WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.647
Volume 6, Issue 6, 144-152 Research Article ISSN 2278 – 4357

IN-VITRO CYTOTOXICITY OF THREE SELECTED MEDICINAL PLANT EXTRACTS FROM KENYA.

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Article Received on
03 April 2017,

Revised on 23 April 2017,
Accepted on 13 May 2017

DOI: 10.20959/wjpps20176-9263

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ABSTRACT

In Kenya, medicinal plants have been used by the local people as traditional medicine to treat different human ailments from time immemorial. However, safety of most of these plants has not been determined hence the present study seeks to determine the *in vitro* cytotoxicity of the three medicinal plants namely; *Ehretia cymosa* Thonn, *Dovyalis abyssinica* (A. Rich.) Warb. and *Periplocca linearifolia* Dill & A. Rich that are commonly used to treat various diseases. The ethnobotanical survey was carried out using a structured questionnaire. Fresh plants were collected from the field and air dried under shade at 25^{o}C and later ground into powder and extracted using acetone and water. *In vitro* cytotoxicity test was carried out following a modified rapid calorimetric assay using actively dividing sub-confluent Vero E6 cells. Cell toxicity showed that most of the plant extracts tested were not cytotoxic having a CC_{50}(µg/ml) of ≥100 against Vero cell lines except the acetone extracts of *P. linearifolia* that were moderately toxic with a CC_{50}(µg/ml) of 12.5, suggesting former extracts may be safe as antimicrobials.

KEYWORDS: Cytotoxicity, Extracts and Medicinal Plants.

INTRODUCTION

The use of herbs and medicinal plants as primary health care is a universal phenomenon. Today, as much as 80% of the people in the world depend on traditional medicine as primary health care (Khaleel & Sudarshanam, 2011). However, there is need to investigate such plants to understand their properties and safety (Arunkumar & Muthuselvam, 2009). For instance, the unpleasant side effects caused by medicinal plants and conventional medicine are due to the toxicity exerted to the normal cells in patients .The safety of the medicinal plants should only be accepted after the plants products has been analyzed through toxicity testing

scientific methods such as MTT assay. MTT 3-(4, 5-dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Mosmann,1983 is a one of the common methods that is used to determine cytotoxicity because it is sensitive and a reliable quantitative colometric assay that measures the viability ,proliferation and activation of cells. The method works based on the capacity of the mitochondrial dehydrogenase enzyme found in living cells that convert the yellow water soluble substrate into a dark blue precipitate. The living cells (viable) are able to reduce the yellow MTT to a dark or purple blue precipitate in cytosol and dissolves when the cell lyses while the dead cells due to toxicity cannot transform MTT. This therefore provides the information on the viability number and degree of cytotoxicity after the addition of the drug in test. This reaction is mediated by the dehydrogenase enzyme associated with the endoplasmic reticulum and the mitochondrial (Fotaski and Timbrell, 2006).

In Kenya traditional medicine is widely practiced as documented in ethnobotanical surveys (Miaron *et al.*, 2004; Njoroge and Bussman, 2007). The high cost of imported conventional drugs and/or inaccessibility to western conventional health care system has led to over reliance on traditional medicine. On the other hand, even when conventional health care facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective (Miaron *et al.*, 2004).

Although, plants have been extensively used, assessment of their toxicity and scientific proof of their evidence is not readily available and could lead to serious complications (Rahman *et al*., 1996). For instance, *Periploca linearifolia* stem bark and leaves are used traditionally to treat stomach problems in human and animals having bloody diarrhea, small quantities normally are used because it is feared to be toxic (Jeruto *et al*., 2011). *Ehretia cymosa* bark is boiled and used traditionally in the treatment of epilepsy and menstruation problems. The bark decoction is applied externally in treating skin diseases. The extract are known to be poisonous, but with apparent inconsistency they have a reputation as an aphrodisiac (Lemmens, 2009). While *Dovyalis abyssinica* leaves are used traditionally to treat gonorrhea, brucellosis and teeth problems in humans, in animals it is used to treat mastitis (Jeruto *et al.*, 2011).Despite, the wide usage of these medicinal plants, only a small proportion of these plants have been studied so far. The study is therefore aimed at determining the cytotoxicity test of water and acetone extracts.

