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Phytochemical Analysis, Cytotoxicity Activity And Acaricidal Activity Of Aqueous Crude Extract Of Phytolacca dodecandra Against Larvae Of Rhipicephalus appendiculatus

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Abstract: *Ticks are acari responsible for severe losses in the livestock industry. This study evaluated the larvicidalproperties of aqueous crude extract of Phytolaccadodecandra against larvae of Rhipicephalusappendiculatus. Contact toxicity was used in the bioassay and mortality data was obtained at 6, 12, 24 and 48 hrs. The data obtained during bioassay was then subjected to probit regression analysis to estimate concentration dependent mortality for LC_{50} and LC_{90} values in mg/ml.The LC_{50} and LC_{90}were 17.3 (15.2-19.4) and 26.8 (23.3-34.4) mg/ml respectively. Phytochemical screening of aqueous extract revealed presence of saponins, steroids, flavonoids and terpenoids. The activity observed in this extract was attributed to the presence of saponins. The plant demonstrated no cytotoxicity against vero cells hence the extract was considered safe for use in controlling R. appendiculatuslarvae infestation in livestock.*

Keywords:*Phytochemicals, Cytotoxicity, Bioassay,*

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I. Introduction

Ticks belong to phylum *Arthropoda,* class *Arachnida* and subclass *Acari*. It is a large and diverse group with an estimated 50,000 described tick species (Hallida*yet al.,* 2000). They are ectoparasites responsible for severe losses in the livestock industry. Studies by Wall (2007) found that 80% of 1200 million cattle were at risk of getting tick-borne diseases causing a global annual loss of US $ 7000 million. It transmits *Theileriaparva* which causes East coast fever, a threatening disease in the livestock industry in Eastern, South-eastern and Central Africa (Olwoch*et al.,* 2008).

The use of synthetic chemical acaricides to control ticks in cattle is expensive, ineffective due to development of resistance and it leads to environmental pollution (Grombon*iet al.,* 2007). Medicinal plants have been in use since ancient times as a remedy to several ailments affecting man and animals. Thus, ethnomedicinal knowledge and practices plays an important role in complementing modern approaches of disease and vector management. According to Kaaya, (2000) pasture grasses are capable of repelling, trapping and killing ticks, since they possess hairs called trichomes that retard ticks from climbing to the top of the grasses in order to attach themselves on passing animals. Leaf extracts of *Tephorosiavogelii* have been shown to be highly toxic to one, two, and three host ticks (Kaposhi, 1992).

P. dodecandra belongs to *Phytolaccaceae*family commonly called endod in some parts of the world (Misganaw*et al.,* 2012). It is native to sub-Saharan Africa (Schemelzer and Gurib-Fakim, 2008). It is a perennial climbing plant and grows rapidly in highlands that lie at (1600-3000 m above sea level). Previous studies on *P. dodecandra* have shown the berries are used widely as molluscicides (Allen-Gil and Aldea, 2003). Leaves of this plant is also used for treating ringworms, while roots and stem are used to alleviate dysentery and other stomach disorders (Kisangau, 2007). Phytochemical analysis of *P. dodecandra*byMekonnen*et al.,*(2012).showed the plant has flavonoids and saponin. To the best of our knowledge, studies on acaricidal efficacy has not been done on aqueous extract of *P. dodecandra* and this study aimed at determining effect of this extract on larvae of *Rhipicephalusappendiculatus*.

II. Materials And Methods

Sample collection

Leaves of *P. dodecandra*were collected from botanical-garden of Egerton University in Kenya which is at altitude of 2,127 meters above sea level. A voucher specimen was deposited at the department of biological sciences, Egerton University

Extraction of aqueous extract

The leaves of *P. dodecandra*were air-dried under a shade for a period of 7-14 days while turning them periodically to expose all the leaves to air. They were weighed frequently until a constant weight was obtained and thus considered dry. Dried leaves were grounded using a blending machine (Thomas-Wiley Laboratory Mill Model 4) at Kenya agricultural research institute (KARI), Njoro. The powdered material was weighed with each 500 g being extracted with 1.8 liters of 95% methanol at room temperature for 72 hrs. It was then filtered through a Buchner funnel and the filtrate was concentrated to dryness under reduced pressure using rota-vapor machine (BUCHI – R 205). The concentrated crude methanol extract was placed in a separating funnel then suspended in water. It was then followed by sequential extraction with ethyl acetate and hexane repeatedly until both ethyl acetate and hexane were colorless, meaning they were no more compounds present in the methanol extract that could be extracted by both hexane and ethyl acetate. The hexane extract carried least polar phytochemicals while the ethyl acetate extract carried medium polar phytochemicals. The remaining extract in the separating funnel after hexane and ethyl acetate removed their compounds was the aqueous extractwhich was then storedat8ºC until qualitative determination of phytochemicals and evaluation of the larvicidal properties against *R. appendiculatus* larvae and cytotoxicity analysis against vero cells.

