Tinctures, ointments are produced from marigold and infusions to treat wounds and reduce inflammation of the skin and mucous membranes. (Zhang et al., 2020).

Table 1 Flower used for phytochemical screening

No.	Plant Name	Scientific Name
1.	Marigold	Tegetes Erecta Linn.
2.	Pink Periwinkle	Clitoria Ternatea
3.	Butterfly Pea	Catharanthus Roseus

II. Material And Method

Material

Collection of plant

For this study, different flowers were collected from the nursery of village Baloda, District – Janjgir-Champa of Chhattisgarh state. Then washed all flowers one by one thoroughly with running tap water for about 2-3 times to remove soil traces and other dirt. Properly washed plants were dried under shade for three weak. Completely dried flowers were grind with electric grinder to make fine powder and stored in air tight container at room temperature. (K.Sahira et al., 2015)

Preparation of plant extract

Extraction was done with solvent methanol by cold maceration process. For making plant extract 10 gm. Plant sample were soaked in 100 ml. methanol for 48 hours at room temperature. Then filter the solution with whatmann filter paper no. 42. Then solution was concentrated to remove solvent from hot air oven at 40˚ C and then heating mental at 20˚ C for complete drying. Then the completely concentrated crude samples were stored in airtight container at 4˚ C for further use (K.Sahira et al., 2015).

Qualitative analysis of phytochemicals

Test for Alkaloids

To detect alkaloids, a few milliliters of dilute hydrochloric acid (HCl) are mixed with the solvent-free crude extract and then filtered. The filtrate is used for Mayer's and Wagner's tests.

Mayer's Test:

Two drops of Mayer's reagent are added to a few milliliters of the filtrate along the side of the test tube. The formation of a creamy white or yellow precipitate indicates the presence of alkaloids.

Wagner's Test:

One to two drops of Wagner's reagent are added to a few milliliters of the filtrate along the side of the test tube. A brown-reddish precipitate confirms the presence of alkaloids.

Test for Flavonoids

Alkaline Reagent Test:

Two milliliters of 2% sodium hydroxide (NaOH) solution are added to two milliliters of the plant extract. The appearance of an intense yellow color, which becomes colorless upon adding a few drops of dilute HCl, confirms the presence of flavonoids.

Concentrated Sulfuric Acid (H_2SO_4) Test:

A few drops of concentrated H_2SO_4 are added to the plant extract. The appearance of an orange color indicates the presence of flavonoids.

Test for Carbohydrates

Molish Test:

In two milliliters of the plant extract, a few milliliters of distilled water and two drops of ethanolic α-naphthol (Molish reagent) are added. Two milliliters of concentrated H_2SO_4 are then added along the side of the test tube. The appearance of a reddish-violet ring at the junction confirms the presence of carbohydrates.

Benedict's Test:

The crude extract is mixed with two milliliters of Benedict's reagent and boiled. The formation of a reddish-brown precipitate confirms the presence of carbohydrates.

Test for Phenolic Compounds

Ferric Chloride Test:

A few drops of 5% ferric chloride solution are added to the aqueous solution of the crude extract. The appearance of a dark green or bluish-black color indicates a positive test for phenolic compounds.

Lead Acetate Test:

The plant extract is dissolved in five milliliters of distilled water, and three milliliters of 10% lead acetate solution are added. The formation of a white precipitate confirms the presence of phenolic compounds.

Test for Tannins

Test with 10% NaOH:

Four milliliters of 10% sodium hydroxide (NaOH) are mixed with 0.4 milliliters of the crude extract. The formation of an emulsion indicates a positive test for tannins.

Test for Proteins/Amino Acids

Biuret Test:

Two milliliters of the extract are mixed with one drop of 2% copper sulfate ($CuSO_4$) solution and one milliliter of 95% ethanol. Potassium hydroxide (KOH) pellets are then added. The solution turning pink indicates a positive test for proteins and amino acids.

Millon's Test:

A few drops of Millon's reagent are added to two milliliters of the extract. The formation of a white precipitate indicates the presence of proteins and amino acids.

