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ANTIOXIDENT, ANALGESIC AND CYTOTOXIC ACTIVITY OF METHANOLIC EXTRACT OF VANDA ROXBURGHII ROOT

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ABSTRACT: The crude methanolic study of *Vanda roxburghii* root was investigated for antioxidant, analgesic and cytotoxic activity. The extract of *Vanda roxburghii* root showed antioxidant activity in DPPH radical scavenging activity, nitric oxide scavenging power assay. In DPPH and NO scavenging assay the extract shown adequate antioxidant activity and the IC₅₀ values in DPPH radical scavenging and NO scavenging assays were found to be 113.35±1.27 and 127.31±0.26ug mL⁻¹ while the IC₅₀ values of ascorbic acid were 12.30±0.11 and 18.64±0.22 ug ml⁻¹. Reducing power activity of the extract increased in a dose dependent manner. Analgesic activity of the crude extract was evaluated using acetic acid induced writhing model of pain in mice. The crude extract at 200 and 400 mg kg⁻¹b.wt. doses displayed significant (p<0.001) reducing in acetic acid induced writhing in mice with a maximum effect of 75.89% reduction at 400 mg kg⁻¹b.wt. Which is comparable to the standard diclofenac sodium (86.52%). The extract was also investigated for cytotoxic activity using Brine Shrimp lethality bioassay. In this the extract showed significant toxicity of Brine Shrimp nauplii with the LC₅₀ value of 25.19±0.98 ug mL⁻¹. The study indicates that the extract possesses good analgesic and cytotoxic activity along with moderate antioxidant activity.

INTRODUCTION: Free radicals formed during metabolism or due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress can interact with bio-molecules and ultimately lead to cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases.

Free radicals contribute to more than one hundred disorders in humans including arteriosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer, AIDS, Diabetes, autoimmune disorders and aging^{1, 2, 3}.

Although the body possesses such defense mechanisms as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS *Tessellate*⁴, continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage⁵. A free radical is a chemical species,

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which is capable of independent existence and posses one or more unpaired electrons that bestow it with immense reactivity. This reactivity is inversely related to their stability³. To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system, the so-called antioxidant defensive system. It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals⁵.

Vanda roxburghii Br. (Family: Orchidaceae) is an epiphytic perennial, stem 30-60 cm long, stout, scandent by the stout, simple or branching aerial roots. Leaves succulent, 15-20 cm, long, linear, recurved, complicate. Flowers in 6-10 flowered racemes, reaching with the peduncle 15-25 cm long. *Vanda roxburghii* plants have been used in the indigenous medicine such as Ayurveda and local traditional medical practices⁶. The leaf juice is used for the treatment of certain inflammatory conditions. It is also instilled into the ear as a remedy for Otitis. The leaves in the form of a paste are applied to the body to bring down fever⁷. The roots were used in rheumatism, nervous problems, bronchitis and dyspepsia⁸. Unani practitioners hold it to be laxative and tonic to the liver. It is also used to treat hiccough, piles, and boils on the scalp. *V. tessellate* has not been evaluated in depth for its pharmacological properties, in spite of its traditional use in numerous medical conditions⁹. It is also a remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in Otitis and the paste as febrifuge.

The roots possess significant anti-inflammatory activity. The plant has an alkaloid, flavonoids glycoside, tannins, β -sitosterol, γ -sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosylferrulate and β -sitosterol-D-glucoside¹⁰. It also enters the composition of several medicated oils for external application in rheumatism and diseases of the nervous system. Roots were reported to possess antibacterial and antitubercular properties¹¹. The methanol extract of this plant root also showed remarkable anti-inflammatory activity against carrageenan – induced oedema in

rodents¹². The traditional use indicates that various parts of this plant are likely to have several pharmacological properties. Lawler reported that several Ayurvedic type preparations containing this plant (root or whole plant) were used as aphrodisia¹³. According with all the function of *V. tessellate* this plant root may also have some medicinal effects also. In this study we investigate the antioxidant, analgesic and cytotoxic activity of plant *Vanda roxburghii* root MeOH extract.

