Changes in essential oil composition, phenolic and pigment content, and antioxidant activity of *Peganum harmala* L. leaves during different phenological stages

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Recieved: July 17, 2025
Accepted: April 8, 2026

The yield and composition of the volatile oil of *Peganum harmala* leaves at different phenological stages were investigated. The yield varied according to the growth stage, specifically, in ascending order, as follows: floral budding (0.06%), flowering (0.08%), and fruit maturity (0.2%). Forty-three components, accounting for 90.6–98.1% of the total oil composition, were characterized. The major constituent was always *n*-pentacosane (2.2–37.9%), followed by phytol (2.2–32.7%) and hexahydrofarnesylacetone (7.5–17.4%). Noticeable variability was observed in diterpene and apocarotene content throughout the different stages. The highest content of total phenols and flavonoids (29.87 and 22.38 mg/g DW) was observed in the leaves at the flowering stage. The antioxidant activity of the extracts varied with phenological stages. Young leaves showed a higher pigment content than adult leaves. Overall, phenological stages had a major effect on the composition of the essential oils from the leaves of *P. harmala*, total phenol and pigment content, and antioxidant capacity. These findings indicate that the phenological stage should be considered when selecting harvest time and suggest that *P. harmala* leaves may represent a promising source of bioactive compounds for further phytochemical and antioxidant investigations.

Keywords: Essential oils; GC-MS; DPPH; *Peganum harmala*; Pigments; Phenological stages

1. INTRODUCTION

Peganum harmala, commonly known as wild rue and Syrian rue, is a flowering plant that is generally widespread in North Africa, Central Asia and the Middle East. It has also been introduced to Australia and America. Other common names are harmel and African rue in North Africa and Turkish rue or Mexican rue in the United States [1]. It belongs to the Zygophyllaceae family, order Zygophyllales, which includes more than 250 species [1]. *P. harmala* has been attributed numerous clinical effects, such as anti-inflammatory, antibacterial and memory enhancement [2]. It has been used clinically for the treatment of Alzheimer's disease, cough, diabetes, colic, jaundice, malaria, lumbago, hypertension and other human diseases [2]. There is an increasing number of reports concerning antiviral, antibacterial, antiparasitic and antifungal activities of *P. harmala* [3]. According to these reports, it is also used in traditional Chinese medicine for the treatment of many human diseases [2]. Currently, *P. harmala* is used as winter forage for cattle and camels. Phytochemical studies have shown that the dominant chemical constituents include flavonoids, volatile oils, alkaloids and some trace elements [4]. Only a few studies on the *P. harmala* essential oils' (EOs) chemical composition and total phenolic and antioxidant activity are available in the literature. Chemical studies of essential oils from different plant parts from Egypt [5], Morocco [6] and Tunisia have been reported [7]. It is

recognized that the chemical composition of plants and their biological activity depends on genetic factors, phenological stage [8] and climatic conditions [9]. Antioxidant compounds are the first line of plant defense against free radicals. These compounds stabilize them before they can attack cells [10]. Phenolic compounds are among the main natural antioxidants, including flavonoids and tannins, which are widely distributed in food plants [10]. Alterations of abiotic factors during the growth period of plants can modulate the secondary metabolism pathways, which in turn can lead to variations in the therapeutic and pharmacological properties of plants collected at different phenological stages [9]. Understanding phenological variation may help identify stages associated with higher levels of desirable chemicals. However, agronomic, toxicological and industrial validation is required before making harvest recommendations. Although literature searches have revealed the presence of reports in the essential oil composition from different plant parts of this species [6, 7], information on the effect of the phenological stage on the composition of the leaves' essential oil composition, phenolic and pigment content, and antioxidant activity remains limited. In this context, the aim of this research was to evaluate changes in volatile oil yield and composition, total phenolic content, leaf pigment content and antioxidant capacity of *P. harmala* leaves collected at different developmental stages. These results provide comparative phenological trends that may be useful for future applications.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

Leaves of Peganum harmala L. were harvested at different stages of development, including floral budding (late April), full flowering (early May) and fruit maturity (early August) in its wild habitat in the Kasserine area (Tunisia), characterized by a semi-arid climate (34° 56' 41" N, 8° 34' 4" E, 710 m altitude). The samples were identified by Pr. Abdelhamid Khaldi from the National Institute for Research in Rural Engineering Water and Forests, Tunisia (INRGREF), and a voucher specimen (number 2023-06) was deposited at the INRGREF Herbarium (Ariana-Tunisia).

