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IN VITRO TOXICITY STUDIES ON THE EXTRACT OF MEDICINAL PLANT *EVOLVULUS NUMMULARIUS* AS A POTENT MICROBICIDAL CANDIDATE

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ABSTRACT

The herb *Evolvulus nummularius* (L). L generally grown as an ornamental plant has found many applications in traditional folk medicine. There was however insufficient scientific data to back its safety to be used on humans. Methanolic extract of *E. nummularius* was used to check for its safety as a vaginal microbicide through various safety tests such as cell viability using MTT assay on three female genital tract epithelial cell lines, vaginal (VK2/E6E7), endocervical (End1/E6E7) and endometrial (HEC-1-A). Quantification of hemolytic activity was done on human red blood cells (RBCs). Determination of cellular integrity was checked by transepithelial electrical resistance (TER) assay and permeability by fluorescent microsphere assay. At 24 hours, application of the extract for cell viability assay showed extensive cell death with cell disruption. IC_{50} of VK2/E6E7 and HEC-1-A cells were found to be 2 mg/ml, $IC_{50}$of End1/E6E7 was 1 mg/ml. For hemolytic assay, with treatment of the extract for one hour did not show hemolysis till the concentration of 2.5mg/ml. In TER and microsphere permeability assays, polarized HEC-1-A monolayer 24 hours post treatment had significant drop in TER and enhanced fluorescence from passage of microspheres implying disruption of the epithelial monolayer. The study revealed that the crude methanolic extract appeared to be toxic towards human RBCs and female genital tract epithelial cells. Due to its toxic nature, its direct applications to the human vaginal tissue *in vivo* should be done with caution.

Keywords: Medicinal plants; Microbicide; *Evolvulus nummularius* (L). L; MTT assay; Transepithelial electrical resistance; Fluorescent microsphere assay.

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INTRODUCTION

Before synthetic drugs became popular, medicines were obtained from natural sources such as plants, animals and microbes. Herbal medicines have passed down through ages being considered as safe. Even today plant parts are eaten, used for cosmetic purpose and as medicines without proper knowledge if they are toxic. As per WHO estimates at present 65-80% of the world population relies on herbal medicines[1]. It is naive to consider however, that since these plants have been consumed as food or are being used as herbal products they are safe. As per the review of Lanini *et al.*[2] "if herbs have an effect, they are also likely to have a side effect". The toxicity or safety of any substance derived from nature depends on several factors such as its route of administration, dosage and period of time the medicine is active. In most countries plant based medicines are sold as dietary supplements and therefore

safety and regulatory studies essential for drugs have never been applied for many a medicinal plant.

Evolvulus nummularius (L). L (*Syn. Convolvulus nummularius* L., L.) though generally grown as an ornamental plant, has found many applications in traditional folk medicines to cure burns, cuts, hysteria, scorpion stings[3]. In Nepal, its paste has been used to treat scabies.[4] It is also known to possess wound healing properties.[5] Study dating back to 1964 revealed anticonvulsant and sedative properties in *E. nummularius*.[6] A paste made of *E. nummularius* along with other plants is used in the treatment of peptic ulcer.[7] The herb is also known to be applied to legs to reduce edema during pregnancy.[8] Activity of *E. nummularius* against standard Gram-positive and negative bacteria has been reported by Pavithra, Sreevidya and Verma.[9] Dash *et al.*[10] reported its antihelminthic activity, and Saha, Dinda, and Sil[11] reported its activity against parasite *Leishmania donovani*.

Since the plant has shown extensive medicinal properties, we intended to test its usefulness as a vaginal microbicide against sexually transmitted pathogens. There was however insufficient scientific data to back its safety to be used on human tissues. *E. nummularius* therefore was found to be an interesting candidate requiring detailed safety studies both for application as a microbicide, as well as to know the safety of its application for its reported other medicinal activities.

