RESEARCH ARTICLE
JIVA 23 (1) April 2025
https://doi.org/10.55296/JIVA/23.1.2025.31-40

Received on 15.03.2025
Accepted on 21.04.2025

ANTI-NEOPLASTIC ACTIVITY OF *SEMECARPUS ANACARDIUM* NUT MILK EXTRACT (*BHALLATHAKA KSHEERAPAKA*) IN DALTON'S LYMPHOMA ASCITES CELLS

Shyju O.[1], Bindya Liz Abraham[2*], Suresh N. Nair[3], Dhanush Krishna B[4] and Muhasin Asaf V N[5]

[1]Senior Medical Officer, Department of AYUSH, Mavoor, Kozhikkode,
[2]Professor and Head, Department of Animal Genetics and Breeding, [3]Associate Professor, Department of Veterinary Pharmacology and Toxicology,
[4]Assistant Professor, Department of Veterinary Pathology,
[5]Assistant Professor, Department of Animal Genetics and Breeding,
College of Veterinary and Animal Sciences, Pookode
Kerala Veterinary and Animal Sciences University, Pookode, Wayanad-673576

*Corresponding author: bindya@kvasu.ac.in

ABSTRACT

The present study aimed at investigating the cytotoxicity and apoptotic activity of the human clinical formulation of *Semecarpus anacardium* (SA) nut milk extract known as *Bhallathaka ksheerapaka* on Dalton's Lymphoma Ascites cells, in comparison to cisplatin. Phytochemical analysis of SA nut milk yielded protein, carbohydrates, phenols, terpenoids, glycosides and saponins. MTT assay revealed the mean percent cell inhibition at the test concentrations of 5, 10, 20, 40, 80, 160, 320, 640 µg/mL to range from 26.052 ±0.01 to 70.909 ±0.13 for SA nut milk extract and from 29.129 ±0.13 to 80.902 ±0.05 for cisplatin, respectively. The IC_{50} estimates of SA and cisplatin were 54.01 µg/mL and 65.44 µg/mL respectively. Trypan blue dye exclusion revealed the mean percent cell viability as 50.33±1.93 and 55.17 ±0.01 for SA nut milk extract and cisplatin respectively. The mean percent cell viability and inhibition differed significantly ($p<0.01$) in comparable range between the extract and cisplatin at IC_{50} as well as at any given concentration. At any given point of time, apoptosis was faster in the SA-treated cells than the cisplatin-treated cells. The results indicated that *Bhallathaka ksheerapaka* induced cytotoxicity and apoptosis in DLA cells to a comparable extent with cisplatin and hence anti-neoplastic against lymphomas.

Keywords: *Semecarpus anacardium*, cytotoxicity, apoptosis, anti-neoplastic

INTRODUCTION

Semecarpus anacardium (SA) is a well-known plant used in the Ayurvedic

and Siddha systems of medicine for various ailments like nervous debility, rheumatism, epilepsy, sciatica, asthma and cancer (Adams *et al*., 2007; Semalty *et al*., 2010). Commonly known as "*Bhallathaka*" in Sanskrit and *Cherkuru* in Malayalam, SA belongs to family Anacardiaceae and is a medium-sized deciduous tree of 10-15 m in height in the outer Himalayas, hotter parts of India and Northern Australia. It is categorized under "*upavisha" group* (toxic, but not lethal for human health) in Ayurveda classics and described as a poisonous medicinal plant in Drugs and Cosmetics Act (India), 1940 (Rabinarayan, 2012). The fruit of *Semecarpus anacardium* is about one inch long, ovoid and smooth lustrous black. In raw form, it has been reported to cause irritation, blisters, toxicity and contact dermatitis and hence used for medicine preparation only after the specific traditional purification process known as "*shodhana*" advocated in "*Charaka Saṃhita"* (Mishra *et al*., 2017) that imparts chemical and pharmacological changes to the SA fruit and increases its safety and efficacy (Deepak *et al*., 2021). "*Bhallathaka ksheerapaka*" is a classical formulation of SA nut milk extract mentioned in *Sarngadhara Samhita* (Chandramurthy, 2007) and *Ashtanga Sangraham* (Kaviraj, 2019) and used widely in human clinical practice as per *Siddha Yoga Samgraha* (Acharya, 2006) against neoplasms. Since tumor-micro-environment is characterized by proliferation, invasion and metastasis, cancer therapies generally depend on anti-tumor properties like cell inhibition and apoptosis for the mitigation of cancer progression. In this context, the present study aimed at investigating the therapeutic merit of nut milk extract of *Semecarpus anacardium* (*Bhallathaka ksheerapaka)* in the treatment of lymphomas through the evaluation of cytotoxicity and apoptotic potential in Dalton's Lymphoma Ascites cells, in comparison to the standard anti-cancer drug, cisplatin.