2.2. ESSENTIAL OIL ISOLATION

Essential oil samples were obtained from 250 g of P. harmala leaves by hydrodistillation for 4 hours, using a Clevenger-type apparatus with 500 mL of distilled water. The essential oils were then dried using over anhydrous sodium sulfate, transferred to dark glass vials, and stored at +4°C until analysis.

2.3. CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

Gas chromatography electron ionization mass spectrometry (GC/EI-MS) analyses were carried out with an Agilent 7890B gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and an Agilent 5977B single quadrupole mass detector. The analysis was performed under the following conditions: the oven temperature was programmed to increase from 60°C to 240°C at a rate of 3°C per minute; the injector temperature was maintained at 220°C, and the transfer line temperature was set to 240°C. Helium was used as the carrier gas at a flow rate of 1 mL per minute. A 1 µL injection volume of a 10% solution in n-hexane was used, with a split ratio of 1:25. The acquisition parameters were as follows: full scan; scan range: 30–300 m/z; scan time: 1.0 s. The peak identification relied on a comparison between the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons (C8-C27) and a computer matching against commercial (NIST 14) and laboratory-developed mass spectra libraries built up from pure substances and components of mixtures of known composition and MS literature data, and by comparison of the calculated linear retention indices with literature data reported in Adams [11] and NIST Chemistry WebBook [12].

2.4. ETHANOL EXTRACTION

Room temperature maceration with ethanol was used for the extraction of phenolic compounds from plant samples. Briefly, 5 g of plant sample was homogenized in 50 mL of 80% ethanol solution at room temperature under stirring for 48 h. Subsequently, the macerate was filtered and the filtrate was centrifuged at 6000 rpm for 20 min at room temperature.

2.5. TOTAL PHENOL CONTENT

Folin–Ciocalteu reagent was used to assay the total phenol content [13]. The reaction mixture consisted of 50 µL of ethanolic extract of P. harmala leaves, 400 µL of Folin–Ciocalteu reagent (10%) and 50 µL of distilled water. After 5 min, 500 µL of sodium carbonate (Na_2CO_3 7.5%) was added. The solution was kept in the dark for 30 min. The absorbance of the samples was read at 765 nm against a blank using a Shimadzu UV-2401PC spectrophotometer. Results were expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW). All experiments were conducted in triplicate.

2.6. TOTAL FLAVONOIDS CONTENT

Flavonoids were quantified using the $AlCl_3$ colorimetric method described by Toit Loots et al [14]. Briefly, 0.5

ml of leaves extract were mixed with 2 mL of distilled water, and 0.15 mL of a 5% $NaNO_2$ solution was added. After 5 min, 0.15 mL of a 10% $AlCl_3$ solution was added. Another 5 min later, the mixture was neutralized with 2 mL of 4% NaOH and the final volume was adjusted to 2.5 mL with distilled water. After 15 min, the absorbance was read at 510 nm against blank. The flavonoid content was calculated using the standard curve of quercetin (0–500 µg/ml) and expressed as mg per g dry weight.

