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ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC CHARACTERISTICS OF A BIOACTIVE COMPOUND ISOLATED FROM THE AERIAL PART OF *DICKSONIA ANTARCTICA* LABB.

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ABSTRACT

The ethanolic extract of *Dicksonia antartica* Labb leaves was extracted in order to explore and evaluate the pharmacological value in terms of antioxidant property, antimicrobial potential, anthelmintic activity and cytotoxic activity on the basis of brine shrimp lethality bioassay, which could provide reference to future first line investigation. The gummy concentrate obtained from ethanol extraction was designated as crude extract with a yield of 18.8%. The extract exhibited a significant inhibition of DPPH radical scavenging activity with IC_{50} of 54.78 µg/ml. Total phenolic content of extract was 7.1 mgGAE/g, total tannin content was 4.33 mgGAE/g. The reducing power of extract revealed strong reducing power in concentration dependent manner. The total flavoid content of extract was 41.93 mgQE/g. Ferric reducing antioxidant power of extract was 25.56 µg/ml. Scavanging activity of extract in term of hydrogen peroxide, hydroxyl ion, superoxide radical and nitric oxide were 66.20 µg/ml, 125.88 µg/ml, 135.49 µg/ml and 41.18 µg/ml respectively. Extract displayed broad spectrum antibacterial activity against *Staphylococcus aureus*, *Vibrio cholerae* and *Shigella dysenteriae* and also showed mild activity against *Escherichia coli*, *Staphylococcus epidermidis*, and *Proteus species*. Ethanol extract also possessed anthelmintic activity. Extract at higher concentration (50 mg/ml) is more effective against *Paramphistomum cervi*. Increased mortality rate of brine shrimp with increased sample concentration demonstrated an approximate linear correlation between them. Cytotoxicity result also correlates the phytochemical component present. The result showed that the extract has some potential bioactivities which require further investigations to isolate bioactive compounds and to identify its underlying mechanisms.

KEYWORDS: Antioxidant, DPPH, Antimicrobial, *Dicksonia antartica* Labb, Cytotoxicity.

INTRODUCTION

Plant and its products have been used as effective therapeutic tools for the treatment of disease and injuries from very early days of civilization to modern days.[1] According to Cragg et al, classification of drugs of natural origin as; original natural products, products derived semi-synthetically from natural products, or synthetic products based on natural product models.[2] In cancer therapy performed from 1940s to 2007, almost 155 drugs have been used. Among 155 drugs used, 73% was drugs of natural origin and solely 47% being natural products or derived from them.[3]

Oxidative stress is the major causative agents for establishment of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, and many others.[4] Oxidative process is the vital way for producing free radicals in foods, drugs and sometimes in living systems.[5] Antioxidants are considered to be those substances that possess free radical chain reaction breaking properties. Ascorbic acid, carotenoids and phenolic compounds are more effective naturally occurring antioxidants available.[6] Mechanism of action is known by inhibiting lipid peroxidation (inactivating lipoxygenase), scavenging free radicals and active oxygen species by propagating a reaction cycle and chelating heavy metal ions.[7]

Bioactive agents which interact with other microorganism in the environment are produce by various aromatic and medicinal plants which are known for antimicrobial activities.[8, 9] These plants are of diverse nutrient and non-nutrient ingredients, of which displayed antimicrobial properties that can protect the human body from pathogens. Therefore, it is necessary to characterize medicinally important for their antimicrobial properties.[10] Utilizing brine shrimp bioassay for natural product research is highly used. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products.[11]

Out of 500,000 species of higher plants available, only less than 15% have been investigated pharmacologically. It is a matter of surprise that large quantity of modern drugs available come from less than 15% of the plants. This present study was done to find out the efficacy of ethanolic extract of *Dicksonia antartica* Labb leaves in terms of antioxidant property,

antimicrobial potential, anthelmintic activity and cytotoxic activity on the basis of brine shrimp lethality bioassay.

MATERIALS AND METHODS

Plant materials

The aerial part of plant *Dicksonia Antarctica* Labb was collected from Chittagong hill tracks in January 2013 and was identified by experts at Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen has been submitted (Accession no. 36077 DACB) for future reference. Adulteration was strictly prohibited during collection.