MATERIALS AND METHODS

Plant collection and authentication

Fresh whole plants including roots of *E. nummularius* were collected from the Tripura University campus at Suryamaninagar, Agartala, Tripura, India. The plant was initially identified by Prof. B. K. Datta, Taxonomist, Department of Botany, TU and finally authenticated by Dr. H. J. Chowdhery, Joint Director, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal and voucher specimen No. (#BD-05/05) was deposited in the Central National Herbarium, Botanical Garden, Shibpur, Howrah, West Bengal, India.

Preparation of extract

Collected whole plants including roots were washed and air dried for three weeks. They were then ground and 5 kilo grams of ground plant parts were macerated with 15 liters of methanol for 48 hrs. The extract was then filtered and evaporated in a rotary evaporator (Hahn Shin Scientific Corporation, South Korea). At the end of this the extracts were left to air dry for a week at 4°C to remove more traces of the solvent. Finally, methanolic extract of *E. nummularius* (MEEN) was dissolved in double distilled water at a stock concentration of 100 mg/ml.

Cell lines and cultural conditions

VK2/E6E7 and End1/E6E7 two keratinocyte cell lines developed from vaginal and endocervical tissues respectively were kind gifts from Dr. Fichorova. HEC-1-A cell line established from human endometrial adenocarcinoma[12] was procured from National Center for Cell Science, Pune, India. VK2/E6E7 and End1/E6E7 cell lines were maintained in keratinocyte serum-free medium and HEC-1-A was cultured in McCoy's 5A (modified) medium[13].

Determination of cell viability using MTT assay

Cell viability was determined using MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} as per previous protocol of Sathe and Reddy.[14] Briefly, VK2/E6E7, End1/E6E7 were incubated in 100 µL KSFM and HEC-1-A cells in 100 µL McCoy in 96-well plates for 24 hrs. Then, 100 µL from 24 mg/ml stock concentration of plant extract was added in row A. The 200 µL of solution (100 µL drug + 100 µL media) were mixed and 100 µL from row A were added into next row (row B) by using micropipette and a serial dilution was done up to row G. Finally, excessive 100 µL from row G were discarded. The final volume for each well was 100 µL. As a positive control for cytotoxicity, 10 µL of 0.1% Triton X-100 (TX) was added. An untreated negative control (UT) well with only cells and media was also included. At the end of the 24 hrs treatment time point cells were washed thrice with PBS (pH 7.2). MTT (0.5 mg/ml) was added next for 3 hrs at 37°C, following which cells were morphologically observed for analyzing changes induced by the extracts. Cells in each well were then solubilized in 100 µL dimethyl sulfoxide (DMSO). Reduction of MTT to formazan crystals within cells was quantified by measuring optical density at 570 nm using Synergy H1 microplate reader (BioTek Instruments) and percentage cell viability calculated as percentage of average absorbance of duplicate drug wells divided by average absorbance of control wells. IC_{50} was calculated as per the protocol of Vijayarathna and Sasidharan.[15]

Determination of transepithelial electrical resistance (TER)

In vitro determination of cellular integrity was done through TER measurement of HEC-1-A cell monolayer as per the protocol of Sathe and Reddy.[14] HEC-1-A cell line was used to prepare cell monolayer. 200 µL and 900 µL of McCoy media with supplements were added in the apical and basal compartments respectively and changed every alternate day. Transwell inserts were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 7- 8 days until confluent monolayer of HEC-1-A cells were formed. TER (Millicell ERS Ohmmeter, Millipore) of the established polarized monolayer was measured daily till the 8th day. The net TER was calculated by subtracting the background and multiplying the resistance by the area (1.12 cm^2) of the filter. Upon formation of confluent monolayer on the 8th day, HEC-1-A cells were treated with the extract. Each Transwell insert was subjected to one treatment: untreated control (UT), MEEN (T-En1.0) at a concentration of 1 mg/ml, and positive control for cytotoxicity treated with 0.1% Triton X-100 (TX). The treatments were given for 24 hrs, post which TER was measured to observe for any drop in resistance.

