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Invitro Antioxidant Studies and Characterisation of Anthocyanin using UV, FTIR and UPLC in Nerium Oleander Flowers, Collected as Temple Floral Waste.

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KEYWORDS

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ABSTRACT:

The present study has been focused on the investigation of the phytochemical characteristics and antioxidant properties of *Nerium oleander* flowers, collected as temple floral waste. Preliminary phytochemical analysis of the methanolic flower extract revealed the presence of diverse components including alkaloids, flavonoids, phenolic compounds, tannins, terpenoids, and anthocyanin. UV-Vis and IR spectroscopic analyses confirmed the presence of key compounds such as anthocyanins, flavonoids, polyphenols, and tannins . The presence of anthocyanins was conclusively confirmed through Ultra-Performance Liquid Chromatography (UPLC), providing robust evidence of their occurrence in the extract.The methanolic extraction was able to scavenge free radicals as tested through DPPH radical scavenging assay.The use of temple floral waste as a source material highlights the study's focus on sustainable resource utilization.

1. Introduction

Nature is still an important reservoir of new molecules with potential therapeutic interest [1]. In recent times, focus on plant research has increased all over the world and a large body of evidence has been accumulated to highlight the immense potential of medicinal plants used in various traditional systems of medicine[2]. Medicinal plants are essential natural resources which constitute one of the potential sources of new products and bioactive compounds for drug development[3].Numerous examples from medicine impressively demonstrate the innovative potential of natural compounds and their impact on progress in drug discovery and development[4]. As the focus of medicine shifts from treatment of manifest disease to prevention, increasing awareness on herbal remedies as potential sources of phenolic antioxidants have grown in recent years, and several plants are being screened for their antioxidant properties using different assays[5].Antioxidants play an important role to protect the human body against damage by reactive oxygen species [6].

Natural dyes , extracted from plants, animals etc. shows minimum environmental impact and used not only in the coloration of textiles [7] but also in the food ingredients [8] and cosmetics [9].Anthocyanins , the natural water-soluble and non-toxic pigments widely existing in plant kingdom is a group of phenolic compounds imparting orange to blue colours to the plants [10]. Flavonoids, carotenoids, and chlorophylls are the major pigments that provide various colouration to flowers and fruits. Of which flavonoids are the most significant flower colour pigments, especially the anthocyanins [11]. Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. The differences in the position of glycosidic bond binding, the type and number of sugars, the number of hydroxyl groups, the type and number of aromatic acids made the anthocyanin in six major groups namely, cyanidin(Cy), petunidin(Pt), peonidin(Pn), pelargonidin (Pg), delphenidin(Dp), and malvidin(Mv) [38].The present study focus on metabolite profiling on the flowers of *Nerium oleander* using various spectroscopic analyses techniques with a view to isolate and characterize the pharmacologically active

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compounds and to ascertain the antioxidant potency of the flower extract using *in vitro* methods.

2. MATERIALS AND METHODS

Plant material

Nerium oleander L. flowers were collected from the floral wastes of the temple Kodimoottil Sree Bhadrakali temple in Parippally, Kollam, Kerala. Kodimoottil Sree Bhadrakali temple in Parippally, Kollam was selected based on the popularity and number of visitors. Regular visit to the temple was made during the normal days and on special occasions like paduka a ritual during the temple maholsav, Navratri, Janmashtami, Shivaratri, Ganesh Chaturthi, etc. for collecting the red flowers of *N. oleander* L. The collected red flowers of *N. oleander* L. were washed thoroughly, air dried and subjected to extraction. A total of 50g of powdered plant material was extracted using 500ml of methanol.

The extractions resulted in yields of 0.65g, 3.19g and 2.2g for hexane, Chloroform and methanol respectively. The extracts were filtered, concentrated under reduced pressure using a rotary evaporator and stored at 4^0C for further analysis.

Qualitative phytochemical investigation of *N.oleander* flower

The methanolic extract of N. oleander was subjected to preliminary phytochemical screening to detect the presence of different types of phytochemicals. The qualitative phytochemical screening was carried out by using standard procedures of Harborne, Raaman and Kokate [12,13,14] .All the qualitative tests were replicated thrice.