Extraction of plant materials

Plant materials were grinded (Capacitor start motor, Wuhu motor factory, China) into powder after proper handling and cleaning. The extraction of plant material was carried out by hot extraction method by using a Soxhlet apparatus. About 250 gm of powered material was taken and ethanol was used as solvent for extraction. Each time about 25gm of powder material was taken in a container prepared by Whatman filter paper (Bibby RE200, Sterilin Ltd, UK) and placed in the chamber of the apparatus for extraction. In this way, the total powder material was extracted by ten times repetition. The filtrate obtained was evaporated under ceiling fan until dried.

Phytochemical assessment

Procedures for identifying different chemical groups were adopted according to Sofowara A.[12] Different tests were performed for identifying different chemical groups. Such as for identification of carbohydrates test (Molisch's test), reducing sugar (Benedict's test and Fehling's test), Alkaloids (Mayer's test, Dragendroff's test, Hager's test, and Wagner's test), phenolic compounds (Ferric chloride test, Lead acetate test, and Dilute nitric acid test), flavonoids (General test, Lead acetate test, Alkaline reagents test, Ferric chloride test, and Ammonia test), Tannins (Ferric chloride test, Potassium dichromate test, Lead acetate test, and Potassium hydroxide test), glycoside (Molisch's test, Conc. H_2SO_4 test, Legal's test, Keller-Kiliani test, and Borntrager's test (Anthraquinol glycoside: O-glycosides)), steroid (Salkowski's test and Libermann-Burchard's test), saponin (Forth test and Foam test) and gums (Xantho Protein test, Ninhydrin test, and Biuret test) were performed.

Antioxidant property

Stable free radical DPPH (1, 1-Diphenyl-2-pycrylhydrazyl) scavenging method was applied for the estimation of antioxidant activity of the hexane, ethyl acetate and methanol plant extract.[13] The following equation was used to calculate the capability to scavenge the DPPH radical.

$$Percentage\ scavenging = \frac{(Ao - As)}{Ao} * 100$$

A_o = absorbance of DPPH radical.

A_s = absorbance of test or reference sample.

The percentage scavenging was then plotted against concentration and regression equation was obtained to calculate IC_{50} (micro molar concentration required to inhibit DPPH radical formation by 50%). The experiment was conducted in triplicate.

The ferric reducing antioxidant power assay of the different extract was carried out according to the Benzie.[14] Hydroxyl radical scavenging activity was measured based on the method of Halliwell[15] with a slight modification according to Jiang.[16] Measurement of Superoxide radical scavenging activity was done using standard method[17] followed by slight modification.

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch.[18] The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated as follows.

% Scavenged (H_2O_2) = [(Ao-A_1)/Ao] x 100 where Ao is the absorbance of the control and A_1 is the absorbance in the presence of the sample of extract and standard.

Total phenolic and tannin content was determined by Folin-Ciocalteu's reagent. Grinded plant material (0.5 g) was mixed with 50 ml of 80% aqueous methanol and sonicated for 20 min. An aliquot of 2ml was taken from it and centrifuged for 15 min at 14,000 rpm. Standard gallic acid solutions were prepared by serial dilution and finally calculated via standard curve analysis.

The total flavonoid content was estimated using aluminum chloride colorimetric assay. The 0.5 mL of test sample' solution in methanol (5 mg/100 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml (150 µl) of 15% w/v sodium nitrate ($NaNO_2$). After 6

min, 0.15 ml (150 µl) of 10% w/v aluminum chloride ($AlCl_3$) and 2 ml of 1M sodium hydroxide (NaOH) were added and left at room temperature for 15 min. Absorbance of the mixtures was measured at 510 nm (UV-Visible Ultraspec 2000 spectrophotometer, England) and total flavonoid contents were calculated as Quercetin equivalents from a calibration curve of Quercetin. The calibration curve was prepared in the same manner using 0.01562-1 mg/ml of Quercetin solutions in ethanol.

