

Original Research

Ultrasonic-Assisted Extraction and Characterization of Natural Colorants from Plants and Evaluation of Their Therapeutic Properties and Cytotoxicity

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Abstract

Natural colorants as substituents for synthetic colors have gained popularity in the food, textile, pharmaceutical, and cosmetic industry. This study aimed to document the cytotoxicity and bioactivities of the natural pigment extracts from five different plant sources i.e. *Rosa indica L.*, *Brassica oleracea*, *Lawsonia inermis*, *Daucus carota L.* and *Calendula officinalis*. Different activities were measured in this study, like antifungal and antibacterial (through disc diffusion method), antioxidant (through DPPH free radical scavenging % inhibition), and cytotoxicity (through hemolytic activity). We used the carrageenan-induced inflammation model for the determination of anti-inflammatory activity. Extracts were analyzed for the determination of main coloring constitutions by reverse phase HPLC-DAD analysis. Separation was performed on the Zorbax SB-C18 column 150 mm 4.6 mm ID, 5 μ m. Best antioxidant activity was shown by *Daucus carota L.* with IC_{50} (μ g/mL) 9.15 ± 0.5 μ g/mL. The best antibacterial activity was shown by *Calendula officinalis L.* is 20 ± 1.5 mm against gram (+ve) and for the gram (-ve) is 10.3 ± 1.5 mm. *Calendula officinalis L.* showed high anti-inflammatory activity i.e. $75.33 \pm 1.20\%$ and the lowest value of cytotoxicity i.e. $3.4 \pm 0.05\%$. HPLC-DAD analysis showed the presence of cyanidin derivatives in *Rosa indica L.* and *Brassica oleracea*, carotenoids like lutein as a major component in *Calendula officinalis L.* Major colorant in *Daucus carota* was β -carotene and, 2-hydroxy-1,4-naphthoquinone in *Lawsonia inermis*. The results showed that the natural pigments are non-cytotoxic, and they have good potential to act as multipurpose active agents for various applications in the food, pharmaceutical, and cosmetic industries.

Keywords: plant pigments, ultrasonic assisted-extraction, characterization & HPLC, bioactivities, cytotoxicity

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Introduction

Due to their eco-friendly nature, biodegradability, and sustainability, the applications of natural colorants have increased. Natural colorants are obtained from renewable sources like plants, microbes, and insects. These coloring agents are present as secondary metabolites in vegetables and fruits [1]. There are four major classes of plant-based colorants: chlorophylls, carotenoids, betalains, and anthocyanin which are abundant in nature. Chlorophylls are usually present in green/olive and bluish-green color in almost all green leaves; betanins are pink/red in color and found in excess amounts in beetroot. Carotenoids are yellow/orange, reddish-orange, orange, and golden yellow in shades and are present in some flowers such as *Calendula officinalis L.* and *Daucuscarota L.* In most red, blue, or purple plants especially flowers, fruits, and tubers, anthocyanins are the natural pigments. Anthocyanin is the cause of the beautiful appearance of some flowers i.e. *Rosa indica L.* and *Brassica oleracea* [2]. One of the important factors that emphasized the use of natural pigments is their therapeutic applications. Natural pigments can show antioxidant, anti-bacterial, anti-inflammatory, and antidiabetic activities [3-8]. Due to their therapeutic properties, these natural pigments have found applications in agriculture, paper, textile, food, and pharmaceutical industries [9]. However, for the applications of crude extracts of these colorants in the formulations of skincare or coloring cosmetics, their biological properties and cytotoxicity need to be investigated.

Colorants can be extracted from their plant source using various extraction methodologies. The most commonly used solvent extraction techniques are maceration, soxhlet extraction, microwave-assisted extraction, extraction using ultrasonic radiation, and supercritical fluid extraction [10-18], etc. The selection of extraction method also depends on the application of natural colorants [1]. For skin care applications, it is important to use extraction procedures that enhance the extraction yield and sustain the integrity of chemical constituents present in the extract. Furthermore, it is also essential to prevent the degradation of the color fastness of natural colorants. Some external factors can decrease color stability such as the nature of the solvent, light, temperature, reactive oxygen species, and pH of the extract. For the extraction of hydrophilic colorants, we can use water and ethanol, and for the extraction of lipophilic pigment, organic solvents are preferred.

To the best of our knowledge, little information is available about the bioactivities of crude extracts of the natural pigments i.e. anthocyanins, carotenoids, and Lawsone from plant sources. In this work, five different plant sources like *Brassica oleracea*, *Rosa indica L.*, *Calendula officinalis L.*, *Daucus carota L.*, and *Lawsonia inermis L.* were analyzed by HPLC-DAD. Keeping in view the biodegradability and eco-friendly behavior of the natural sources, the main purpose of

the work was to study the different bioactivities and cytotoxicity of colored extracts of the above-mentioned plant sources, to facilitate their incorporation in various bio-based natural cosmetics.

