

Evaluation and Comparison of the Antioxidant Effect of Different Landraces of Henna (*Lawsonia inermis* L.) Using the Lipid Peroxidation Method

Running Title: Antioxidant properties of different populations of Henna

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Abstract

Introduction: Henna (*Lawsonia inermis*) has various properties, including antibacterial, antiparasitic, and anticancer effects. Considering the different henna landraces in Iran, this study aimed to investigate and compare the antioxidant effects of henna landraces (and their fractions) from Shahdad, Rudbar-e-Jonub, Salmaniyeh, Dalgan, Kohnuj, Jiroft, Ghale Ganj, and Ordougah using the lipid peroxidation method.

Methods: Henna was extracted by the percolation method, and different concentrations of the henna extract were prepared. Total phenols, tannins, and saponins were measured in the different landraces and fractions. We used the lipid peroxidation method to assess antioxidant activity.

Result: Dalgan showed the highest total phenol content, at 190.04 µg/mL of extract. The saponin content in Dalgan was 5.2 ± 0.02 µg/mL, and the tannin content in Salmaniyeh was 6.3 µg/mL.

Discussion and conclusion: The total phenol and saponin contents in Dalgan, as well as the tannin content in Salmaniyeh, were higher than those in the other landraces. The lipid peroxidation assay showed that Dalgan had the lowest absorbance at 532 nm. Based on this test, Dalgan had the highest antioxidant effect, whereas Jiroft had the lowest.

Keywords: Lwasonia Inermis, Lipid Peroxidation, Total Phenol.

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Introduction

Oxidative stress plays a pivotal role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurological diseases, and renal disorders (1, 2). Antioxidants are compounds that delay or prevent oxidative stress by neutralizing free radicals (1, 3, 4). The human body combats oxidative stress—and thereby reduces disease risk—through various mechanisms involving antioxidants produced endogenously or obtained from the diet and supplements (1, 2). These antioxidants help protect and repair lipids, proteins, and DNA, thereby limiting oxidative damage (5). Numerous studies indicate that antioxidants confer protective effects against radiation and conditions such as cancer, renal disease, heart disease, and stroke, ultimately lowering their incidence (3).

Lipid peroxidation refers to the oxidative degradation of lipids. In this process, free radicals extract electrons from fats (such as cell membrane lipids), generating radicals. These radicals initiate further lipid peroxidation, culminating in harmful byproducts—most notably aldehydes. Aldehydes exacerbate atherosclerosis and aging and contribute to the pathogenesis of various diseases. Antioxidants, whether endogenous or exogenous, inhibit lipid peroxidation and mitigate these detrimental effects (6).

Many modern pharmaceuticals originate from traditional herbal remedies, and herbal medicine experts have recognized the therapeutic benefits of numerous native plants. Natural products remain a valuable source of both synthetic drugs and

traditional herbal therapies and play a vital role in primary healthcare systems worldwide. Iran's unique climatic and ecological diversity supports a rich, nearly unparalleled flora of approximately 8,000 plant species, offering vast reservoirs of biologically active compounds (7).

Henna has been used for millennia as a coloring, cosmetic, and hygienic plant worldwide. Scientifically classified as *Lawsonia inermis* L., a dicotyledonous species in the Lythraceae family, it is commonly known as Mehndi or henna. One of the principal constituents of henna leaves, used in plant identification and standardization, is lawsone (2-hydroxy-1,4-naphthoquinone) (8, 9).

Studies have shown that henna leaf extracts inhibit the growth of burn-causing microorganisms and promote wound healing (10). Extensive research has also shown broad-spectrum antimicrobial activity against many Gram-positive and Gram-negative bacteria (13). Methanolic extracts of henna leaves also modulate the immune system by stimulating T-lymphocyte proliferation (7,14).

Although henna is believed to have originated in North Africa and the Middle East, it is cultivated commercially in countries such as India, Egypt, Somalia, Sudan, Morocco, Iran, Yemen, Nigeria, and Pakistan. Major exporters include India, Pakistan, Iran, Sudan, and Egypt, while primary importers of Iranian henna are Saudi Arabia, the United Arab Emirates, North African countries, and France. In Iran, key production centers are located in Kerman Province, including the cities of Shahdad, Bam, Rigan, Jiroft, Kahnuj, and Rudbar-e Jonub, as well as Sistan and Baluchestan Province, including Dalgan, Iranshahr,

Nikshahr, Qasr-e Qand, Fanuj, and Chabahar, as well as Hormozgan and Bushehr provinces (8).