MATERIALS AND METHOD:

Plant Material: The fresh roots of the plant, *Vanda roxburghii* was selected for the chemical and biological investigations. Since the plant is parasitic, therefore, the whole plant was collected from Dhaka University Campus during the month of August, 2015 and identified by an expert taxonomist. A voucher specimen was submitted to the herbarium of the Department of Botany, Dhaka University, Dhaka.

Preparation of plant material:

The collected roots were first washed with water to remove adhering dirt and then sun dried for 15 days and finally dried in an electric oven for 24 hours at a temperature below 50°C. The roots were pulverized into coarse powder with the help of a grinder and stored in an airtight container for further use. The coarse powder is preliminary extracted with methanol. The dried coarse powder (600gm) was soaked in 2L of methanol in a clean flat bottomed glass container for 7 days with occasional shaking and stirring. The extract was filtered through several means, e.g. cotton, cloth, filter paper (Whatman No 1). The filtrate was then concentrated with a rotary evaporator under reduced pressure and finally obtained crude extract (12.75gm).

Chemicals:

Sodium nitroprusside were purchased from E. Merck (Germany). 1,1 -diphenyl-2-picryl-hydrazyl (DPPH), sodium nitroprusside, ascorbic acid, quercetin and potassium ferric cyanide were purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). Diclofenac-Na was collected from Square Pharmaceuticals Ltd., Bangladesh. All other chemicals and reagents were of analytical grade.

Animal:

For the experiment, twenty swiss albino mice of either sex, 3-4 weeks of age, weighing between 20-25 g, were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). Animals were maintained under standard environmental conditions (temperature: (24.0±1.0°C), relative humidity: 55-65% and 12 h light/dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to experiments. The experimental processes were approved by the Institutional Ethics Committee (SUB/IAEC/12.01)

Acetic acid-induced writhing test:

The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% v/v acetic acid but Diclofenac-Na was administered intraperitonally 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min¹⁴.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay:

DPPH was used to evaluate the free radical scavenging activity of various compounds and medicinal plants¹⁵. 2 ml of methanol solution of plant extract or standard at different concentration was taken in a test tube. 3 ml of methanol solution of DPPH was added into the test tube. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution. The percentage (%) inhibition activity was calculated from the following equation.

$$\text{Scavenging activity \%} = (1 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard.

Reducing power capacity assessment:

The reducing power of different extractives of *V.roxburghii* was evaluated by the method of Oyaizu¹⁷. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. 1.0 ml of plant extract or standard of different concentration solution was taken in a test tube. 2.5 ml of potassium buffer (0.2 M) and 2.5 ml of Potassium ferricyanide [K₃Fe (CN)₆], (1%) solution were added into the test tube. The reaction mixture was incubated for 20 minutes at 50°C to complete the reaction. 2.5 ml of trichloro acetic acid, (10%) solution was added into the test tube.

The total mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml supernatant solution was withdrawn from the mixture and mix with 2.5 ml of distilled water. 0.5 ml of ferric chloride (FeCl₃), (0.1%) solution was added to the diluted reaction mixture. Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution mixture without plant extract or standard and it was incubated under the same conditions as the rest of the samples solution. Also the absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation. Increased absorbance of the reaction mixture indicated increase reducing power.

Phytochemical screening:

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann-Burchard reagent. Reducing sugars with Benedict's reagent. These were identified by characteristic color changes using standard procedures¹⁹.

Nitric oxide radical scavenging assay:

The procedure is based on tire method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered solution (pH 7.4) was mixed with different concentrations of plant extract of *Vanda roxburghii* dissolved in 10% DMSO and incubated at room temperature for 150 min.

The same reaction mixture without the extract but the equivalent amount of the solvent used served as the control. After incubation, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N-(l-naphthyl) ethylenediaminedihydrochloride was added. The absorbance was measured at 546 nm and the percentage inhibition activity was calculated from [(A₀-A₁/A₀] × 100, where, A₀ is the absorbance of the control and A₁ is the absorbance of the extract/ standard¹⁸. IC₅₀ value was calculated from the equation of line obtained by plotting a graph of concentration (ug mL⁻¹) versus % inhibition.

