Journal of the Andaman Science Association Vol. 27(1):62-67 (2022)
ISSN 0970-4183, Printed in India

Colorimetric MTT assay with Peripheral blood mononuclear cell for safety test of medicinal plants

T. Sujatha, S.N. Joardar, D.Bhattacharya, Neha Haldar, Gayatri Samaddar, S.C. Mayuri, A.K.De, Jai Sunder and E.B.Chakurkar
ICAR-Central Island Agricultural Research Institute, Port Blair-744105, India

Abstract

The present study was conducted on peripheral blood mononuclear cell (PBMC) to study the cell viability test for the ethno veterinary medicinal plants that are used among Nicobarese tribal farming community. Medicinal plants used for ailments related to eye and alimentary tract were collected from Nicobarese tribes and their acetone, methanolic and aqueous extracts were prepared. Using Peripheral blood mononuclear cell (PBMC) of poultry bird, the cell viability tests of extracts was studied through MTT assay. Irrespective of medicinal plants, the highest mean cell viability index was recorded with methanolic extract (1.9) followed by acetone extract (1.62) and aqueous extract (1.00). The findings of the present study revealed that all the documented medicinal plants of tribal ethno veterinary medicine *viz*., *Tabernamontana crispa, Sida acuta, Crotolaria alata, Leea indica, Spondias pinnata, Ehretia laevis, Psidium gujava, Fleugga virosa, Ageratum conyzoides* and *Abutilon indicum* are safe for ethno-veterinary.

Key words: *MTT assay, peripheral blood mononuclear cell, Medicinal plants, cell viability test*

Introduction

Herbal medicines are time-tested and comparatively safe for both human and animal use. Due to their immense pharmacological activities, they are considered as a source of new antibacterial drugs since antiquity and mostly used by indigenous people for the management of some health problems of livestock and poultry. However, the drug in the form of synthesized or natural source needs to be examined for its cytotoxicity effect or safety to the host cell by cell viability test. Herbal extracts possessing good pharmacological activities with low toxicity are potential to address health issues and to improve productivity of animal husbandry (Dzoyem *et al.*, 2014). Cytotoxic effect of medicinal plants is an essential and desirable pharmacological action against cancerous cell lines. Preliminary screening for cytotoxicity of medicinal plants usually provides an idea about its anticancer effect because of bioactive compounds which are toxic to living body at higher doses and pharmacologically beneficial at lower doses (Emran, et al., 2012). There are many reports on the cytotoxic activity of the plant materials through MTT assay (Shridhar et al., 2017; Gomaa et al.,2018; Li et al., 2020; Abubakara, et al., 2016). Biological lethality test with Brine shrimp lethality test (BST) has been reported by McLaughlin *et al.* (1991) for monitoring the live and death in the organism by plant extracts.

The viability tests also vary from simple technique to complex one. Among the commonly used identification methods to study the cell viability *viz*., trypan blue staining (Strober, 2001) and lactate dehydrogenase (LDH) quantification in the extra cellular membrane, MTT (Di-methyl Thiazol Diphenyl Tetrazolium bromide) assay is more sensitive and most frequently used method to assess the cell viability (Fotakis and Timbrell, 2006) including cellular functions using colorimeter (Mosmann *et al*., 1983). The mechanism of action of MTT reagents depend on livability of cells as the reagent react with metabolically active cells leading to actual quantification of dead cells or growing cells (van de Loosdrecht *et al*., 1994). The mechanism of MTT assay involves reduction of MTT yellow colour by proliferating cells to purple color formazan that is dissolved in DMSO (Di-methyl sulphoxide) and measuring using colorimeter at 540 nm (Berridge *et al*., 2005). The cell viability is defined by the rate of intensity of purple colorand has direct relationship with development of purple colour. In the present study, peripheral blood mononuclear cell (PBMC) which are highly active cells were used to study the cell viability test for the ethno veterinary medicinal plants that are used among Nicobarese tribal farming community.

Material and methods

Medicinal plants used for ailments related to eye and alimentary tract were collected from Nicobarese tribes and their botanical identification was done. Plant material was collected and collected samples were washed with tap water and powdered after drying under shade. Samples were then soaked with periodical manual shaking in acetone, methanol and water at the ratio of 1:10 dilution for 3 days to prepare acetone, methanolic and aqueous extracts respectively. It was filtered using Whatman No.1 filter paper and kept at 40 – 50^0 C in water bath until solvents are completely evaporated. Subsequently, extracts of those medicinal plants were dissolved in dimethyl sulfoxide (DMSO (10% w/v) at the ratio of 1: 5 (Mishra and Padhy, 2013)to arrive at final concentration of 50 µg/µl. The diluted extracts were stored at 4°C till further usage for the work.