MATERIALS AND METHODS

Preparation of the aqueous extract of SA nut milk

The nuts of *S. anacardium* collected

Fig 1. The tree, nuts and nut-milk extract of *Semecarpus anacardium*

from Chelannur, Calicut, Kerala were authenticated at the Centre for Medicinal Plants Research, Arya Vaidyasala, Kottakkal, Kerala. The nut milk extract of *Semecarpus anacardium* (SA) mentioned as *"ksheerapaka*" in Ayurvedic treatises was prepared in the traditional way as per *Siddha Yoga Samgraha* (Acharya, 2006) from the nuts purified with milk and water in the ratio 1:8:32 (Chandramoorthy, 2007; Kaviraj, 2019). A 100 g of good quality SA nuts after removing the thalamus portions was soaked in cow urine for seven days and in cow milk for another seven days and finally washed with water and powdered (Deepak *et al.,* 2021). A decoction of SA was prepared with milk to get the nut milk extract, filtered and cooled for use as an aqueous extract.

Phytochemical analysis of aqueous SA nut milk extract

Phytochemical analysis of a 10% stock solution of the aqueous SA nut milk extract was carried out using different tests. The different tests were Mayer's test for alkaloids and Keller-Killiani test for glycosides (Ugochukwu *et al*., 2013), Molish's test for carbohydrates (Foulger, 1931), Biuret test for proteins (Wokes and Still, 1942), Foam test for saponins (Kareru *et al*., 2008), Alkaline reagent test for flavonoids (Ugochukwu *et al*., 2013), Ferric Chloride test for phenols (Soloway and Wilen, 1952), Salkowski test for terpenoids (Soni and Sosa, 2013), Borntrager's test for anthraquinones and Libermann – Buchard test for steroids (Nath *et al*., 1946). The qualitative assessment for alkaloids was based on colour development and classified as present or absent.

Daltons Lymphoma Ascites (DLA) cells and the experimental animals

The DLA cells authenticated by the National Centre for Cell Science (NCCS), Pune and propagated at Amala Cancer Research Centre, Thrissur for nearly 20 years were used for the study. Ten adult Swiss albino mice, six to eight weeks old, weighing 25-30 g each procured from the Small Animal Breeding Station of College of Veterinary and Animal Sciences, Mannuthy (KVASU) were used to maintain the DLA cells *in vivo* as ascitic fluid intra-peritoneally and counted using a cell counter as 2.5 million cells/ mouse as per Thummar *et al*. (2016). The animals were housed in polypropylene cages under standard management, feeding and optimal environmental conditions of air and illumination, acclimatized for one week before the start of the experiment with routine clinical examinations throughout the experiment. The DLA cells maintained in the mice were used for *in vitro* studies. The procedures for animal experimentation were approved by the Institutional Animal

Ethics Committee of the College of Veterinary and Animal Sciences, Mannuthy, Thrissur as per proposal No. IAEC/22/01 dt. 06/04/2022.

MTT reduction assay

Cytotoxicity is indicated by the characteristic feature of reduction in cell viability (cell inhibition). The per cent cell inhibition of SA nut milk extract and cisplatin (Batch No. HHBL 2109CI, Hetero Health Care Limited, Hyderabad) in DLA cells was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl tetrazolium bromide assay (MTT) performed in triplicate (Mosmann, 1983; Riss *et al.*, 2016). Viable cells were detected by the presence of purple formazan crystals formed through the reduction reaction by succinate dehydrogenase enzyme. The aqueous extract of SA was suspended in distilled water at a concentration of 1.3 mg/ml to get a stock solution, further diluted with RPMI medium to the desired concentrations. The DLA cells aspirated from the peritoneal cavity of tumor-bearing mice were washed thrice with PBS and seeded in 96-well plates at a density of $5x10^3$ cells per well in 100 µL RPMI medium. Cells were then exposed to different test concentrations (5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, 160 µg/mL, 320 µg/mL and 640 µg/mL) of SA nut milk extract and cisplatin for 24 hours. Six wells seeded with DLA cells and left untreated were used as the experimental control. After 24 hours, 10 µL of MTT (5 mg/mL) was added and the cells were incubated at 37□ for four hours. The formazan crystals developed were solubilized with an organic solvent (200 µL of DMSO) and the absorbance or optical density (OD) at 595 nm was measured in an ELISA plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). The percent cell inhibition in treated and control cells was calculated by the formula:

