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Research Article

EVALUATION OF ERYTHROCYTE MEMBRANE STABILIZING ACTIVITY, HAEMOLYTIC ACTIVITY AND CYTOTOXIC EFFECT OF THE AREAL TUBERS OF *DIOSCOREA ALATA* L OF NORTH-EASTERN REGION OF INDIA

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ABSTRACT
D. alata is a staple food in many regions of the world and also extensively consumed in the North-Eastern Region of India. Medicinal properties of the tubers of *D. alata* are well established, but this is the first time that the possible toxicological evaluation of the areal tubers has been done. Hydro-methanolic extract of the areal tubers were prepared and used for all the experiments. EMSA was estimated at 562 nm against quercetin as standard. The haemolytic activity was assayed at 540 nm against Triton-X as the standard. The cytotoxic effect of *D. alata* was studied on murine splenocytes, thymocytes, hepatocytes and peritoneal macrophage by MTT cytotoxic test. All the results were statistically analysed. The half maximal haemolytic concentration for *D. alata* and triton-X were 1312.94 ± 180.15µg/ml and 189.24 ± 1.54 µg/ml, respectively. The half maximal inhibitory concentration of the *D. alata* was 216.79 ± 8.73 µg/ml for EMSA and 2905.66 ± 538.34 µg/ml, 3997.91 ± 1873.94 µg/ml, 5201.23 ± 226.38 µg/ml and 4284.62 ± 1256.16 µg/ml, respectively, for MTT assay. The results suggest the presence of potent EMSA and negligible cytotoxic and haemolytic activity of *D. alata*.
Keywords: *Dioscorea*, Yam, haemolytic activity, cytotoxicity, erythrocyte membrane stabilising activity, macrophage, immunostimulatory

INTRODUCTION

Dioscorea alata L, commonly known as Greater yam (family *Dioscoreaceae*) is a staple food in south-eastern Asia and Africa[1] and also extensively consumed in the north-eastern region of India. Works on the medicinal properties of Chinese and Taiwanese variety of *D. alata* have been done by various groups of researchers. Hepatoprotective activity and anti-ulcer activity of *Dioscorea* on rat model has already been established[2]. The chief storage protein dioscorin exhibited as trypsin inhibitor and carbonic anhydrase activator[3] apart from dehydroascorbate reductase and monodehydroascorbate - reductase activities[4]. Two groups of researchers[5,6] have reported the hypoglycemic activities of *Dioscorea* species. The tuber mucilage of *D. Alata* has potent immunostimulatory effect as demonstrated by Shang *et al*., 2007[7].

Considering the fact that *D. alata* is extensively consumed in the north-eastern region of India, the safety aspects of *D.alata* as food must be taken into account. Therefore, the objective of the present study was to evaluate the possible haemolytic and cytotoxic activity of 70% hydro-methanolic extract of the areal tubers (figure 1) of *D. alata*. In addition, we have also studied the erythrocyte membrane stabilizing activity of the plant extract.

MATERIALS AND METHODS

Chemicals

All solvents and materials were obtained from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). RPMI-1640, fetal bovine serum (FBS), antibiotics and EZcount™ MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Assay Kit were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

Collection of the Plant

D. alata was cultivated in the garden of the Department of Zoology, University of North Bengal and the areal tubers were collected during the month of November. The tubers were authenticated from Botanical Survey of India, Shillong, Meghalaya, India with an accession number of 78051.

Preparation of plant extract

The tubers were washed properly with double distilled water and shade dried at room temperature for 2 weeks followed by grinding to powder. The powder (100 g) was mixed with 70% methanol (1000 ml) and kept at 37° C in a shaking incubator (160 rpm) for 18 h. The mixture was centrifuged at 2850 x g for 15 min. The pellet was mixed with 70% methanol (1000 ml) and kept in a shaking incubator as previously described. The supernatant was collected from both phases, filtered and the filtrate was concentrated under reduced pressure in a rotary evaporator. The resultant was dehydrated and stored at -20°C until further use.