Peripheral blood mononuclear cell (PBMC) of poultry bird was separated by density gradient centrifugation (Boyum, 1968) with slight modification made by Joardar*et al*. (2002). Briefly, blood was collected from wing vein of poultry birdinto sterilized tubes containing 2.7% EDTA solution. The mixture was diluted by PBS (pH 7.2) and was layered carefully onto Hisep-1.084™ (Himedia, India) in a proportion of 1:3. After centrifugation at 300g for 30 min, the interface ring, rich in mononuclear cells, was collected and washed. Viability of the cells was ascertained by trypan blue exclusion method using Neubauer's counting chamber.The cells were suspended in RPMI-1640 growth medium at a concentration of 2 X 10^6 cells/ ml, and 100 µl of this suspension was put into each well of a 96 well flat bottom tissue culture plate. The ultimate volumes of the wells were brought to 200µl with the test samples or with ConA (10µg/ml) or with growth medium (un-stimulated controls). The plates were incubated at 37°C containing 5% CO_2 tension.After incubation for 24 h, 20 µl of MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution (dissolved in PBS @ 5 mg/ml and sterilized by filtration) was added into each well and left to further incubation for 4 h at 37^{0}C.

The formazan production was determined by the method of Plumb *et al*. (1989). The supernatant was removed without disturbing the formazan precipitate and 200 µl of dimethyl sulphoxide (DMSO) followed by 25 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added into each well to dissolve the formazan crystals. The contents of the well were mixed thoroughly with micropipette and incubated at room temperature for 10 min. The colour development was read at 595 nm using a plate reader (ECIL, India). Cytotoxicity of the test materials assessed by MTT method was done as per Nandi *et al*. (2014) with slight modification and expressed in terms of O.D. at 595 nm. The test values were expressed by subtracting the values of treated wells from control wells (i.e. without any treatment). More O.D. values mean more cell viability that indirectly depicts less cytotoxicity. In other words, more cytotoxicity means less O.D. values obtained.

Cytotoxicity/ Cell Viability Assay:

$$\text{Cell viability Index (CVI)} = \frac{\text{OD of Test Cells}}{\text{OD of Control Cells}}$$

Sample	Cell viability Index
Positive Control (Concanavalin – A)	0.145

Results and Discussion

A total of ten medicinal plants were documented from tribal farming community of Car Nicobar and Hutbay, South Andaman that were meant for ophthalmic infection and gastrointestinal problems in animals (Table 1).All the solvent extracts exhibited positive cell viability index (Table 2). Formation of formazon has depicted in plate 1. Highest cell viability index was observed with aqueous extract of *Fleugga virosa*(2.61) and methanolic extract of *Tabernamontana crispa(2.60)* and subsequently, all the methanolic extracts of *Fleugga virosa (2.41), Ageratum conyzoides (2.41), Leea indica (2.42)* and *Crotolaria alata (2.1.)*and aqueous extract of *Abutilon indicum*(2.1). Irrespective of medicinal plants, the highest mean cell viability index was recorded with methanolic extract (1.9) followed by acetone extract (1.62) and aqueous extract (1.00). The lowest cell viability index was found with aqueous extracts of *Leea*

indica, Crotolariaalata and *Sidaacuta*.Irrespective of solvents, the mean cell viability index was highest with *Fleugga virosa* (2.3) followed by *Abutilon indicum* (1.9), *Ageratum conyzoides* (1.91),*Tabernamontana crispa (1.61)Spondia spinnata*(1.60) and lowest was with *Ehretia laevis (1.0)*. The findings of the present study revealed that all the documented medicinal plants of tribal ethno veterinary medicine *viz*., *Tabernamontana crispa, Sidaacuta, Crotolaria alata, Leea indica, Spondia spinnata, Ehretia laevis, Psidium gujava, Fleugga virosa, Ageratum conyzoides*and *Abutilon indicum*are safe for ethno-veterinary uses as listed in Table 1.Based on shrimp lethality bioassay and MTT assay, various solvents extracts of *Leeaindica, Abutilon indicum, Spondia spinnata*, *Ageratum conyzoides*, *Tabernaemontana sps*. L *has cytotoxicity effect (Emranet al., 2012;Shridhar et al., 2017;*Gomaa *et al*.,2018; Li *et al.,* 2020; Adebayo, *et al*., 2010; Abubakara, *et al*., 2016).

