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Original Article

BRINE SHRIMP LETHALITY ASSAY IN TWO SPECIES OF *BIOPHYTUM* DC.(OXALIDACEAE)

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ABSTRACT

Objectives: The present study focuses on the detection of the major classes of phytocomponents and the appraisal of the cytotoxic potential of the ethanol extracts of the plant parts of two species of *Biophytum, B. veldkampii* Shanavas *et al*. and *B. reinwardtii* (Zucc.) Klotzsch using Brine Shrimp Lethality Assay (BSLA).

Methods: Individual plant parts of both the species [roots, stems, leaves & flowers (including seeds and fruits)], shade dried and ground to fine powder, were subjected to Soxhlet extraction using 70% ethyl alcohol. Preliminary phytochemical screening of the ethanol extract of the plant samples were done according to the standard biochemical procedures. Cytotoxicity was assessed using BSLA (Meyer, 1982).

Results: The preliminary phytochemical analysis showed that**,** alkaloids, flavonoids, tannins, phenols, saponins, quinones, glycosides, cardiac glycosides, fixed oils and fats, steroids, terpenoids, were present in all the parts of both the species. The BSLA revealed that both the plant extracts have potent activity against brine shrimp nauplii, comparable to the positive control potassium permanganate.

Conclusion: The ethanol extracts of *B. veldkampii* leaves and *B. reinwardtii* stems appear to be more effective than the other test samples, as they showed LC_{50} values 3.73 and 2.87µg/ml respectively, comparable to the standard, potassium permanganate (LC_{50} - 3.920µg/ml). The positive response obtained in this assay suggests that the extracts of both *B. veldkampii* and *B. reinwardtii* may contain bioactive compounds.

Keywords: Phytoconstituents, Cytotoxicity, Brine Shrimp, *Biophytum*, Ethanolic extracts Potassium permanganate

INTRODUCTION

Phytochemicals are secondary metabolites produced by the plants for their protection, repair processes and survival in the natural environment. The determination of the phytochemical profile of a plant provides evidence for the major classes of compounds in that plant. The secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins and steroids produced naturally in plants are also known to possess curative properties. Experimental validation shows that the bioactive components could be localized in specific plant parts like roots, stems, barks, leaves, flowers or seeds [1]. Thus, the determination of the phytochemical profile of plants/plant parts is of great significance to human health.

Cancer is a serious health problem and is the major cause of human mortality all over the world. Difficulty in early detection and subsequent delay in treatment results in tragic consequences. Cancer is a complex disease, which is manifested in a number of ways. Treatment requires adoption of appropriate strategies. One mode of cancer treatment is through the use of cytotoxic drugs. Cytotoxic drugs have the potential to kill the cancerous cells and are usually developed after initial screening of thousands of lead compounds from various sources.

Among the many recent advances in cancer chemotherapy, it has been noticed that plant derived compounds play an important role in development of chemotherapeutic drugs. The earth is a vast repository of medicinally important plants. It is imperative that such plants must be subjected to exhaustive studies for screening the bioactive compounds.

Assay systems are available which give a preliminary idea on the cytotoxicity and therefore the anticancer potential of plant extracts. Among the available cytotoxicity screening assays, Brine Shrimp Lethality Assay (BSLA) appears to be the most rapid (24 hours), simple (no aseptic techniques are required), easily mastered, and inexpensive methods. Moreover, it requires only small amount of test material (2 or 20 mg or less) [2]. BSLA has been routinely used in the primary screening of the extracts as well as isolated compounds to provide an indication of possible cytotoxic properties of the test materials [3].

The genus *Biophytum* is represented by about 80 species in tropical and subtropical regions of the world [4]. It is one among the 'Dasapushpas', - the group of ten flowers considered sacred in the traditional and cultural folklore in the state of Kerala, India.

Two species of *Biophytum* DC., (family Oxalidaceae) namely, *B. veldkampii* and *B. reinwardtii* are considered for the present study. Both the species are very common on the roadsides, wastelands, forests and habitats with moist soils.

The medicinal value of species of *Biophytum* is quite evident from the fact that these plants have been used in traditional folk medicine for a long time. In Ayurveda, the plant is used as a tonic and stimulant and also for the treatment for chest complaints, convulsions, cramps and inflammatory tumors, arthritis, back pain, bone spur, cervical spondylosis, degenerative joint disease, degenerative neck disease, leg cramps, leg pains, osteoarthritis, and rheumatoid arthritis.

