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Phytochemical screening and antioxidant activity of rare medicinal orchid *Luisia zeylanica* Lindl

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Abstract
The present investigation deals with phytochemical screening and antioxidant activity of a rare medicinal orchid *Luisia zeylanica* Lindl. of Bangladesh. Extraction of the leaf, stem and root of the plant were fractionated into four fractions *viz*. Methanol, n-Hexane, Butanol and Dichloromethane (DCM). Qualitative assessment of phytochemicals mainly alkaloids and other secondary metabolites viz. flavonoid, steroid, saponin, phlobatannin, terpinoid, tannin, glycoside, anthroquinone, quinine and coumarin were screened out in this study. Among the three studied parts, leaf extract was found superior to others in terms of ten other studied secondary metabolites. Antioxidant activity of four different fractions of root, stem and leaf of *Luisia zeylanica* Lindl were also studied. Based on the acquired results it is concluded that, stem and root have the highest antioxidant activity. Present study provides novel findings on the efficacy of these orchid species and promotes the continued research of medicinal orchids in Bangladesh.

Keywords: *Luisia zeylanica* Lindl, Phytochemical, Antioxidant, Secondary metabolite

Introduction
Orchids are not only important for their attractive horticultural and commercial value but also important for medicinal properties. Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine [1]. Bangladesh is rich in orchids, with 159 species and 2 varieties under 63 genera [2] and later on the number of species updated into 178 [3]. These species are distributed mainly in the hilly areas of greater Sylhet, Chittagong, Chittagong Hill Track and Mymensingh district [4]. Fifty-three orchid species belonging to 34 genera have exhibited various medicinal properties [5]. In Bangladesh about 26 species of the orchids also been used by the tribal people of Bangladesh to treat different diseases [6]. Orchid species contain different types of alkaloid e.g. dendrobine (derived from *Dendrobium nobile*) which provokes violent uterine contraction, progressively paralyzes peristalsis, low blood pressure and as analgesic and vanillin (*Vanilla sp*) is reputed to have aphrododisiac, carminative, tonic, antispasmodic and stimulant properties [7]. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians [8]. Pharmacological studies conducted on orchids indicate the immense potential of these plants in treatment of conditions such as neurodegenerative disorders, anticonvulsive, anticancer, antidiabetic etc. [9]. *Bulbophyllum lilacinum* Ridl. is a member of the family Orchidaceae having flowers of wonderful beauty and also medicinal value. Extracted juice from the pseudobulbs of *Bulbophyllum* species are used for restoration of adolescence and also as tonic [10].

Recently there has been observed an increase interest in the therapeutic potential of medicinal plants as in reducing of free radical induced tissue injury [11,12]. An antioxidant is a molecule capable of showing or preventing the oxidation of other molecules such as free radicals or reactive oxygen species (ROS). Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species [13].

Bangladesh, though is a small country has a valuable heritage of herbal remedies. Its rural and tribal people living in remote areas still depends on the indigenous system of medicine. Both Ayurvedic, Unani and homeopathic systems are exist in the country. Use of medicinal plants are getting importance day by day. There are substantial numbers of medicinal plants in Bangladesh [14, 15] documented more than 500 plants. Most of them have more of less antimicrobial properties. In our country these medicinal plants were extensively used in both raw and semi processed forms as medicines in various pharmaceutical dosage forms while some of these medicinal plants are procured from indigenous sources through imports from other countries although many of these imported plants grow naturally or under cultivation in

this country. Further, many medicinal plants suitable for commercial development as therapeutic agents are readily available in this country and many of them are highly efficacious and are internationally recognized as official drugs [16]. Some medicinal plant are used in the preparation of Kabiragi, Hakimi, Unani, Ayurvedic, Homeopathic and Allopathic system of medicine [17]. Disease like cancer, AIDS (Acquire Immune Deficiency Syndrome) etc. are still unimpeded. No specific active properties of medicine have been yet discovered against to treat these diseases. It is alarming that twelve hundred plant species is being extinct in every year and this figure is gradually increasing. There might be many valuable components, which would help us to combat against the above mentioning fatal diseases. Therefore, we should keep our restless effort to find out the valuable components before extinction for the incoming generation so that they can lead their lives peacefully and happily. So aim of the present investigation is to discover important photochemical components from rare orchids of Bangladesh.

Material and Methods

Collection of plant materials

The Root, leaf and stem of *Luisia zeleynica* Lindl. were used for the qualitative estimation of alkaloids and other secondary metabolites *viz.* flavonoid, steroid, saponin, phlobatannin, terpinoid, tannin, glycoside, anthroquinone, quinine and coumarin. The plant was collected from Kaptai National Park, Rangamati. Samples were thoroughly washed with water and dried in oven at 65^0C for 48 hours. It was then ground into coarse powder by using grinding machine and stored in airtight container for further investigation. Mixing of one part with another was carefully avoided. The voucher specimen of the orchid species preserved at the herbarium of Chittagong University.