Materials and Methods

Materials

Acetone, distilled water, ethyl acetate, methanol, acetonitrile, formic acid, acetic acid, and 1, 1- diphenyl 2-picryl hydrazyl (DPPH) was purchased from Sigma-Aldrich. Cyanidin, lutein, lawsone, and beta carotene standards for HPLC-DAD analysis were purchased from Oxoid (UK). All the chemicals were of analytical grade.

Selection of Plant

Different vegetables, flowers, and leaves were collected from the local market of Faisalabad, Punjab Pakistan. Petals of flowers were separated, dried under shade, and ground in the form of fine powder. *Brassica oleracea* and *Dacus carota* vegetables were dried under the shade and ground into powder form.

Ultrasonic Assisted Extraction

All the dried plant sources (2 g) were weighed and then placed in 100 mL sealed vessels having 80 mL extraction solution ethanol in a ratio (1:40). The vessels were placed in the ultrasonic bath (using a Rohns US bath (100 W, 40 kHz) for 40 minutes at room temperature (25°C). The ultrasonication was applied for better extraction of maximum coloring constituents in the extract. The extract was filtered using Whatman No. 42 filter paper and the residue was washed with 10 mL of extracting solvent in a volumetric flask. The extract was refrigerated till further analysis [19].

Evaluation of Biological Activities of Extracts

Antioxidant Activity

The antioxidant activity of natural extracts was measured by following the method reported in the literature [20]. The extract 100 μ L (mg/mL concentration) was mixed with one mL of 0.1 mM DPPH solution in methanol. After that, the prepared mixture was kept in dark at least for 30 min. Ascorbic acid was used as the reference standard. A mixture of methanol and DPPH (1 mL+1 mL) was taken as control. We used UV-Vis Spectrophotometer to measure the absorbance at 517 nm and the IC_{50} was calculated. The percentage inhibition was calculated using the following formula:

$$\% \text{ of DPPH } \cdot \text{ radical inhibition} \\ = \text{Control} - \text{Sample} / \text{Control} \times 100$$

Antibacterial Activity

Inhibitory action of all the extracts was evaluated against both gram (-ve) and gram (+ve) bacteria. The inhibitory action was reported against *Ballicus subtilis* (+) and *Escherichia coli* (-). Anti-bacterial activity of the selected pigment sources was done by the method reported [21].

Antifungal Activity

The same protocol was followed for the anti-fungal activity of the pigments as has been discussed for the anti-bacterial activity.

Anti-Inflammatory Activity

To elucidate *in vivo* anti-inflammatory effects of the natural pigments' carrageenan-induced acute, the inflammatory model was used. 0.1 mL of 1% carrageenan in normal saline was used to cause acute inflammation in the hind paw. We allocated thirty mice into 6 groups (n = 5). The experimental dose was prepared in 2% Tween 80. Group 1 is those which injected with carrageenan injection. Group 2 (negative control) without carrageenan injection and not treated. Group 3 (standard group) treated with diclofenac (dose of 10 mg/kg + 0.1 mL (1%) carrageenan injection. Groups 4, 5, 6, 7, 8, and 9 are the pigments at the dose of 50mg/kg respectively. By a digital Vernier caliper, paw edema was measured consecutively for 5 hours. Percentage inhibition was calculated by using the formula:

$$\text{Percentage of inhibition (\%)} = \frac{(K_t - K_o) \text{ disease control} - (K_t - K_o) \text{ treated}}{(K_t - K_o) \text{ disease control}}$$

Where K_t : paw thickness (mm) at time t, K_o : paw thickness (mm) before carrageenan injection, ($K_t - K_o$) disease control: increase in paw diameter after carrageenan injection in disease control group at time t. ($K_t - K_o$) treated: increase in paw diameter

after carrageenan injection in natural pigments treated groups at time t.

Cytotoxicity through Hemolytic Activity

The hemolytic activity of plant extracts was assayed through the method reported in the literature [22]. In the 15 mL, falcon tube three millimeters of human blood cells were poured and washed at last three times with chilled buffer saline (5 mL) by centrifuging the tubes for 5 min. 180 μ L of (RBCs) suspension was mixed with the 20 μ L of extracts and mixed in 2 mL Eppendorf tubes. The mixture was centrifuged at least for 5 min. 100 μ L of supernatant was taken and diluted with chilled phosphate buffer saline (900 μ L). The positive control was 0.1% Triton X-100 and phosphate buffer saline (PBS) was taken as a negative control. ELISA plate reader was used to measure the absorbance at 576 nm.