Test for Glycosides

Borntrager's Test:

A few grams of the plant extract are hydrolyzed with concentrated hydrochloric acid (HCl) for two hours in a water bath and then filtered. Three milliliters of chloroform are added to two milliliters of the filtrate, shaken well, and the chloroform layer is separated. Ammonia solution is then added, and the appearance of a pink-colored solution confirms a positive test for glycosides.

Test for Terpenoids

Salkowski's Test:

Two-tenths of a gram of the plant extract is mixed with two milliliters of chloroform. Three milliliters of concentrated sulfuric acid (H_2SO_4) are carefully added to form a layer. The appearance of a reddish-brown color at the interface indicates the presence of terpenoids.

Test for Saponins

Foam Test:

One milliliter of the plant extract is mixed with one milliliter of water and heated. The formation of froth indicates a positive test for saponins.

Test with Olive Oil:

One milliliter of olive oil is added to one milliliter of the plant extract. The formation of an emulsion confirms the presence of saponins.

Test for Steroids

Two milliliters of acetic anhydride are added to 0.5 grams of the plant extract along with two milliliters of sulfuric acid. A color change from violet to blue or green indicates the presence of steroids.

Test for Carotenoids

One gram of the extract mixed with ten milliliters of chloroform in a test tube, vigorously shaken, and filtered. A small amount of concentrated sulfuric acid (H_2SO_4) is then added to the filtrate. The appearance of a blue color at the interface confirms a positive test for carotenoids. (Yadav et al., 2014)

Phenolic acid profiling by LC-MS

Sample Preparation

For extraction, standardize a known weight of the sample in 80% methanol. Then centrifuge the standardized solution and adjust the volume to 20 ml. Evaporate the extract to about dryness under vacuum at 45°C. After drying the extract, add 5 ml of water to dilute the residue. Again, the aqueous solution has to be extracted with petroleum ether thrice. Now extract the solution with ethyl acetate with using a separating funnel, collect the aqueous layer and discard it. Now again, evaporate the ethyl acetate extract to in vacuum at room temperature until almost dryness. In dry residue, add 4 mL of 2N NaOH and let it hydrolyze overnight. Then add 5 ml of 2N HCl to acidify the solution to pH 2 and again extract the solution with 10 ml of ethyl acetate. Finally, evaporate the ethyl acetate layer under vacuum to complete dryness. 1ml of MS-grade methanol added into residue and dissolved it. Filter the solution using the 0.2 µm nylon filter before injecting it into the LC-MS/MS system. (Weidner et al. 2000 & Chen et al. 2001).

LC-MS/MS Conditions

1. Column: BEH-C18 (2.1 x 50 mm, 1.7 µm) analytical column, protected by a Vanguard BEH C-18 guard column.
2. Mobile Phase:
   - For solvent A: 0.1% formic acid in water.
   - For solvent B: 0.2% formic acid in methanol.
3. Run Rate: 0.1 mL/min.
4. Temperature of column: 25°C.
5. Injection Volume: 6 µl.
6. Detection: Monitor the eluted phenolic acids and flavonoids using a PDA detector.
7. MS/MS System: The UPLC column effluent is pumped directly into the TQD-MS/MS system without any split, optimized for phenolic acids and flavonoids analysis (Weidner et al. 2000 & Chen et al. 2001).

DPPH Radical Scavenging Assay

The antioxidant activity was determined by using DPPH radical scavenging assay. (Sarkar et al., 2012)(Razack et al., 2015). The plant extracts were dissolved in methanol solvent to prepare different concentrations and a 0.1 mm DPPH solution was prepared in the same solvent. Mixed the DPPH solution with the plant extracts at 1:1. The mixture was incubated in the dark for 45 minutes at room temperature. Then using a spectrophotometer the absorbance was read at 517 nm. (Razack et al., 2015) The percentage of antioxidant activity was calculated using the following formula:

Percent inhibition = [(A control - A sample) / A_control] x 100 (Razack et al., 2015)

Where A control is the absorbance of the DPPH solution with the methanol solvent, and A sample is the absorbance of the DPPH solution with the plant sample. (Razack et al., 2015)

III. Results And Discussion

All naturally occurring plants are rich in phytochemicals, these phytoconstituents of plants contain therapeutic properties to treat ailments. They have unique medicinal properties. In this study, the qualitative phytochemical analysis of all three plants has been done.