Preparation of plant extract

25 gm of sample from each of part were taken for further analysis. 50 ml of Methanol was added to the 25 gm of samples in a conical flask. Shaken very well for 30 minutes and then kept overnight and then shaken again and sonnicated for 10 minutes and then filtered using Whatman filter paper No 1. The process was repeated for three times with Methanol and the extract was then rotavaporated. The dried sample was kept as crude sample for each part. The concentrated crude extract was separated into four different solvent systems (Methanol, n-Hexane, Butanol-1, Dichloromethane) by following kupchan modified method.

Phytochemical screening

Qualitative tests were carried out on the fresh sample, powdered specimens and methanol extracted crude using standard procedures to identify the constituents as described [18-20].

Test of alkaloids: For qualitative test of alkaloid, the most reliable and rapid testing method was develop [21] and the method was slightly modified [22]. For the qualitative test of alkaloid, five alkaloid detecting reagents were used. These are Dragendroff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Wagner's reagent (W) and Tannic acid reagent (T). These reagents were prepared following the methods [23].
5 gm fresh finely chopped and pasted plant material was mixed up to moisten with 10 ml 2% HCL and heated in water bath of 60° C for one hour. After cooling the extract was filtered through Whatman No. 1 filter paper. Two drops of extract were put on a microscopic groove slide with one drop of the alkaloid detecting reagent. The relative abundance of precipitate, if any formed in the plant extract with the reagent was considered as an index of the quality of the presence of alkaloid and was expressed by'+', '++' and '+++' signs which mean slight, moderate, substantial to heavy amount respectively. No precipitate was indicated by ' - ' (negative sign) and stood for the absence of alkaloid in the plant extract. Phytochemical screening of *Luisia zeleynica* Lindl. Orchid species for secondary metabolites were analyzed following standard methods.

Test for phlobatannins: Deposition of a red precipitate when an aqueos extract of each plant sample was boiled with 1% aqueous hydrochloric acid (HCL) was taken as evidence for the presence of phlobatannins [24].

Test for flavonoids: A portion of the crude powdered plant sample was heated with 10 ml of ethyl acetate over a stem bath for 3 min. The mixture was filtered and 4 ml of filtrate was shaken with 1 ml of dilute Ammonia solution. A yellow coloration was observed indicating a positive test for flavonoides [24].

Test for saponins: About 2 gm of crude powder was boiled with 20 ml of distilled water in a water bath and filtered. 10 ml of filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The persistent of froth indicates the presence of saponins [25].

Test for tannins: About 0.5 gm of the crude powdered samples boiled in 10ml of distilled water in a test tube and filtered. A few drops of ferric chloride reagent added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins [26].

Test for terpenoids: 0.5 gm of crude powder was dissolved in 5 ml of methanol. 5 ml of the extract was treated with 2 ml of chloroform in a test tube. 3 ml of concentrated sulphuric acid carefully added to the mixture to form a layer. An interface with a reddish brown coloration formed if terpenoid constituent is present [27].

Test for steroids: 0.5 gm of crude powder was dissolved in 5 ml of methanol 1 ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids [27].

Test for glycosides: 0.5 gm crude powder was dissolved in 5 ml of methanol. 10 ml of 50% HCL was added to 2 ml of methanolic extract in a test tube. Then it was heated in a boiling water bath for 30 minutes. 5 ml of Fehling's solution was added to the mixture and the mixture was boiled for 5 minutes. A brick-red precipitate was taken as evidence for the presence of glycosides [26].

Antioxidant activities

The antioxidant activity of the Methanolic, n-Hexane, Butanol-1 and DCM extract of the root, leaf and stem of *Luisia zeleynica* Lindl. and the standard antioxidant ascorbic acid were assessed on the basis of the free radical scavanging effect of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH, MWt.394.32) free radical activity according to the method described [28] with slight modification.

DPPH assay

The reaction mixture contained 3 ml 0.004% DPPH in 100% methanol and 5ml crude extract or ascorbic acid solution in case of experiment or standard control, respectively. After 30 min incubation period at room temperature (19 ^{0}C) in the dark, the absorbance, optical density (OD), was measured against a blank at 517 nm in UV-Visible Spectrophotometer (Shimadzu, Japan). The degree of discoloration of DPPH from purple to yellow following reduction indicated the scavenging efficiency of the extract or ascorbic acid solution. Lower absorbance followed by the degree of discoloration of the reaction mixture indicated the free radical scavenging efficiency of the substances. The percentage of DPPH discoloration (scavenging) activity was calculated with the help of the following formula:

% of scavenging activity $= \left(\frac{A-B}{A}\right) \times 100$

Where, *A* was the absorbance of control (DPPH solution without the sample), *B* was the absorbance of DPPH solution in the presence of the sample. Values are presented as mean with ± SE of the mean of three replicates. The % scavenging activity was plotted against log concentration and the IC_{50} (inhibition concentration 50 µg/ml) value of plant extract was calculated by using linear regression analysis.

