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Estimation of *in vitro* cytotoxicity of *Adhatoda vasica* leaves on monkey kidney cell line

Monica Bothinathan, Ghadhevaru Sarathchandra, KS Vijayarani, M Parthiban, SP Preetha and S Vairamuthu

Abstract
Aim: *Adhatoda vasica* is well known for its effectiveness in treating asthma, chronic bronchitis and other respiratory conditions. This study aims for the estimation of Nephrotoxicity of *Adhatoda vasica* leaves extract by *in vitro* cytotoxicity on Monkey Kidney cell line.
Methodology: The leaves of *Adhatoda vasica* collected and extracted for the active principle Vasicine. *In vitro* cytotoxicity of the extract Vasicine on Monkey kidney cell line was performed by Micro culture Tetrazolium assay.
Results: The study finding infers that the extract of Adhatoda vasica leaves is safe at 10 µg, 100 µg and 250 µg doses and cytotoxic at 500 µg and 1000 µg dose concentration.
Interpretation: This study infers that safe dose of *Adhatoda vasica* plant Extract Vasicine is upto 250ug in normal cell Cell line above which it will be toxic.

Keywords: *Adhatoda vasica*, Vasicine, cytotoxicity, MTT assay, TLC Vasicine, FTIR Vasicine

1. Introduction
Adhatoda vasica, commonly known as Adosa or Vasica, belongs to the medicinal plant family Acanthacea. It is a small evergreen shrub found in many regions of India and throughout the world with multitude of uses in traditional Ayurveda. It has been used for centuries with much success to treat asthma, chronic bronchitis and other respiratory conditions due to its antispasmodic, expectorant and stimulant effect on the respiratory system. The herbal powder boiled with sesame oil used to treat ear infections and to arrest bleeding. Boiled leaves are used in the treatment of rheumatic pain and to recline the pain of urinary tract infection. It is also reported to have abortifacient properties (Atul *et al*., 2014) [3].

Fig 1: Image shows *Adhatoda vasica* plant

Adhatoda vasica is an evergreen shrub of 1-3 feet in height with many long opposite branches (Fig.1). Leaves are large and lance shaped. Stem is herbaceous above and woody below. The flowers of *Adhatoda vasica* are either white or purple in colour.
Adhatoda vasica can be used in anti-asthmatic therapy due to its antibacterial activity on *Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus pyrogene* and *Staphylococcus aureus* causing upper respiratory tract infections (Nilani *et al.*, 2009) [10].
The pectic arabinogalactan isolated from aqueous extract of *Adhatoda vasica* showed

antitussive activity in guinea pigs (Nabanita *et al*., 2011). Essential oil obtained from *Adhatoda vasica* leaves showed antimicrobial activity against *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus* and E.coli (Sarker *et al*., 2011) [14].

According to Vijayanandaraj *et al*. (2013) [18], the aqueous extract from leaves of *Adhatoda vasica nees* has the capability to detoxify the aflatoxin B_1.According to Bharat singh *et al.* (2013) [4], vasicine has the potent anti-inflammatory and antimicrobial activity.

It has the following pharmacological activities like Anti – asthmatic, Bronchodilator, Wound healing, Anti-ulcer, Cholagogue, Anti allergic, Antitubercular, Abortifacient, Uterotonic, Insecticidal, Anti-bacterial, Anticestodal, Anti inflammatory, Anti oxidant, Antitussive, Hepatoprotective, Anti-viral, Thrombolytic, Antifungal, Antidiabetes, Antituberculosis and Immunomodulatory.

In vitro exposure of *Adhatoda zeylanica* lacks acute toxicity to Human renal cells and also lacks proximal tubule nephrotoxicity (Miriam *et al*., 2016) [7]. *In vitro* exposure of Vasaka on African green monkey kidney cell line (Vero) and assessing its cytotoxicity by Micro culture tetrazolium (MTT) assay lacks cytotoxicity (Jethva *et al*., 2016) [5].

Adult Zebrafish were treated with different concentrations of vasicine orally and were periodically assessed for a 14 day acute period and 30 day chronic period and both the organ pathology and cognitive studies showed no toxicity on oral administration at 18.5 ng for 30 days revealed that vasicine is safe at nanogram quantity for a Zebrafish when administered orally (Arthi *et al*., 2016) [1].