The reducing power of ethanol extract of *Dicksonia antartica* Labb. was determined by the slight modification of the method of Oyaizu.[19] Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

$$\text{Potassium ferricyanide + Ferric chloride} \xrightarrow{\text{Antioxidant}} \text{Potassium ferrocyanide + ferrous chloride}$$

The percentage of reduction of the sample as compared to standard was calculated using the formula.

$$\text{Percentage (\%) of reducing power} = [1-(1-\frac{A_s}{A_c})] \times 100$$

A_c= absorbance of standard at maximum concentration tested

A_s=absorbance of sample

Nitric oxide scavenging activity was measured spectrophotometrically according to Govindarajan.[20] Sodium nitroprusside (5 mmol) in phosphate buffered saline was mixed with different concentrations of the extract (5-100 µg/ml) dissolved in suitable solvent system and incubated at 25°C for 30 minutes. After 30 minutes of incubation 1.5 ml solution was taken and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylene diamine dihydrochloride).The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm with a double beam Analykjena UV/Visible spectrophotometer. The process was done for ethanol extract *of D. antarctica* Labb. The nitric oxide (NO) radical scavenging activity was expressed as the inhibition percentage (I%) and calculated as per the equation.

$I\ (\%) = [(A_{Blank} - A_{Sample})/A_{Blank}] \times 100$

Where, A_{Blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the experimental sample with all reagents.

Antimicrobial activity

Preparation of test sample for disc diffusion method

100 mg and 250 mg of the crude extract of *Dicksonia antarctica* were taken in two small volumetric flasks. Then small amount of ethanol was added and triturated in unidirectional manner using a vortex mixer, after proper mixing the volume was adjusted to 4 ml and 5 ml respectively by ethanol and the concentration of obtained solutions were 25 µg/µl and 50 µg/µl.

Antibacterial activity test by disc diffusion assay

Agar diffusion method[21] with some modification was used to determine antibacterial activities. The bacterial cell suspension was adjusted to an inoculation of 1×10^6 CFU/ml from prepared 24 h culture. Both gram positive and gram-negative bacterial strains were taken for the test. Microorganisms used for this test were *Staphylococcus aureus, Escherichia coli, Vibrio cholera, Shigella dysenteriae, Proteus species,* and *Staphylococcus epidermidis*. These organisms were collected from the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDRB). After that these organisms were cultured at microbiology laboratory of pharmacy discipline, Khulna University, Khulna-9208. Sterile blank discs (BBL, Cocksville, USA) were impregnated with the test extract at the concentration of 25 µg/µl and 50 µg/µl on each disc. Standard antibiotic discs (Kanamycin 30 µg/disc, Oxoid Ltd, UK) and control discs were placed along with test discs on nutrient agar medium innoculated with test organism. The petri plates were then incubated at 37 °C for 16 h. The zone of inhibition was measured using digital slide calipers.[22] An average zone of inhibition was calculated for the three replicates.

Evaluation of anthelmintic activity

0.0625, 0.125, 0.25 and 0.5 gm ethanol leaf extract of *Dicksonia antarctica* Labb was taken and triturated with 0.2% v/v of Tween 80 as a suspending agent to make concentrations of 6.25, 12.5, 25 and 50 mg/ml and final volume was adjusted to 10 ml for respective preparation. Live parasites *Paramphistomum cervi* was collected from freshly slaughtered cattle at local abattoirs. After cleaning, parasites were stored in 0.9% phosphate buffered

saline (PBS) of pH 7.4 prepared with 8.01 gm of NaCl, 0.20 gm of KCl, 1.78 gm of Na_2HPO_4 and 0.27 gm of KH_2PO_4 in 1 litre of distilled water at 37±1°C. Anthelmintic activity of the plant extract was investigated on live parasites *Paramphistomum cervi* of cattle. The parasites were divided into different groups consisting of four parasites in each group. 0.2% v/v Tween-80 in water was used as control, standard albendazole at concentrations of 10 and 15 mg/ml was used as positive control and extract at concentrations of 6.25, 12.5, 25 and 50 mg/ml was used as test group. For the preparation of standard albendazole at concentrations of 10 and 15 mg/ml; 100 and 150 mg were taken and triturated with 0.2% v/v of Tween 80 as a suspending agent and final volume was adjusted to 10 ml for respective concentration. Time taken for paralysis and death was recorded for each group. Student's t-test was used to determine significant differences between the control group and test group.

Cytotoxic activity ethanolic leaf extract of *Dicksonia antarctica* Labb.

The eggs of *Artemia salina* were hatched in a solution of 20 g of NaCl and 18 g of table salt in 1 litre of distilled water with constant shaking to make homogeneous mixture and were filtered with cotton plug to get clear solution. Two compartments rectangular tank with porous divider was used to hatch eggs. Of the two compartments, the larger compartment was darkened and eggs were kept to hatch at 25-28°C for 24 hours with continuous supply of air through the application of air pump and air stone was used to spread air uniformly. The nauplii were attracted to light and entered into the illuminated compartment by the small holes of divider from where they were collected by pipette.

