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Estimation of various properties of bioactive compounds isolated from *Catharanthus roseus* leaf

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Abstract

Lupeol, stigmasterol, ursolic acid, myricetin, and naringenin were isolated from n-hexane, ethyl acetate, and methanolic extracts of *Catharanthus roseus* leaves to assess their free radical scavenging, cytotoxic, and antibacterial activities. The determination of the free radical scavenging activity (FRSA) of the isolated compounds was performed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method along with UV-Visible spectroscopy. The brine shrimp lethality bioassay (BSLT) method was applied to determine cytotoxic activity. For antibacterial screening, the disk diffusion method was employed, with streptomycin (10 µg/disc) serving as a control. Among the compounds, myricetin exhibited noteworthy free radical scavenging activity with an IC_{50} 6.18 µg/mL. Ursolic acid had a strong cytotoxic effect in the brine shrimp lethality bioassay, with an LC_{50} of 0.72. Naringenin outperformed streptomycin in terms of antibacterial activity against the pathogenic microorganisms that have been tested. The FRSA, cytotoxicity, and antibacterial activity of isolated compounds were consistent with the traditional uses of this plant.

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Introduction

Medicinal plant products can be very effective in minimizing the deterioration of chemotherapeutic agents and extending longevity by helping maintain good health (Kaushik and Dhiman, 1999). Thus, the interest in plant-based products' curative properties has naturally increased over the past few decades. To detect a wide range of bioactivities which are found in crude extracts, general bioassays like free radical scavenging activity (FRSA), brine shrimp lethality bioassay test (BSLT), and antibacterial screening are often used due to their simplicity, low cost, and low requirement of a test material. In particular, BSLT can efficiently determine cytotoxicity and pesticide activity (Ghisalberti, 2007). Invented in 1982, this test (Meyer *et al*., 1982; Kaushik Rahman *et al*., 1999) has been applied for bioassay-guided fractionation of active cytotoxic and anticancer agents like trilobatin, extracted from the bark of *Asimina triloba* (Zhao *et al*., 1992) and cis-annonacin from *Annona muricata* (Rieser *et al*., 1996). Antioxidant activity can be predicted by FRST, which was first used in 1958 (Blois, 1958), and applied to identify active free radical scavengers such as flavonoids, vitamin E, vitamin C, phenolic acids, phytic acids, carotenes and phytoestrogens, which are often credited for having the potential to lessen the risk of disease (Bendich *et al*., 1995). Some studies indicate that antioxidants, present in vegetables, fruits, tea, and red wine, cause the apparent effectiveness of these foods in lessening the chance of chronic diseases, which include heart diseases as well as a few cancers (Miller *et al.,* 2000).

Catharanthus roseus (Bengali: Nayantara; Common English Names: Periwinkle, Vinca; Synonym: *Vinca rosea*; belonging to the Apocynaceae family), commonly called the Madagascar periwinkle, is a plant rich in antileukemic alkaloids. The plant manifests anticancer activity, which is the main reason for its cultivation (Jaleel *et al*., 2009). As an herbaceous or evergreen subshrub plant, it can grow up to 1 m in height (Huxley *et al*., 1992).