Table 2 Qualitative Phytochemical Screening of Methanol Extract of Different Flowers

No.	Secondary Metabolites	T. erecta linn.	C. roseus	C. ternatea
1.	Alkaloids	+	+	+
2.	Terpenoids	+	-	+
3.	Carbohydrates	+	-	+
4.	Proteins	-	+	+
5.	Tannins	+	+	+
6.	Phenols	+	+	-
7.	Saponins	+	+	-
8.	Steroids	-	-	-
9.	Flavonoids	+	-	+
10.	Glycosides	-	-	-
11.	Carotenoids	+	+	+

(+) = presence of composites, (-) = absence of composites

The result of qualitative phytochemical analysis of methanol extract of Tegetes erecta indicated the presence of Alkaloids, Flavonoids, Terpenoids, Phenols, Carbohydrates, carotenoids, Tannins, saponins, while the methanol extract of Catharanthus roseus showed the presence of Alkaloids, Phenols, Proteins, Tannins, carotenoids saponins, and Methanol extract of Clitoria Ternatea showed the presence of Alkaloids, flavonoids, Terpenoids, Proteins, Carbohydrates, Tannins, carotenoids.

Table 3 Phenolic acid analysis by LC-MS/MS in µg of methanolic flower extract

No.	Phenolic acids	T. erecta linn.	C. roseus	C. ternatea
1.	Benzoic acid	301.8776	**184.6510**	68.3249
2.	p-hydroxy benzoic acid	84.0542	7.2137	45.5629
3.	Salicylic acid	0.9639	0.0223	1.7058
4.	3-hydroxy benzoic acid	146.8258	13.0700	82.8766
5.	t-cinnamic acid	5.4944	4.0893	10.0481
6.	2,4-dihydroxy benzoic acid	111.4210	5.4852	1.7253
7.	Gentisic acid	59.5688	0.0286	2.7152
8.	Protocatechuic acid	0.2639	**0.0033**	**0.0038**
9.	p-coumaric acid	0.3019	0.1977	2.1745
10.	o-coumaric acid	**0.0648**	0.1293	0.2220
11.	Vanillic acid	41.5957	1.9196	1.2995
12.	Gallic acid	129.6011	0.1438	18.3773
13.	Caffeic acid	1.3055	0.1068	0.0104
14.	Ferulic acid	20.9114	0.2008	0.0514
15.	Syringic acid	**903.1227**	0.2552	0.3575
16.	Sinapic acid	0.0780	0.0812	0.0902
17.	Ellagic acid	23.8809	0.1155	**1323.3253**
18.	Chlorogenic acid	14.3054	0.0202	0.2238

All the three flowers have different concentrations of the eighteen phenolic acids that were found using LC-MS phenolic acid profiling. The blue color represents the most abundant phenolic acid, while the red color shows the least abundant. In marigold flowers, syringic acid is most abundant, although o-coumaric acid is less plentiful. Butterfly pea flowers have low levels of protocatechuic acid while high levels of benzoic acid. In pink periwinkle flowers, the quantity of ellagic acid is high and protocatechuic acid is low.

Table 4 Antioxidant activity by DPPH for methanolic extracts

Concentration	10µg	30 µg	50 µg
T. erecta linn.	8.02	9.52	9.76
C. roseous	3.81	5.07	5.91
C. ternatea	3.43	4.53	5.12

The methanolic extract of the three flowers revealed the highest antioxidant activity at a concentration of 50µg. Among the three flowers, marigold showed the greatest antioxidant activity, followed by butterfly pea flower, which had higher activity than pink periwinkle flowers. Consequently, it is evident that marigold flower retains the highest antioxidant properties, while pink periwinkle flower possesses relatively low antioxidant activity.

Qualitative Phytochemical Screening*:* The presence of phytoconstituents was screened by qualitative phytochemical examination of methanol extract in all three flower extracts. The different concentrations of secondary metabolites are found in all three flowers. Marigolds (Tegetes erecta) contained the highest number of metabolites, such as alkaloids, phenols, terpenoids, flavonoids, carbohydrates, tannins, saponins, and

carotenoids. Whereas, the lowest number of different metabolites is shown in C. roseus and C. ternatea. Particularly, steroids and glycosides were absent in all three flowers.