Brine shrimp lethality bioassay was assayed according to Meyer et al to assess cytotoxic potential of the ethanol leaf extract of *Dicksonia antarctica* Labb.[11] Samples were prepared in distilled water with DMSO (not more than 0.01%) and serially diluted at concentrations of 320, 160, 80, 40, 20, 10 and 5 µg/mL in 5 mL containing 10 alive nauplii in each test tube. DMSO in distilled water was considered as control. Anticancer drug vincristine sulphate (5, 2.5, 1.25, 0.625 and 0.312 µg/mL) was taken as standard drug. After incubation at 25-28°C for 24 hours, the number of viable nauplii were counted and compared with control to assess lethal effect. The percentage of lethality of brine shrimp nauplii was calculated by following way;

% Mortality/Lethality = (Avg. no. of alive shrimp of control - Avg. no. of alive shrimp of sample)/ Avg. no. of alive shrimp of control) × 100.

RESULT AND DISCUSSION

The obtained ethanol leaf extract of *Dicksonia antarctica* Labb was evaporated under ceiling fan until completely dried. It rendered a gummy concentrate (47gm) of greenish black color. The gummy concentrate was designated as crude extract. Hence, the yield was 18.8%.

Phytochemical studies showed that Reducing Sugar, Steroid, Tannin, Alkaloids, Saponin, Flavonoid & Glycosides were present in the ethanolic extract of *Dicksonia antarctica* Labb. and also demonstrated that gums was not the part in the ethanolic extract of *Dicksonia antarctica* Labb. The result of phytochemical study of leaves of *Dicksonia antarctica* Labb is shown in table 1.

The ethanolic extract of *Dicksonia antarctica* Labb displayed IC_{50} value of 54.78 µg/ml in compared to standard ascorbic acid IC_{50} value of 7.294 µg/ml. DPPH radical scavenging activity, being a complementary assay for accessing the total antioxidant capacity was displayed well by leaf extract of *Dicksonia antarctica* Labb. Our results showed the presence of a better cocktail of antioxidant compounds in the ethanolic extract. The presence of phyto-constituents; phenols, flavonoids and tannin, indicates the possibility of antioxidant activity and is considered to be preventing a number of diseases through free radical scavenging activity [23]. The total phenolic content of ethanol extract of *Dicksonia antartica* Labb revealed to be 7.1 mg GAE/g of dried plant material. Total Phenolic Content determination of *Dicksonia antarctica* Labb was calculated with the help of gallic acid standard calibration curve (shown in figure 1). Phenolic compounds may be of three types- non flavonoids like hydroxybenzoic acid, flavonoids like flavones, flavonols, flavanones, etc and third is tannins.[24] Mechanism of action is considered to be through scavenging or chelation.[25] The total flavonoid content of ethanol extract of *Dicksonia antartica* Labb revealed to be 41.93 mg QE/g and total tannin content found to be 4.33 mg GAE/g (shown in figure 2) of dried plant material. The reducing capacity of a compound is an important indicator of its antioxidants activity.[6] The reducing activity of a compound usually depends on the presence of reductases which exhibited antioxidant potential by breaking the free radical chain and donating a hydrogen atom. Comparison of absorbance vs. log concentration of ascorbic acid vs. *Dicksonia antarctica* Labb is shown in figure 3.

The ethanolic extract of *Dicksonia antarctica* Labb displayed RC_{50} value of 25.56 µg/ml in compared to standard ascorbic acid RC_{50} value of 9.17 µg/ml. The trends for ferric ions reducing activities of *Dicksonia antarctica* Labb. extracts at different concentrations were