$$\text{Per cent cell inhibition} = \frac{(\text{Mean OD of Control} - \text{Mean OD of treated cells})}{\text{Mean OD of Control}} \times 100$$

The cytotoxicity of SA extract and cisplatin were expressed as their concentration inhibiting cell growth in DLA cells by 50 per cent (IC_{50} values), estimated from the per cent cell inhibition under various concentrations using the AAT Bioquest software (https://www.aatbio.com).

Trypan blue dye exclusion method

The DLA cells aspirated from the peritoneal cavity of tumour-bearing mice were washed thrice with PBS. The cells (5×10^5 cells/mL) were added to tubes containing IC_{50} levels of the SA nut milk extract and cisplatin and the volume made up to one millilitre using PBS. After the incubation of tubes for three hours at 37□

one part of the cell suspension (10 µL) was stained with one part of trypan blue dye (10 µL). Using an automated cell counter (Invitrogen Life Technologies USA), the viable cells were counted. The test was performed in triplicate as per Strober (2015). The cell viability per cent was calculated as:

$$\text{Per cent viable cells} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}} \times 100$$

Acridine Orange / Ethidium Bromide (AO/EB) dual staining

Acridine orange/ethidium bromide (AO/EB) staining was performed to differentiate the live, apoptotic and necrotic cells (Ribble *et al*., 2005). A concentration of $5x10^5$ DLA cells was seeded into a six-well cell culture plate and treated with the MTT-estimated IC_{50} concentrations of SA nut milk extract and cisplatin for 24 hours. Twenty-five microlitres of the cells were stained with five microlitres of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analyzed under a trinocular research fluorescence microscope (DM 2000 LED, Leica) with blue excitation (488 nm) and emission (550 nm) filters at 40X magnification. The vital dye, acridine orange stained both live and dead cells whereas ethidium bromide stained only those cells that lost their membrane integrity. Cells in green shades were identified to be either viable or in early apoptosis. Orange color indicated late apoptosis whereas red color indicated necrosis (Kasibhatla *et al.,* 2006).

Statistical analysis

The descriptive statistics for mean and standard error estimation as well as the means comparison using Duncan Multiple Range Test was undertaken with SPSS V. 2.1.

RESULTS AND DISCUSSION

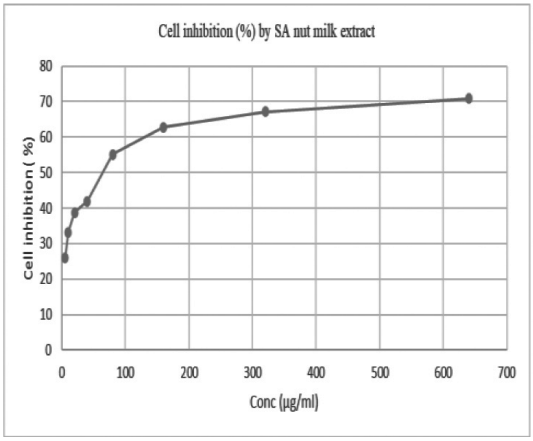
Qualitative phytochemical analysis of SA nut milk extract

Phytochemical screening of the traditional ayurvedic form and formulation of *Semecarpus anacardium* (SA) nut milk extract widely used in human clinical practice, revealed the presence of protein, carbohydrates, phenols, terpenoids, glycosides and saponins in agreement with earlier reports (Kaundal and Ranote, 2024). Alkaloids, quinones, anthra-quinones, coumarins and steroids were absent in the aqueous extract of SA nut milk.