Catharanthus roseus can be prescribed as a cooling medicine. The herb is useful for chest diseases, throat infections, malaria fever, treatment of diabetes, etc. It can also be applied as a remedy for regulating menstrual cycles and as a euphoriant (Phondke, 1992). The plants contain indole alkaloids found in all parts of the plant. In addition, antihypertensive alkaloids, namely reserpine, serpentine, and ajmalicine, are also found in the herb roots, whereas antineoplastic alkaloids, which are physically essential, named vincristine and vinblastine, are mostly found in the leaves (Mishra *et al*., 2001). Vincristine and vinblastine are two alkaloids that are used to treat a variety of diseases such as lymphoma, leukemia, nonmalignant diseases, and platelet-associated disorders (Farnsworth *et al*., 1968; Svoboda *et al*., 1975). Previous studies show that phytochemical investigation can effectively isolate Kaemferol (Forsyth *et al*., 1957), Kaemferol trisaccharides (Nishibe *et al*., 1996), Syringetin glycosides (Bruneton, 1999; Auriola *et al*., 1990), Petunidin (Svoboda *et al*., 1975), Petunidin 3-o-glucosides (Filippini *et al*., 2003), Petunidin 3-o-(6-o-p-coumaroyl) (Filippini *et al*., 2003), Quercetin (Svoboda *et al*., 1975), Quercetin trisaccharides (Forsyth *et al*., 1957), Malvidin (Svoboda *et al*., 1975), Malvidin 3-o-glucosides (Filippini *et al*., 2003), Malvidin 3-o-(6-o-p-coumaroyl) (Filippini *et al*., 2003), Hirsutidin (Svoboda *et al*., 1975), Hirsutidin 3-o-glucosides (Filippini *et al*., 2003) and Hirsutidin 3-o-(6-o-p-coumaroyl) (Filippini *et al*., 2003). The alkaloids collected from this plant, which are antineoplastic medicines, have important pharmaceutical activity, while the mono indole alkaloids ajmalicin and serpentine are effective against hypertension (Rahman *et al*., 1988; Rahman *et al*., 1984; Rahman *et al*., 1983; Rahman *et al*., 1984; Rahman *et al*., 1985; Zhao *et al*., 2007).

As a result, the current study was designed to determine the free radical scavenging activity, cytotoxic and antibacterial activities of compounds (lupeol, stigmasterol, ursolic acid, myricetin, and naringenin) isolated from n-hexane, ethyl acetate, and methanolic extract of the leaves of *Catharanthus roseus* to support the traditional medicinal uses of this important plant.

Materials and methods

Plant material collection

Fresh *Catharanthus roseus* leaves were collected in June 2018 from the herbarium of the Department of Botany, University of Dhaka, Bangladesh, and a voucher specimen (No. = 39512) was submitted to the Bangladesh National Herbarium, Dhaka, where the appointed taxonomist identified the plant.

Equipment

The UV-Vis spectrometric analysis was carried out using a Perkin Elmer Shelton, CT 06484 USA, Lambda 25 UV-Vis spectrometer. To evaporate solvents, a vacuum rotary evaporator (BUCHI, Rotavapor R-210, Switzerland) was employed. All of the solvents used, without further purification, were of analytical grade and purchased commercially (Sigma-Aldrich, St. Louis, MO, USA).

Solvent extracts preparation (Cold extraction)

510 gm leaves were dried, ground and screened, followed by extraction with methanol for five consecutive days at room temperature. With the help of a rotary evaporator under reduced pressure, the resulting filtrate was made into a sticky mass by evaporating it. Subsequently, the residue (40.0 g) was extracted with solvents like n-hexane (100 mL × 3), ethyl acetate (100 mL × 3), and n-butanol (100 mL × 3). After that, the resultant extracts were evaporated with a rotary evaporator, yielding n-hexane extract (11.0 g), ethyl acetate extract (9.0 g), and n-butanol extract (8.5 g). Finally, the remaining methanol soluble part (11.5 g) was denoted as methanol extract.

Isolation of compounds from the different extracts of leaf Catharanthus roseus

From n-hexane extract

The column grade silica gel was used to adsorb the dehydrated mass of n-hexane extract (5.0 g). Under a UV lamp, the TLC study of the n-hexane extract showed a number of spots. Then spray reagent development on a TLC plate was analyzed. In the VLC apparatus, on top of the column bed filled with silica gel (chromatography grade). Elution was carried out with solvents (mixtures) of increasing polarity, consecutively with 100n-hexane, n-hexane/ethyl acetate (EA), ethyl acetate/methanol. Twenty-two collection (200-ml) each were obtained and amalgamated, for further purification, into 8 fractions based on their TLC results.