UV-VIS Spectrum analysis

The extract was centrifuged at 3000 rpm for 10 min and filtered through Whatmann No.1 filter paper. The sample was diluted to 1:10 with the same solvent. The extract was scanned at wavelength ranging from 200 to 1100 nm using UV spectrophotometer and the characteristic peaks were detected.

FTIR Spectrum analysis

The shade dried flowers of *N. oleander* were powdered in mechanical grinder. 20 g of whole flower powder was weighed; 150 ml of methanol was added and kept for 3 days. The extract was filtered using Whatman No.1 filter paper and the supernatant was collected. The residue was again extracted two times (with 3 days of interval for each extraction) and supernatants were collected. The supernatants were pooled and evaporated (at room temperature, 28 ± 1°C). The prepared extract (whole plant powder with methanol) was stored in airtight bottles for subsequent analysis. Dried powder of test sample was used for FTIR analysis. 1 mg of the dried powder was encapsulated in10 mg of KBr pellet, to prepare translucent sample discs. The powdered sample of the pellet was loaded in FTIR spectroscope (Shimadzu, Japan), with a scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

Anthocyanin Determination by Ultra Performance Liquid Chromatography (UPLC)

For UPLC analysis, the anthocyanin extracts of *N. oleander* floral methanolic extract was concentrated and dried extracts were dissolved in 1 ml methanol. UPLC of these anthocyanin extracts was performed using Waters Acquity Ultra Performance. LC system, equipped with a quaternary pump system [15]. An Acquity BEH C-18 (50 x 2.1 mm id, 1.7 µm particle size) column (Waters) was used for analysis. Detection was carried out using a photodiode array (PDA) detector with the absorbance wavelength of 520 nm. The injection volume was 2 µL, column temperature was set at 50^OC, and the flow rate was 0.5 ml min^{-1}. Eluent A comprised 5% (v/v) formic acid and eluent B comprised HPLC grade acetonitrile. The gradient run was 0–5.0 min: 95% A and 5% B; 5.0–5.1 min: 85% A and 15% B; 5.1–6.0 min: 84.5% A and 15.5% B; 6.0–6.1 min: 84.5% A and 15.5% B; 6.1–6.8 min: 0% A and 100% B. The separation was carried out for 6.8 min in the gradient elution. The identification of anthocyanins present in the extracts was performed by comparing the retention times of the anthocyanin peaks in sample extracts with the peaks in standard solutions.

Determination of anthocyanin content by pH differential method

The pH differential method for determining monomeric anthocyanin was used. 10 ml of buffer solution (pH 1, potassium chloride 0.025 M) were added in a 50 ml volumetric flask. Subsequently, 1 ml of liquid extract (or 10 mg of dry extract) was added. The volume was completed by using the same buffer solution. In another flask with the same capacity, 10 ml of buffer solution (pH4.5; sodium acetate 0.4 M) and 1 ml of the

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liquid extract was added. The volume was completed using a buffer solution of pH 4.5. Both flasks were gently stirred manually, and let it in repose for 5 min, protected from light. Afterward, both solutions were filtered through a Whatman paper. After that, the absorbance of both samples was recorded using a spectrophotometer at 520 nm and, 700 nm. The total content of anthocyanins (AT) was calculated as cyanidin-3-glucoside (mg/l) equivalents, using the following expression:

$$AT\left(\frac{mg}{ml}\right) = A \times 834.9 (1)$$

Determination of Antioxidant Potential

The methanolic extract of *N. oleander* were used for the analysis of antioxidant activity.