Table 1 Traditional uses of the tribal ethno veterinary medicinal plants collected

Name of the plant	Nicobari language	Traditional uses
Tabernamontana crispa	Tökuròtòng	Treatment of gastro intestinal problems
Sida acuta	Meuitameuyo	Eye infection
Crotolaria alata	Raneúl	Treatment of ophthalmic infection & dewormer
Leea indica	Tokiteuny	Treatment of all gastro intestinal problems
Spondias pinnata	Amra	Treatment of ophthalmic infection & dewormer
Ehretia laevis	Pööcho	Dewormer
Psidium gujava	Kuyavö	Treatment of all gastro intestinal problems in particularly dysentery and respiratory problems
Fleugga virosa	Hingot	Dewormer
Ageratum conyzoides	Hakonpookore	Eye infection
Abutilon indicum	Marvalu	Diarrheoa

The present results have been opined by various researchers (Virkar*et al*., 2011; Akapa *et al*., 2014; Kaushik *et al*., 2011; Dashputre *et al*., 2010) through *in vivo* trial that the oral administration of aqueous, ethanol and methanolic extract of *A. indicum*and the fresh juice of leaves at doses of 2, 4, 6, 8 and 10 g/kg body weight in mice, rabbit and rats showed that the plant was found to be safe. Moreover, it has been proved that there wasno mortality in rats administered with the plant extract of *Sidaacuta*up to a dose level of 2 gm/Kg body weight (Tcheghebe, *et al*., 2017) which is in conformity with our present trial.Further biological safety study with *Flueggea virosa* indicated that *Flueggea virosa* extract (FVE) given orally up to the dose of 10,000mg/kg caused no deathin rats (Ezeonwumelu, *et al*., 2012).The oral feeding of aqueous extract of *Ageratum conyzoides*i n rats had induced liver toxicity at 1 gm per kg for long term feeding (Diallo *et al*., 2014), however, the extract in the present study is used for eye infection as external application for few days to treat secondary bacterial infection. The *in-vivo* study on feeding of seeds of *Crotalaria alata* in chick had recorded nontoxic due to less content of pyrolizidine alkaloids (Williams and Molyneux, 2017) and in the present study, the extracts of leaves of *Crotalaria alata* is used as external application for ophthalmic infection and is found safe according to present methodology. The safety tests either *in-vivo* or *in-vitro* for the medicinal plants *viz*., *Tabernamontana crispa, Sida acuta, Leea indica, Spondias pinnata, Ehretia laevis, Psidium gujava* could be untraceable for comparison.

Phytochemistry and pharmacological activities of documented medicinal plants havediversity of secondary metabolites such as flavonoids, phenolic acids, sterols, triterpenes, quinones, coumarins, alkaloids, sphingolipids, megastigmanes, iridoids and others which are responsible for various biological activities and cytotoxic activities Gomaa *et al*. (2018)indicating the biological activity of the compound present in the extract (Joysree*et al*., 2011,). According to present study, crude extracts of medicinal plants up to 1 mg have good cell viability index and safe for administering. The positive cell viability of these medicinal plants in the present study strengthens the scientific base of medicinal plantsfor various pharmacological activities and the findings of the present study may be the base for their recommendation in the herbal formulations as safe and proven phytotherapiesfor the present phyto scenario, an integral part of one health programme.

Conclusion

The safety test of the documented plants indicated that aqueous, methanolic and acetone extracts are safe for biological trials for further processing for development of veterinary herbal products.

Table 2: Cell cyto-toxicity of various solvent extracts of Ethno Veterinary Medicinal plants

Sl.No	Plants	Aqueous extract		Methanol extract		Acetone extracts	
		Viability index	Remarks	Viability index	Remarks	Viability index	Remarks
1	TöKURòTòNG (*Tabernamontana crispa)*	0.297	+	2.6	+	1.93	+
2	Meuitameuyo (*Sida acuta)*	0.079	+	1.2	+	1.5	+
3	Raneúl (C*rotolaria alata)*	0.047	+	2.1	+	1.5	+
4	Tokiteuny (*Leea indica)*	0.003	+	2.42	+	1.7	+
5	Amra (*Spondias pinnata)*	0.102	+	1.63	+	1.7	+
6	Pööcho (*Ehretia laevis)*	0.172	+	1.4	+	1.4	+
7	KUYAVö (*Psidium gujava)*	1.92	+	1.2	+	0.9	+
8	Hingot (*Fleugga virosa)*	2.61	+	2.41	+	1.9	+
9	Hakonpookore (*Ageratum conyzoides)*	1.82	+	2.41	+	1.51	+
10	Marvalu (*Abutilon indicum*)	2.1	+	1.9	+	1.7	+
	Mean value	1.001	+	1.903	+	1.62	+