Erythrocyte membrane stabilizing activity

A standard method[8] was followed to assay the erythrocyte membrane stabilizing activity of *D. alata* extract. The reaction mixture contained 50 mM phosphate buffer (0.5 ml; pH 7.2), 1 ml distilled water, 0.25 ml of 10% RBC suspension (in PBS), 100µl EDTA (12 mM), 150µl of 1% NBT (nitro blue tetrazolium), 100 µl riboflavin and varying concentrations of *D. alata* extract (0-200 µg/ml). The reaction mixture was kept under bright light for 30 sec and incubated for 30 min at 50ºC. After the incubation, the mixture was centrifuged at 1000 rpm for 10 min and the absorbance of the supernatant was measured at 562 nm. Quercetin was taken as reference standard. The erythrocyte membrane stabilizing activity was measured by the following equation:

$$\% \text{ of protection} = (A_s / A_b) * 100$$

Where, A_s and A_b are the absorbance value of sample and blank, respectively.

Haemolytic activity

Haemolytic effect of *D. alata* extract was evaluated according to the previously standardised method[9] after slight

modifications. Briefly, mouse blood was collected in citrated tubes and the cells were then washed (150 x g for 5 min) three times with 20 mM Tris–HCl containing 144 mM NaCl (pH 7.4). Erythrocyte suspension (2%) was prepared with the same solution and 100 µl of the cell suspension was plated into each well of the 96-well plate. One hundred millilitres of 0.85% NaCl solution containing 10 mM $CaCl_2$ was added to each well. The first lane served as the negative control containing no plant extract. From the second well, 100 µl of *D. alata* extract of various concentrations (0-200 µg/ml, dissolved in 0.85% NaCl solution containing 10 mM $CaCl_2$) were added. In another set of lanes, 100 µl of Triton X-100 of various concentrations (0-200 µg/ml) in 0.85% saline were plated which served as standard. The plate was incubated for 30 min at 37°C in a humidified atmosphere. After incubation, the suspension was centrifuged (604 x g for 5 min) and the supernatant was used to measure the absorbance of the liberated haemoglobin at 540 nm. The percentage of haemolytic activity was calculated using the following equation:

$$\% \text{ of haemolytic activity} = [(H_o - H_1) / H_o] * 100$$

Where H_0 was the absorbance of the blank and H_1 was the absorbance in the presence of the samples and standard (triton-X).

MTT Cytotoxicity assay

Swiss albino mice were sacrificed and the spleen, liver and thymus were separated. Peritoneal exudate macrophages were collected by washing the mouse peritoneal region with RPMI-1640. Cell suspension was prepared in RPMI-1640 medium supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, 50 U/ml nystatin and 10% FBS and adjusted as 2 x 10^6 cells/ml. One hundred microliters of the cell suspension was added with 100 µl of different concentrations (0-200 µg/ml) of *D. alata* extract (dissolved in RPMI-1640) to the wells of 96-well plate. The cytotoxicity assay was performed in six sets using EZcount™ MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions.

All the experiments were performed after the approval from the departmental ethical committee of the Department of Zoology, University of North Bengal and according to the principles in the Declaration of Helsinki.

Statistical analysis

All data are reported as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} (half maximal inhibitory concentration) and HC_{50} (half maximal haemolytic concentration) values were calculated by the formula Y = 100 * A1/(X + A1), where A1 = IC_{50} or HC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. Data were statistically analysed using one-way analysis of variance (ANOVA) with Dunnett's test and $p<0.05$ was considered significant.

Figure 1: Photograph of areal tubers of *D. alata* which was cultivated in the garden of Department of Zoology, University of North Bengal.