Detection of Antioxidant Activity by Thin Layer Chromatography

TLC plates were prepared by using silica gel (G-60 Merck) as the adsorbent. The glass plates were carefully cleaned with acetone to remove grease. A slurry of silica gel was prepared by mixing it with distilled water in 1:2 (w/v) ratio and was spread over 1 mm thick glass plates (75 X 25mm) with the help of a TLC applicator, which was adjusted to a thickness of 0.5mm. The plates were activated at 110°c for 30 minutes in a thermoregulated hot air oven before use (Harborne,1998). The concentrated extracts were dissolved in 100% methanol and spotted on the plates and developed in methanol: ethyl acetate (2:1; w/v) solution. The plates were air dried and sprayed with 0.04% solution of stable DPPH (2,2-diphenyl1-picrylhydrazyl hydrate) radical [16] and visualized in visible light and the R.F value was calculated.

$$R.FValue = \frac{Distancetravelledbythecomponentfromthespot}{Distancetravelledbythesolvent} (2)$$

Determination of Antioxidant activity Using DPPH Radical Scavenging Assay

The Scavenging activity for DPPH free radicals was measured according to the procedure[17]. Methanol extract of the sample at various concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 & 1.4 mg/ml) was added separately to each 5ml of 0.1mM methanolic solution of DPPH and allowed to stand for 20 min at room temperature. After incubation, the absorbance of each solution was determined at 517 nm using spectrophotometer. Ascorbate was used as standard. The corresponding blank was also taken, and DPPH radical scavenging activity was calculated by using the following formula:

$$\%ofinhibition = \frac{(Absorbanceofcontrol - Absorbanceofsample)}{Absorbanceofcontrol} \times 100 (3)$$

Statistical analysis

The data obtained from the studies were represented as Mean ± SD using the GraphPad InStat version 3.05 and Microsoft Excel 2010 were used for statistical analysis

3. Results & Discussion

The preliminary qualitative phytochemical screening of the crude methanolic flower extracts of *N .oleander* was carried out to assess the presence of bioactive compounds. The crude extract revealed the presence of alkaloid, flavonoids , phenolic compounds, tannins , terpenoids and anthocyanin compounds. The qualitative UV-VIS spectrum was carried at wavelengths from 229 to 741 nm due to sharpness of the peaks and proper baseline. The profile at the liquid phase showed the peaks at 229, 288, 327, 546 and741 nm with the absorption of 3.148, 2.117, 2.398, 0.022 and 0.001 respectively (Fig.1). Meanwhile, at solid phase the extract displayed only two peaks i.e., at 537 and 352 nm with the corresponding absorptions 0.351 and 0.447 respectively (Fig.2).

FT-IR measurement was carried out to identify the possible biomolecules in *N. oleander* extract (Fig.3). The spectrum showed 21 absorption bands which indicated the presence of many active functional groups. The intensity peaks 3441.01, 2916.37, 2636.69, 964.41, 1033.85, 1155.36, 1257.59, 1367.53, 1454.33, 1519.91, 1614.42, 1741.72, 2636.69 cm−1 and corresponding area were 11.066, 3.682, 3.702, 2.242, 3.693, 2.34, 4.278, 2.391, 1.67, 2.714, 9.067, 7.556 and 3.702. The decreased intensity peaks were 1830.45, 2299.15, 2736.99, 2856.58 cm^{-1} and corresponding area were 0.648,0.573, 0.41, and 0.941. The peak at 3441.01 correspond to N-H, O-H stretching vibrations, amide, alcohol and H– bonded to phenols. The peak at 1614.42cm^{-1} indicate to C=C, C=O stretching vibrations to alkenes and amide. The peak at1367.53 represents to C-H in plane bend to alkenes. The peak at 677.01 cm^{-1} corresponds to C- Cl, C-Br stretching vibrations to alkyl

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halides. The band at 1830.45 corresponds to C-N stretching vibration. The weak band at 1033.85 cm^{-1} indicates C-O, C-N stretching vibrations and it corresponds to the presence of alcohols, carboxylic, acids, ethers, esters and aliphatic amines in the extract. The presence of active functional groups in the extract results in the swift reduction. The spectrum shows a band at 3441.01cm^{-1} associated with the stretching vibration of OH bonds. At 2916.37cm^{-1} was observed a characteristic band of the C–H stretching vibration and at 1741.72cm^{-1} the characteristics stretching vibration of the ester carbonyl group were observed. The band at 1614.42 cm^{-1} corresponds to C- C of aromatic rings stretching vibration. In the same way, it was observed a band at 1519.91cm^{-1} corresponding to the axial deformation of the C -C bond in aromatic rings. The spectrum also showed a band at 1454.33 cm^{-1} related to the deformation of C–H bonds and, another band at 1033.85 cm−1corresponding to the C–O bonds stretching of phenol .The resulted functional groups by FTIR indirectly predict the presence of anthocyanins, flavonoids, polyphenols and tannins in the extract.