Result and Discussion

Photochemical screening

Root, stem and leaf of *Luisia zeylanica* gave different test for alkaloids with one or another type of reagents. In root, Dragendroff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Tannic acid (T) and wagner's reagent (W) gave '++', '+', '++', '+++' and '+++', respectively whereas in stem, Dragendroff's reagent (D) gave '+', Hager's reagent (H) gave '+', Mayer's reagent (M) gave '++', Tannic acid (T) gave '+++' and Wagner's reagent gave '+++', indicating the presence of alkaloids and in leaf, Dragendroff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Tannic acid (T) and wagner's reagent (W) gave '+', '+', '+', '+++' and '+++', respectively. All the test parts of the species gave positive response for alkaloids. Mayer's reagent (M), Tannic acid (T) and wagner's reagent (W) gave '+++', '++', '+++', '+++' and '+++', respectively. All the test parts of the species gave positive response for alkaloids (Table 1). Theng [29] who found similar type of results and noted the presence of alkaloid in *Geodorum densiflorum* on orchid.

Table 1: Qualitative test for alkaloids of *Luisia zeylanica* Lindl.

Plant parts used	Qualitative estimation of alkaloids by different reagents				
	D	H	M	T	W
Root	++	+	++	+++	+++
Stem	+	+	++	+++	+++
Leaf	+	+	+	+++	+++

Note: Name of the reagents, D-Dragendroff's reagent, H-Hager's reagent,M-Mayer's reagent, T-Tannic acid reagent and W-Wagner's reagent.

In addition to alkaloids, qualitative assessment for ten other secondary metabolites, *viz.* tannin, flavonoids, steroids, phlobatannins, saponins, glycosides, terpinoids, anthroquinones, Quinine and coumarin were also done in root, stem and leaf of *Luisia zeylanica* Lindl. In the present work, root of *Luisia zeylanica* Lindl gave three '+++' test with tannin, anthroquinone and quinine. Four'++' test with phlobatannin, saponin, steroid, and coumarin whereas three '+' test with glycosides, flavonoids and terpinoids. Stem of this test species gave five '+++' test with glycoside, saponin, tannin, steroid, quinine and coumarin, four '++' test with glycosides, flavonoids, terpinoids and anthroquinone while one '+'with phlobatannins. On the other hand, leaf extract of *Luisia zeylanica* gave eight '+++' test with flavonoids, saponins, tannins, terpinoids, steroids, Anthroquinone, quinine and coumarin. One '++' test with glycosides and one '+' phlobatannins (Table 2). Johnson and Janakiramam [30] found the presence of steroid, terpenoid, alkaloids, tannins, phenols and flavonoides in *Dendrobium panduratum* Theng [29] reported that *Geodorum densiflorum* leaves contain alkaloid, glycoside, steroids, saponins, carbohydrates, tannin and flavonoids. Bhattacharjee [31] showed the analysis of alkaloid, terpenoid, flavonoids, phenols, tannins, steroids and glydosides of the orchid *Vanda tessellata.* Harshitha [32] showed the presence of alkaloids, tannin, phenol and reducing sugar in *Bulbophyllum neilgherrense.*

Table 2: Qualitative test for ten Secondary metabolites of *Luisia zeylanica* Lindl

Plant parts used	Secondary metabolites (% of coloration)									
	Gly.	Flv.	Phl.	Sap.	Tan.	Ter.	Str.	Ant.	Qui.	Cou.
Root	+	+	++	++	+++	+	++	+++	+++	++
Stem	++	++	+	+++	+++	++	+++	++	+++	+++
Leaf	++	+++	+	+++	+++	+++	+++	+++	+++	+++

Note: Gly. = Glycosides, Flv. = Flavonoids, Phl.= Phlobatannins, Sap.= Saponins, Tan.= Tanins. Ter.= Terpinoids, Str.= Steroids, Ant.= Anthroquinone, Qui.= Quinine, Cou.= Coumarin.