observed. This strong ferric reducing power indicates the hydrogen donating ability of the extract. This assay is implied for determining hydrophilic antioxidants.[26] *D. antarctica* leaves extract displayed hydrogen peroxide radical scavenging activity is (SC_{50} = 66.20µg/ml) and standard Ascorbic Acid is (SC_{50} = 43.22µg/ml), a well-known antioxidant. This scavenging activity may be due to the presence of various phytochemicals including phenolics and flavonoids in ethanol extract of *D. Antarctica*. Eventhough, hydrogen peroxide itself is not a free radical, it is a precursor to certain radical species such as peroxyl radical, hydroxyl radical, superoxide. These hydroxyl radicals can readily react and can damage vital cellular components especially of the mitochondria.[27] *D. antarctica* leaves extract showed potent hydroxyl radical scavenging activity. In the assay, *D. antarctica* leaves extract displayed hydroxyl radical scavenging activity (SC_{50} = 125.88 µg/ml) and standard ascorbic acid is (SC_{50} = 97.12 µg/ml). Hydroxyl radical is the major reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and harms to cells.[28] The hydroxyl activity may be due to the presence of various phytochemicals including phenolics and flavonoids in ethanol extract of *D. antarctica*. The *D. antarctica* leaves extract displayed superoxide radical scavenging activity (SC_{50} = 135.49 µg/ml) which is comparable to that of ascorbic acid (SC_{50} =99.14 µg/ml). Superoxide radical is well known to be harmful to cellular components as a precursor of the more reactive oxygen species which contribute to the tissue damage and various diseases. Nitric oxide scavenging activity of *D. antarctica* leaves extract showed 41.18 µg/ml where standard ascorbic acid showed 17.85 µg/ml. Extract showed a moderate NO scavenging activity. Direct tissue toxicity and vascular collapse related with septic shock could result from a sustained production of the nitric oxide radical. Chronic expression of the radical also contributes in many carcinomas and inflammatory conditions such as juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.[29]

The crude extract of *Dicksonia antarctica* Labb displayed moderate antibacterial activity against the bacterial strains *Staphylococcus aureus, Vibrio cholerae* and *Shigella dysenteriae* but showed mild activity against *Escherichia coli, Staphylococcus epidermidis,* and *Proteus species* in comparison with standard drug kanamycin (30 µg/disc). Further investigation is required to identify the active compounds and to determine the mechanism of action of the compounds present in the plant extract. The antibacterial activity of crude extract of *Dicksonia antarctica* Labb is shown in table 2. The extract showed broad spectrum antibacterial activity ultimately relating its application for new drug development.

In the present study the ethanolic leaf extract of *Dicksonia antarctica* Labb was found to show anthelmintic activity. Ethanol extract at concentrations of 6.25, 12.5, 25 and 50 mg/ml showed paralysis at 90, 75, 57.5, 36.5 min and death time found at 101, 88, 73 and 48.5 min respectively. Standard albendazole also showed paralysis time 25 and 21 min and death time at 33 and 29 min for concentration of 10 and 15 mg/ml, respectively. Extract at higher concentration (50 mg/ml) is more effective against *Paramphistomum cervi*. Anthelmintic activity of leaf extract *of Dicksonia antarctica* Labb is shown in figure 4. From the results it is concluded that, ethanol leaf extract of *Dicksonia antarctica* Labb possesses anthelmintic activity that compared to albendazole. The possible mechanism of the anthelmintic activity of *Dicksonia antarctica* Labb cannot be explained on the basis of our present results. Further studies are required to isolate and revealed the active compound in order to establish mechanism(s) of action.

The crude extract showed lethality indicating the biological activity of the compound present in the extract. Test sample showed different mortality rate at different concentrations. The mortality rate of brine shrimp was increased with the increase in concentration of the sample and plot of percent mortality versus log concentration (figure 5) demonstrated an approximate linear correlation between them. Cytotoxicity result also correlates the phytochemical component present. Brine shrimp lethality bioassay is one of the widely used technique to assess bioactivities like enzyme inhibition, ion channel interference, antimicrobial and cytotoxic properties.[11, 30, 31] Many researches have been shown correlation between cytotoxic activity and brine shrimp lethality.[32, 33] This indicates a further investigation of any possible novel compounds in the *D. antarctica* leaves, being extracted with ethanol, for their structures and functional aspects as anticancer agents.

Figure legends

Figure 1: Total Phenolic Content determination of *Dicksonia antarctica* Labb with the help of gallic acid standard calibration curve.

Figure 2: Standard gallic acid calibration curve for total tannin content.

Figure 3: Comparison of absorbance vs. Log concentration of ascorbic acid vs. *Dicksonia antarctica* Labb for reducing power assay.

Figure 4: Anthelmintic activity of leaf extract of *Dicksonia antarctica* Labb.

Figure 5: Graphical Representation of lethality test for the ethanolic extract of *Dicksonia antarctica* Labb.