Brine shrimp lethality bioassay:

TABLE 1: RESULT OF CHEMICAL GROUP TESTS OF THE METHANOL EXTRACT OF VANDA ROXBURGHII ROOT

Sample	Alkaloid	Steriod	Flavonoid	Sugar	Tannin	Saponin	Glycoside
<i>Vanda roxburghii</i>	+++	++	-	-	+	-	+++

ME: Methanol extract, + Present, - Absent, +++ Reaction intensity is high, ++ Reaction intensity is medium, + Reaction intensity is normal

In vivo Analgesic screening:

Acetic acid-induced writhing test:

The effect of the extract of on acetic acid induced writhing in mice (**Table 2**). The oral

The cytotoxic activity of the plant was evaluated using Brine Shrimp lethality bioassay method²⁰ where 6 graded doses (viz, 5, 10, 20, 50,100 and 200 ug mL⁻¹) were used. Brine shrimps (*Artemiasalina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 h. The nature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. The median lethal concentration LC₅₀ of the test sample after 24 h was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. Vincristine sulfate was used as a reference standard in this case.

Statistical analysis:

All the *in vitro* experimental results were Mean ±SEM of three parallel measurements. Results of *in vivo* study were given as Mean ±SEM. and data were evaluated by using student's test. P values<0.001 were regarded as significant.

RESULT:

Phytochemical screening: Phytochemical analysis of the crude extract revealed the presence of steroid, alkaloid, tannin and glycoside (**Table 1**).

administration of both doses of *V.roxburghii* root extract significantly (p<0.001) inhibited writhing response induced by acetic acid in a dose dependent manner.

TABLE 2: EFFECTS OF THE METHANOLIC EXTRACT OF VANDA ROXBURGHII ROOT ON ACETIC ACID INDUCED WRITHING IN MICE

Groups	Dose (mg kg ⁻¹)	No of writhing	%inhibition
I	Vehicle	28.2±0.748	-
II	10	7.8±1.008**	86.52
III	200	11.8±0.765**	0.16
IV	400	6.8±0.637**	68.89

Values are Mean ± SEM, (n=5), **p<0.001, student's test as compare to vehicle control.

Group-I animals received vehicle (1% Tween 80 in water), Group-II received Diclofenac Sodium 10 mg kg⁻¹ body weight, Group-III and Group-IV were treated with 200 and 400 mg kg⁻¹ body weight (p.o) of the crude extract of *Vanda roxburghii* root.

In vitro antioxidant activity:

DPPH radical scavenging activity:

The percentage (%) scavenging of DPPH radical was found to be concentration dependent i.e., concentration of the extract between 10-200 µg mL⁻¹ greatly increasing the inhibition activity. The IC₅₀ value of the extract was 113.35±1.27 µg mL⁻¹, as opposed to that of ascorbic acid (IC₅₀ 12.30±0.11 µg mL⁻¹) which is a well-known antioxidant (**Table 3**).

TABLE 3: ANTIOXIDENT ACTIVITIES OF THE VANDA ROXBURGHII ROOT ON DPPH AND NO

Sample	DPPH IC ₅₀ (µg mL ⁻¹)	NO IC ₅₀ (µg mL ⁻¹)
Methanolic extract	113.35±1.27*	127.31±0.26*
of <i>Vanda roxburghii</i>		
Ascorbic acid	12.30±0.11	18.64±0.22
Quercetin		27.69±0.57

DPPH is the free radical scavenging activity (IC₅₀ µg mL⁻¹), NO is the inhibition of nitric oxide production (IC₅₀ µg mL⁻¹), *p<0.001 by student's test for values between the sample and the control.

Nitric Oxide (NO) scavenging activity:

Table 3 represent the result of nitric oxide scavenging activity (IC₅₀ value) of the methanolic extract of *Vanda roxburghii* compared with various reference compounds such as ascorbic acid and quercetin. IC₅₀ value of the extract of was 127.31±0.26 µg mL⁻¹ respectively.