MATERIALS AND METHODS

Collection and Identification of plant materials

The information on the medicinal plants were gathered from the traditional practitioners, herbalists using a structured questionnaires in order to obtain information on the medicinal plants that are traditionally used for management of infectious diseases. The plant parts collected for identification consisted of flowers, roots, stems and leaves that were obtain from their natural habitats and identified by a taxonomist at the Department of Botany herbarium University of Eldoret where the Voucher specimens were deposited. Various parts for instance, leaves and stem-bark a for *Periploca linearifolia*, bark and leaves for *Ehretia cymosa* and leaves for *Dovyalis abyssinica* that are used for treatment of various diseases were collected from their habitats. Selection of the plants was based on available ethnobotanical information from traditional health practitioners consulted during the pilot study as well as available literature.

Preparation of plant extracts

The Parts used by the herbalists were collected and transported to Kenya Medical Research Institute (KEMRI) Phytochemistry laboratory and washed thoroughly with running tap water. They were then chopped into small pieces and air-dried for two weeks at room temperature by spreading evenly in the open drying area. The dry samples were ground separately into fine powder using a Willy mill and labeled appropriately using their voucher numbers. Fifty grams of the ground bark powder for each plant was exhaustively extracted with acetone to obtain the organic extracts for cytotoxicity test. The extracts were filtered through Whatman No. 1 filter paper and the solvents were removed using a rotary evaporator.

Water extraction was done by weighing (50 g) of dry powder of each considered plant soaked by adding distilled water to cover the materials then shaken for five minutes and put in a water bath at 65^{o}C for one hour. The suspension was then filtered using filter paper and the filtrate was freeze dried using dry ice until they were semi-solid before it was evaporated to dryness for three days using freeze drying machine. The lyophilized dry powder was then put in a stoppered sample vial, weighed and kept in desiccator to avoid absorbing moisture. The later was stored in sterile airtight vials at 4^{o}C in readiness for Cytotoxicity test .

Cytotoxicity Assay

The cytotoxic concentration causing 50% cell lysis and death (CC_{50}) was determined for the extracts by following a modified rapid calorimetric assay, a method described by Mosmann (1983) using Vero E6 Cell lines obtain from KEMRI.

Procedure

The extracts of the plants were tested for *in vitro* cytotoxicity, using actively dividing sub-confluent Vero E6 cells. Briefly, preparation of plant extracts involved 100µg of each extract was dissolved in 1ml of Dimethyl sulfoxide (DMSO) because it is a good stabilizer for both organic and water extracts to come up with a concentration of 100 µg /ml. The extracts were then diluted in minimum essential medium (MEM) to reduce the toxicity of DMSO.

The cells were thawed to revive them from the stock cultures by placing on warm water bath. Once thawed they were poured into a 75ml culture flasks containing minimum essential medium (MEM) supplemented with 10% Fetal bovine serum (FBS) and incubated at 37^0C and 5% CO_2. Upon attainment of confluence, cells were detached by trypsinization and pooled in a 50 ml centrifuge tube to aid in cell density count to attain 2×10^5. This was determined using tryphan blue exclusion test by placing the cells centrifuged in four wells and tryphan blue added then transferred to a haemocy-tometer (each) to aid in counting viable cells the unstained(viable) cells while blue-stained(dead) was seen under 40x objective lens of an inverted microscope to obtain a field containing cells in 4x4 columns. The cell average density was obtained from the formula C_1 = A× dilution factor$\times10^4$ as below. Where A = 32.5

$C_1=32.5\times2\times10^4$

The volume of cells (V_1) was calculated by multiplying a constant (C_2) $2x10^5$ by volume of the MEM used and that of cells used in the setting (V_2) (96 wells ×100) 9600 µl and divided by (C_1) $65x10^4$ this gave 2.9 as the Volume of cells to be diluted with MEM to make 9.6 ml, the total volume that was put in the 96 well plate.

The volume of MEM to be added to the cells was obtained by subtracting the number of cells obtained from the centrifuge (V_1) from the total volume of cells used in the setting and that of MEM added (V_2) that is V_2- V_1, 9.6-2.9=6.7. The calculated volumes were then mixed and transferred to the 96 well plate using micro-titre pipette.