Variations in altitude and climate across these regions influence the ratios of bioactive compounds in henna and, consequently, its pharmacological effects. Considering the diversity of henna landraces in Iran and the significance of oxidative stress, this study aimed to assess and compare the antioxidant potential of henna landraces from Shahdad, Rudbar-e Jonub, Salmaniyeh, Dalgan, Kahnuj, Jiroft, Qaleh Ganj, and the Ordougah using the lipid peroxidation assay.

Methods

Plant Collection

Henna leaves were collected from eight locations in Kerman Province, Iran: Shahdad (Kerman, 448 m altitude), Qaleh Ganj (Kerman, 405 m altitude), Rudbar-e Jonub (Kerman, 488 m altitude), Ordougah (Kerman, 405 m altitude), Kahnuj (Kerman, 516 m altitude), Jiroft (Kerman, 680 m altitude), Salmaniyeh (Kerman, 418 m altitude), and Dalgan (Sistan and Baluchestan, 19 m altitude). Specimens were air-dried at room temperature and protected from direct sunlight. Herbarium voucher numbers were as follows: Shahdad: SSU 0062, Salmaniyeh: SSU 0080, Jiroft: SSU 0059, Kahnuj: SSU 0060, Qaleh Ganj: SSU 0066, Dalgan: SSU 0081, Ordougah: SSU 0079, Rudbar-e Jonub: SSU 0061.

Preparation of Hydroalcoholic Extracts (Percolation)

Thirty grams of each powdered henna landrace was mixed with 300 mL of 70% ethanol. The mixture was placed in a percolation funnel at room temperature for 24 hours. The funnel stopcock was adjusted to 40 drops/minute, and percolation continued for 14 days.

Approximately 10 mL of fresh 70% ethanol was added daily to maintain the solvent volume.

Fractionation

The dried crude extract from Dalgan, which showed the highest lipid peroxidation activity, was suspended in 50 mL of n-hexane in a separatory funnel, shaken vigorously, and the n-hexane layer was removed. This step was repeated five times. The remaining residue was successively partitioned with dichloromethane, ethyl acetate, and water using the same procedure. Each fraction was evaporated to dryness under ambient conditions in separate Petri dishes and stored at room temperature (11).

Total Phenolic Content

Different concentrations of gallic acid were prepared in 60% methanol (10, 40, 60, 80, 100, 150, and 300 µg/mL). Then, 0.4 mL of each prepared concentration was transferred to a test tube, and 2 mL of Folin-Ciocalteu reagent was added. After 8 minutes, 1.6 mL of 7.5% sodium bicarbonate was added to the test tubes. The absorbance was measured after 30 minutes using an ELISA reader at 760 nm. For the blank, 0.4 mL of 60% methanol was mixed with 2 mL of Folin-Ciocalteu reagent, left for 8 minutes, and then combined with 2 mL of 7.5% sodium bicarbonate before incubation for 30 minutes. For the samples, extract concentrations of 1 mg/mL were prepared in 60% methanol, and the same steps were repeated (12, 13).

Saponin Content Assay

One gram of standard saponin was dissolved in 50 mL of 65% sulfuric acid to obtain a 20 mg/mL stock solution. The stock was then diluted to 0.05, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL in 65% sulfuric acid. Absorbance was measured using a UV-visible

spectrophotometer at 469 nm, and the calibration curve was plotted. First, 0.7% vanillin was prepared in 65% sulfuric acid because this reagent is unstable and must be freshly prepared. Then, 2 mg of the extract was weighed, and 5 mL of the vanillin solution was added, followed by vortexing for 5 seconds. The mixture was placed in a 60°C water bath for 1 hour, then cooled on ice for 10 minutes. Finally, absorbance was measured at 469 nm. For the blank, 5 mL of 0.7% vanillin solution and 65% sulfuric acid were placed in a 60°C water bath for 1 hour, then cooled on ice for 10 minutes (14).