Assessment of cell inhibition of SA nut milk extract under MTT reduction assay

After 24 hours of treatment, the MTT reduction assay revealed the mean

per cent inhibition of DLA cells at the concentrations of 5, 10, 20, 40, 80, 160, 320, 640 µg/mL to increase from 26.052 ± 0.10 to 70.909 ±0.04 per cent for SA nut milk extract and from 29.130 ±0.13 to 80.902 ±0.05 for cisplatin, respectively (Table 1). The IC_{50} values estimated for SA nut milk extract and cisplatin in DLA cells based on MTT assay and AAT Bioquest were 54.01µg/mL and 65.44 µg/mL respectively. Based on the IC_{50} and the concentrations used in the study, the optimum cytotoxic level for SA nut milk extract in DLA cells was deduced as 40 µg/ml. The mean per cent cell inhibition differed between SA and cisplatin at any given concentration significantly ($p<0.01$) and are represented as dose-response curves (Fig. 2). The cytotoxicity of SA nut milk extract on DLA cells is in agreement with the earlier reports (Phatak *et al*., 1983; Chakraborty *et al*., 2004).



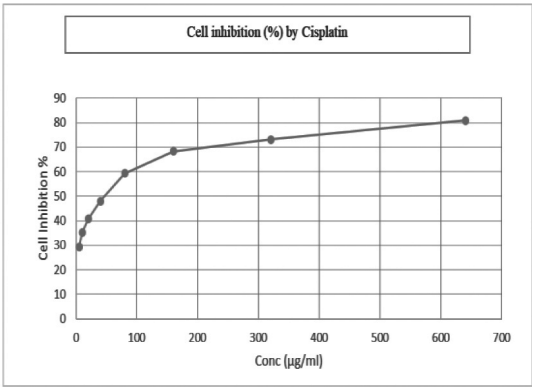


Fig 2. Dose-response curve of the per cent cell inhibition of DLA cells against various concentrations of SA nut milk extract and cisplatin under MTT assay

Table 1. The mean ± SE of per cent cell inhibition of DLA cells with various concentrations of SA nut milk extract and cisplatin under MTT assay

Concentration (µg/ml)	Cell inhibition %	
	SA nut milk extract	Cisplatin
5	26.052 ± 0.10 ^{b}	29.130 ±0.13^{a}
10	33.138 ± 0.09 ^{b}	35.155 ±0.05^{a}
20	38.780 ± 0.08 ^{b}	40.577 ±0.13^{a}
40	41.979 ± 0.08 ^{b}	47.964 ±0.03^{a}
80	55.188 ± 0.06 ^{b}	59.345 ±0.04^{a}
160	62.825 ± 0.05 ^{b}	68.357 ±0.03^{a}
320	67.125 ± 0.00 ^{b}	73.077 ±0.03^{a}
640	70.909 ±0.04 ^{b}	80.902 ±0.05^{a}

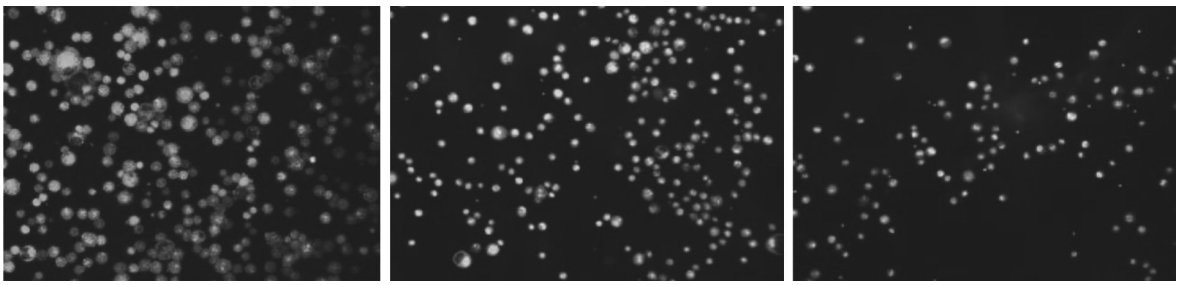
Means with different superscripts within each row differ significantly ($p<0.01$)

Assessment of cell viability by Trypan blue dye exclusion method

Trypan blue dye exclusion revealed the number of viable cells ($\times 10^5$) on treatment with IC_{50} concentrations to be 6.133 ± 0.03, 3.167 ±.0.09, 3.167 ±.0.09 and 3.467 ±0.03 respectively for the untreated control, SA nut milk and cisplatin in DLA cells. The viable cell count was found to be significantly higher with cisplatin than SA extract ($p<0.01$). The per cent cell viability on an average as against that of the control (92 %) following three hours of incubation with IC_{50} concentrations was found to be 50.33±1.93 and 55.17 ±2.23 for SA nut milk extract and cisplatin respectively. The results indicated the potent cytotoxicity of SA nut milk extract.