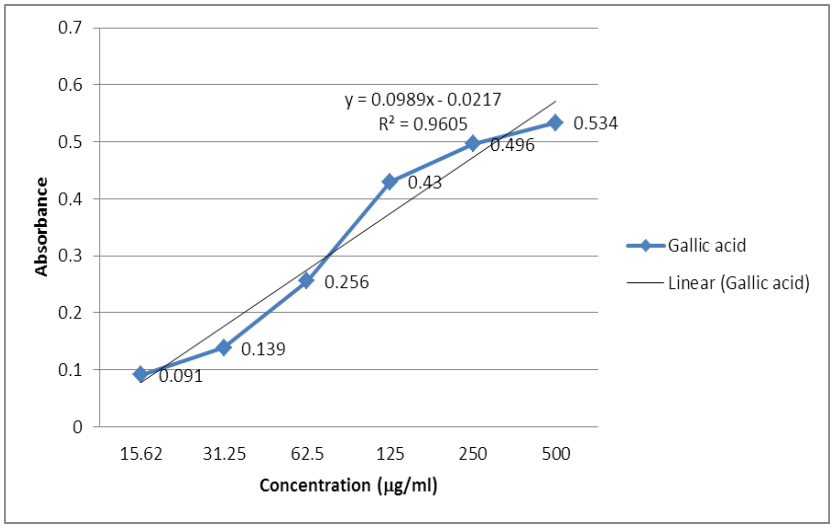

Figure 1

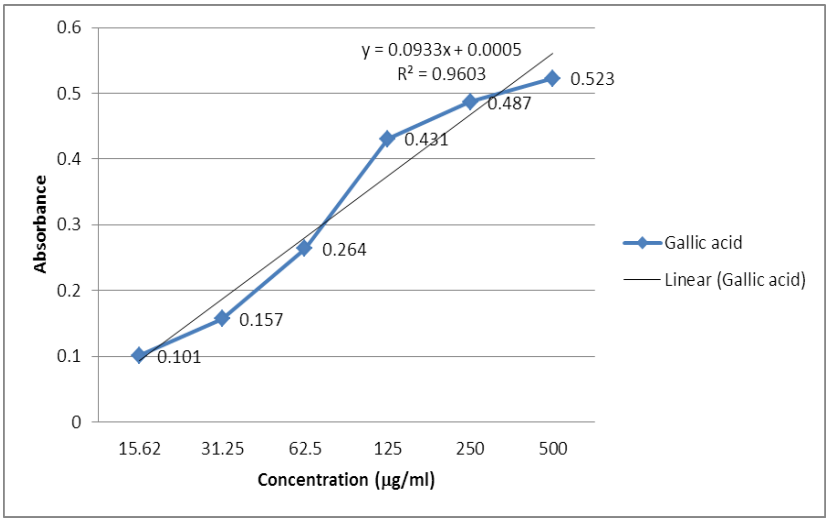

Figure 2

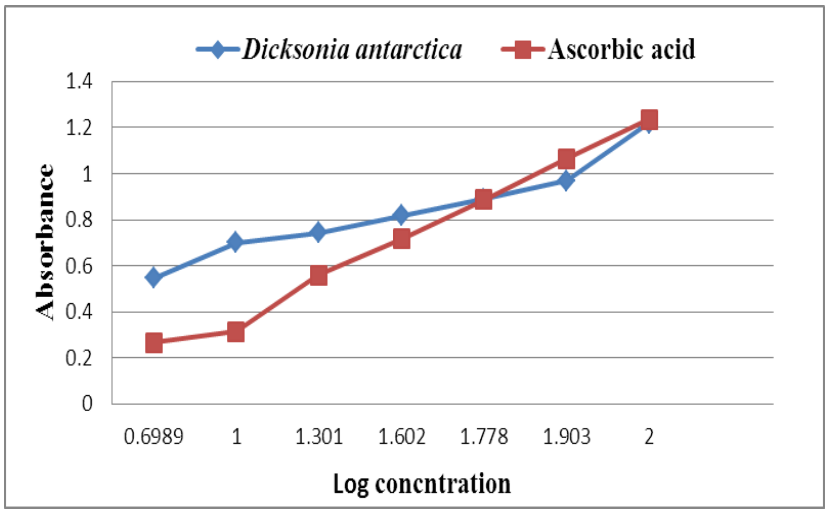

Figure 3.