Tannin Content Assay

Extracts with a concentration of 2 mg/mL were prepared from different henna landraces. Then, 50 µL of each extract was added to test tubes, followed by 3 mL of 4% vanillin and 1.5 mL of concentrated HCl. The tubes were kept at room temperature for 15 minutes, and absorbance was measured at 290 nm. For the blank, 3 mL of 4% vanillin and 1.5 mL of concentrated HCl were added to a test tube (15).

Antioxidant Activity: Lipid Peroxidation Assay

Extract solutions at 1.0 and 1.6 mg/mL were prepared for each henna landrace. For phosphate buffer (0.2 M, pH 7.0), Solution A was prepared by dissolving 2.75 g NaH_2PO_4 in 100 mL distilled water, and Solution B was prepared by dissolving 3.83 g Na_2HPO_4 in 100 mL distilled water. Then, 19.5 mL of Solution A was mixed with 30.5 mL of Solution B; the final volume was adjusted to 100 mL, and the pH was confirmed using a pH meter.

For the Linoleic Acid Emulsion, 0.2804 g linoleic acid, 0.2804 g Tween 20 and 50 mL phosphate buffer were mixed and vortexed for 5 seconds. The mixture

was homogenized at 19,000 rpm for 6 minutes to obtain a milky emulsion. For the peroxidation reaction, 5 mL emulsion, 2 mL phosphate buffer, 250 µL extract, 70 µL 0.05 M CuSO_4 , 30 µL H_2O_2 , and 150 µL methanol were combined and incubated in a water bath at 50 °C for 5 hours.

For the TBA–TCA (Thiobarbituric acid-Trichloroacetic acid) assay, 100 µL of reaction mixture was mixed with 2 mL TBA–TCA solution containing 15 g TCA in 100 mL water and 0.2883 g TBA added to the TCA solution. The mixture was vortexed for 5 seconds and heated at 100 °C for 15 minutes. After cooling to room temperature, 2 mL chloroform was added, followed by another 5-second vortex. Absorbance was measured at 532 nm using an ELISA reader (16). Negative control (without extract), positive control (quercetin, 250 µmol), and blank were included for comparison.

Statistical Analysis

Data were expressed as mean $\pm$ SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's test in SPSS. A P value <0.05 was considered statistically significant.

Results

The total phenolic content, saponin content, tannin content, and lipid peroxide inhibition of the hydroalcoholic extracts from various henna landraces are summarized in **Table 1**.

Variables in each column that differ significantly from one another are indicated by the same letters, with significance set at $P < 0.05$.

In each column, the same letters indicate significant differences ($P < 0.05$).

Table 1: Phytochemical contents and lipid peroxidation inhibition of hydroalcoholic extracts

Extract	Total Phenol Content (µg/ml)	Saponin Content (µg/ml)	Tannin Content (µg/ml)	Lipid Peroxide Inhibition (%)
Shahdad	172.8±0.01	4.2±0.02 ^{b,c,e}	5.5±0.002 ^c	71.9±0.26 ^{d1}
Qaleh Ganj	188.1±0.06	3.4±0.02 ^{a,c,d}	5.4±0.002 ^c	58.1±0.57
Rudbar-e Jonub	144.8±0.03	2.5±0.2 ^{*b,e}	5.6±0.002 ^{b,c}	65.5±0.06 ^b
Ordougah	171.5±0.05	2.09±0.02 ^{*a,e}	5.2±0.002 ^{a,c}	48.6±0.38 ^c
Dalغان	190.04	5.2±0.02 ^{b,a,c,d,f}	6.1±0.003 ^b	74.3±0.12 ^{c,d}
Kahnuj	171.6	1.5±0.02 ^{*a,c,d,e}	5.5±0.003 ^c	64.9±0.25 ^c
Jiroft	143.2	2.2±0.2 ^f	5.6±0.003 ^c	43.8±0.12 ^{a,b,d}
Salmaniyeh	189.8	5.03±0.2 ^{*c,f}	6.3 ^{a,c}	73.1±0.19 ^a
Quercetin	---	---	---	23.8±2.3

Table 2 shows the results of lipid peroxide inhibition of henna fractions (n-Hexane, Dichloromethane, Ethyl acetate, Aqueous extract) at a concentration of 1.6mg/ml.