The leaf extract of *Adhathoda vasica* has anticestodal activity against *Hymenolepis diminuta* (Arun *et al*., 2017) [2].

Keesara *et al*. (2017) [6] documented the vast pharmacological uses of Adhatoda, believed to be due to high concentration of alkaloids. The prominent alkaloid found in Adhatoda leaves is the quinazoline known as vasicine. In addition to vasicine, the leaves and roots also contain the alkaloids 1-vasicine, maintone, vasicinolone and vasicinol which are responsible for Adathoda's bronchodilator effect. Recently (Tapan *et al*., 2018) [16] reported the antiproliferative activity of Vasicinone on lung carcinoma cells.

However literature on its action in kidney is scarcely available, therefore the *in vitro* activity of *Adhatoda vasica* on kidney cell has been selected for this study.

2. Materials and methods

2.1 Materials

2.1 A) Chemicals used for extraction

1. Chloroform
2. Methanol
3. Petroleum ether
4. Dichloromethane
5. Hexane

2.1 B) Thin Layer chromatography

1. TLC plates (precoated with silica)
2. Anisaldehyde sulphuric acid reagent

2.1 C) MTT Assay

2.1 C) a) Cell line

Vero cell line available at Department of Animal Biotechnology, Madras Veterinary College, Chennai was used for this study.

2.1 C) b) MTT dye

3-(4,5 –dimethyl –thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) yellow dye available at Department of Animal Biotechnology, Madras Veterinary college, Chennai was used for MTT assay.

2.2 Methodology

a) Collection and extraction

The leaves of *Adhatoda vasica* were collected from Medicinal Botanical Garden, Pharmacovigilance Laboratory for Animal Feed and Food safety, DCAHS, Madhavaram were dried under shade and coarsely powdered with the help of mechanical grinder. The methanolic extract from the *Adhatoda vasica* leaves was prepared (Keesara *et al*., 2017) [6] 200g of the powdered Adhatoda vasica leaves were added to 750 ml of methanol and refluxed at 60 °C for 90 min. The contents were filtered and to the filtrate added 800ml 0f 75% Methanol and again refluxed at 60°C for 4 hours. The contents were allowed to stand cool and filtered. The obtained 750 ml methanolic extract was collected and extracted in separation funnel with 100ml water followed by 200ml Dichloromethane thrice. The extracted 600ml lower layer was added with 8-10gms of activated charcoal and kept in water bath for 10 minutes. The contents were filtered using Whatmann filter paper. The yellowish colour solution obtained was evaporated on water bath and cooled at room temperature. A blackish coloured semisolid material obtained is washed and shaken well with 70-80 ml of n-Hexane, then allowed to stand for 5 min and decanted to other dish.

Again 70ml hexane added and washed. Yellowish green powder obtained which contains vasicine (Fig.2).

Fig 2: Extracted Vasicine

2.2 b) Characterisation of Vasicine

Characterisation of *Adhatoda vasica* was done by Fourier Transform Infrared spectroscopy (FTIR) (Fig.3).

Chemical Names: vasicine, Peganine, Vasicin

Molecular Formula: C11H12N20

Molecular weight: 188.23 g/mol

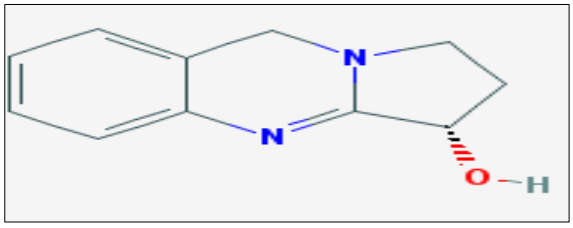

Fig 3: Vasicine structure

2.2 c) Thin layer chromatography

The extracted vasicine was confirmed by Thin layer chromatography (Keesara *et al*., 2017) [6]. The extract was dissolved in methanol and 10, 20 and 30 µl of the dissolved extract were applied on the TLC plate precoated with silicagel and the bands were developed using the solvents chloroform: methanol (9.0:1.0) as the mobile phase. The plates were dried and examined under 254 nm. The spraying reagent anisaldehyde sulphuric acid was sprayed to the plate. The plate was heated at 110 celsius for about 5 min for the formation of bands.