It is a good medicinal herb to clean the uterus after delivery. It is also used for treating heavy bleeding seen in women. Therefore it is also called "Teendanaazhi".

The present study is concerned with the detection of the major classes of phytochemicals and the evaluation of the cytotoxic potential of the ethanol extracts of the roots, stems, leaves and flowers of two species of *Biophytum* using Brine Shrimp Lethality Assay.

MATERIALS AND METHODS

In the present study, two commonly available species of *Biophytum, Biophytum veldkampii* Shanavas *et al* and *Biophytum reinwardtii* (Zucc.) Klotzsch were used. The two species of *Biophytum,* were collected from Thiruvananthapuram, Kerala.

Methods

Preparation of plant extract: Fresh plants of *B. veldkampii* and *B. reinwardtii* were collected; the roots, stems, leaves & flowers (including seeds and fruits) of the two species were separated and shade dried. The shade dried parts were ground to fine powder and subjected to Soxhlet extraction using 70% ethyl alcohol.

The extract was concentrated under reduced pressure and preserved in the refrigerator until further use.

(i). Preliminary phytochemical screening of the ethanol extract of the plant samples were done according to the standard procedures [5].

Table 1: Phytochemical screening of the ethanol extracts of the plant samples

Name of the phytoconstituent	Crude extract + test solution added	Indication of the compounds
Alkaloids	Five drops of Dragendroff's (Potassium bismuth iodide) and shaken well	Formation of an orange red or yellow precipitate
Morphine alkaloids	0.6 ml of 1% H_2SO_4 + 2 ml of distilled water, followed by 10 % $NaNO_3$	Formation of reddish brown color
Phenols	1% aqueous $FeCl_3$	Formation of green, purple, blue or black color
Fixed oils	Poured through paper	Oil stains
Tannins	Hot water. Mixture filtered and added 2ml of 2% solution of $FeCl_3$	A dark green coloration
Phlobatannins	Hot boiling water, and 1% HCl	Formation of red precipitate
Quinones	Concentrated H_2SO_4, heated on water bath	Formation of violet color
Anthraquinones	About 5ml of chloroform in a test tube, shaken vigorously. The mixture was filtered and added an equal volume of 10% NH_3	Bright pink color
Coumarins	Alcoholic NaOH	An yellow color which disappears on adding concentrated HCl (5-10 drops)
Flavonoids	Few fragments of magnesium ribbon and concentrated HCl added drop wise	Pink scarlet colour
Saponins	About 5ml of distilled water in a test tube and the mixture shaken vigorously	Foam produced, persists for ten minutes
Glycosides	Boiled with a drop of Fehling's A and Fehling's B solution for 2 minutes	Red precipitate
Cardiac glycosides	Glacial acetic acid containing trace of $FeCl_3$ and shaken well	The presence of brown ring at the interphase and violet ring beneath this layer and a green upper layer
Terpenoids/Steroids	About 5ml of acetic anhydride	Terpenoids confirmed by pink color and steroids by green color.

(ii). Cytotoxicity study

Cytotoxicity study of crude ethanol extracts was done using *Artemia salina* (Brine Shrimps) commonly known as 'sea- monkeys' [6]. The freeze-dried cysts were procured from Department of Aquatic Biology and Fisheries, University of Kerala, Kariavattom. The lethality test involves the culture of brine shrimp larvae.

The cysts hatch to nauplii when deposited in saline sea-water. Sea water was kept in a glass trough and one spoon of cyst (shrimp eggs) was put into the trough, properly sealed with aluminium foil and few pores were made for constant air supply and maintained at 37°C. Two days were allowed for the shrimp to hatch and mature as nauplii. The larvae were allowed another 48 h in sea water to ensure survival and maturity before use. These nauplii were taken for the bioassay.