2.7. DPPH ASSAY

The free radical scavenging activity was measured by 2, 2-diphenyl-1-picrylhydrazil (DPPH) according to the method described by Sadraoui Ajmi et al [15]. Briefly, 375 µL of various concentrations (25, 50, 100, 250, 500 µg/mL) of extracts were mixed with 725 µL of DPPH solution (0.024 g/l). Then, the mixture was thoroughly vortexed and incubated for 30 min in the dark. The absorbance of the mixture was measured at 517 nm, using 95% ethanol as blank. Ascorbic acid was used as a positive control. All determinations were accomplished in triplicate. Lower absorbance of the reaction mixture revealed a greater free radical scavenging activity. The results of the antioxidant activity were expressed using the following equation:

$$I\% = (A_{blank} - A_{sample})/A_{blank} \times 100$$

Where:
A_{blank}: Absorbance of the blank
A_{sample}: Absorbance of the sample

The concentration of the extract that could scavenge 50% of the DPPH radicals (IC_{50}) was then calculated.

2.8. REDUCING-POWER ASSAY (FERRICYANIDE METHOD)

The extract's ability to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was assessed as defined by Sadraoui Ajmi et al [15]. Briefly, 500 µL of extract or BHT at increasing concentrations (100, 200, 300, 400, 500 µg/mL), was mixed with 625 µL of sodium phosphate buffer (0.2 M, pH = 6.75) and 625 µL of $K_3Fe(CN)_6$ in a test tube and incubated for 20 min at 50 °C. Subsequently, 625 µL of TCA (10%, w/v) was added and the mixture was centrifuged at 3000 rpm for 10 min. Finally, an aliquot of the supernatant (625 µL) was combined with 625 µL of distilled water and 250 µL of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm with a spectrophotometer. BHT was used as a positive control. The experiments were performed in triplicate. The results are expressed as effective concentration (EC_{50}), which was the concentration of the extract corresponding to an absorbance equal to 0.5 [16].

2.9. CHLOROPHYLL AND CAROTENOID DETERMINATION

Powdered dry leaves were extracted using 95% ethyl alcohol for 24 h in the dark. The extract was centrifuged at 26000 rpm for 10 min and the supernatant was then removed and the concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) were determined spectrophotometrically (Shimadzu UV-2401PC) at 470, 649 and 665 nm according to Lichtenthaler [17]. The chlorophyll a and b ratio (Chl a/b) and the Chl a + b to Car ratio (Chl a + b/Car), as well as the Chl a + b concentration have been calculated.

2.10. STATISTICAL ANALYSIS

All tests were carried out in triplicate and the results were expressed as mean ± standard deviation (SD). Data were subjected to One-Way ANOVA using IBM SPSS Statistics 25 and means (n=3) were compared using Duncan's multiple-range test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. ESSENTIAL OIL CONTENT AT DIFFERENT GROWTH STAGES

The oils of air-dried samples were obtained by hydrodistillation. The essential oil yield (w/w) from the leaves of P. harmala at the different phenological stages followed this order: floral budding (0.06%), flowering (0.08%) and fruit maturity (0.2%). To the best of our knowledge, comparable data on the essential oil yields of P. harmala leaves remain scarce. Relevant studies nevertheless show that volatile oil yield and composition can vary markedly among aromatic taxa and experimental conditions, as reported for Garcinia urophylla, Listea spp. and Syzygium [18-20]. The results obtained led to the conclusion that the growth stage could affect the yield of essential oil. Thus, the harvesting time needs to be carefully chosen to ensure the highest oil content and/or the desired composition [21].

3.2. CHEMICAL COMPOSITION OF ESSENTIAL OIL AT DIFFERENT GROWTH STAGES

The composition of the essential oils from the leaves of P. harmala at different growth stages has not been previously reported in a phenological framework, and comparative information remains limited. Figure 1 shows representative GC-MS chromatograms of the essential oil obtained from P. harmala leaves at different phenological stages. The components identified by GC–MS in these volatile oils are shown in Table I, ordered on the basis of their linear retention indices. In total, 43 volatile components were characterized, accounting for 90.6–98.1% of the total volatile oil composition. The number of essential oils components of the leaves collected at the floral budding and flowering stages was higher compared to those at the fruiting stage (Table I).