2.2 d) *In vitro* cytotoxicity assessment

In vitro cytotoxicity assay (Microculture Tetrazolium assay MTT) of *Adhatoda vasica* leaf extract on African green Monkey kidney cell line was performed. African green Monkey kidney cell line which was available at Department of Animal Biotechnology, Madras Veterinary College, Chennai was used for the study.

MTT assay: The 3- (4, 5 –dimethyl –thiazoyl) -2,5-diphenyl-SH -tetrazolium bromide (MTT) assay was performed (ural *et al*., 2006) [17] for the viability of the cells.

Target cells were resuspended in medium after subculturing and 100µl of cell suspension was dissolved into each well for 24 hours. Wells with 200µl of cells containing medium alone without reagents was used as the negative control.

After treatment with different doses of 1mg, 500 µg, 250 µg,100 µg and 10 µg leaf extracts of *Adhatoda vasica* for the stated incubation time, 20µl of MTT solution (5mg/ml) was added to each well, and the microplates were further incubated at 37 °C for 4 hrs. The unreactive supernatants in the wells was discarded and 100 µl of DMSO was added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. The absorbance values of each well were recorded with a microplate enzyme –linked immunoassay reader equipped with a 570 nm filter.

The results are presented as the percentage viability determined as,

$$\text{Cell Viability\%} = \frac{\text{OD Sample}}{\text{OD Control}} \times 100$$

3. Results

3.1 a) FTIR Confirmation of Vasicine: a) The active principle vasicine was confirmed with the C-N in it's structure by Fourier Transform Infrared spectroscopy which showed the absorbance peak spectrum at 1023.97 cm^{-1} (Fig.4).

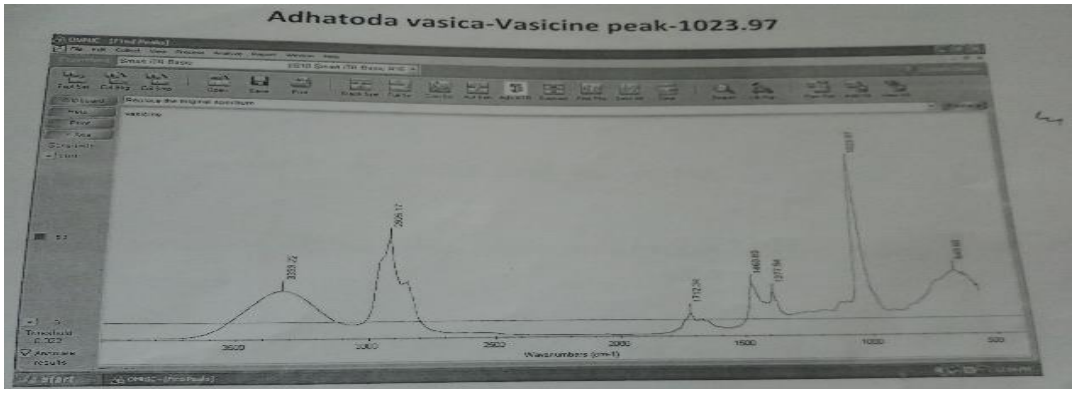

Fig 4: FTIR absorbance spectrum

3.1 b) Thin layer chromatography confirmation of Vasicine

The bands were clearly visible and the Vasicine was spotted in violet colour with anisaldehyde sulphuric acid reagent in the Thin layer chromatography plates (Fig.5 & 6). The spots were separately scrapped off and also confirmed in Fourier transform infrared spectroscopy with the chemical names as Peganol, Peganate and Pegameen.

Peganine is the chemical name of Vasicine which forms co crystal with alkaloid Peganol which was confirmed in Fourier transform infrared spectroscopy.