Phenolic Acid Analysis*:* LC-MS/MS analysis of phenolic acids detected 18 different acids in different amounts across the three flower samples. IN T. erecta syringic acid and benzoic acid are found in the highest quantities while o-coumaric acid is found in the lowest amount. Benzoic acid is found in the highest amount but is found in low levels of protocatechuic acid. C. ternatea was plentiful in ellagic acid whereas meagre in protocatechuic acid. These findings suggest that considerable variation in phenolic acid compositions among various flower species.

Antioxidant Activity by DPPH*:* The antioxidant activity of methanolic extracts was examined by DPPH assay at concentrations of 10 µg, 30 µg, and 50 µg. among the three samples tested, T. erecta showed the highest antioxidant effects, with C. ternatea and C. roseus showing lesser antioxidant effect. The antioxidant activities of T. erecta, C. ternatea, and C. roseus were 9.76%, 5.12%, and 5.91% respectively, at a concentration of 50µg. this confirms that Tegetes erecta showed the most powerful antioxidant properties among the three flowers.

The phytochemical analysis of methanolic extract indicates a rich presence of secondary metabolites, perticularly in T. erecta. The presence of these compounds is significant as they contribute to the biological activities of the plants, including antioxidant, anti-inflammatory, and antimicrobial properties. The absence of certain metabolites, such as steroids and glycosides, across all three flowers suggests that these compounds are not prominent in the studied species, potentially influencing their medicinal properties. (Siddham et al., 2023)

The phenolic acid analysis highlights the different profiles of phenolic acids among the three flowers. Antioxidant effect of the plant is attributed to the presence of Phenolic acids and their concentrations in plants are different. It may influence the overall antioxidant capacity of the extracts. The high levels of syringic acid in T. erecta and ellagic acid in C. ternatea are particularly noteworthy, as these compounds have been associated with strong antioxidant and anti-cancer activities. (Adeyi et al., 2023 & Abijeth et al., 2020)

The antioxidant activity results corroborate the phytochemical and phenolic acid findings. *T. erecta*, with its diverse array of secondary metabolites and high syringic acid content, demonstrated the most robust antioxidant activity. The lower antioxidant activity of *C. roseus* and *C. ternatea* may be attributed to their less diverse phytochemical profiles and lower concentrations of potent phenolic acids. The study underscores the potential of T. erecta as a source of natural antioxidants, which could be further explore for pharmaceutical and nutraceutical applications. The variability in phytochemical and phenolic acid profiles among the flowers also suggests the need for targeted studies to explore their specific health benefits. (Karagöz et al., 2015, Matei et al., 2015, & Randive et al., 2023)

IV. Conclusion

Phenolic acid profile and antioxidant capacity tests, as well as a comparative study of phytochemical compositions, have demonstrated notable variations in the methanol extracts of Tagetes erecta, Catharanthus roseus, and Clitoria ternatea. T. erecta showed the greatest diversity of secondary metabolites among the three species. T. erecta is a prospective option for further investigation in antioxidant-rich medicines or nutraceuticals because of its wide spectrum of phytochemicals, which are linked to its outstanding antioxidant activity as shown by the DPPH experiment.

The phenolic acid analysis further supports the unique phytochemical attributes of each flower. *T. erecta* exhibited high levels of syringic acid, a compound known for its potent antioxidant properties, which likely contributes to its overall high antioxidant capacity. On the other hand, *C. ternatea* showed a significant concentration of ellagic acid, while *C. roseus* was characterized by high benzoic acid content, each contributing uniquely to the antioxidant potential of these flowers.

In conclusion, the methanolic extract of *T. erecta* emerges as the most promising among the three flowers in terms of antioxidant activity, attributed to its diverse phytochemical and phenolic acid composition. The findings underscore the potential of *T. erecta* in developing antioxidant-rich products, while also highlighting the need for further research into the specific health benefits of the distinct phenolic acids and secondary metabolites present in *C. roseus* and *C. ternatea*.

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