The column-grade silica gel was used to absorb the dried mass of leaf n-hexane extract (7.0 g), which was dissolved in a little volume of n-hexane. When subjected to a UV lamp and then spray reagent on the TLC plate, the n-hexane extract revealed a variety of spots in various solvent systems on the TLC plate. The silica gel bed (TLC grade) in the VLC apparatus was then topped with the adsorbed sample. It was eluted with the low polarity n-hexane and methanol mixes first, followed by 100% n-hexane, and then with n-hexane and dichloromethane (DCM) mixtures which is of the highest polarity. The eluents were collected in 23 conical flasks of 200 mL each, and according to their TLC pattern, they were divided into five fractions. Due to its high quality resolution, fractions 3 (collection nos. 9–13) and 4 (collection nos. 14–18) were chosen for more research based on the TLC behavior of VLC fractions.

Using column chromatographic separation and elution with a hexane-ethyl acetate solvent gradient, the compound lupeol (9.8 mg) was recovered from the VLC fraction 3 (collection no. 9-13) of n-hexane extract. It was discovered as a white, amorphous solid that was entirely soluble in chloroform. By repeatedly separating VLC fraction 4 (collection no. 14-18) using a hexane-chloroform solvent system in a gradient manner as the mobile phase, compound stigmasterol (8.5 mg) was discovered as a white crystalline solid. Chloroform can dissolve the pure (with respect to TLC) compound.

From ethyl acetate (EA) extract

A minimum amount of the same solvents were used to dissolve the leaf part's ethyl acetate extract (4.0 g), which was then absorbed on column-grade silica gel. On top of a bed of TLC-grade silica gel packed in a VLC apparatus, the adsorbed sample was initially eluted with 100% n-hexane. Then, solutions of ethyl

acetate and methanol (higher polarity) were used to elute it, followed by combinations of n-hexane and ethyl acetate. Based on their TLC activities, 26 200-mL specimens were gathered and divided into six fractions.

VLC fractions 2 (collection nos. 4-6), 3 (collection nos. 7-8), and 4 (collection nos. 9–15) were chosen for more research based on the TLC behavior. Using a similar technique, the chemical ursolic acid (9.3 mg) was extracted in pure form as crystals from subsequent collections (collection nos. 32–36) of the same column. Both the compounds were found to be TLC pure and were soluble in chloroform mixed with a few drops of methanol.

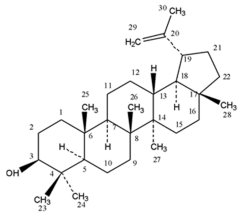
The VLC fraction 4 (collection no. 9-15, 110 mg) was subjected to column chromatography to separate the compound myricetin. The column was made by silica soaked in 50% $CHCl_3$ in n-hexane and was eluted using n-hexane/chloroform followed by chloroform/methanol solvent system (increasing polarity). The compound was collected almost in pure form from the collection no. 20-25 (10 mL each) of the column. The compound myricetin (7.6 mg) was finally purified as yellow crystals by recrystallization from a minimum amount of ethyl acetate. The crystals were completely soluble in methanol.

From methanol (MeOH) extract

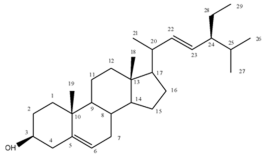
The lowest concentration of solvent was used to dissolve the methanol extract of the leaves (7.0 g), which was then absorbed on column-grade silica gel. The adsorbed substance was placed on top of a packed silica gel bed in the VLC device (TLC grade). It was first eluted with 100% n-hexane, then with blends of n-hexane and ethyl acetate, and finally, with mixtures of ethyl acetate and methanol, with increasing polarity. Based on their TLC behaviors, a total of 26 collections of 200 ml each were gathered and divided into six fractions.

Fractions 2 (collection numbers 4-9) and 4 (collection numbers 14-17) of the VLC separation were chosen for further purification based on the TLC behavior. The VLC fraction 2 (collection no. 4-9) of the methanol extract of leaves was comparatively a non-polar fraction that was subjected to a column made of 100% n-hexane. The column was eluted with an n-hexane-chloroform solvent gradient, yielding 25 collections of 20 ml each.