The volatiles of P. harmala leaves at all three phenological stages were dominated by long-chain alkanes, mainly n-pentacosane (2.2-37.9%). Other major components were phytol (2.2-32.7%), hexadecanoic acid (1.3-33.0%), hexahydrofarnesylacetone (7.5-17.4%), (E, E)-farnesylacetone (2.7–5.5%), and (E)-geranylacetone (1.3-8.2%) (Table I). Given the predominance of long-chain hydrocarbons and phytol, which may partly originate from leaf cuticular waxes and chlorophyll-derived diterpenes co-extracted during hydrodistillation, the obtained product is more appropriately referred to here as a hydrodistilled "volatile oil" rather than a classical terpene-rich essential oil. Accordingly, the findings of this study differ from those reported in the literature for P. harmala cultivated in other geographical regions. The extraction technique significantly influences the yield and chemical composition of the oil [22]. Shahini et al [23] reported that hydrodistillation showed variability in the composition of specific components. Traditional techniques are often associated with prolonged extraction times, which can degrade thermally sensitive compounds [24]. Tahrouch et al [6] noticed significant variations in the composition of essential oil extracted from P. harmala leaves in Morocco, including mainly N-acetylaniline, aniline, isoquinoline, 2,3-dihydrobenzofurane and propylic acid. Yang et al [25] reported that the major constituents of the essential oils of P. harmala leaves were limonene and thymol. Saleem et al [26] concluded that the oil of the leaves of P. harmala consist mainly of hexadecanoic acid methyl ester, 11-octadecenoic acid methyl ester, (E)-9-octadecenoic acid methyl ester and (Z,Z)-9,12-octadecadienoic acid methyl ester. This quantitative and qualitative variability could be attributed to harvest time, environmental factors and genetic differences, as well as the presence of various chemotypes within this species [27]. According to Mirahmadi et al [28], the expression of genes related to the biosynthesis and accumulation of essential oil compounds varies considerably between the various growth stages. With regard to the chemical classes identified in the oils of P. harmala leaves, this study showed that their composition was especially rich in non-terpene derivatives, diterpenes and apocarotenes. The highest content of non-terpene derivatives (mainly represented by n-pentacosane) and apocarotenes (mainly represented by hexahydrofarnesylacetone) were observed at the fruit maturity stage. Nonetheless, the diterpene compounds, including phytol and isophytol, exhibited their highest levels during the floral budding stage (Table I). The presence of high levels of diterpenes and apocarotenes have been associated with antimicrobial, anticancer, antioxidant and anti-inflammatory effects [29]. Oxygenated monoterpenes were initially present at low levels and increased during the growth stages (Table I). In the younger stages there was probably an inability to fully activate the enzymes essential for the biosynthesis of some constituents [30]. Saeb et al [31] reported that monoterpenes were the main group of compounds in essential oil from Melissa officinalis L leaves (80%) during different stages of plant growth, whereas sesquiterpene was estimated at 20% [31]. Chemical class differences were also reported for other taxa, i.e. oxygenated monoterpenes were the dominant components in the essential oil of P. amboinicus, representing 56.6% of the total composition [32]. It has been stated that the impact of various growth stages on volatile oil content and composition might be due to effects of enzyme activity and metabolism [33].