Fluorescent microsphere assay

Permeability assay using fluorescent microspheres of the size of HIV virus particles was done as per the protocol of Gali *et al.*[16] To the polarized HEC-1-A cell monolayers set up in Transwell units upon reaching confluency, treatments were divided into three groups: untreated control (UT), MEEN (T-En1.0) in concentration of 1 mg/ml, and positive cell death induced by 0.1% Triton X-100 (TX). Yellow green fluorescent microspheres of diameter 0.1 µm were then added in the volume of 100 µL (approx. 1.8 x 10^4 beads). Post treatment for 24 hrs, 200 µL of media from the basal chamber was harvested and fluorescence was measured at 486 nm. Fluorescence emitted by beads that crossed the treated monolayer as compared to the no treatment control with intact monolayer was plotted as percentage fluorescence and level of significance analyzed.

Hemolytic assay

5 mL of fresh human red blood cells (HRBCs) was obtained from a healthy donor in a polycarbonate tube containing heparin. The hemolytic activity of MEEN was evaluated as described previously.[17] The release of hemoglobin after 1 hour of incubation was measured by monitoring optical density at 576 nm. Untreated HRBC suspension was used as a negative control (UT), and the HRBCs treated with 0.1% Triton X-100 (TX) was employed as a control positive to bring about hemolysis. Percent hemolysis was calculated using the equation given below and the concentration of extract which caused 10% hemolysis was taken as the minimum hemolytic concentration.

$$\text{Hemolysis (\%)} = \frac{\text{O.D.}_{576nm} \text{ of the treated sample} - \text{O.D.}_{576nm} \text{ of the negative control)}}{(\text{O.D.}_{576nm} \text{ of the positive hemolysis inducer} - \text{O.D}_{.576nm} \text{ of the negative control)}} \times 100$$

Statistical analysis

All data were calculated as the mean value ± standard deviation (SD) from three independent experiments performed on different days. The significant effect of each treatment with respect to untreated control was carried out through t test at various significance levels (*$p < 0.05$; ** $p < 0.01$).

RESULTS

Determination of cell viability by MTT Assay

The effects on viability of three cell lines after treatment with MEEN were assessed through the MTT assay. Multiple concentrations of MEEN were used and the results of percentage viability at each concentration are as shown in **Table 1**. Dose-response curve was plotted against each concentration to enable the calculation of the concentrations that kill 50% of the cells (IC_{50}) as shown in **Fig. 1**. MEEN showed cell viability in the range of 34% - 54%, and its IC_{50} was determined to be 2 mg/ml for VK2/E6E7 and HEC-1-A cell lines and 1 mg/ml for End1/E6E7 cell line.

Fig. 2 shows microscopic observations of VK2/E6E7 cells with various treatment post MTT assay.

Table 1: Viability assay of vaginal, endocervical and endometrial cell lines after 24 hrs treatment with MEEN as determined through MTT assay. Absorbance was measured at 570 nm. Values are calculated as mean ± SD of three independent experimental trials.

Concentration of MEEN (mg/ml)	Absorbance (Mean ± SD)			Viability (%)		
	VK2/E6E7	End1/E6E7	HEC1A	VK2/E6E7	End1/E6E7	HEC1A
1.00	0.292 ± 0.03	0.239 ± 0.06	0.289 ± 0.01	51.14%	50.05%	54.12%
2.00	0.286 ± 0.01	0.238 ± 0.01	0.28 ± 0.03	50.03%	49.89%	52.46%
3.00	0.276 ± 0.01	0.215 ± 0.03	0.264 ± 0.01	48.29%	45.17%	49.38%
6.00	0.225 ± 0.07	0.2 ± 0.02	0.252 ± 0.01	39.37%	41.88%	47.14%
12.00	0.195 ± 0.01	0.173 ± 0.02	0.25 ± 0.02	34.15%	36.24%	46.90%
Untreated	0.571 ± 0.02	0.477 ± 0.02	0.534 ± 0.03	100%	100%	100%
Triton X-100	0.111 ± 0.03	0.05 ± 0.03	0.095 ± 0.04	19.37%	10.46%	17.78%
IC_{50} (mg/ml)				**2.00**	**1.00**	**2.00**