Table 2: Lipid peroxide inhibition of henna fractions (concentration:1.6 mg/ml)

Fractions	Inhibition (%)
n-Hexane	17.5
Dichloromethane	49.1
Ethyl acetate	30.7
Aqueous extract	23.5

Discussion

According to the results of the lipid peroxidation assay, the henna landrace from the Dalغان region exhibited the highest antioxidant activity among the tested landraces. In the lipid peroxidation test at a concentration of 1.6 mg/mL and a wavelength of 532 nm, the lipid peroxide inhibition percentage of the Dalغان landrace was 74.3%. Statistical analysis

showed that the inhibition percentage of the Dalغان landrace did not differ significantly from that of quercetin, indicating that the antioxidant effect of the Dalغان landrace is comparable to that of quercetin (P value > 0.05).

Reports indicate that flavonoids inhibit lipid peroxidation. These compounds are described as strong antioxidants against free radicals and as free radical scavengers, with their activity attributed to their ability to donate hydrogen atoms. Studies have reported 31.2% free radical scavenging activity in basil. They also stated that the greater total phenolic and flavonoid content in a plant is associated with higher antioxidant activity (17).

Premalatha Muniandy and colleagues (2016) investigated the antioxidant effects of white, black, and green teas using DPPH and FRAP methods. They found that green tea contained higher total phenolic content than white and black teas and exhibited greater antioxidant activity than the other two. The study concluded that green tea's antioxidant properties contribute to reducing cardiovascular diseases and certain cancers, confirming its antioxidant effects (18). The impact of environmental factors on the production of active compounds in medicinal plants is highly complex and not fully understood. The most important ecological factors affecting the quantity and quality of active compounds in medicinal plants include climatic conditions (such as light, rainfall, temperature, and wind); soil characteristics (such as texture, acidity, and nutrient content); and geographical factors (such as altitude, slope magnitude, and direction). One key factor is altitude, which plays a fundamental role in plant growth and

production across different ecosystems and natural habitats and is considered an important determinant of both plant quantity and quality. Overall, environmental factors in the plant habitat influence medicinal plants in three main ways: affecting the total amount of active compounds, influencing the composition of those compounds, and altering the plant's dry weight production (19).

The results also indicate that the antioxidant effect of highly polar fractions of henna, such as water, and non-polar fractions, such as n-hexane, was not significant. In contrast, the antioxidant activity was higher in fractions with intermediate polarity, such as dichloromethane and ethyl acetate.

Hasan and colleagues (2016) investigated the hepatoprotective potential of different fractions of the ethanolic leaf extract of *Lawsonia inermis* against chemically induced hepatitis in rats. The antioxidant effect of henna was evaluated by measuring the reduction in elevated serum bilirubin, SGPT, SGOT, and SALP levels. They concluded that different fractions exhibited varying antioxidant activities in the following order: ethyl acetate fraction > chloroform fraction > petroleum ether fraction (20). In this study, the antioxidant activity of the relatively polar ethyl acetate fraction was higher than that of the non-polar chloroform fraction. Similarly, our results showed that the antioxidant effect of the relatively polar ethyl acetate fraction was greater than that of the non-polar n-hexane fraction, although the antioxidant activity of henna in our study was assessed using the lipid peroxidation method.

Chaudhary Gagandeep and colleagues (2010) studied the photopharmacological properties of *Lawsonia*

inermis and found that the chloroform extract of henna exhibited the highest antioxidant activity (87.6%) using the FTC method based on the TBA assay (21). According to this study, the chloroform fraction of henna had the strongest antioxidant effect. Similarly, in our results, the dichloromethane fraction of henna, which is a chlorinated fraction, showed the highest antioxidant activity. This could be due to the presence of lawsone in this fraction; however, the antioxidant effect in our study was evaluated using the lipid peroxidation method.

Conclusion

Based on the results of this study, the Dalgan landrace showed a stronger antioxidant effect than the other landraces, which may be attributed to its higher phenolic compound content. Among the fractions, dichloromethane showed the highest antioxidant effect. It is recommended that future studies investigate the individual plant parts of henna—particularly lawsone—and examine the effects of each component separately.

Declarations

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Authors' contribution: Rahele Zareshahi and

Hamid Nadri conceptualized the study and contributed to methodology. Zahra Mahdavianpoor and Fatemeh Ajak contributed to data acquisition, and all authors contributed to drafting and editing the manuscript.

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