3.3. PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY

Table II summarizes the total phenol and flavonoid contents and antioxidant activity of P. harmala leaves extracts at different phenological stages using gallic acid as standard. This is the first study about the phenol content and the antioxidant activity of the leaf extracts of P. harmala at various stages of growth. Total phenols varied from 19.00 to 29.87 mg GAE/g DW with the highest value observed at the flowering stage (29.87±3.92 mg GAE/g DW) ($P \leq 0.05$). Total flavonoids were also significantly ($P \leq 0.05$) influenced by phenological stage, ranging from 10.29±0.34 to 22.38 mg QE/g DW, and the highest level was recorded at the flowering stage. Phenols are among the most abundant antioxidant species found in different parts of plants. Changes in their levels can be attributed to several factors, such as plant species, the organ and the age of plants [10]. The results of Hayet et al [34] indicate that among the tested extracts of the leaves of P. harmala at the full ripening fruit stage, the methanol and butanol extracts had the highest total phenolic content (112.5 and 63.0 mg catechin equivalents/g DW, respectively). In this study, a 1.2 times increase in the phenolic content of the leaves was observed at the flowering stage compared to the floral budding stage (Table II). Many other studies have demonstrated that some plants have a peak of phenolic compounds at the flowering stage [10, 35]. An increase in the activity of the "phenylalanine ammonia lyase" enzyme (PAL) in the reproductive phase could be the main reason for the phenolic content increase at the flowering stage [36]. The variability of the total flavonoids content was influenced by the phenological stage. The highest level of total flavonoid ($P \leq 0.05$) was recorded at the flowering stage (22.38 mg/g DW), increased to 28% compared to the floral stage, while the lowest level ($P \leq 0.05$) was observed at the fruit maturity stage (10.29±0.34 mg/g DW). The effect of the phenological stages on the levels of phytochemical constituents has also been reported for other plant species, such as Achillea millefolium [27], Portulaca oleracea L [10] and Valeriana jatamansi [37]. Generally, phenological stages may affect the enzymes involved in phenolic

Table I - Chemical composition of the essential oil of *Peganum harmala* leaves at different phenological stages.

No	Compounds (%)	LRI[a]	LRI lit.[b]	Floral budding	Flowering	Fruit maturity
1	linalool	1101	1101	-	0.2	-
2	α-terpineol	1191	1189	-	0.1	-
3	*p*-menth-4-en-3-one	1250	1251	0.2	-	-
4	nonanoic acid	1270	1268	-	-	1.7
5	dihydroedulan IA	1292	1292	0.1	0.2	1.4
6	undecanal	1308	1306	0.1	0.3	-
7	*p*-vinylguaiacol	1314	1312	-	-	0.5
8	4-acetylanisole	1355	1355	0.2	-	0.6
9	decanoic acid	1379	1380	-	-	1.4
10	*trans*-β-damascenone	1384	1383	-	0.4	0.3
11	*n*-tetradecane	1400	1400	0.2	0.3	0.5
12	dihydro-γ-ionone	1401	1397	0.3	0.3	1.2
13	dihydrodehydro-β-ionone	1424	1425	-	-	0.4
14	*(E)*-α-ionone	1427	1428	0.1	-	-
15	γ-elemene	1433	1434	0.1	-	-
16	nerylacetone	1436	1435	0.1	0.2	-
17	*trans*-6-hydroxy-*p*-menth-1-en-3-one	1438	1435	-	-	1.0
18	dihydro-β-ionone	1443	1444	0.1	-	-
19	*(E)*-geranylacetone	1456	1453	1.3	1.3	8.2
20	1-dodecanol	1476	1473	0.1	-	-
21	*(E)*-β-ionone	1490	1487	1.7	2.3	1.6
22	*n*-pentadecane	1500	1500	-	-	1.4
23	tridecanal	1511	1509	0.1	-	-
24	dihydroactinidiolide	1532	1532	-	1.4	4.4
25	dodecanoic acid	1568	1565	-	2.6	2.6
26	boronia butenal	1585	1584	-	0.2	-
27	*n*-hexyl salicylate	1682	1684	0.8	1.1	-
28	*n*-heptadecane	1700	1700	-	-	0.7
29	pentadecanal	1712	1715	0.9	-	-
30	tetradecanoic acid	1764	1762	-	2.4	-
31	hexahydrofarnesylacetone	1845	1843	14.1	7.5	17.4
32	*(E,E)*-farnesylacetone	1920	1918	2.7	5.5	2.7
33	methyl hexadecanoate	1929	1927	-	0.6	-
34	isophytol	1949	1946	0.3	0.4	-
35	hexadecanoic acid	1968	1971	-	33.0	1.3
36	ethyl hexadecanoate	1994	1992	0.7	-	-
37	*n*-eicosane	2000	2000	0.7	-	-
38	*n*-heneicosane	2100	2100	1.4	1.3	0.8
39	phytol	2114	2112	32.7	23.8	2.2
40	*n*-docosane	2200	2200	3.0	1.0	0.4
41	*n*-tricosane	2300	2300	6.4	1.9	-
42	*n*-tetracosane	2400	2400	10.3	1.4	-
43	*n*-pentacosane	2500	2500	19.4	2.2	37.9
Oxygenated monoterpenes				**0.2**	**0.3**	**1.0**
Sesquiterpene hydrocarbons				**0.1**	**0.0**	**0.0**
Diterpenes				**33.0**	**24.2**	**2.2**
Apocarotenes				**20.5**	**19.3**	**37.6**
Non-terpene derivatives				**44.3**	**48.1**	**49.8**
Total				**98.1**	**91.9**	**90.6**