One hundred (100) µl of the cell suspension at 2 x 10^5 cells per ml were seeded into each well of a 96- well plate and incubated at 37^0 C in 5% CO_2 for 24 hours to attach. The test sample extracts diluted with MEM at a ratio of 1:99 to a starting concentration of 100 µg/ml and a volume of (10+990) 1000 µl were seeded in duplicate in columns in a 96 well plate while the third column was left blank as the control; Columns 1, 2, 4, 5, 7, 8 and 10, 11 "had drug extracts and cells whereas columns 3, 6, 9 and 12 that served as Controls only had cells but no drug extracts.

Row H had the highest drug concentration and a volume of 150 µl a serial dilution of 50 µl was carried out upwards from row H to row B, (row A had no drug extracts). Chloroquine drug with an Initial concentration of 10 µg/ml in 1% DMSO was used as the control standard for the experiment. The plates were then incubated for 48 hours at 37^oC in a 5% CO_2 incubator. At the end of the incubation time, 10 µl of Thiazolyl blue tetrazolium bromides (MTT) dye was added into each well and cells were incubated for another 4 hours, (with 0.8 mg/ml of MTT), dissolved in Phosphate buffered saline (PBS). After 4 hours of incubation with MTT, the cells were observed for dye intake, after which all media was removed from the plates and 100 µl of DMSO added into each well. The plates were then read on a scanning multiwall spectrophotometer (Mullikan Ex labs systems) at 562 nm and 620 nm as reference. Data was analyzed as follows:

$$\% \text{ Cell viability } (CC_{50}) = \frac{[OD_{sample562} - OD_{620}]}{[OD_{control562} - OD_{620}]} \times 100$$

Where OD = optical density

CC_{50} = Concentration or the dose of the extract that kills 50% of the cells.

RESULTS

To evaluate cytotoxicity of the three plant extracts against African green monkey vero kidney cells lines (Vero) were incubated for 24hours to observe cell viability after carrying out serial dilution starting from the highest drug concentration and a volume of 150 µl a serial dilution of 50 µl.

Plants collected for Cytotoxicity test

Three medicinal plants belonging to three different families namely; *Dovyalis abyssinica, Periplocca linearifolia* and *Ehretia cymosa,* parts were collected (Table 1).

Table 1: Ethnobotanical information of the selected medicinal plants

Botanical name	Part collected	Family Name	Dosage per day
Dovyalis abysinicca (A. Rich.) Warb.	Leaves	Flacourtiaceae	Half spoon X 3
Periplocca linearifolia Dill & A. Rich	Leaves & stem	Asclepiadaceae	Half spoon X 1
Ehretia cymosa Thonn	Leaves, bark	Boraginacea	Half spoon X 2

Table 2: showing cytotoxicity (CC_{50}) of the active plants extracts to VERO E6 cells

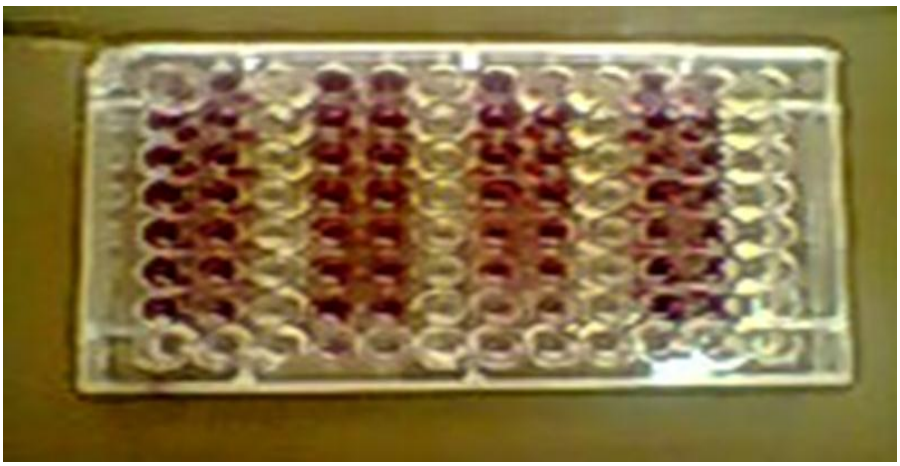
Plant	Plant part	Type of extract	CC_{50}(µg/ml)
Periplocca linearifolia	Leaves & stem	Acetone Water	12.5 ≥100
Eheretia cymosa	Bark	Acetone Water	≥100 ≥100
Dovyalis abyssinica	Leaves	Acetone Water	≥100 ≥100
$_{+}$Control	Chloroquine		24.78

Key.