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100$$

Characterization of Colorants

TLC of the pigments is performed by TLC pre-coated silica gel (Merk-60 F254, 0.25 mm thick). Different solvents were used as mobile phase listed in the Table 1. These solvents of different polarities and ratios were able to identify the active components in the extracts.

R_f = Distance moved by the solute/ Distance moved by the solvent.

UV- visible analysis of the pigments was done by making 1 mg/10 mL solution. The absorbance was measured within 1 hour at wavelength range i.e. 200-800 nm.

HPLC-DAD Analysis

HPLC-DAD analysis of the natural colorants was performed in the high-tech lab of the University of

Table 1. Thin layer Chromatography Mobile phase.

Sr. #	Extracts	Mobile phase	References
1	<i>Brassica oleracea L.</i>	hydrochloric acid–glacial acetic acid–water 40:4:12	[25]
2	<i>Rosa indica</i>	Acetone: ethyl acetate 1.5 : 0.5	[26]
3	<i>Calendula officinalis L.</i>	Hexane: ethyl acetate 70 : 30	[27]
4	<i>Daucusa carota L.</i>	hexane: acetone 3 : 2	[28]
5	<i>Lawsonia inermis L.</i>	n-hexane	[29]

Table 2. HPLC-DAD analysis mobile phase.

Sr. #	Extracts	Mobile phase	Gradient elution
1	<i>Brassica oleracea L.</i> <i>Rosa indica L.</i>	(A) 0.1% Formic acid in aqueous solution (B) 0.1 % Formic acid acetonitrile solution	Liner Gradient: 0–15 min, 10% to 35% B; 15–35 min, 35% to 45% B; 35–45 min, 45% to 100% B; 45–55 min, 100% B
2	<i>Calendula officinalis L.</i> <i>Daucusa carota L.</i>	Acetonitrile:Metahmol:ethylacete 88:10:2	Same as above
5	<i>Lawsonia inermis L.</i>	0.1 % acetic acid :methanol 35:65	Same as above

Karachi, Sindh, Pakistan. Analysis was performed with an HPLC system equipped with Shimadzu Prominence Auto Sampler (SIL-20A), (Shimadzu, Kyoto, Japan), with Shimadzu LC-20AT reciprocating pumps that are connected to a DGU 20A5 degasser and a CBM 20A integrator, SPDM20A diode array detector and LC solution 1.22 SP1 software. C18 column was used for the separation at the temperature 30°C. Respective standards like cyanidin, Lawsone, lutein, alpha carotenoids and beta carotenoids were also run at the same conditions. Mobile phases with the column preparation are listed in the table below. The injection volume was 10 μ L. Spectra were recorded from 200 to 600 nm. The chromatograms were recorded at 520 nm for anthocyanins at 280 nm, 360, and 420 nm.

Statistical Analysis

All the experiments were carried out in three replicates. The data obtained were presented as mean \pm Standard deviation. Statistical analysis was carried out using Analysis of Variance (ANOVA) and Graphed paid prism software for the evaluation of Carrageenan-induced inflammation model.

Results and Discussions

Characterization of Colorants

Characterization of isolated natural pigments was done by UV-Visible spectroscopy, thin layer, and high-performance liquid chromatography. The R_f value can be used for the identification of unknown compounds compared with the standards. Matching the R_f value of a spot with the standard, confirm the identity of the unknown. The chromatogram of *Brassica oleracea* showed three spots with R_f value of 0.3 which is glucobrassicin-1 sulphonate and the second spot with R_f value of 0.6 for glucobrassicin. Our results are in accordance with the literature [23]. *Rosa indica* extract showed two active spots with R_f values of 0.38 and 0.46 are active compounds that have been reported in the literature [24]. *Calendula officinalis* showed two spots of active components with R_f values of 0.18 and 0.79. *Daucusa carota L.* with the developing system

hexane: acetone (3:2) showed two spots of active components with R_f values of 0.95 and 0.31 for carotene and xanthophylls respectively. Our findings are in accordance to the literature [25], and pigment extracted from the *Lawsonia inermis L.* with n-hexane showed three spots with the R_f values of 0.43, 0.73, and 0.76 which are active components.