Reducing power ability:

For the measurement of the reductive ability preset study investigated the Fe³⁺ to Fe²⁺ transformation in the presence of methanolic extract. Like the antioxidant activity, the reducing power of *Vanda roxburghii* increased with increasing concentration of the sample. (**Table 4**) shows the reductive capabilities of the *Vanda roxburghii* compared with ascorbic acid, galic acid and quercetin.

TABLE 4: REDUCING ABILITY OF V.ROXBURGHII

Sample	% Reducing power Mean±SE
<i>Vanda roxburghii</i>	263.69±1.12
Ascorbic acid	485.653±0.19
Gallic acid	736.301±0.36
Quercetin	763.01±0.54

In vitro cytotoxicity activity:

Brine Shrimp lethality bioassay:

The result of Brine Shrimp lethality bioassay is given in **Table 4**. *Vanda roxburghii* root extract displayed strong toxic potentiality. LC₅₀ value for the extract was found to be 27.32±1.21 µg mL⁻¹.

TABLE 4: BRINE SHRIMP LETHALITY BIOASSAY OF CRUDE METHANOL EXTRACT OF VANDA ROXBURGHII ROOT

Test sample	Concentration (µg mL ⁻¹)	Log Conc.	No. of dead shrimps (out of 10)	Mortality (%)	LC ₅₀ of test sample (µg mL ⁻¹)	LC ₅₀ of Vincristine sulphate (µg mL ⁻¹)
Mathanolic extract of <i>Vanda roxburghii</i>	320	2.303	10	100		
	160	1.042	10	100		
	80	0.903	9	90		
	40	0.602	7	70		
	20	0.301	4	40		
	10	1.000	2	20	27.32±1.21	0.25±0.22

DISCUSSION: The oral administration of both doses of *Vanda roxburghii* extract significantly (p<0.001) inhibited writhing response induced by acetic acid in a dose dependent manner. Acetic

acid-induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipid¹⁴.

The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The response is thought to be mediated by peritoneal mast cells²¹, acid sensing ion channels (Vogel and Vogel, 1997) and the prostaglandin pathways²². Preliminary phytochemical screening showed the presence of tanin, alkaloid and glycoside in the plant extract. However, tetrrandrine and fangchinoline alkaloids were isolated from *S. japonica* and showed anti-inflammatory effect through decrease leukotriene and prostaglandin generation.

Furthermore tetrandrine has been shown to inhibit the production of TNF-alpha and IL-6²³. It was demonstrated that aconitum and *S. tetrandra* combinedly showed remarkable analgesic activity in rabbits and mice model²⁴. So, the observed analgesic activity may be attributed to these compounds. Moreover, recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites²⁵. Again the plant extracts demonstrated antioxidant action in the tested models.

So it can be assumed that Cyclooxygenase (COX) inhibitory activity together with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relieve pain-sensation. Polyphenolic compounds, like flavonoids, tannins and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity^{26, 28}.

Fangchinoline and cepharantheine isolated from *Stephan rotunda* showed antioxidant activity in different *in-vitro* model²⁹. Tannic acid present in the plant extract, as evident from phytochemical screening, may be responsible for the antioxidant action. NO scavenging capacity of the extract may help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to die human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions³⁰. A direct correlation between antioxidant capacity and reducing power of certain plant extracts has been reported³¹. The reducing

properties are generally associated with the presence of reductase, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom³². The extract also showed significant cytotoxicity on Brine Shrimp nauplii. This may be due to the fact that *Vanda roxburghii* contains tetracosylferrulate and β-sitosterol-D-glucoside²⁷ which was previously reported work on antibacterial and antitubercular properties¹¹.

CONCLUSION: The study clearly indicates that the extract possesses antioxidant and cytotoxic substances. At the same time its ability to suppress abdominal writhes confirms the analgesic property of the extract. These findings justify the traditional uses of this plant in the treatment of heart disease and high cholesterol. It is also used for boosting the immune system and for preventing colon cancer, as well as for gallstones, the common cold and flu, asthma, hair loss, migraine headache, and chronic fatigue syndrome. Further research is necessary for elucidating the active principles.

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