Lethality bioassay

Five concentrations of both plant extracts (2, 4, 6, 8 and 10 µg/ml) in 5% DMSO were prepared and tested. Each extract preparation was dispensed in 10 ml volumes and tested in triplicate. After labeling the glass vials properly, ten living shrimps were added to each vial with the help of a Pasteur pipette. About 10 ml of DMSO in sea water and different concentrations of potassium permanganate (as in the sample vials) were taken as negative and positive controls respectively. The vials were kept for 24 hours. Larvae were considered dead if they did not exhibit any internal or external movement during the several seconds of observation. The larvae were not provided with any food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not due to starvation; the dead larvae in each treatment were compared with the dead larvae in the negative control.

Counting nauplii and analysis of data

After 24 hours, the vials were inspected using a magnifying glass and the number of surviving larvae were counted. The percentage of mortality was calculated at each concentration. The concentration-mortality data were analyzed statistically. The effectiveness or the concentration-mortality relationship of the plant product is usually expressed as a median lethal concentration (LC_{50}) value which was determined using the Probit analysis method described by [7]. The LC_{50} value represents the concentration of the chemical that produces death in half of the subjects after a certain exposure period. The percentage of mortality at each test dose and the control was determined by using the formula:

$$\% \text{ of mortality } = \frac{\text{No.of dead nauplii}}{\text{Total number}} 100 \ldots\ldots\ldots\text{(a)}$$

The percentage of mortalities for 0 and 100% was then corrected by the following formulas (b & c) proposed by [8] before the determination of Probit.

For 0 % mortality: $100 \times 0.25/n \ldots\ldots\ldots$(b)
For 100% mortality: $100 \times (n - 0.25)/n \ldots\ldots\ldots$ (c)

Where, n = total number of animals in each group.

In many experiments, it is desirable to correct the mortality in the experimental treatments by the mortality that occurs in the control treatment. When there are a small number of treatments, correction of control mortality has traditionally involved the use of Abbot's formula. It is a mathematical formula used to correct mortality in the untreated check. The adjusted value is permissible when mortality in control does not exceed 20% [9].

$$\text{Corrected \% mortali} = (M_{obs} - M_{control})/(100 - M_{control}) \times 100 \ldots \ldots (d)$$

Where, M_{obs} = observed % mortality; $M_{control}$ = control % mortality

RESULTS

Preliminary phytochemical screening of the ethanol extract of the plant samples

The Preliminary phytochemical studies of two species of *Biophythm* are summarized in the table 2.

The data shows that**,** alkaloids, flavonoids, tannins, phenols, saponins, quinones, glycosides, cardiac glycosides, fixed oils and fats, steroids, terpenoids, were present in all the parts of both the species. Morphine alkaloids, anthraquinone, coumarins were completely absent in all the samples tested. Phlobatannins were absent only in the leaves of both the species.

(ii). Cytotoxicity study

In the present study, the brine shrimp lethality assay of extracts of two species of *Biophytum* used in traditional medicine was determined using the procedure of Meyer *et al.* (1982).

The results of the BSLA using the crude ethanol extracts from the different parts of *B. veldkampii (B.v)* and *B. reinwardtii (B.r)* and details about the percentage mortality and Probit percentage are given below (Tables 3, 4). The LC_{50} values obtained for extracts of these two species and that of the positive control, Potassium Permanganate are given in table 5.

Table 2: Phytocompounds in the ethanol extracts of plant parts of *B. veldkampii* and *B. reinwardtii*

S. No.	*Biophytum veldkampii*					*Biophytum reinwardtii*			
	Compounds	**Leaf**	**Stem**	**Root**	**Flower**	**Leaf**	**Stem**	**Root**	**Flower**
1	Alkaloids	+	+	+	+	+	+	+	+
2	Flavonoids	+	+	+	+	+	+	+	+
3	Morphine alkaloids	–	–	–	–	–	–	–	–
4	Tannins	+	+	+	+	+	+	+	+
5	Phlobatannins	–	+	+	+	–	+	+	+
6	Phenols	+	+	+	+	+	+	+	+
7	Saponins	+	+	+	+	+	+	+	+
8	Quinones	+	+	+	+	+	+	+	+
9	Anthraquinone	–	–	–	–	–	–	–	–
10	Coumarins	–	–	–	–	–	–	–	–
11	Glycosides	+	+	+	+	+	+	+	+
12	Cardiac glycosides	+	+	+	+	+	+	+	+
13	Fixed oils and fats	+	+	+	+	+	+	+	+
14	Steroids	+	+	+	+	+	+	+	+
15	Terpenes	+	+	+	+	+	+	+	+
	Total	11	12	12	12	11	12	12	12

Table 3: Preliminary cytotoxicity study in crude ethanol extracts of *B. veldkampii* against Brine Shrimps.