Structurally, anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts, being composed of an aglycon moiety called anthocyanidin and carbohydrate residues (glucose, rhamnose, xylose, galactose, arabinose, rutinose)[18]. At low pH, anthocyanins are predominantly present in the flavylium cation form, giving a reddish color to aqueous solutions [19]. Flavylium cation is stable in acid solutions, being the only groups of phenolic compounds that can be favored by increasing the acidity of the extracted solution. For this reason, mineral and organic acids are used for anthocyanins extraction [20].However, mineral acid can hydrolyze the acyl groups of anthocyanins, losing stability and activity. In this work, it was observed, a strong effect of the volume of acetic acid in the anthocyanin content. This occurs, probably, because the oxygen atom in the aromatic ring of anthocyanins has a high electronic density allowing to accept a proton (H+) from the acetic acid, getting a positive charge (cation flavylium). Thus, anthocyanins are dissolved preferably, to the rest of the phenolic substances, that are usually extracted using a more basic and polar solution. The FTIR spectra obtained for the extract (Fig.3) showed all characteristic bands of anthocyanins by those observed by other authors for different anthocyanin extracts and a standard of anthocyanin-3-O-glycosidic [21]. Others bands observed at 1456–1419; 1377–1340and 1155–889 $cm^{-1}$are associated with monosaccharide such as glucose and galactose that are commonly present in the structure of anthocyanins [22].

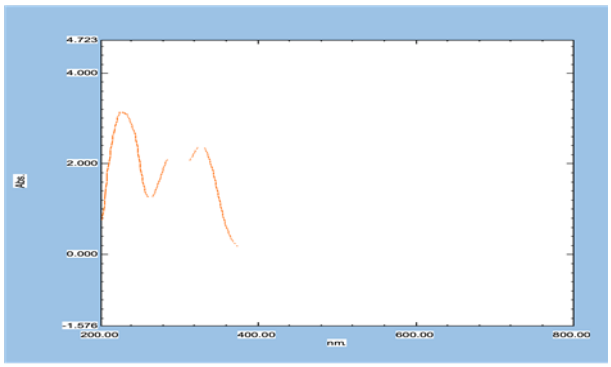

Fig.1. UV-VIS spectra of liquid phase of *N. oleander* extract

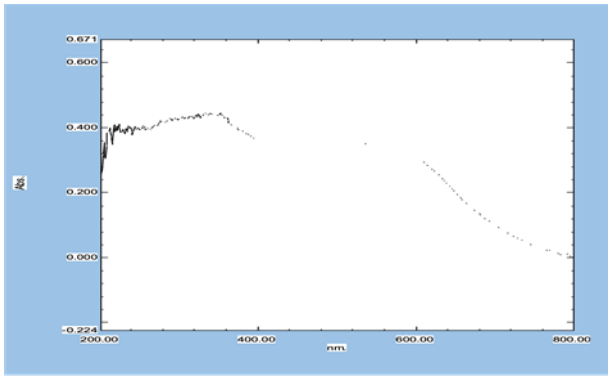

Fig.2. UV-VIS spectra of solid phase of *N. oleander* extract

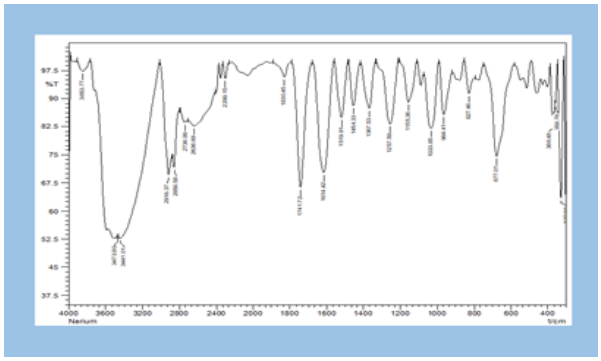

Fig.3. FTIR Spectrum analysis of *N. oleander* L.