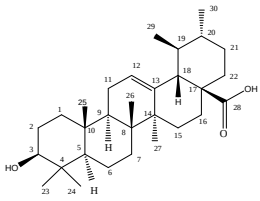
Compound naringenin (9.6 mg) was extracted from the VLC fraction 4 (collection no. 14-17) of the methanol extract using a silica gel column consisting of 100% ethyl acetate.Ethyl acetate and methanol mixtures in various polarity ratios were used to elute the compounds. A yellow crystalline compound naringenin was found in pure form from the collections 10-14 (20 mL each) of the column after crystallization and was soluble in methanol. The structures of the isolated compound are in Fig. 1.



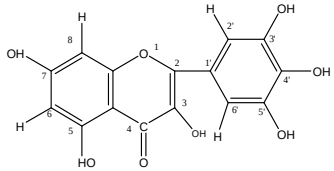
Lupeol



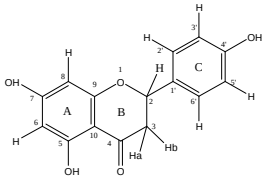
Stigmasterol



Ursolic Acid



3,3',4',5,5',7-hexahydroxy flavone or Myricetin



4',5,7-trihydroxyflavanone or Naringenin

Fig. 1. Isolated compounds

Bioassays of the isolated compounds

Free radical scavenging activity

The antioxidant potential was measured spectrophotometrically via the DPPH technique (Auriola *et al.*, 1990). Because of its unpaired electron, the DPPH radical displays a profound violet color, and radical scavenging power can be tracked using spectrophotometry by a drop (at 517 nm) of the absorbance. Variable concentrations (5, 10, 25, 50, 100, 200, 400 µg/mL in methanol) of ascorbic acid solution (1 mL) and *C. sophera* flavonoid components extracted from methanol extract of floral solutions (1 mL) were mingled individually with 3 ml of DPPH solution (0.4 mM). A UV-Visible spectrophotometer was used to detect absorbance (517 nm) after 30 minutes in the absence of light, and ascorbic acid was used as control. For each test solution, the entire procedure was repeated three times. The reaction mixture's lower absorbance suggested stronger free radical cleansing potential. The extent of DPPH color transformation (from purple to yellow) showed the extract's scavenging activity. Scavenging activity with respect to DPPH was computed from the equation scavenging activity (%) = [(A-B)/A] × 100. Where A signifies the absorbance of the control, and B signifies the absorbance of the DPPH solution with sample. Following a plot of scavenging activity (%) vs concentration, linear regression analysis was used to determine the IC50 (concentration 50% inhibition) value. Three times of each procedure were completed, and the results were averaged.

Cytotoxicity

The cytotoxic activity of the compound was evaluated using the brine shrimp lethality bioassay method (Oladimeji *et al.*, 2006). The following serial dilutions of DMSO were used to dissolve the isolated compound test samples: 150, 75, 37.5, 18.5, 9.375, 4.684, 2.344, 1.172, 0.586, and 0.292 g/mL. A test tube containing 10 shrimp and simulated brine water was then filled with the test solutions, and it was left to incubate for 24 hours at room temperature. After 24 hours, the test samples' median lethal concentration (LC_{50}) was determined by graphing the proportion of shrimps versus the logarithm of the sample concentrations (Finney method). Vincristine sulfate (LC_{50}=0.57) was used in this assay as a positive control to compare the cytotoxicity of the test substances.

Antibacterial screening

Isolated compound test samples were dissolved separately in precise volumes of chloroform or methanol, depending on how soluble they are. The disk diffusion method was then used for antibacterial screening (Barry, 1986; Bayer *et al.*, 1966). For this test, diluted samples were applied to sterile discs (100 µg/disc, Oxford, UK), with streptomycin (10 µg/disc, Oxford, UK) serving as the reference.

Results and discussion

The IC_{50} values of isolated compounds (lupeol, stigmasterol, ursolic acid, myricetin and naringenin) from n-hexane, ethyl acetate and methanolic extract of leaves of *Catharanthus roseus* are shown in Table 1. The results represented the noteworthy antioxidant activity of flavonoid compound myricetin with IC_{50} values of 9.60 and 10.86 µg/mL, respectively, which is quite comparable to the IC_{50} value (4.64 µg/mL) of the positive control, L-ascorbic acid.