Tested samples	No: of dead shrimps	% of mortality	Corrected mortality (%)	Probit %
$KMnO_4$ (positive control)	3	30	30	0.265
	5	50	50	0.509
	7	70	70	0.750
	9	90	90	0.907
	10	100	97.5	0.976
B. v root	2	20	20	0.173
	2	20	20	0.261
	4	40	40	0.369
	5	50	50	0.488
	6	60	60	0.608
B. v stem	3	30	30	0.318
	4	40	40	0.427
	6	60	60	0.542
	7	70	70	0.654
	7	70	70	0.753
B. v leaves	4	40	40	0.318
	5	50	50	0.427
	6	60	60	0.542
	9	90	90	0.654
	10	100	97.5	0.753
B. v flowers	3	30	30	0.242
	3	30	30	0.374
	5	50	50	0.523
	7	70	70	0.669
	8	80	80	0.793

Five concentrations of plant extracts were tested = 2, 4, 6, 8 and 10 µg/ml, Number of living shrimps added =10

Table 4: Preliminary cytotoxicity study in crude ethanol extracts of *B. reinwardtii* against Brine Shrimps.

Standard	No: of dead shrimps	% of mortality	Corrected mortality (%)	Probit %
$KMnO_4$ (positive control)	3	30	30	0.265
	5	50	50	0.509
	7	70	70	0.750
	9	90	90	0.907
	10	100	97.5	0.976
B. r roots	3	30	30	0.242
	4	40	40	0.374
	5	50	50	0.523
	7	70	70	0.669
	8	80	80	0.793
B. r stems	4	40	40	0.450
	6	60	60	0.563
	7	70	70	0.671
	8	80	80	0.766
	8	80	80	0.844
B. r leaves	2	20	20	0.128
	3	30	30	0.300
	4	40	40	0.536
	7	70	70	0.759
	10	100	97.5	0.906
B. r flowers	4	40	40	0.363
	5	50	50	0.512
	6	60	60	0.660
	8	80	80	0.786
	9	90	90	0.880

Five concentrations of plant extracts were tested = 2, 4, 6, 8 and 10 µg/ml, Number of living shrimps added=10

Table 5: Calculation of LC_{50}, Regression equation, Confidence limit and Chi square by Probit analysis.

Tested samples	LC_{50} (µg/ml)	95% confidence limit (µg/ml)	Regression equation	R^2
B.v – root	8.19	5.74-22.71	5.5x +5	0.945
B.v – stem	5.25	3.72-8.86	5.5x+21	0.916
B.v – leaves	5.25	0.815-5.202	7.75x+21	0.951
B.v – flower	5.68	2.70-8.20	7x+10	0.942
B.r – root	5.93	2.70-8.20	7x+10	0.942
B.r – stem	2.87	5.48-41.55	5x+36	0.892
B. r – leaves	5.70	4.19-7.18	9.75x-7	0.94
B.r – flower	3.83	2.10-5.80	6.5x+25	0.982
Potassium permanganate	3.920	1.78 – 5.21	y= 8.75x+15	0.980

Following the procedure of Mayer *et al* [6], the lethality of the extracts of *Biophytum veldkampii* and *Biophytum reinwardtii* to brine shrimp was determined on *Artemia salina* after 24 hours of exposure of the samples and comparing them relative to the positive control, potassium permanganate. The study reveals maximum mortality took place at a concentration of 10 microgram /ml. The degree of lethality was found to be directly proportional to the concentration of the extracts. The LC_{50} values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentration of the extracts and the best-fit line was obtained from regression analysis. The ethanol extract of *B. veldkampii* leaves and *B. reinwardtii* stems appear to be more effective as it showed an LC_{50} value of 3.73 and 2.87µg/ml respectively which can be considered to be comparable to the standard, potassium permanganate (LC_{50} value of 3.920µg/ml). The R^2 values obtained in the present study were almost close to 1.