The UV-Visible spectra of isolated natural pigments are shown in Fig. 1a) (A, B, C, D, and E). In these figures, A and B are the UV -Visible spectra of the anthocyanin's pigments. The maximum wavelength in the visible region has revealed that cyanidin derivatives show maximum absorbance at 515-526 nm. So, the *Brassica oleracea*, *Rosa indica L.* contain the cyanidin derivatives which exhibited a maximum wavelength of 516 nm. Two absorption bands are seen in the chromatogram (A and B) in the range of 200-300 nm and 500-600 nm. These can be tentatively identified as cyanidin derivatives. From the chromatogram, the two kinds of carotenoids are isolated one is lutein and another one is beta-carotene. Two sharp absorption bands are seen in the chromatograms D and E at 445 nm for lutein and at 458 nm for beta carotene in the *Daucus carota L.* extract.

HPLC-DAD analysis was performed on extracted colorants for the identification of main coloring constituents using the gradient elution method. It provided excellent separation of anthocyanins, carotenoids, and lawsone pigments (Fig. 1(b,c), Figs 2, 3 and 4). The comparison of retention time of the peaks in the chromatogram and the UV-Visible spectra of the associated components with those of standards available confirmed the presence of 3-glucoside and 3,5-diglucoside derivatives of delphinidin, cyanidin, in the red rose and red cabbage. Lutein is a major component in the marigold petals extract and alpha and beta carotene as major components in the carrot extract.

Evaluation of Biological Activities of Plant Colorants

Antioxidant Activity

Results of antioxidant activity of natural extracts are based on measuring the electron donor capacity of

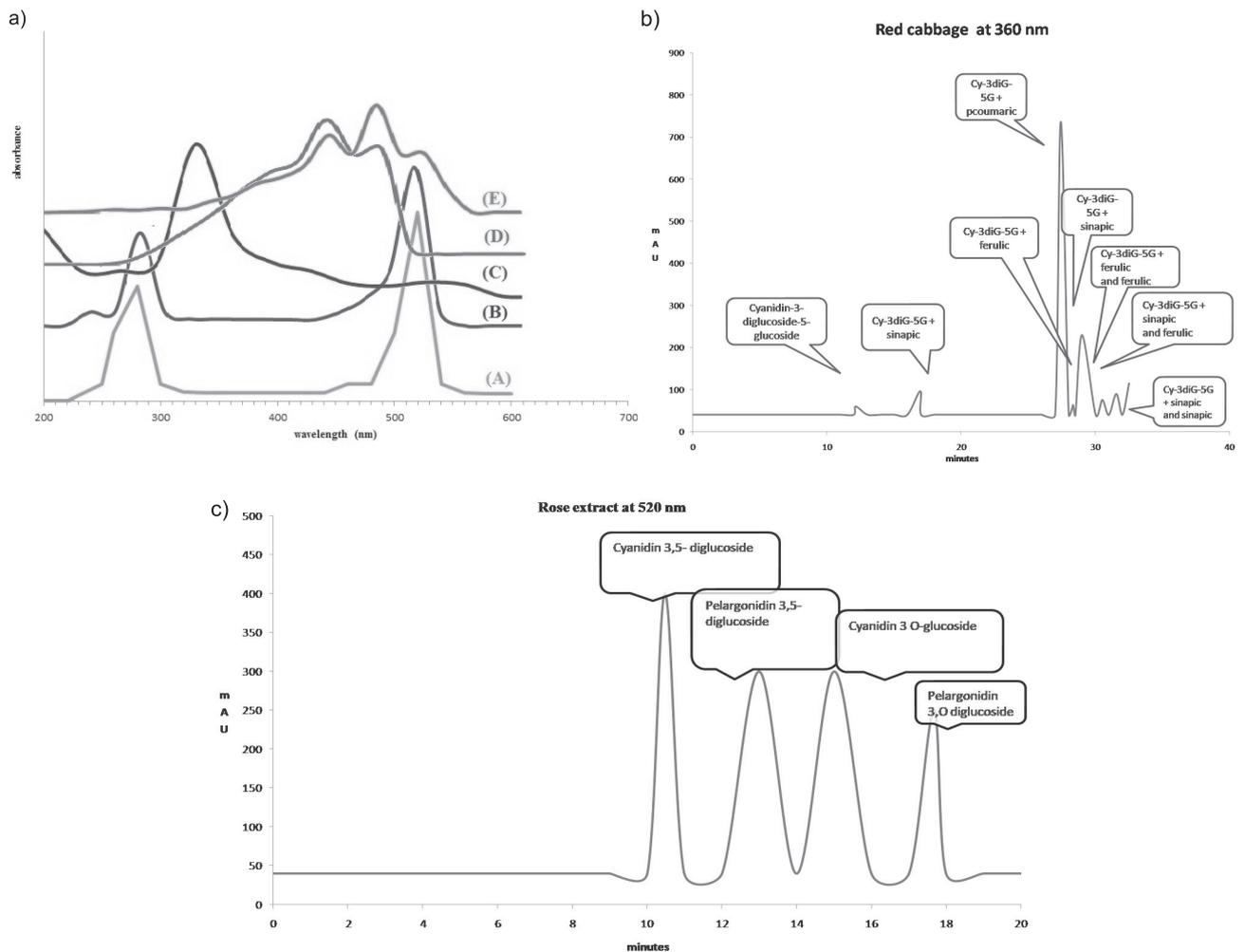


Fig. 1. UV-Visible Spectra of the extracts a); HPLC-DAD chromatogram of *Brassica oleracea* b) and *Rosa indica* c).