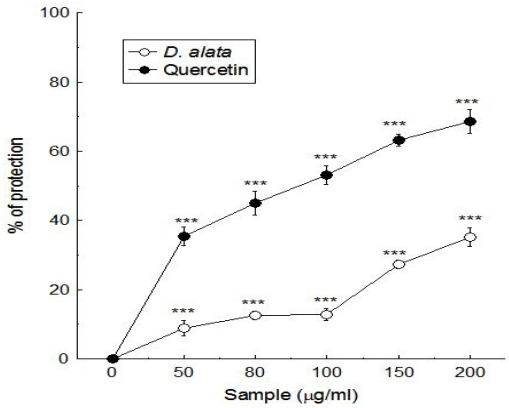

Figure 2: Demonstrates erythtocyte membrane stabilizing activity of the plant extract and standard quercetin. The value represents mean ± S.D of six sets of experiments. ***p < 0.001 vs 0 µg/ml

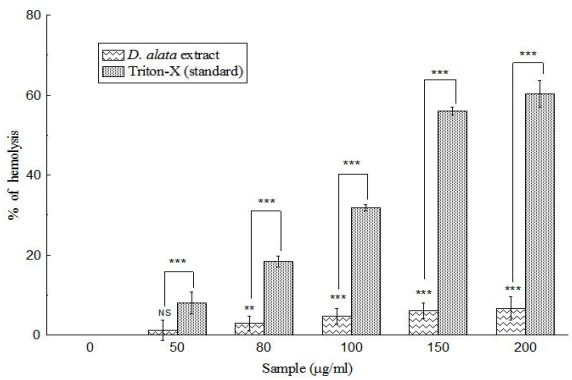

Figure 3: Represents hemolytic activity of the areal tubers of *D. alata* compared to the standard triton-X. The value represents mean ± S.D. (n = 6). NS= non-significant, *p < 0.05, P<0.01 and ***p < 0.001 vs 0 µg/ml.

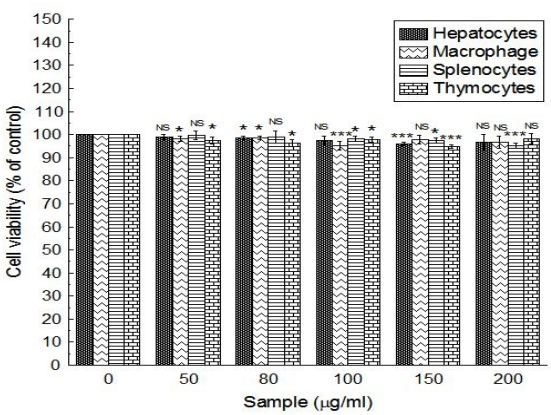

Figure 4: Represents the cytotoxic effect of the areal tubers of *D. alata* on murine splenocytes, hepatocytes, thymocytes and peritoneal macrophages which was assayed by MTT cytotoxic assay. The value represents mean ± S.D. (n = 6). NS= non-significant and *p < 0.05, ***p < 0.001 vs 0 µg/ml.

RESULTS AND DISCUSSION

Erythrocytes are packed with haemoglobin and their membranes are composed of highly unsaturated lipid materials. In the erythrocyte membrane stabilizing assay, haemolysis of RBC was induced by superoxide radical which was generated by auto oxidation of riboflavin in presence of light. Superoxide ($O_2^{\cdot-}$) radical induced free radical chain oxidation in erythrocytes leads to haemolysis. Being a natural source, the activity showed by *D. alata* areal tuber extract was quiet convincing. At 200 µg/ml concentration (figure 2), the plant extract showed 35.11 ± 2.64 % protection; whereas, the standard quercetin displayed 68.50 ± 3.45% protection. The IC_{50} value of the plant extract and quercetin were calculated to be 427.51 ± 53.82 µg/ml and 91.20 ± 2.61 µg/ml respectively. Our present study demonstrated that *D. alata* inhibited lysis of erythrocytes in a dose dependent manner, probably through scavenging superoxide radical.