Assessment of apoptosis under AO/EB staining

The microscopic examination (40X) of DLA cells on acridine orange / ethidium bromide staining under different treatments are presented in Fig. 3 (A to C). All the treated DLA cells, within a given span of time, showed prominent morphological changes like cell shrinking, rounding of cells, formation of membrane blebs and the appearance of apoptotic bodies which were characteristic of cytotoxicity and apoptosis reported on herbal extracts of *Semecarpus anacardium* and others in tumor cells (Mathivadhani *et al*., 2007; Dolai *et al*., 2016). The untreated cells (A) were live with green fluorescence. Majority of the DLA cells with cisplatin (B) showed apoptosis of early stages and as a result of chromatin condensation and nuclear fragmentation, they stained green seen as bright green dots. The cells subjected to SA nut milk extract (C) stained orange and red, characteristic of late apoptosis. The necrotic cells also stained red with a nuclear morphology of viable cells, but without a condensed chromatin. The results of the staining indicated that the SA-treated cells moved faster towards apoptosis than cisplatin-treated cells in a given time period pointing out the possibility of IC_{50} concentration



A B C

Fig 3. (A to C). DLA cells under SA nut milk extract and cisplatin at their IC_{50} stained with AO/EB (40X magnification); A- Untreated cells (control); B- Cisplatin-treated; C- SA-treated

of SA nut milk to exert a faster apoptosis when compared to cisplatin.

SUMMARY

The anti-neoplastic activity of the clinical formulation of *Semecarpus anacardium* (SA) nut milk extract (*Bhallathaka ksheerapaka*) used in ayurveda medicine was evaluated in Dalton's Lymphoma Ascites cells based on MTT assay, trypan blue exclusion dye method and AO/EB staining, in comparison to the anti-cancer drug, cisplatin. The study revealed that SA nut milk extract induced considerable degree of apoptosis and cell inhibition in DLA cells indicative of its anti-neoplastic action. The cytotoxicity exhibited by SA nut milk extract may be attributed to the presence of various phytochemicals of anti-proliferative nature. The findings of the study may be used to leverage the therapeutic properties of *Semecarpus anacardium* (*Cherkuru*) for the prevention and cure of lymphomas.

ACKNOWLEDGEMENT

The authors duly acknowledge the technical and financial support provided by KVASU, Pookode, Wayanad for the smooth conduct of the experiment.

REFERENCES

AAT Bioquest, Inc. 5775 W Las Positas Blvd. Pleasanton, CA 94588, USA. Available: https://www.aatbio.com/.

Acharya, V.Y.T. 2006. *Siddha Yoga Samgraha, Jwaradhikara*1.Shri Baidhnath Ayurveda Bhavan, Varanasi. 4p.

Adams, M., Gmunder, F., Hamburger, M. 2007. Plants traditionally used in age related brain disorders - a survey of ethnobotanical literature. *J. Ethnopharmacol*. **113**: 363–381.

Chakraborty, S., Roy, M., Taraphdar, A.K. and Bhattacharya, R.K. 2004. Cytotoxic effect of root extract of *Tiliacora racemosa* and oil of *Semecarpus anacardium* nut in human tumour cells. *Phytother. Res*. **18:** 595-600.

Chandramurthy, P.H. 2007. *Sarngadhara samhitha*. (2nd Ed.) Choukambha sanskrit series, Varanasi, 139p.

Deepak, M., Salman, M. and Balachandran, I. 2021. Purification of "*bhallathaka"* (*Semecarpus anacardium*) enhanced anti-cancer activity. *Regul. Toxicol. Pharmacol.* **122**: 04898.

Dolai, N., Islam, A. and Haldar, P.K. 2016. Antiproliferative activity and apoptosis inducing mechanism of *Anthocephalus cadamba* on Dalton's lymphoma ascites cells. *Iran. J. Pharm. Sci.* **15**: 505.