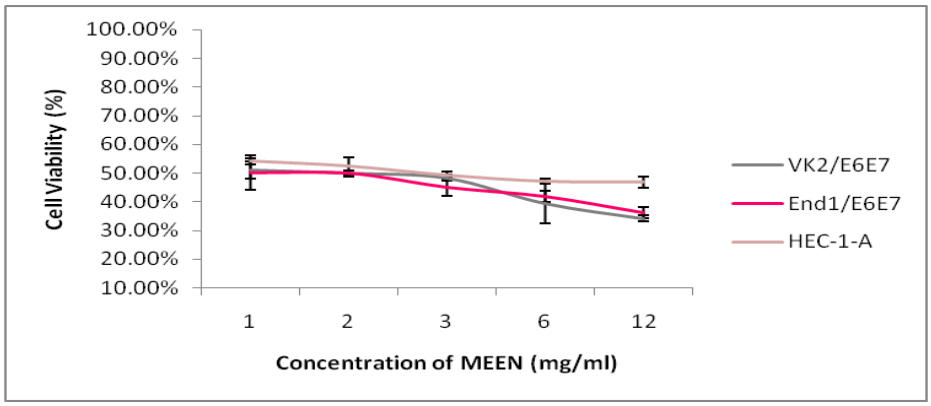

Figure 1: Effect of MEEN on the viability of vaginal, endocervical and endometrial epithelial cell lines as tested through MTT assay after 24 hrs of treatment. Percentage viability of cells in presence of various concentrations of MEEN was measured after 24 hours of treatment. Values are calculated as mean ± SD of three independent experimental trials where each condition was tested in duplicate.

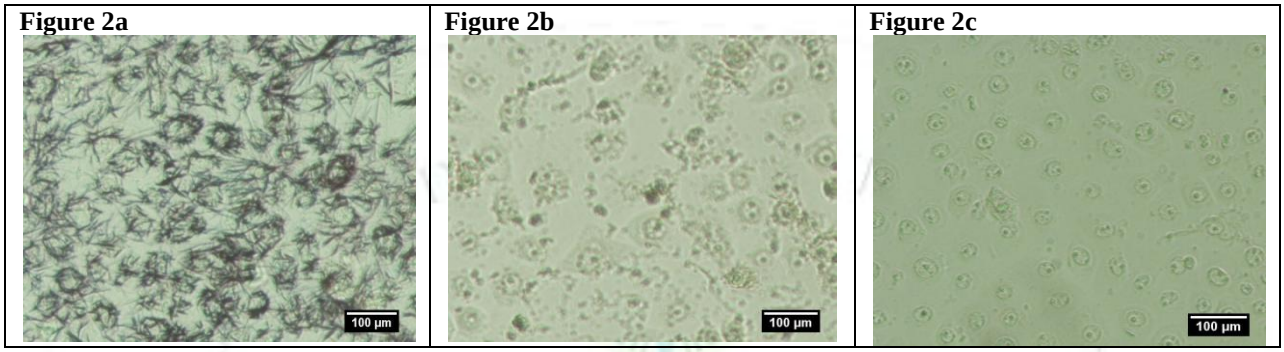

Figure 2: Microscopic observation of viability of vaginal epithelial cell line (VK2/E6E7) as tested through MTT assay after 24 hrs of treatment. (a) Untreated control, (b) MEEN (1 mg/ml), (c) Positive control of cytotoxicity (0.1% Triton X-100) (Magnification: 20X; Scale: 100 µm). When compared to untreated control, MEEN and Triton X-100 treated cells can be seen to have shrunken, rounded cells with lot of cell debris and ruptured cell membrane, formazan crystal formations are also rarely observed.