Heme is liberated routinely in case of destruction of hemoglobin. It also happens in case of certain pathological conditions such as hemoglobinopathies, oxidative stress and glucose-6-phosphate dehydrogenase deficiency[10]. Certain components of food materials often contain varying degree of haemolytic activity, like saponins from alfalfa possess haemolytic activity[11]. Such compounds make the erythrocyte lipid bilayer permeable irreversibly and thus, hemoglobin is released into the medium[12]. According to another hypothesis, saponins elevate the water carriage by aquaporins, resulting in hemolysis[13]. The haemolytic activity of *D. alata* areal tuber extract on murine erythrocytes was negligible as presented in figure 3. The standard triton-X displayed very high ($P<0.001$) haemolytic activity on murine erythrocytes in all the doses and its potent haemolytic activity is evident from the high HC_{50} value. It might sound alarming that the plant extract have displayed high haemolytic ($P<0.001$) activity at higher doses (above 100 µg/ml), but the overall very high HC_{50} value shown by the areal tubers signifies that upto the highest dose (200 µg/ml), the haemolytic activity of the areal tubers were negligible. The HC_{50} value for *D. alata* and triton-X were calculated to be 1312.94 ± 180.15 µg/ml and 189.24 ± 1.54 µg/ml, respectively. For each of the concentrations, the haemolytic activity of *D. alata* extract was significantly ($p<0.001$) less than that of the triton-X. *D. alata* areal tubers displayed several fold lesser haemolytic activity than that of the triton-X at all the doses, and therefore, may be considered safe from the haemolytic perspective.

To study the cytotoxic effect of *D. alata,* the suspension of the murine splenocytes, hepatocytes, thymocytes and macrophages were incubated with varying concentrations of plant extract and their viability were tested by MTT method. The succinate-tetrazolium reductase enzyme from the mitochondrial respiratory chain of metabolically active cells reduced the tetrazolium salt to form purple formazan which was solubilized and the absorbance (540 nm) was proportional to the viable cells.

Our results indicated that upto certain dose (200 µg/ml) there was insignificant cytotoxic effect of *D. alata* areal tuber extract on various murine cell types. Figure 4 demonstrates the results of MTT cytotoxic assay on the murine cells. Cytotoxicity has been demonstrated ($P<0.001$) on macrophage at 100 µg/ml, on hepatocytes and thymocytes at 150 µg/ml and on splenocytes on 200 µg/ml. But in reference to very high IC_{50} value and the % of viable cells at the highest dose (200 µg/ml), it can be considered that the tubers contained negligible cytotoxic activity upto the highest dose. At 200 µg/ml concentration the viability of cells were 95.09 ± 0.95 %, 96.72 ± 3.36 %, 98.34 ± 2.07 % and 95.09 ± 2.80 % for mouse splenocytes, hepatocytes, thymocytes and peritoneal macrophages, respectively. The IC_{50} value of *D. alata* for splenocytes, hepatocytes, thymocytes and peritoneal macrophages were found to be 5827.32 ± 286.86 µg/ml, 6490.23 ± 482.29 µg/ml, 5254.13 ± 435.79 µg/ml and 7588.91 ± 513.79 µg/ml, respectively. There was no drastic decrease in metabolic activity of the cells as evident from high degree of cell viability at all the doses. The results donot indicate any cytotoxic effect of the areal tubers on the murine model.

CONCLUSION

According to Paracelsus, the father of toxicology, "the dose makes the poison"[14], but up to certain high dose, the areal tubers of *D. alata* showed no marked toxic effect on the murine cells; rather it demonstrated significant EMSA. The underground consumable tubers and some compound isolated from the plant have already demonstrated some medicinal properties as well. Being cultivated extensively in the north-eastern region of India, the toxicological study was essential. Therefore, we may conclude that the plant material possess no hemotoxic or cytotoxic activity and the areal tubers must also be studied thoroughly for its medicinal properties as it has demonstrated potent erythrocyte membrane stabilizing activity.

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