DPPH. It has been reported that the phenolic compounds have antioxidant potential by donating electrons to the free radicals due to strong redox properties [26, 27]. The presence of phenolic compounds is directly linked to the radical scavenging activity [28, 29].

The natural pigments are phenolic compounds; therefore, they show antioxidant activities. Extract with the smaller IC_{50} value has greater antioxidant potential. The inhibitory concentration (IC_{50}) values of each extract were calculated and presented in Table 3. The extract

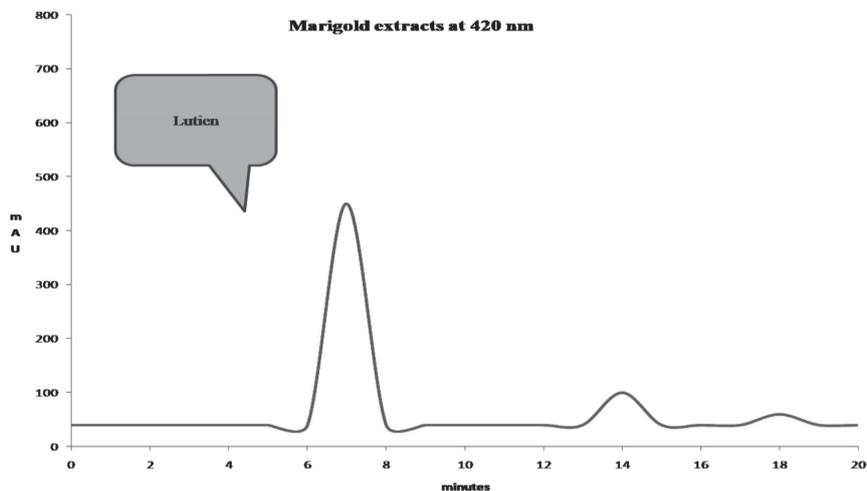


Fig. 2. HPLC-DAD chromatogram of *Calendula officinalis* L.