Determination of transepithelial electrical resistance

By growing HEC-1-A cells on Transwell inserts, a polarized epithelial cell monolayer with both apical and basolateral compartments was established (**Table 2**). On day 8th, confluent monolayer of HEC-1A cells were treated with various treatments for 24 hrs and changes in TER values in comparison to a known cell death inducer (0.1% Triton X-100) were determined. In this test as well MEEN at a concentration of 1.0 mg/ml (T-En1.0) showed significant ($p < 0.01$) reduction in TER values, comparable to the cell death inducer, indicating considerable cell death and monolayer disruption. **Fig. 3a and 3b.** shows the recording of TER values as the confluency increased and effect on TER values post treatment.

Table 2: Time dependent effect of MEEN on transepithelial electrical resistance (TER) on HEC-1-A cell monolayer. Monolayer integrity was measured through changes in TER values upon treatment with plant extracts. Values are calculated as mean ± SD of three independent experimental trials.

		Resistance (Ω/cm^2) (Mean ± SD)		
Treatments		**UT**	**T-En1.0**	**TX**
Time (days)	Day 1	32.67 ± 8.39	30.33 ± 5.03	32.67 ± 3.21
	Day 2	39.00 ± 2.00	37.67 ± 4.51	44.00 ± 3.61
	Day 3	44.00 ± 3.00	60.00 ± 7.21	70.67 ± 2.52
	Day 4	61.33 ± 4.04	72.33 ± 2.08	71.00 ± 2.65
	Day 5	67.33 ± 2.52	76.67 ± 5.69	74.33 ± 4.04
	Day 6	70.67 ± 4.51	82.00 ± 6.56	75.67 ± 5.86
	Day 7	85.67 ± 1.53	83.67 ± 2.52	85.33 ± 3.51
	Day 8 (before treatment)	93.67 ± 5.13	98.00 ± 4.00	91.33 ± 4.04
	Day 9 (after treatment)	92.67 ± 3.06	73.33 ± 5.13	47.00 ± 2.00

UT, Untreated; T-En1.0, MEEN (1mg/ml); TX, 0.1% Triton X-100.The significant effect of each treatment as compared to untreated control was carried out through t test at significance level (*** $p < 0.01$).