Table 1. *Catharanthus roseus* leaves isolated compounds from various extract (free radical scavenging activity)

Test compound	IC_{50} µg/mL
L-Ascorbic acid	4.64
Lupeol	16.80
Ursolic acid	22.34
Stigmasterol	26.20
Myricetin	6.18
Naringenin	7.34

The availability of phenolic hydrogens and the potential for stabilizing the phenoxy radicals generated by hydrogen bonding or extended electron delocalization are two factors that influence the free radical scavenging activity of flavonoids (Rechner *et al.*, 2002). Although the separated compounds' DPPH radical scavenging properties were less than those of ascorbic acid, the study found that all flavonoids and flavones exhibit proton-donating capability and could serve as free radical inhibitors or scavengers, feasibly

acting as major antioxidants. Thus, the isolated substances from *Catharanthus roseus* leaves may be a promising candidate for the therapy of free radical damage. From the detailed analysis of isolated compounds from various extracts of *Catharanthus roseus*, it can be concluded that the isolated compounds myricetin and naringenin (flavonoids and flavones) showed more potent activity as DPPH free radical scavengers.

The LC_{50} values of isolated compounds ursolic acid (triterpenoids) were found to be 0.72 in comparison to the positive control (vincristine sulfate) (LC_{50} = 0.571 g/mL) in the brine shrimp lethality test. Whereas flavonoids like myricene showed an LC_{50} = 10.69 µg/mL value (Table 2).

Table 2. *Catharanthus roseus* leaves isolated compounds from various extracts (Cytotoxic effect on brine shrimp nauplii)

Tested material	LC_{50} = 0.571 µg/mL
Vincristine sulphate	0.571
Lupeol	5.24
Stigmasterol	9.20
Ursolic acid	0.72
Myricetin	10.69
Naringenin	9.31

Compared with the positive control (vincristine sulfate) (LC_{50} = 0.571 µg/mL), all the test samples were deadly to brine shrimp nauplii. Nonetheless, both the triterpenoids (ursolic acid and lupeol) from leaves demonstrated strong efficacy in the brine shrimp lethality bioassay. These encouraging findings showed that they could have anticancer or pesticide properties.

The antibacterial activities of the isolated compounds from the leaves of *Catharanthus roseus* were tested at 100 µg/disc against a few pathogenic bacteria, where streptomycin (10 µg/disc) served as a standard antibiotic disc. The results are shown in Table 3.

From the investigated compounds, naringenin showed a moderate zone of inhibition against almost all pathogenic microorganisms. Additionally, it was shown that the active substances were less effective against gram-negative bacteria than gram-positive bacteria, which may be related to variations in the chemical composition and cell wall construction of both types of microorganisms.

Table 3. *Catharanthus roseus* leaves isolated compounds from various extracts (Antibacterial screening)

Test microorganism	Diameter of zone inhibition (mm)					
	Lupeol	Stigmasterol	Ursolic Acid	Myricetin	Naringenin	Streptomycin
Gram-positive bacteria*						
Bacillus cereus FM1042	NA	4	5	5	9	16
Staphylococcus aureus FM1011	5	4	5	6	10	15
Bacillus megaterium FM1047	NA	3	NA	NA	11	21
Staphylococcus sp. FM1018	NA.	NA	NA	NA	15	17
Gram-negative bacteria*						
Vibrio cholerae FM2022	4	NA	3	5	10	14
Escherichia coli FM2087	NA	5	Na	5	6	10

Streptomycin (Std., 10.0 µg/disc)

* The mentioned Gram-positive and Gram-negative pathogenic microorganisms were collected as pure cultures from the Institute of Food Science and Technology (IFST), BCSIR, Dhaka, Bangladesh.

Conclusion

Free radical scavenging activity, cytotoxicity, and antibacterial activity of isolated compounds (lupeol, stigmasterol, ursolic acid, myricetin & naringenin) from n-hexane, ethyl acetate, and methanolic extract of leaves of *Catharanthus roseus* were found to be congruent with local people's traditional use of this plant. Further investigation is needed into more isolated compounds of this medicinally active plant to evaluate active phytoconstituents from *Catharanthus roseus*.

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