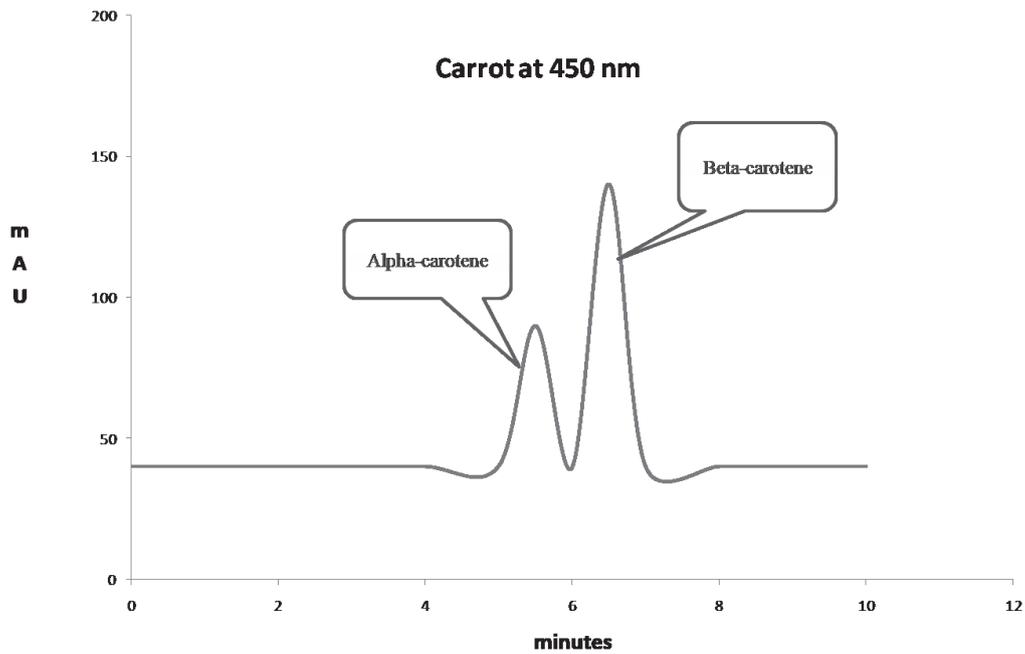


Fig. 3. HPLC-DADchromatogram of *Daucus carota L.*

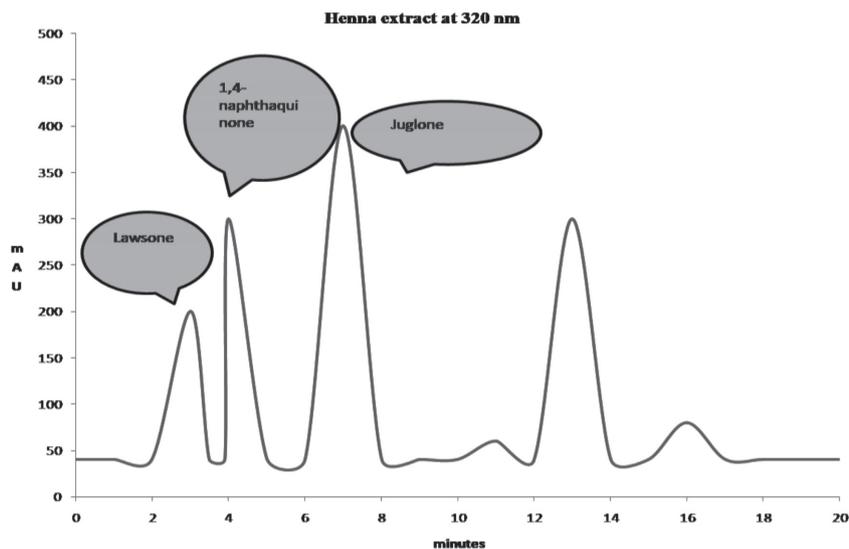


Fig. 4. HPLC-DADchromatogram of *Lawsonia inermis L.*

obtained from *Daucus carota L.* has an inhibitory concentration (IC_{50}) close to the ascorbic acid standard. It confirms that carotenoids can act as antioxidants and thus protect cells against photo-oxidation. The *Daucus carota L.* extract showed the maximum antioxidant activity of $9.4 \pm 1.00 \mu\text{g}$ ascorbic acid/mL followed by the *Rosa indica L.* ($64.89 \pm 1.00 \mu\text{g}$), *Calendula officinalis L.* ($67.43 \pm 1.00 \mu\text{g}$), *Lawsonia inermis L.* ($74.01 \pm 0.5 \mu\text{g}$), and *Brassica oleracea* ($111 \pm 0.5 \mu\text{g}$).

Antibacterial Test by Disc Diffusion Method

In the present study, five sources of natural colorants were screened for their antibacterial activity. Results

have been presented in Table 4. Antibacterial activity of *Bacillus subtilis* (gram + ve) and *Escherichia. coli* (gram – ve) was determined through the agar disc diffusion method. A comparison of the inhibition zone of the extracts and the standard streptomycin has been shown in Table 4. In recent research work maximum inhibition was shown by *Calendula officinalis* against *Bacillus subtilis* i.e. 20 ± 1.5 mm much closer to the standard, followed by *Brassica oleracea* (18 ± 1.5 mm), *Rosa indica* (16 ± 1.5 mm), *Lawsonia inermis* (14 ± 0.5 mm), and *Daucus carota* (12 ± 1.00 mm). Maximum inhibition is shown by the *Calendula officinalis* extract for both the gram-positive and gram-negative bacteria. The active agent is lutein which exerts

Table 3. Antioxidante activity of Extracts.

Sr. #	Extract	IC ₅₀ (µg/mL) Values	Results
Standard	<i>Ascorbic acid</i>	11.98±1.00	
1	<i>Brassica oleracea L.</i>	111±0.5	Good
2	<i>Rosa indica L.</i>	64.89±1.00	Good
3	<i>Calendula officinalis L.</i>	67.43±1.00	Good
4	<i>Daucusa carota L.</i>	9.4±1.00	Excellent
5	<i>Lawsoniai nermis L.</i>	74.01±0.5	Good

IC₅₀: Inhibitory concentration. The values are presented as mean ±standard deviation under the same experimental conditions

Table 4. Antibacterial activity of Extracts.