Figure 3a

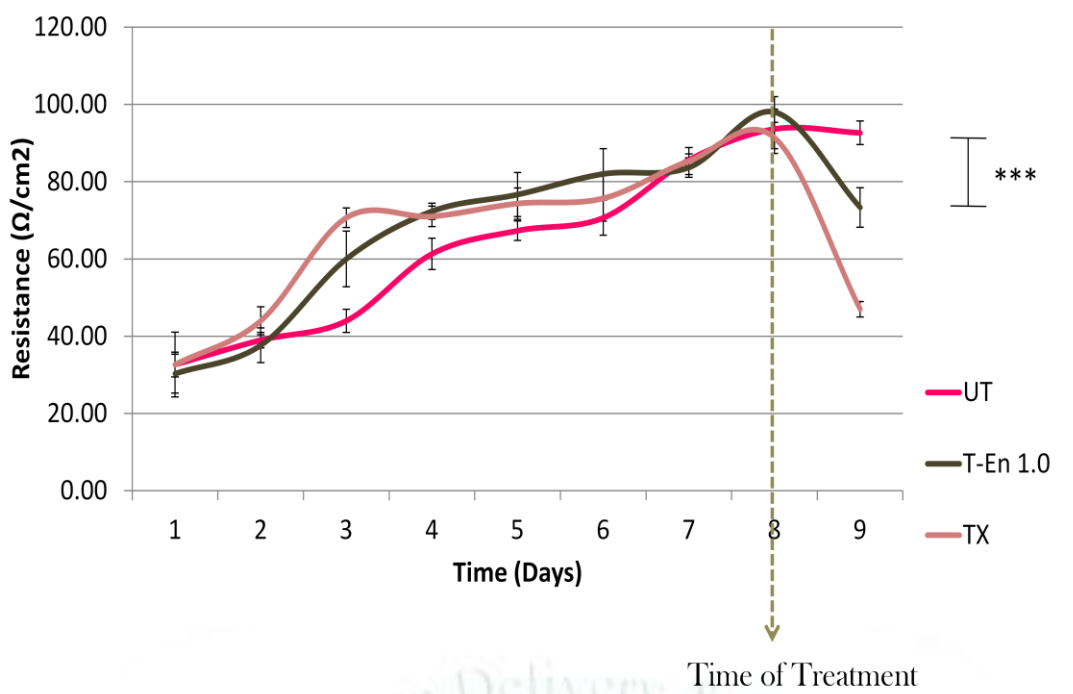

Figure 3b

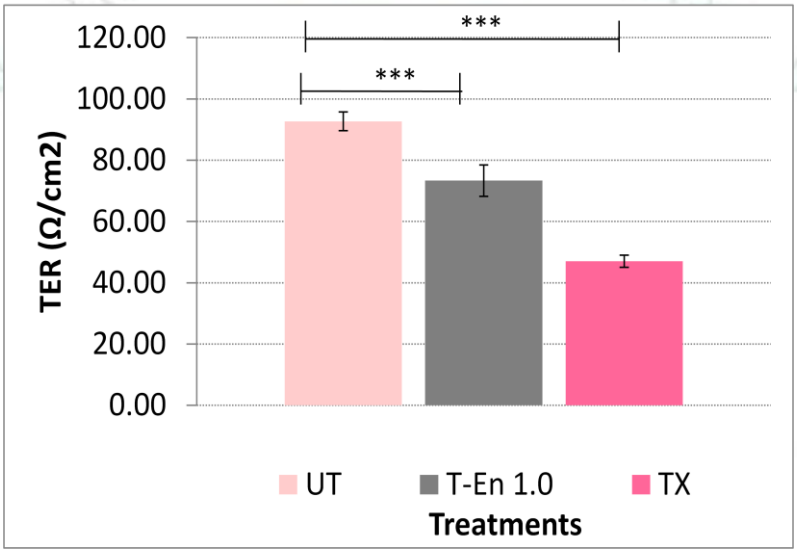

Figure 3: Time dependent effect of MEEN on transepithelial electrical resistance (TER) of HEC-1-A cell monolayer. a) TER values as measured on a daily basis till the 8th day when monolayer was formed and TER values peaked. On 8th day treatments were given and on 9th day (24 hrs) TER values were noted again to check for any drop in values signifying disruption of monolayer integrity. b) Comparison of TER values between untreated and treated groups on 9th day. Values are calculated as mean ± SD of three independent experimental trials. The significant effect of each treatment as compared to untreated control was carried out through t test at significance level (*** $p < 0.01$).

UT, Untreated; T- En1.0, MEEN (1mg/ml); TX, 0.1% Triton X-100

Permeability of fluorescent microspheres

Enhanced fluorescence was observed for the background well containing media only without cell and cell death inducer 0.1% Triton X-100, indicating TX to have extensively disrupted the confluent monolayer formed by HEC-1-A cells. As seen in **Fig. 4,** percentage fluorescence resulting from passage of fluorescently labeled microspheres at the 24 hour treatment time point were 29% for MEEN, indicating significant ($p < 0.01$) disruption in cellular integrity.

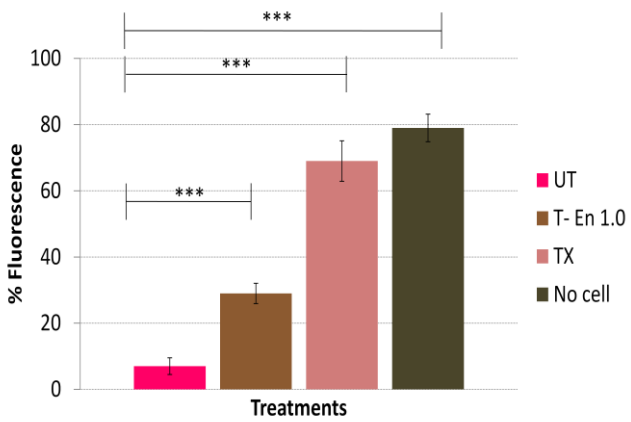

Figure 4: Graphical representation showing percentage fluorescence measured in the basal media due to permeation of fluorescent microsphere beads across polarized HEC-1-A monolayer. Values are calculated as mean ± SD of three independent experimental trials. The significant effect of each treatment as compared to untreated control was carried out through t test at significance level (*** $p < 0.01$).