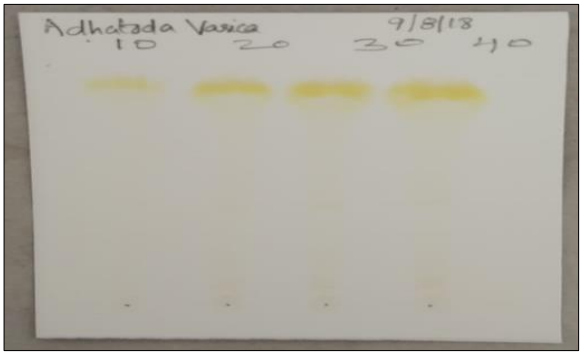

Fig 5: TLC plate before spraying reagent

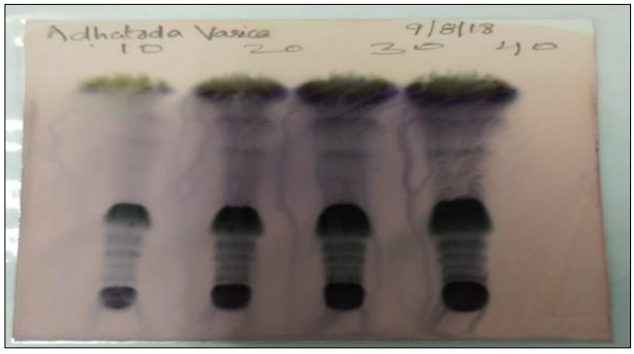

Fig 6: TLC plate after spraying reagent

3.2 *In vitro* cytotoxicity

The absorbance values of each well was observed with a microplate enzyme–linked immunoassay reader equipped with a 570 nm filter (Fig 7 and 8).

MTT Assay Plate

Fig 7: Micro wells before incubation.

Fig 8: Micro wells after incubation.

The results (Table.1 & Fig.9) were presented as the percentage viability determined as, Cell Viability% = OD Sample x100 and the results are as follows: OD Control

Table 1: Cell Viability % of Monkey kidney cell line by MTT assay

S. No	Dose concentration	Cell Viability %
1	10 µg	100%
2	100 µg	77%
3	250 µg	45%
4	500 µg	16.8%
5	1000 µg	6.28%

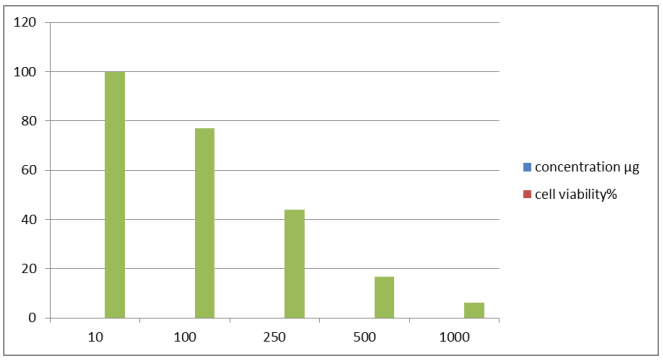

Fig 9: Cell Viability % of Monkey kidney cell line by MTT assay

Cell Viability %
Concentration (µg)

The dose concentration 10 µg,100 µg and 250 µg of the *Adhathoda vasica* extract showed more than and nearing to 50% cell viability which was considered as non toxic to cell whereas the dose concentration of 500 µg and 1000 µg showed cell viability less than 50% which revealed cytotoxicity.

4. Discussion

4.1 FTIR confirmation of Vasicine

The *Adhathoda vasica* leaf were extracted for the active principle Vasicine having the chemical name Peganine which was confirmed in Fourier Transform Infrared spectroscopy and Thin layer chromatography.

The C-N in Vasicine structure was characterised by Fourier Transform Infrared spectroscopy which showed the absorbance peak spectrum at 1023.97 cm^{-1}.

The FT-IR spectrum of purified extract from suspension cultures of Justicia Adhatoda L showed the characteristic absorption bands for various functional groups at 1470 cm^{-1} (CN bond); 1660,1684 cm^{-1} (stretching vibration of C=O); 2871, 3163 cm^{-1} (stretching vibration of O-H group) (Rashmi *et al*., 2012). Thus the Vasicine structure were confirmed with their CN bond showing absorbance at 1023.97 cm^{-1}.

4.2 Thin layer chromatography confirmation of Vasicine

The TLC bands were clearly visible and Vasicine was spotted in violet colour with anisaldehyde sulphuric acid reagent in the thin layer chromatography plates which confirmed the vasicine. Peganine is the chemical name of Vasicine which forms co crystal with alkaloid Peganol which was confirmed in Fourier transform infrared spectroscopy.