Sr. #	Extracts	<i>E. coli</i>	Results	<i>B. subtilis</i>	Results
0	Streptomycin	28±1.19 mm	Highly active	23±1.17 mm	Highly active
1	<i>Brassica oleracea L.</i>	9.5±0.5 mm	Highly active	18±1.5 mm	Highly active
2	<i>Rosa indica L.</i>	8±1.5 mm	Active	16±1.5 mm	Highly active
3	<i>Calendula officinalis L.</i>	10.3±1.5mm	Highly active	20±1.5 mm	Highly active
4	<i>Daucusa carota L</i>	10±1.00 mm	Higly active	12±1.00 mm	Highly active
5	<i>Lawsonia inermis L</i>	7.75±0.1 mm	Active	14±0.5 mm	Highly active

The scale was as follows: inactive, 1-4.5 mm; partially active, 4.5-6 mm; active, 6.5-9 mm and greater than 9 mm means highly active against bacterial strains. The values are presented as mean ±standard deviation under the same experimental conditions.

Table 5. Antifungal activity of Extracts.

Sr. #	Extracts name	<i>Flavus</i>	<i>Black Niger</i>	Results
1	<i>Brassica oleracea L.</i>	8±0.5 mm	7±0.1 mm	Active
2	<i>Rosa indica L.</i>	6.6±1mm	6.8±0.1mm	Active
3	<i>Calendula officinalis L.</i>	6.5±0.5 mm	6.6±0.1mm	Active
4	<i>Daucuscarota L.</i>	15±0.5 mm	16±0.1mm	Highly active
5	<i>Lawsoniainermis L.</i>	13±0.15 mm	15±0.15 mm	Highly active

The scale was as follows: inactive, 1-4.5 mm; partially active, 4.5-6 mm; active, 6.5-9 mm and greater than 9mm means highly active against fungal strains.

antibacterial activity. The antibacterial activity of plant extracts could be related to the presence of bioactive compounds such as tannins, terpenoids, polyphenols, and flavonoids [30]. The mechanism of the antibacterial activity involves inhibition of various cellular processes, followed by the rise in plasma membrane permeability that causes finally ion leakage from the cells. Generally, the inhibition of samples is more effective against gram (+ ve) bacteria as compared to the gram (-ve), because of the difference in the composition of their cell walls from each other. Foreign molecules have easy excess to the *B. subtilis* bacteria because of the porous peptide polyglycogen cell wall. Table 5 represents the antifungal activity. All the methanolic and ethanolic extracts

showed inhibition zone against fungus strain. Natural pigments have the ability to act as antifungal agents.

Anti-Inflammatory Activity

Natural colorants were examined against the carrageenan models and the results have been presented in Table 6. This model was mostly utilized to evaluate the anti-inflammatory activity of the drugs and plant extracts. The carrageenan model was used to produce biphasic edema. Two stages are involved in the mechanism. During the primary stage (60 min.) histamine and serotonin arrived, and in the second stage (>60 minutes) they were interceded by the

Table 6. Effect of pigments on carrageenan induced paw edema in rates.

	Hour 0	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5
Sample						
Carrageenan	5.26±0.04	6.64± 0.01	8.30±0.04	8.37± 0.03	8.21± 0.10	7.86± 0.01
Control	4.8 ± 0.01***	4.78 ±0.01***	4.74 ±0.12***	4.7 ± 0.2***	4.78±0.11***	4.75±0.18***
Standard (Diclofenac)	4.18±0.01***	5.22±0.19***	5.38±0.02***	5.58± 0.01***	5.19±0.04***	4.65±0.04***
<i>Brassica oleracea</i> L.	4.64±0.11***	5.64±0.03***	6.34±0.02***	6.04± 0.01***	5.64±0.11***	5.24±0.12***
<i>Rosa indica</i> L.	4.60±0.01***	5.50±0.02***	6.00±0.01***	5.70± 0.12***	5.40±0.03***	5.30±0.01***
<i>Calendula officinalis</i> L.	4.22±0.01***	5.22±0.02***	5.62±0.02***	5.42±0.12***	5.32±0.05***	4.92±0.04***
<i>Daucusa carota</i> L	4.20±0.01***	5.20±0.03***	5.60±0.04***	5.40±0.01***	5.00±0.05***	5.00±0.01***
<i>Lawsonia inermis</i> L	4.16±0.01***	5.16±0.02***	5.74±0.01***	5.26±0.03***	5.06±0.01***	4.76±0.01***

Values are expressed as (Mean±SEM) and two ways ANOVA was performed followed by Bonferroni *post hoc test* (n = 5), *** p<0.001 and ns (non – significant) in comparison to carrageenan induced inflammatory model.

cyclooxygenase substances. Kinin causes congruity between two stages. In the present study, pigments controlled the edema development in both the primary and secondary stages. The anti-edematous action of the pigments was preserved in the second stage, with the maximal impact found at 5hrs [30]. Results showed a significant ($p<0.001$) decrease in paw inflammation compared to the carrageenan-induced inflammatory

model in rats. All the colorant samples showed high inhibition at the 5th hour. Table 7 shows maximum inhibition exhibited by *Calendula officinalis* L. i.e. 75.33±1.20%. It is very close to the standard value 79±0.57%. The high anti-inflammatory activity of carrot extract is due to the presence of lutein that which has chemoprotective potential, followed by the *Brassica oleracea* (74.71±0.64%), *Rosa indica* L.