UT, Untreated; T- En1.0, MEEN (1mg/ml); TX, 0.1% Triton X-100

Hemolytic activity of MEEN

Hemolytic activity of MEEN against HRBCs was evaluated at serial concentrations in the range of 0.1–20 mg/ml (**Fig. 5**). MEEN showed no hemolytic activity in the range of 0.1–2.5 mg/ml, on the other hand at higher concentrations of 5–20 mg/ml MEEN showed considerable hemolysis in the 19%–31% range. Minimum hemolytic concentration that caused 10% hemolysis of HRBCs was determined to be 5.0 mg/ml for MEEN (**Table 3 & Fig. 6**).

Table 3: Determination of Minimum hemolytic concentration. The concentration of MEEN which caused 10% hemolysis in human RBCs was taken as the minimum hemolytic concentration.

Concentration of extract (mg/ml)	% Hemolytic
0.1	0% ± 0.00
0.2	0% ± 0.00
0.3	0% ± 0.00
0.6	0% ± 0.00
1.2	0% ± 0.00
2.5	0% ± 0.00
5	**19.25% ± 0.03***
10	28.92% ± 0.02
20	31.47% ± 0.06
Untreated	0% ± 0.00
0.1% Triton X-100	100% ± 0.00

*Minimum hemolytic concentration

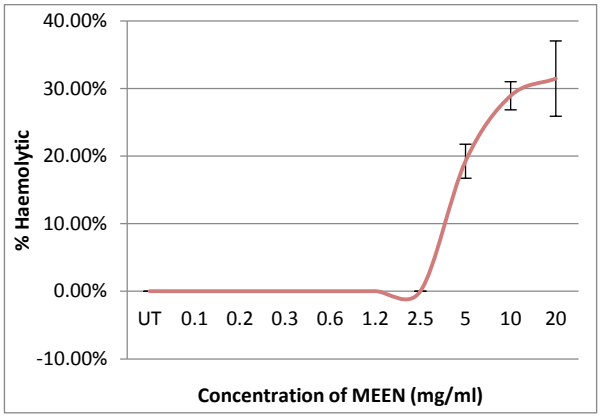

Figure 5: Effect of MEEN in inducing hemolysis in human RBCs. Values are calculated as mean ± SD of three independent experimental trials.

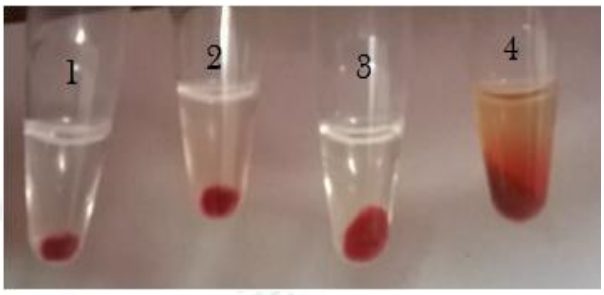

Figure 6: Effect of MEEN treatment on hemolysis of human RBCs. 1. Untreated control, 2. MEEN (2.5 mg/ml), 3. MEEN (5 mg/ml), 4. MEEN (10 mg/ml). Hemolysis was observed in the 3rd and 4th MEEN treated tubes as can be seen through diffused RBC pellets.

DISCUSSION

The study was undertaken with the aim of developing a microbicide from the herb *Evolvulus nummularius*, which is well-known for its medicinal properties.[3-11] Since safety study is an essential early step in preclinical microbicide development, the safety of the methanolic crude extract of *E. nummularius* (MEEN) was tested in this paper.