Keesara *et al*. (2017) [6] documented that the prominent alkaloid found in Adhatoda leaves is the quinazoline known as Vasicine. In addition to Vasicine, the leaves and roots also contain the alkaloids 1-vasicine, Maintone, Vasicinolone and Vasicinol which are responsible for Adathoda's bronchodilator effect. The phytochemicals present in Adhatoda leaves and roots were also confirmed using thin layer chromatography (TLC) and the plate was examined under 254 nm with anisaldehyde sulphuric acid as spraying reagent which is in accordance with the study.

4.3 *In vitro* Cytotoxicity

In the study the extracts of *Adhathoda vasica* were able to produce a significant decline in cell viability in the dosage concentration of 500 µg and 1000 µg to 16% and 6.28% respectively while the cell viability in the dosage concentration of 10 µg, 100 µg and 250 µg showed the cell viability as 100%, 77% and 44% respectively.

The Cytopathic effect (CPE) was noticed in 500 µg and 1000 µg with the disappearance of the cells and clogging of the cells (Fig.12 &13). The changes in the cell size and shape was noticed.

This finding reveals that the extracts of *Adhathoda vasica* dose concentration of 500 µg and 1000 µg are cytotoxic to Vero cells and dose concentration of 10 µg, 100 µg and 250 µg are non-cytotoxic shows the toxicity dose of Vasicine (Fig.10 &11).

In vitro exposure of *Adhatoda zeylanica* lacks acute toxicity to Human renal cells and also lacks proximal tubule nephrotoxicity (Miriam *et al*., 2016) [7]. *In vitro* exposure of Vasaka on African green monkey kidney cell line (Vero) and assessing its cytotoxicity by Micro culture tetrazolium (MTT) assay lacks cytotoxicity (Jethva *et al*., 2016) [5].

The study finding infers that the crude extracts of Adhatoda vasica is non Cytoxic at 10, 100 and 250 µg dose concentration and cytotoxic at 500 and 1000 µg dose concentration.

4.4 Micrographic photos of Mt assay

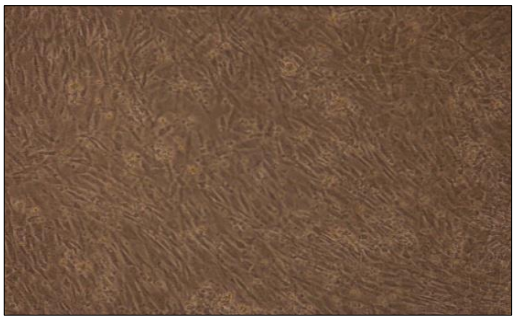

Fig 10: Microscopic photo of control well showing normal cells 10x.

Fig 11: Microscopic photo of 10ug Conc well showing normal cells 10x.

4.5 Cells showing cytopathic effect

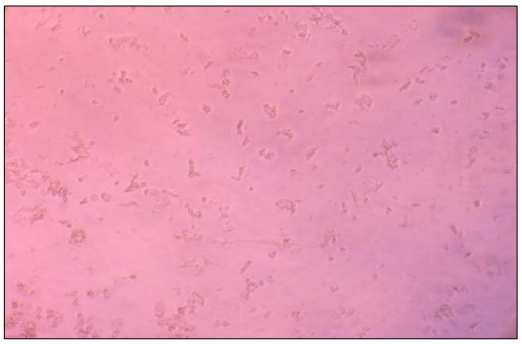

Fig 12: Microscopic photo of 500 ug Conc well showing shrinkage of cells 10x.

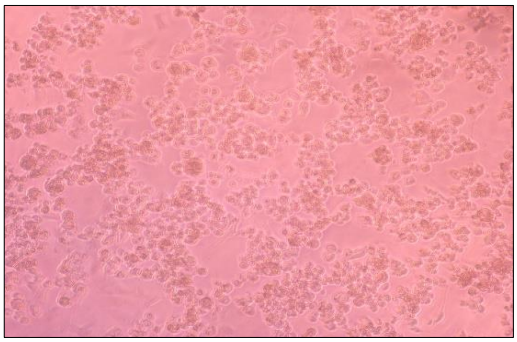

Fig 13: Microscopic photo of 1000ug Conc well showing clogging of cells 10x

5. Conclusion

The finding study shows that safe dose of *Adhathoda vasica* plant extract Vasicine is upto 250 µg in African monkey kidney cell line above which it will be toxic.

6. Acknowledgement

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