Foulger, J.H. 1931. The use of the Molisch (d-naphthol) reactions in the study of sugars in biological fluids. *J. Biol. Chem*. **92:** 345-353.

Kareru, P.G., Keriko, J.M., Gachanja, A.N. and Kenji, G.M. 2008. Direct detection of triterpenoid saponins in medicinal plants. *Afr. J. Tradit. Complement. Altern. Med.* **5:** 56-60.

Kasibhatla, S., Amarante- Mendes, G.P., Finucane, D., Brunner, T., Bossy-Wetzel, E. and Green, D.R. 2006. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. *Cold Spring Harb. Protoc.* **38**: 438-443.

Kaundal, P. and Ranote, S. K. 2024. Extraction and analytical study of *Semecarpus anacardium*

L. seed oil. *Int. J. Ayur. Pharma Res.* **12**:14-16. https://doi.org/10.47070/ijapr.v12i3.3162

Kaviraj, A. G. 2019. *Ashtangasangraha*, *Vol II*. Choukamba Krishnadas academy, Varanasi, 169p.

Mathivadhani, P., Shanthi, P. and Sachdanandam, P. 2007. Apoptotic effect of *Semecarpus anacardium* nut extract on T47D breast cancer. *Cell Biol. Int.* **31**: 1198-1206.

Mishra, S.K., Doshi, G.M., Chaskar, P.K. and Sahu, P.K. 2017. *Shodhana* attenuates cytotoxicity of methanolic extract of *Semecarpus anacardium* nuts. *Res. J. Pharm. Technol.* **10**: 567-574.

Mosmann T. 1983. Rapid calorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. methods*. 65: 55-63.

Nath, M.C., Chakravorty, M.K. and Chowdhury, S.R., 1946. Liebermann-Burchard reaction for steroids. *Nature* **157:** 103-104.

Phatak, M.K., Ambaye, R.Y., Indap, M.A. and Bhatia, K.G. 1983. Cytotoxicity of the acetylated oil of *Semecarpus anacardium* Linn. *Indian J. Physiol. Pharmacol.* **27:** 166-170.

Rabinarayan, A. 2012. Urushiol-induced contact dermatitis caused during *shodhana* (purificatory measures) of *bhallataka* (*Semecarpus anacardium Linn.)* fruit. *Ayu*. **33**: 270.

Ribble, D., Goldstein, N.B., Norris, D.A. and Shellman, Y.G. 2005. A simple technique for quantifying apoptosis in 96-well plates. *BMC biotechnol.* **5**: 1- 7.

Riss, T.L., Moravec, R.A., Niles, A.L., Duellman, S., Benink, H.A., Worzella, T.J. and Minor, L. 2016. Cell viability assays. *Assay Guidance Manual*. Available*:* https://www.ncbi.nlm.nih.gov/books/NBK144065/ [07 Mar.2023].

Semalty, M., Semalty, A., Badola, A., Joshi, G. and Rawat, M.S.M. 2010. *Semecarpus*

anacardium L.: a review. *Pharmacog. Rev.* **4**: 88–94.

Soloway, S. and Wilen, S.H., 1952. Improved ferric chloride test for phenols. *Anal. Chem*. **24:** 979-983.

Soni A. and Sosa, S. 2013. Phytochemical analysis and free radical scavenging potential of herbal and medicinal plant extracts. *J. Pharmacog. Phtyochem.* **2**: 22-29.

Strober, W.2015. Trypan Blue Exclusion Test of cell viability. *Curr. Protoc. Immunol.* Appendix 3: Appendix 3B. doi: 10.1002/0471142735. ima03bs111. PMID: 26529666.

Thummar, V.R., Parasuraman, S., Basu, D. and Raveendran, R. 2016. Evaluation of *in vivo* antitumour activity of cleistanthin B in Swiss albino mice. *J. Tradit. Complement. Med.* **6**: 383-388.

Ugochukwu, S.C., Arukwe, U. I. and Ifeanyi, O. 2013. Preliminary phytochemical screening of different solvent extracts of stem bark and roots of *Dennetia tripetela. Asian J. Plant Sci. Res*. **3**: 10-13.

Wokes, F. and Still, B.M. 1942. Estimation of protein by biuret and Greenberg methods. *Biochem. J.* **36:** .797.