\+ Samples not showing cytotoxicity

\- Samples may have cytotoxicity

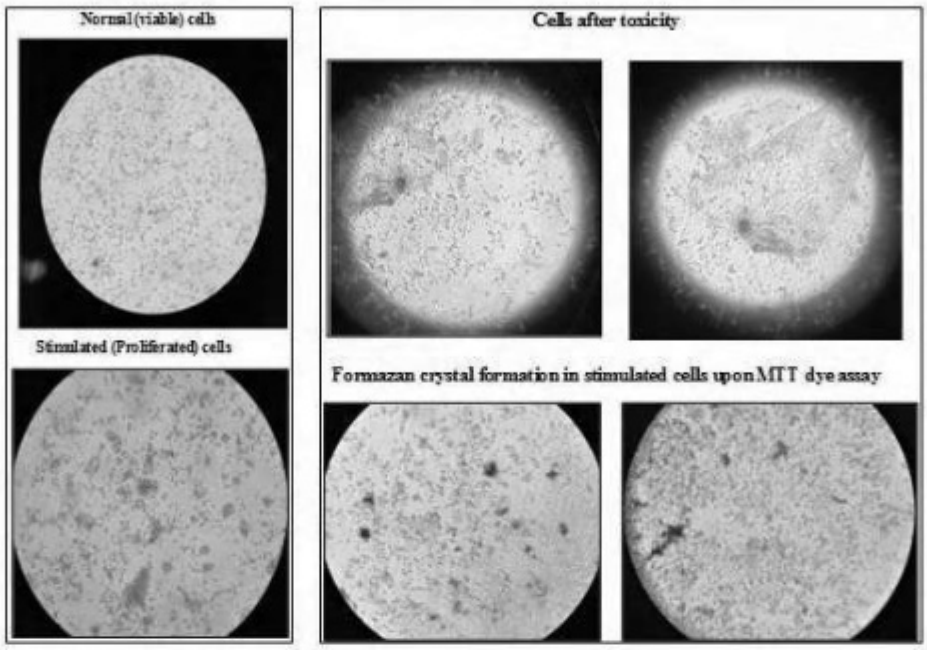

Plate 1: Microscopic image of cell viability

References

Abubakara, I.B. & Hwei-San Loha, B. (2016). A review on ethnobotany, pharmacology and phytochemistry of Tabernaemontana corymbosa. J. Pharmacy and Pharmacology, 68. 423-432.

Adebayo, A.H., Tan, N H., Akindahunsi, A. A., Zeng, G.Z. & Zhang, Y. M. (2010). Anticancer and antiradical scavenging activity of Ageratum conyzoides L. (Asteraceae). Pharmacogn Mag. 6:62-66.

Akapa, T.C., Kehinde, A.O., Beatrice, O.O. & Olajide, O. (2014). Antipyretic activity of *Abutilon mauritianum* (Jacq.) roots in Wistar Rats. Inter. J. Pharmaceu. Sci. Res.5:44-56.

Berridge, M.V., Herst, P.M. & Tan, A.S. (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev. 11: 127-52.

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood: Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifuge and sedimentation at 1g. Scand. J. Clin. Lab. Inves. 21: 77-89.

Dashputre, N. & Naikwade, N. (2010). Immunomodulatory activity of *Abutilon indicum* linn on albino mice. Inter. J. Pharma. Sci. Res. 1:178-84.

Diallo, A., Eklu-Gadegbeku, K., Amegbor, K., Agbonon, A., Aklikokou, K., Creppy, E. & Gbeassor, M. (2014). In vivo and in vitro toxicological evaluation of the hydroalcoholic leaf extract of Ageratum conyzoides L. (Asteraceae). J Ethnopharmacol. 55:1214-1218.

Dzoyem, J.P., McGaw, L.J. & Eloff, J.N. (2014). In vitro antibacterial, antioxidant and cytotoxic activity of acetone leaf extracts of nine under-investigated Fabaceae tree species leads to potentially useful extracts in animal health and productivity. BMC Comple. Altern. Med.. 14:147-151.