- CC_{50} - 2< µg/ml- Cytotoxic

-CC_{50} between 2-89 µg/ml- Moderately toxic

- CC_{50}>90 µg/ml- Not toxic



Control **Experiment**

Plate.1: Microtitre plates showing cytotoxicity test

DISCUSSION

A total of three plants were studied which are known for their healing properties and are used for the treatment of various human diseases ranging from stomachache, headache, diarrhoea, chest infection, skin diseases and wound infections among others. The information on these

medicinal plants was obtained from the medical practitioners that includes; the parts used, dosage and how common they are used in the region, (Table 1). Majority of the studied plants was cultivated and the herbalists collect only a few from the wild, for example is *Ehretia cymosa*.

Toxicity studies are very important during the screening of medicinal plants in order to determine their safety. Cell toxicity of water and acetone extracts of the three medicinal plants was determined. All the extracts for *E. cymosa, Dovyalis abyssinica* and water extracts of *P. linearifolia* were considered to be safe because it recorded a CC_{50} which was greater than 90 µg/ml except the acetone extracts of *P. linearifolia* that was moderately cytotoxic with a CC_{50} of 12.5 µg/ml (Table 2).This was in agreement with the dosage used, the traditional practitioners used half as spoon per day because they consider them being poisonous when taken in large quantities, (Table 1). However, *In vitro* cytotoxicity does not mean that an extract cannot be used in humans (Kokwaro, 2003) as there is potential for isolation of save non-toxic compounds. For instance, *galega officinalis* is a plant that has proved too toxic for widespread agricultural use, with the potential to induce tracheal frothing, hypertension, paralysis and even death and yet Metroformin the current gold standard for management of Type II diabetes was isolated from it. Experimental and clinical evaluation of Galegine, a substance produced by the herb *G. officinalis* provided the pharmacological and chemical basis for the subsequent discovery of Metformin (Bailey *et al*,2007). Acetone and water were chosen as the extractant because it is traditionally used by the medical practitioners while acetone was chosen because it is the best extractant with low toxicity and very volatile,(Eloff *et al*,1999) .Acetone extracts of *P. linearifolia* was found to be moderately toxic while water extracts were not toxic. The cytotoxic activity could be explained by the ability of acetone being an organic solvent to extract more polar compounds in the extracts that contributed to the cytotoxic activity as compared to water extract. This result agrees with the findings of Kigondu *et al*. (2009) which indicated that organic extracts were cytotoxic as compared to water extracts. Chloroquine which was a positive control had a CC_{50} of 24.78. Water extracts were not cytotoxic while the acetone extracts of *P. linearifolia* were moderately toxic (Table 2). These results are in agreement with other work done by Muthaura *et al.*, (2007) on antimalarial properties of *Boscia angustifolia* water extracts, which he found to have no cytotoxicity. Cepleanu *et al.*, (1994) also found out that the water stem/bark extracts of *B. angustifolia* had neither cytotoxicity nor brine shrimp lethality. *In vivo* cytotoxicity should be done because cells have the ability to metabolize

chemicals to more or less toxic compounds which is normally expressed in a small extent by the cultured cells ,which results in limited activation or deactivation of test chemicals or *in vivo* accumulation that does not occurs in vitro, (Walum,1998). These results seem to confirm the validity of their traditional uses, since traditionally herbs are boiled in water (Gessler *et al.,* 1995). Therefore, the non-toxic effects of the water extracts screened indicates the concentrations of the plant extracts at the doses will be safe, hence justify the continued use of these plants as medicinal.

ACKNOWLEDGEMENTS

I would like to thank the staff of the Centre for cytotoxicity Research, the Centre for Traditional Medicine and Drugs Research of the Kenya Medical Research Institute and the Department of Biological Science Masinde Muliro University of Science and Technology for their tremendous contribution towards the development of this journal.

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