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Anthocyanin Determination by Ultra Performance Liquid Chromatography (UPLC)

In the study, the determination of anthocyanins through UPLC authenticated and provided an insight into the composition and characterization of anthocyanins. Cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin are the most common anthocyanidins distributed in the plants [23]of which except malvidin all the others are present in the present extract . Delphinidin has a chemical characteristic like most of the anthocyanidins. It appears as a blue-reddish or purple pigment in the plant. The blue hue of flowers is due to the delphinidin pigment [24]. In nature, cyanidin is a reddish-purple (magenta) pigment [25] .Pelargonidin differs from most of the anthocyanidins. In nature, it appears as red-colored pigment. Methylated anthocyanidin such as peonidin is another type of anthocyanidin abundantly found in plants. It has the visible colour magenta [26]. Pelargonidin gives an orange hue to flowers [27] red to some of the fruits and berries [28].

Determination of anthocyanin content by pH differential method:

The anthocyanin content of methanolic extract of *N. oleander* as determined through pH differential method yielded a value of 3.78 mg/ g cyanidin- 3-glucoside. Aruna *et al*., reported the quantification of anthocyanin in both red and pink cultivars and are approximately equivalent [29]. The maximum anthocyanin content (3.54mg/k 3 g eq) was recorded in red flowers extracted by 100% ethanol followed by red flower (3.40 mg/k 3 g eq) extracted by 100% methanol, while the minimum anthocyanin is recorded in pink flower (1.93mg/k 3 g eq) extracted by 100% distilled water.

Determination of Antioxidant activity Using DPPH Radical Scavenging Assay

The present study utilized Thin Layer Chromatography (TLC) and the DPPH free radical scavenging assay to evaluate antioxidant activity. The TLC plate was treated with DPPH solution, and the intensity of yellow coloration increased with concentration, indicating a rise in antioxidant activity. The antioxidant potential, as measured by the DPPH radical scavenging assay, was notable .The extract exhibited concentration-dependent inhibition of radical scavenging, with the highest recorded inhibition being 71.4% at a concentration of 1.4 mg/mL. The IC_{50} value was calculated as 1.0 ±0.05 mg/mL.DPPH, astable free radical at room temperature, accepts an electron or hydrogen atom to form a stable diamagnetic molecule [30]. The ability of plant extracts to donate hydrogen atoms was assessed by the decolorization of the methanolic DPPH solution, which shifts from deep violet to yellow in the presence of antioxidants.The reduction of the DPPH radical was quantified by the decrease in absorbance at 517 nm. This reduction occurs due to the reaction between antioxidant molecules and free radicals, which involves hydrogen donation and indicates radical scavenging activity. The findings revealed that the extract is a significant source of antioxidants.

Conclusion

The present study successfully highlighted the potential of *Nerium oleander* flowers, collected as temple floral waste, as a source of anthocyanins with significant antioxidant properties.In vitro antioxidant assays demonstrated the remarkable free radical scavenging activity of the anthocyanin extracts, indicating their potential as natural antioxidants. Characterization using UV-visible spectroscopy, FTIR, and UPLC confirmed the presence of anthocyanins and provided insights into their structural properties and purity.This research not only emphasizes the value of temple floral waste as a sustainable source of bioactive compounds but also opens avenues for their application in pharmaceuticals, nutraceuticals, and cosmetics. Furthermore, the study supports the growing interest in utilizing natural antioxidants for health benefits and highlights the role of advanced analytical techniques in elucidating phytochemical profiles.Future studies should explore the scaling-up of extraction processes, detailed toxicological evaluations, and potential therapeutic applications to fully harness the benefits of anthocyanins from Nerium oleander flowers. This approach aligns with sustainable development goals by promoting waste valorization and contributing to eco-friendly practices in research and industry.

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