DISCUSSION

Analysis of the ethanol extracts of all the plant parts of *B. veldkampii* and *B. reinwardtii* revealed the presence of phytochemicals such as alkaloids, flavonoids, tannins, phenols, saponins, quinones, glycosides, cardiac glycosides, fixed oils and fats, steroids, terpenoids and the occurrence of the above phytochemicals are probably responsible for the use of these plants in the indigenous systems of medicine. Diverse uses of plants in treatment of wide variety of diseases are attributable to the presence of the phytochemicals [10]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity [11]. Several workers have also reported the anti-inflamatory [12], analgesic [13], anti-spasmodic and anti-bacterial [14, 15] properties of alkaloids. The phenols, flavonoids and tannins are reported to be potential antioxidant substances [16] and prevent or control oxidative stress related disorders [17]. Phenolics are employed in adaptive or defense mechanism [18, 19]. Flavonoids are potent water soluble anti-oxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity [20]. Tannins are antiseptic in nature; they have astringent properties and can hasten healing of wounds in an inflamed membrane. The plant extracts contained saponins which are known to produce inhibitory effect on inflammation [21]. Saponins have the

property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions and haemolytic activity [22, 23].

The other notable groups of compounds in the plant extracts were steroids. Steroids interact with hormone and their regulations [24]. Glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group *via* a glycosidic bond. In living organisms glycosides play numerous important roles in self medication. Many plants store inactive glycosides in their cells; which can be activated by enzyme hydrolysis.

Since morphine alkaloids, anthraquinone and coumarins are completely absent in all the tested samples, the associated activities are not recorded here. On the whole, the present results show that both the species of *Biophytum* are a valuable reservoir of bioactive compounds of substantial medicinal merit and further suggest that the identified phytochemical compounds may be the bioactive constituents. Secondary metabolites like alkaloids, tannins, and saponins contained in the plant are of equal interest in pharmacy [25].

Brine Shrimp Lethality Aassay indicates cytotoxicity as well as a wide range of other pharmacological activities such as anti-microbial, pesticidal and anti-tumor [26]. The cytotoxic activity of ethanol extracts of different parts of *B. veldkampii* and *B. reinwardtii* were tested using BSLA.

In the present investigation, varying degrees of lethality were observed with exposure to different dose levels of the test samples. The degree of lethality was found to be directly proportional to the concentration of the extracts tested. The ethanol extract of *B. veldkampii* leaves and *B. reinwardtii* stems appear to be more effective as it showed an LC_{50} value of 3.73 and 2.87µg/ml respectively which can be considered to be comparable to the standard, potassium permanganate (LC_{50} value of 3.920µg/ml).

The R^2 values obtained in the present study were almost close to 1 (Table 5). R^2 values determine how closely a certain function fits a particular set of experimental data. R^2 values range from 0 to 1, with 1 representing a perfect fit between the data and the line drawn through them, and 0 representing no statistical correlation between the data and a line. The variation in BSLA results observed for different parts of *B. veldkampii* and *B. reinwardtii* may be due to the difference in the amounts of cytotoxic substances present in these extracts. The inhibitory effect of the extract might be due to the toxic compounds present in the active fractions that possesses ovicidal and larvicidal properties.

CONCLUSION

The results of the preliminary phytochemical screening of ethanol extracts of the two species of *Biophytum* revealed the presence of alkaloids, phenols, tannins, flavonoids, quinones, saponins, glycosides, cardiac glycosides, fixed oils and fats, steroids and terpenoids in all the samples studied. In the present study, both the plant extracts were found to show potent activity against brine shrimp nauplii comparable even to the positive control potassium permanganate. Mortality increased gradually with an increase in concentration of the test sample. Thus the brine shrimp lethality assay proved useful for the scientific validation of the bioactivity of the two species of *Biophytum*. Further work should be carried out to isolate, purify, and characterize the active constituents responsible for the specific activity of these plants. Also, additional work is necessary to elucidate the possible mechanism of action of these extracts.