[a]LRI: Linear Retention Index

[b]LRI lit: values from the literature (Adams [11]; NIST Chemistry WebBook [12])

metabolism [10]. In this study, the antioxidant activities of the extracts obtained from plant material collected during the three phenological stages were evaluated by the DPPH and the Reducing power assays (Table II). It should be noted that the lower the IC_{50} and EC_{50} values, the higher the antioxidant activity of the sample. As shown in Table II, the flowering and the fruit maturity stages exhibited the same activities (IC_{50} = 0.26 mg/ml). The best activity was observed for the extract of the leaves harvested at the floral budding stage (IC_{50} = 0.23 mg/ml). This extract also exhibited the highest reducing power value (0.42 mg/ml) ($P \leq 0.05$). The extract probably contains different types of phenolic compounds, which have different antioxidant activities [38]. Elansary et al [39] reported that the extract of the leaves of P. harmala showed an IC_{50}

Table II - Total phenol and flavonoid content and antioxidant activity of *Peganum harmala* leaves extract at different phenological stages.

Phenological Stages	Total Phenols (mg/g DW)	Total flavonoids (mg/g DW)	DPPH IC_{50} (mg/ml)	Reducing power EC_{50} (mg/ml)
Floral budding	25.00±2.85 [b]	16.12±0.25 [b]	0.23±0.01 [a]	0.42±0.02 [b]
Flowering	29.87±3.92 [b]	22.38±0.25 [c]	0.26±0.02 [a]	0.32±0.01 [a]
Fruit maturity	19±0.10 [a]	10.29±0.34 [a]	0.26±0.02 [a]	0.33±0.01 [a]
Synthetic antioxidants				
Ascorbic acid			0.060±0.004	-
BHT			-	0.106±0.005

Means followed by different letters are significantly different ($p \leq 0.05$). Mean of three replications with standard deviation SD.

Table III - Chlorophyll and carotenoid content (mg/g DW) in *Peganum harmala* leaves at different phenological stages.

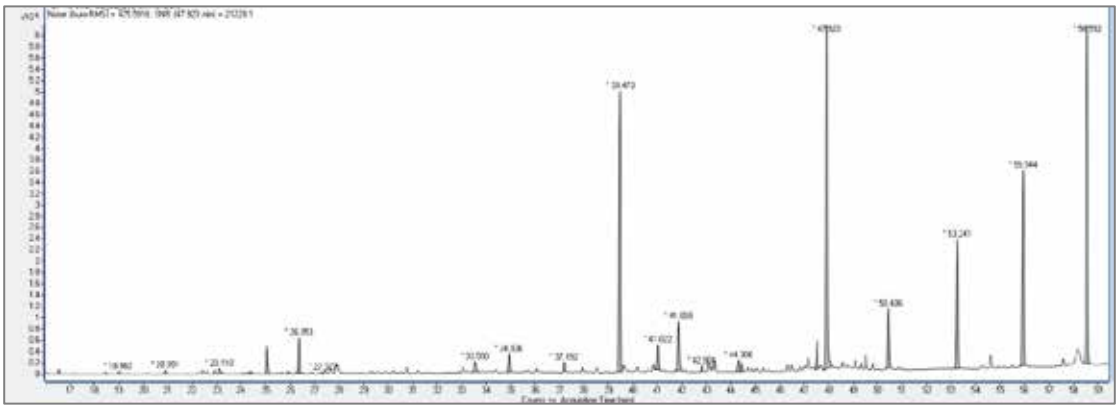
	Floral budding	Flowering	Fruit maturity
Chl a	2.93±0.14 [c]	1.08±0.07 [b]	0.23±0.01 [a]
Chl b	1.61±0.10 [c]	0.72±0.04 [b]	0.14±0.01 [a]
Total Chl a + b	4.54±0.24 [c]	1.80±0.11 [b]	0.37±0.02 [a]
Car	0.93±0.02 [c]	0.35±0.01 [b]	0.11±0.01 [a]
Chl a/b	1.82±0.04 [a]	1.50±0.18 [a]	1.67±0.21 [a]
Chl a+b/Car	4.90±0.35 [b]	5.11±0.19 [b]	3.42±0.33 [a]