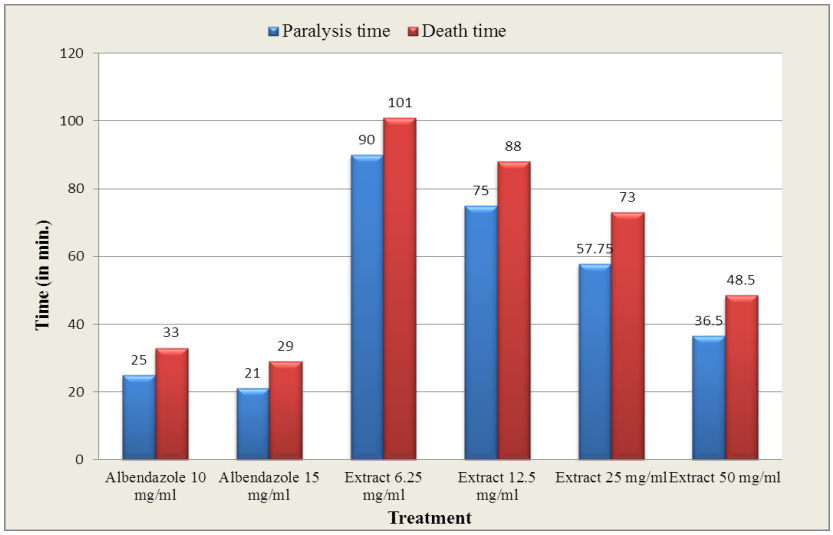

Figure 4.

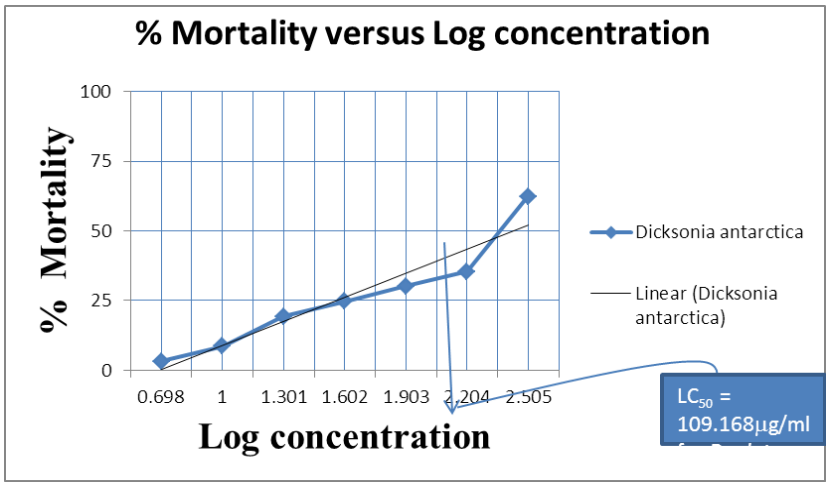

Figure 5.

Table 1: Different Chemical Groups of *Dicksonia antarctica* Labb.

Components	Result
Reducing Sugars	+
Saponins	+
Alkaloids	+
Glycosides	+
Flavonoids	+
Tannins	+
Gums	-
Steroids	+

Table 2: Determination of antibacterial efficiency of *Dicksonia antarctica* Labb leaves extract.

S.No.	Bacterial Strains	Gram's	Diameter of Zone of Inhibition in mm

		property	Control	Kanamycin (30µg/disc)	Extract (250 µg/disc)	Extract (500 µg/disc)
1	*Staphylococcus aureus*	Gram(+)	-	23.59	4.41	9.29
2	*Escherichia coli*	Gram(-)	-	18.42	-	6.28
3	*Vibrio cholerae*	Gram(-)	-	20.94	6.23	10.21
4	*Shigella dysenteriae*	Gram(-)	-	20.64	8.14	7.09
5	*Proteus species*	Gram(-)	-	19.74	-	3.36
6	*Staphylococcus epidermidis*	Gram(+)	-	17.88	-	8.59

Gram (-): Gram Negative Bacteria; Gram (+): Gram Positive Bacteria; (-): No inhibition

CONCLUSION

Simple in extraction and board range of activities under tested conditions of leaf extract from *Dicksonia antarctica* Labb which might possesses compounds with biological activity such as enzyme inhibition, ion channel interference, antimicrobial, pesticidal and cytotoxic activity. Further studies are required for the establishment of bioactive compound present in aerial part of *Dicksonia antarctica* Labb plant.

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