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REFERENCES

1. Ara N, Nur H. *In vitro* antioxidant activity of methanolic leaves and flowers extract of *Cippia alba.* Res J Medicine & Med Sci 2009; 4: 107 -110.
2. Colegate SM, Molyneux RJ. Bioactive natural products: Detection, isolation and structure elucidation. 2nd ed. Boca Raton: CRC Press; 1993.
3. McLaughlin JL, Chang CJ, Smith DL. "Bench-Top" bioassays for the discovery of bioactive natural products: An update. Stud Nat Prod Chem 1991; 9: 101.
4. Lourteig A. Flora of Panama. Part IV: Family 84, Oxalidaceae. Ann. Missouri Bot. Gard 1980; 67: 823-850.
5. Harborne JB. Phytochemicals Methods. 2nd ed. London: Chapman and Hall Ltd; 1979.
6. Meyer BN, Ferrigni NR, Putnam JE, Jacobson IB, Nichols DE, Melaughlin JL. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med 1982; 45: 31–34.
7. Finney DJ. Probit analysis. 3rd ed. Cambridge: Cambridge University Press; 1971.
8. Ghosh MN. Fundamentals of Experimental Pharmacology. 2nd ed. Calcutta: Scientific Book Agency; 1984.
9. Abbot WS. A method of computing the effectiveness of an insecticide. J Econ Entomol 1925; 18: 265-267.
10. Ayodele SQ. The effects of herbal remedies. Paper presented at the 12th Annual Conference of Botanical Society of Nigeria. South Africa: University of Logos; 2003.
11. Nobori T, Miurak K, Wu DJ, Takabayashik LA, Carson DA. Deletion of cyclin -dependent kinase -4 inhibitor gene in multiple human cancers. Nature 1994; 46: 753-756.
12. Singh S, Ehana NM, Dhar MM. Solaplumbin: An anti-cancer glycoside from *Nicotiana plumbaginifolia*. Phytochemistry 1974; 13: 2020 – 2022.
13. Antherden LM. Textbook of Pharmaceutical Chemistry. 8thedn. London: Oxford University Press; 1969.
14. Stray F. The Natural Guide to Medicinal herbs and Plants. London: Tiger Books International; 1998.
15. Okwu DE, Okwu ME. Chemical composition of *Spondias mombin* linn. plant parts. J Sustainable Agriculture and Environment 2004; 6: 140-147.
16. Agbor AG, Ngogang JY. Toxicity of herbal preparations. Cam J Ethnobot 2005; 1: 23-28.
17. Vinson JA, Dabbagh YA, Serry MM, Jang J. Plant flavonoids, especially tea flavonoids are powerful antioxidant using an *in vitro* antioxidant model for heart disease. J Agric Food Chem 1995; 43: 2800-2802.
18. Boilley JPB, Lavaga C, Cagnon R, Duran JC, Salvdo P. Phenolic pattern of Bean (*Phaseolus vulgris* L.,) as an indicator of chromiozone stress. Water Air Soil Pollut 1998; 106: 355-368.
19. Giertych MJP, Karolewski LO, De Temmernan. Foliage age and pollution alter content of phenolic compounds and chemical elements in Pinus nigra needles. Water Air Soil Pollut 1999; 110: 363-377.
20. Okwu DE, Josaiah C. Evaluation of the chemical composition of two Nigerian medicinal plants. Afr J Biotechnol 2006; 5: 357 – 36.
21. Just MJ, Recio MC, Giner RM, Cueller MU, Manez S, Billia AR, Rios JL. Anti-inflammatory activity of unusual lupine saponins from *Bupleurum fruticescens*. Planta Med 1998; 64: 404–407.
22. Sodipo OA, Akiniyi JA, Ogunbamosu JU. Studies on certain characteristics of extracts of bark of *Pansinystalia macruceras* (K schemp) picrre Exbeille. Global J Pure Appl Sci 2000; 6: 83-87.
23. Okwu DE. Phytochemicals and vitamin content of indigenous species of south eastern Nigeria. J Sustainable Agriculture and Environment 2004; 6: 30-37.
24. Okwu DE. Evaluation of chemical composition of medicinal plants belonging to Euphorbiaceae. Pak Vet J 2001; 14: 160-162.
25. Longanga OA, Vercruysse A, Foriers A. Contribution to the ethnobotanical, phytochemical and pharmacological studies of traditional used medicinal plants in the treatment of dysentery and diarrhoea in Lomela area. Democratic republic of Congo (DRC). J Ethnopharmacol 2000; 71: 411-423.
26. Anderson JE, Chang CJ, McLaughlin JL. Bioactive components of *Allamanda nodiflora* Linn. J Nat Prod 1988; 51: 307-308.