For a future microbicidal candidate to be successful, *in vitro* results should mimic future *in vivo* results. For this reason a logical step is to select such cell types for cytotoxicity tests which are most likely to be involved during *in vivo* infection cycles. Since the exact mechanism of infection is still a mystery for most STI pathogens and also because an ideal microbicidal candidate is required to be effective against more than one STI pathogen, it is best to test the candidate compound on a variety of cell types focusing on those known to be targets for *in vivo* infection. Hence, vaginal (VK2/E6E7), endocervical (End1/E6E7) and endometrial (HEC-1-A) cell lines were chosen, which will likely be involved in *in vivo* infection cycles[18] and are the target cells for developing a vaginal microbicide.

A microbicide for optimal activity needs to be applied frequently over a long period of time and it is essential that the compound does not cause abrasion or inflammation to the host tissue. Therefore cell viability assay using MTT assay on vaginal epithelial cell lines and a hemolytic assay on human RBCs were performed.

Following MTT treatment, after a period of 24 hrs with MEEN at concentration range of 1 mg/ml – 12 mg/ml, microscopic observation showed extensive cell death with cell disruption, breakage of cell to cell adhesion and very few intracellular formazan crystals. While the IC_{50} of VK2/E6E7 and HEC-1-A cells were 2 mg/ml, IC_{50} of End1/E6E7 was even lower at 1 mg/ml. Cell mortality was comparable to the commonly used cytotoxic compound Triton X-100. In the hemolytic assay, after treating with MEEN for one hour, MEEN did not show hemolysis till the concentration of 2.5 mg/ml. However at higher concentrations ranging from 5 mg/ml to 20 mg/ml 20% to 30% hemolysis were observed.

In TER and microsphere permeability assays, polarized HEC-1-A monolayer were set up in dual chamber Transwell units mimicking the *in vivo* epithelial barrier. Also mimicking natural infection by STI causing pathogens were the fluorescent microspheres used in the permeability assay which were of 100 nm diameter, a dimension similar to that of HIV- 1 virus particles. As would be done in the application of a microbicide, MEEN was applied onto the apical side only for 24 hours. A significant drop in electrical resistance as measured through TER and enhanced fluorescence from passage of microspheres from the apical to the basal chamber compared to the control well implied cell death, which in *in vivo* system would translate to disruption of the epithelial layer and passage of STI pathogens.

Although several plant extracts may have medicinal value, their therapeutic potential is known to depend on various other factors. Secondary metabolites can cause toxicity to cells at concentrations demonstrating inhibition of bacterial growth. In this study, phytochemical analysis of *E. nummularius* was found to contain saponins, triterpenoids and cardiac glycosides in high concentrations. Saponins and triterpenoids have both been studied to have cytotoxic effect. The presence of saponins which if present in high concentration may disrupt cellular membranes and cause cellular toxicity and hemolysis.[19] Triterpenoids in several studies have shown specific toxicity towards cancer cell lines,[20] however, in certain studies they have shown cytotoxicity to normal cells of the human body too.[21-23]

Drawbacks of this study and studies in general on plant extracts as microbicidal study candidates are batch to batch variations on presence of secondary metabolites which may be due to season of collection, phenological age of the plant, climatic and abiotic factors. Future studies therefore should focus on methods to identify the pure compound responsible for its antimicrobial activity, which can avoid such problems as batch to batch variations, and also pure compounds can be expected to be effective at much lower concentrations.

CONCLUSION

In conclusion, though we started out with the aim of developing a microbicide from the plant *E. nummularius* whose medicinal properties have been well documented, *in vitro* safety studies showed the methanolic extract of the plant to be highly toxic towards human red blood cells and female genital tract epithelial cells. *E. nummularius* extract has many applications in folk medicine. Due to its highly toxic nature, its applications where applied directly to the human epithelium such as to treat burns and cuts,[3] as a treatment for scabies when applied as a paste,[4] as a wound healing compound,[5] and as a paste to treat peptic ulcer[7] should be done with extreme caution and safety studies should be conducted using appropriate cell types.

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Conflict of Interest: Nil

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