Means followed by different letters are significantly different ($p \leq 0.05$). Mean of three replications with standard deviation SD.

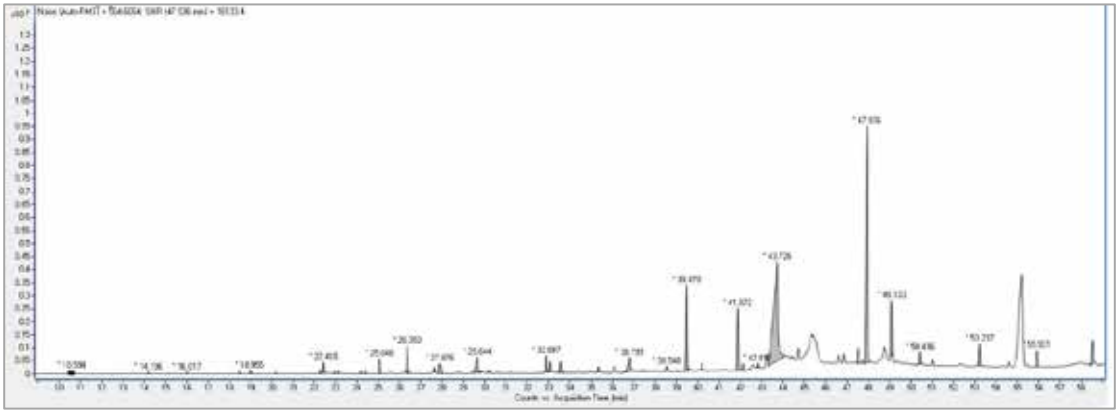
= 0.021 mg/ml and the EC_{50} recorded was 32.4±3.5 (mM TEAC/g extract) at the fruit maturity stage. The results presented in Table II further showed that all extracts have a lower antioxidant activity compared with ascorbic acid (IC_{50} = 0.060 mg/ml) and BHT (IC_{50} = 0.106 mg/ml). The antioxidant activity of the extracts showed varied results during the plant growth period. The effect of phenological stages on antioxidant activities was previously reported by Farhadi et al [27] and Saffaryazdi et al [10]. In this research, no significant correlation was observed between the antioxidant activity and the total phenolic content. Rahiman et al [38], also reported no correlation between antioxidant activities and total phenolic content of a number of medicinal plant extracts. This lack of correlation between antioxidant activity and total phenolic content in the leaf extracts may be due to the presence of the following factors: the antioxidant activity observed may not be only due to the phenolic levels, but could possibly be due to the presence of some other antioxidants such as tocopherols, ascorbic acid and pigments, as well as the synergistic effects among them, which also contribute to the total antioxidant activity. On the other hand, total phenolic content measured according to the Folin-Ciocalteu method is not an absolute determination of the amount of phenolic compounds [38].