Antioxidant activity

Free radical assay is one of the most widely used methods and has become a routine work in establishing the antioxidant activity of herbal extracts and phytochemicals. Among the five different concentrations used in the present study (50, 100, 150, 200 and 250 µg/ml) ascorbic acid showed 99.833%, 98.671%, 98.837%, 98.671% and 98.172% scavenging activity respectively, where highest scavenging activity was 99.833% at concentration 50 µg/ml, while, Methanolic fraction of root where highest scavenging activity was 88.206% at concentration 150 µg/ml (Fig. 1). The fraction n-Hexane of root showed the highest activity 92.359% at concentration 250 µg/ml (Fig. 1). The Butanol fraction of root showed highest activity 92.359% at concentration 200 µg/ml (Fig. 1). The DCM fraction of leaf (Fig. 3) exhibited the highest activity 92.53% at concentration 200 µg/ml. Methanolic fraction of stem showed activity 90.70% at concentration 250 µg/ml and n-Hexane fraction of stem where showed the highest activity 92.53% at concentration 250 µg/m (Fig. 1). The Butanol fraction of stem showed activity 89.53% at concentration 250 µg/ml (Fig. 2). The DCM fraction of stem where highest scavenging activity was 87.87% at concentration 100 µg/ml (Fig. 2).The Methanolic fraction of leaf showed scavenging activity 89.04% at concentration 200 µg/ml (Fig. 3). The n-Hexane fraction of leaf showed scavenging activity 92.03% at concentration 50 µg/ml and the Butanol fraction of leaf where showed scavenging activity 90.86% at concentration 50 µg/ml

whereas the DCM fraction of leaf showed highest scavenging activity 89.04% at concentration 200 µg/ml (Fig. 3). Ghosh [33] described the *in vivo* antioxidant activity of ethanolic extract of *Bacopa monniera*. Aerial parts and the extracts prevented significant elevation of glycosylated hemoglibin in vitro, with IC_{50} value being 11.25 µg/ml. Mishra [34]. tested the antioxidant activity of methanolic extract of *Coccinia grandis* and it was found as 54.6 µg/ml. It may be concluded that *B. monniera* and *C.grandis* possess significant antioxidant activity compared to other well characterized, standard antioxidant systems *in vitro* and may serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants, possibly due to the presence of secondary metabolites like alkaloids, tannins, flavonoids, saponins etc. According Mukherjee [35] aqueous extract of aseptically regenerated *Dendrobium aqueum* was used for *in vitro* estimation of antioxidant and antiglycation potential. *D. aqueum* extract showed a dose dependent DPPH free-radical scavenging potential and exhibited a significant antiglycation potential.

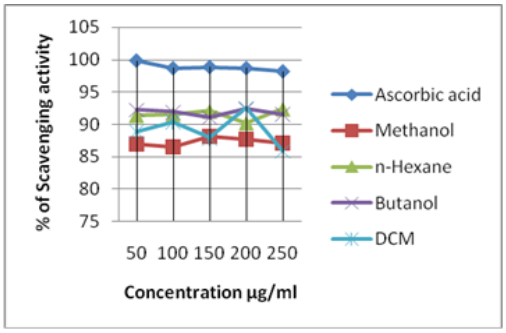

Fig 1: Relative % of Scavenging activity or % inhibition of standard antioxidant Ascorbic acid and Methanol, n-Hexane, Butanol and DCM fraction of root of *Luisia zeylanica* Lindl.

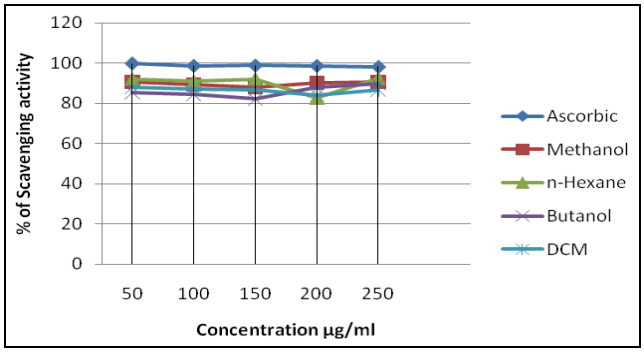

Fig 2: Relative % of Scavenging activity or % inhibition of standard antioxidant Ascorbic acid and Methanol, n-Hexane, Butanol and DCM fraction of stem of Luisia zeylanica Lindl.

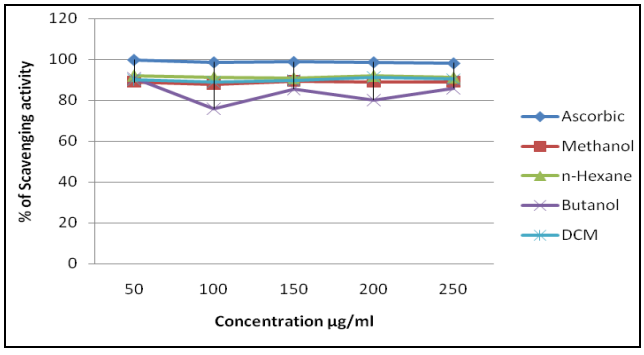

Fig 3: Relative % of Scavenging activity or % inhibition of standard antioxidant Ascorbic acid and Methanol, n-Hexane, Butanol and DCM fraction of leaf of *Luisia zeylanica* Lindl.

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