Emran, T.B., Atiar, R., Zahid Hosen, S. M., Umma, H.K. & Dibyajyoti, S. (2012). Antioxidant, cytotoxic and phytochemical properties of the ethanol extract of *Leea indica* leaf. J. Pharm. Res. 5:2938-2941.

Ezeonwumelu, J.O.C., Omar, A.N., Ajayi, A.M., Okoruwa, A.G., Tanayen, J.K., Kiplagat, D.M., Okpanachi, O.A., Abba, S., Ezekiel, I., Onchweri, A.N., Okonkwo, C.O. & Byarugaba, F. (2012).

Phytochemical screening, acute toxicity, anti-inflammatory and antipyretic studies of aqueous extract of the root of *Flueggea virosa* (Roxb. ex Willd.) in rat. Int. J. Pharm. Biomed. Sci. 3: 128-135.

Fotakis, G. & Timbrell, J.A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett, 160: 171-77.

Gomaa, A.A., Mamdouh, N.S., Samar, Y.D. & Salah, K. (2018). Phytochemistry and pharmacological activities of genus Abutilon: a review (1972-2015). J. Adv. Biomed. Pharm. Sci. 6: 56-74.

Joardar, S. N., Ram, G. C. & Goswami, T. K. (2002). Dynamic changes in cellular immune responses in experimental bovine tuberculosis. Medi. Sci. Monitor. 8: 471-480.

Joysree, D., Adnan, M., Mominur, R., Monsur, D., Erfan, U., Irfan, N.K., R., Habib. & Nahid, H. (2011). Chloroform and Ethanol Extract of *Spondias Pinnata* and its Different Pharmacological activity Like-Antioxidant, Cytotoxic, Antibacterial Potential and Phytochemical Screening through In-Vitro Method. Inter. J. Res. Pharmact. Biomed. Sci. 2: 1805-1812.

Kaushik, P., Kaushik, D. & Khokra, S.L. (2011). *In vivo* antioxidant activity of plant *Abutilon indicum*. J. Pharm. Edu. Res. 2:50-56.

Li,R., Yang, J.J., Xing-Zhen, S., Wang,Y.F., Richard,T.C., Xu,Y.K. & Hu, H.B. (2020). Chemical Composition and the Cytotoxic, Antimicrobial, and Anti-Inflammatory Activities of the Fruit Peel Essential Oil from Spondias pinnata (Anacardiaceae) in Xishuangbanna, Southwest China. Molecules. 25:343-345.

Loosdrecht, A.A., Beelen, R.H., Ossenkoppele, G.J., Broekhoven, M.G. & Langenhuijsen, M.M. (1994). A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J Immunol Methods. 174:311-20.

McLaughlin, J.L. (1991). Brine shrimp: a convenient general bioassay for active plant constituents. Plant Med. 45:31-34.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55–63.

Nandi, S.K., Kundu, B., Mahato, A., Thakur, N.L., Joardar, S. & Mandal, B.B. (2014). In vitro and in vivo evaluation of marine sponge skeleton as a bone mimicking material. Integr. Bio. 7: 250-262

Plumb, J.A., Milroy, R. & Kaye, S.B. (1989). Effects of the pH dependence of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide-formazan adsorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res. 49: 4435-4440.

Shridhar, C., Ghaganea Sridevi, I., Puranika,B., Vijay, M., Kumbarc, R. B. N., Sunil, S. J.E., Murigendra, B., Hirematha, Shivayogeeswar, N. & Ravindranath, A. (2017). In vitro antioxidant and anticancer activity of Leea indica leaf extracts on human prostate cancer cell lines. Integr. Medi. Res.6: 79-87.

Strober, W. 2001. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 21: 3B:A.3B.1–A.3B.2.

Tcheghebe, O.T., Jackson, S. & Ngouafong, T. (2017). Ethnomedicinal uses, phytochemical and pharmacological profiles, and toxicity of *Sida acuta* Burm. f. : A review article. The Pharma Innov.J. 6: 01-06.

Virkar, S.S & Virkar, P.S. (2011). Evaluation of acute toxicity for *Abutilon indicum*. *Der Pharmacia Lettre*. 3:37-42.

Williams, M. C. & Molyneux, R. J. (2017). Occurrence, Concentration, and Toxicity of Pyrrolizidine Alkaloids in *Crotalaria* Seeds. Weed Sci. 35: 476-481.

Received : 20th January 2022 Accepted : 15th June 2022