3.4. CHLOROPHYLL AND CAROTENOID CONTENT

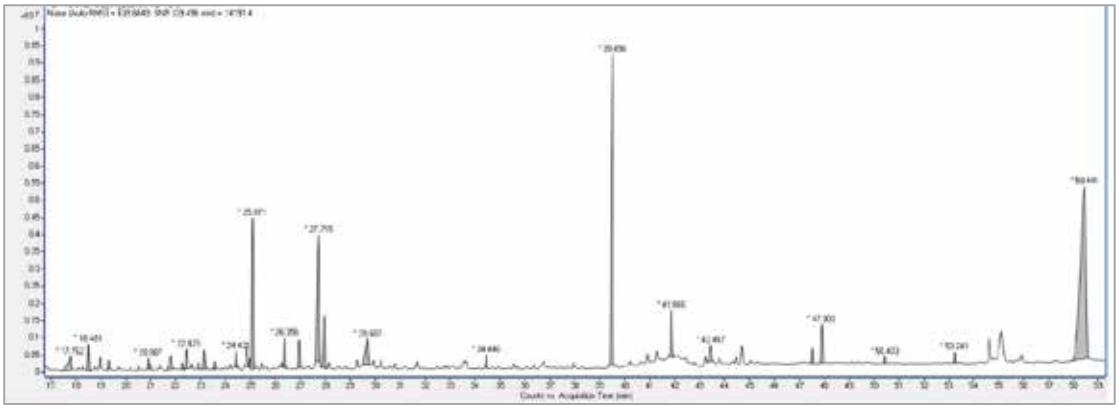
Changes in total chlorophyll and carotenoid content of P. harmala leaves at different growth stages are presented in Table III. The level of Chl a was always higher than that of Chl b, and both pigments followed a similar trend across the phenological stages. The highest contents of Chl a and Chl b were found at the floral budding stage. Total Chl a + b content showed a reduction with progression of the phenological phase, experiencing a 78% decrease at the fruit maturity stage. By contrast, the Chl a/b ratio was not influenced. The reduction of chlorophyll (Chl) content during leaf maturation is a natural part of leaf senescence and involves Chl degradation leading to the yellowing of leaves as other pigments become more visible [40]. Total carotenoid content (mg/g DW) also varied significantly ($P \leq 0.05$) in leaf extracts across the stages (Table III), with the highest values observed at the floral budding stage. The decrease in carotenoids may be related to their conversion into other lipid compounds and/or isomerization [41]. Measurements of the ratio Chl a+b/Car revealed a higher value at the flowering stage. The decrease in carotenoids is linked to Chl a, supporting a relationship between carotenoids and Chl a [42]. In this study, the antioxidant activity of ethanolic leaf extracts tended to be higher (lower IC_{50} values) at stages with higher pigment content. Reducing power EC_{50} values showed a different trend. These observations suggest that pigments may co-vary with other antioxidant constituents and/or contribute to the overall antioxidant response; however, mechanistic interpretations require dedicated enzymatic and metabolomic analyses. Li et al [43] indicated that plant pigments may function as cofactors or substrates for antioxidant enzymes and can modulate their catalytic properties [43].



Floral budding



Flowering



Fruit maturity

Figure 1 - GC-MS chromatograms of *P. harmala* leaves essential oil at different phenological stages

4. CONCLUSIONS

This study investigated for the first time how phenological stages influence the yield and composition of essential oil, total phenolic and flavonoid content antioxidant activity, and pigment content of P. harmala leaves. The highest yield was observed at the fruit maturity stage and GC-MS analyses have highlighted qualitative and quantitative variations linked to developmental stage. The volatile oil profiles were dominated by n-pentacosane and phytol. Leaf extracts obtained at the floral budding and flowering stages showed the highest phenolic content and the strongest antioxidant activity in comparison with the fruit maturity stage; flowering showed the lowest reducing power EC_{50}, whereas floral budding showed the lowest DPPH IC_{50}. P. harmala leaves at earlier stages also exhibited higher chlorophyll and carotenoid content than adult leaves. Overall, these findings provide comparative phenological trends; further agronomic, toxicological and industrial studies are required before recommending a specific harvest stage for practical applications.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
The authors reported that there is no funding associated with the work featured in this article.

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