Table 7. % age inhibition of the natural pigments.

Sr. #	Extracts	Hour 1	Hour 2	Hour 3	Hour 4	Hour 5
	Standard	48±0.57%	49±0.57%	52±0.57%	65±1.73%	79±0.57%
1	<i>Brassica oleracea</i>	25.66±1.20%	44±0.88%	54.66±1.20%	65.72±2.95%	74.71±0.64%
2	<i>Rosa indica</i> L.	33.66±0.88%	54.33±0.88%	65.33±1.76%	72.66±0.88%	73.66±1.26%
3	<i>Calendula officinalis</i>	27.66±2.40%	54.66±3.17%	60.66±0.88%	62±1.73%	75.33±1.20%
4	<i>Daucusa carota</i> L	27.33±2.18%	55.66±0.88%	61.66±1.85%	71±1.54%	70.33±0.88%
5	<i>Lawsonia inermis</i> L	25.66±1.20%	53.33±2.18%	65±2.08%	69.66±1.85%	72.33±1.20%

Table 8. Cytotoxicity of the Extracts.

Sr. #	Extracts	Cytotoxicity (%age)	Results
0	Positive control (Triton X-100)	98.5±0.22	Highly toxic
0	Negative control (PBS)	0.12±0.01	Non-toxic
1	<i>Brassica oleracea</i>	6.5±0.05	Non-toxic
2	<i>Rosa indica</i> L.	5.7±0.05	Non-toxic
3	<i>Calendula officinalis</i> L.	3.4±0.05	Non-toxic
4	<i>Daucusa carota</i> L.	5.5±0.04	Non-toxic
5	<i>Lawsonia inermis</i> L	9.5±0.04	Non-toxic

Table 9. Thin layer chromatography of the Extracts.

Sr. #	Extract	R _f Calculation	R _f Result
1	<i>Brassica oleracea L.</i>	3/8,4.8/8,6.8/8	0.37,0.6,0.85
2	<i>Rosa indica L.</i>	3/8,3.7/8,6.5/8	0.37,0.46,0.81
3	<i>Calendula officinalis L.</i>	1.5/8,6.3/8	0.18,0.79
4	<i>Daucusa carota L.</i>	1.5/8,2.45/8	0.95,0.31
5	<i>Lawsonia inermis L.</i>	3.5/8, 5.9/ 8,6.1/8	0.43, 0.73, 0.76

(73.66±1.26%), *Lawsonia inermis L.* (72.33±1.20%) and *Daucusa carota L.* (70.33 ±0.88 %).

Cytotoxicity of Extracts

The cytotoxicity of the samples was evaluated in terms of hemolytic activity against human red blood cells. Table 8 shows the results of cytotoxicity. Triton X-100 serves as positive control (show 100% lysis) and phosphate buffer saline (PBS) as negative control (no lysis of RBCs). All colorant extracts showed less cytotoxicity. Minimum cytotoxicity %age value means that the pigments have minimum toxic effects. The highest hemolytic activity was observed by *Lawsonia inermis L.* (9.5±0.04%) followed by *Brassica oleracea* (6.5±0.05%), *Rosa indica L.* (5.7±0.05%), *Daucusa carota L.* (5.5±0.04%), and *Calendula officinalis L.* (3.4±0.0 %) as shown in Table 8. The extract of natural sources has been demonstrated to possess biological activities as well as cytotoxic effects. Weak cytotoxicity towards normal human blood cells of the natural pigments showed that all pigments are secondary metabolites and they possess beneficial health effects along with an attractive color appearance. The present data showed that the cytotoxicity value of the natural pigments was very low and within the safe limits for further applications.

Conclusions

These findings suggest that the crude extracts of pigments from natural sources have high antibacterial properties for both the gram (+ve) and gram (-ve) bacteria. These extracts have good antioxidant and anti-inflammatory properties along with less cytotoxicity. On the basis of these findings, we can conclude that the crude extracts of *Rosa indica L.*, *Brassica oleracea*, *Lawsonia inermis*, *Daucusa carota L.* and *Calendula officinalis* has a great potential to be used for different applications, especially in the formulation of skin care products. Our group is working to formulate skin care cosmetics using these extracts of pigments from plant sources whose findings will be published in near future.

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This research study has not received any funding.

Conflict of Interest

There is no conflict of interest among all authors.

Data Availability Statement

The whole data is available in this manuscript.

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