Larval bioassay

The larvae used for the bioassay were reared according to (Bailey, 1960) while acaricidal bioassay was done using contact toxicity according to Pamo *et al.,* (2005) with slight modification. From the preliminary results, 30 mg/ml of the aqueous crude extract dissolved in 2% DMSO, was found to be active against the tick larvae and this concentration was the stock solution. Serial dilution done on the stock solution resulted in 9 concentrations ranging from 30 mg/ml to 7 mg/ml. The concentrations were sprinkled using a pasture pipette on the petri dishes that hadwhatman No. 1 filter paper (15 cm) attached to the bottom using double sided cellophane tape, and contains 20 larvae. During sprinkling, the filter papers was ensured wet, and the larvae were exposed to the sprinkled extracts. The experiment was replicated three times and petri dishes held at 75 % relative humidity at 25°C. From the start of the experiment, mortality data was collected at 6, 12, 24 and 48 hrs. The larvae were considered dead if they couldn't move their appendages when prodded with a pin. A negative control was set consisting of 2% DMSO while a positive control was also set consisting of 0.2% v/v of amitraz.

Phytochemical tests

Chemical tests to identify phytochemical constituents of aqueous extract of *P. dodecandra* were carried out. It was done qualitatively, using standard procedures according to (Edeoga*et al.*,2005; Khan *et al.*,2011). The tested phytochemicals were tannins, phlobatanins, saponins, flavonoids, steroids terpenoids and cardiac glycosides.

Cytotoxicity assay

Aqueous extract of *P. dodecandra* was tested for *in vitro* cytotoxicity using MTT calorimetric assay (Mosmann, 1983). Vero cells (ATCC CCL-81) established from the kidney of a normal African green monkey (*Cercopithecusaethiops*), were used to determine the cytotoxicity of the plant extracts. These cells were obtained from KEMRI Nairobi. The Cells were first grown in Minimum Essential Medium (MEM) Eagle's Base supplemented with 15% Fetal Bovine Serum (FBS), 2.62 g/L $NaHCO_3$, 20 mM L-glutamine, 10 ml/L Penstrep and 0.5 mg Fungizoid using T-75 culture flask.

Culturing of the cells was done at 37^{o}C in 5% CO_2 for 24 hrs and once they attained confluence they were harvested by trypsinization and pooled into 50 ml vial. Cell suspension (1 x 10^5 cell/ml) approximately 100 µl were seeded into the 96-well flat-bottomed micro-titer plate containing100 µl of MEM (growth media) and incubated at 37^{o}C in 5% CO_2 for 48 hrs, to attain confluence. Once confluency was attained, the growth media was aspirated and replaced with 100 µl of maintance media. The cells were then exposed to increased concentrations of the aqueous extract ranging from (500 µg/ml to 0.23µg/ml) and further incubated at 37^{o}C for 48 hours.

After the incubation period, MTT (10 µL of 5 mg/mL) was added into each well and the cells incubated for another 4 hrs until purple precipitates (formazan) were clearly visible under a microscope. Subsequently, the supernatant was removed and replaced with acid-isopropanol (0.04N HCl in isopropanol). The well plate was gently shaken for 15 minutes to dissolve the formazan, followed by measurement of optical density (OD) using ELISA scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 690 nm. The 690 nm was the absorbance of background reference filter while the 562 nm was the absorbance of formazan. Percentage growth inhibition at each concentration was automatically calculated using a graphic program Ms excel, 2003 using the formula below (Ngeny*et al.,* 2013).

$$\%\ \text{growth inhibition} = 100 - \frac{(\text{OD sample } 562 - \text{ OD } 690)}{\text{OD control } 562 - \text{ OD } 690} X100$$

The IC_{50}, which is the concentration of the extracts, that reduced viable cell by 50%, was automatically calculated from graphs generated by the graphic program.

Statistical analysis

The mortality data obtained was subjected to Probit regression analysis to calculate concentration dependent mortality for the LC_{50} and LC_{90} values. SPSS 11.5 statistical software was used to determine the associated 95% confidence interval.

III. Results

Larval bioassay

No mortality was observed on larvae exposed to the extract within the first 12hrs. Insignificant mortality of 3.3% was observed at 30 mg/ml at 24 hrs. Varying mortalities depending on the concentration was observed at 48 hrs. Mean percentage mortalities of the larvae between 0-48 hrs are shown in Table 1. Varying mortality observed at 48 hrs resulted in LC_{50} of 17.3 (15.2-19.4) mg/ml and $LC_{90}$of 26.8 (23.3-34.4) mg/ml. The negative control used was 2% of DMSO which showed no activity against the larvae within 0 to 48 hrs.

Table 1: Mean larval mortalities caused by aqueous extract of *P. dodecandra*.

Concentration in mg/ml	**Mean % larval mortality at the hrs shown below**			
	6 hrs	**12 hrs**	**24 hrs**	**48 hrs**
7	0±0	0±0	0±0	0±0
10	0±0	0±0	0±0	13.3±5.8
13	0±0	0±0	0±0	20±17.3
16	0±0	0±0	0±0	30±10
19	0±0	0±0	0±0	56.7±11.5
22	0±0	0±0	0±0	70±17.3
25	0±0	0±0	0±0	86.7±11.5
27	0±0	0±0	0±0	93.3±5.8
30	0±0	0±0	3.3±5.8	100±0
Amitraz® (0.2% v/v)[P]	0±0	56.7±11.8	90±17.3	100±0
(2% DMSO)[Q]	0±0	0±0	0±0	0±0

[P] positive control;[Q] Negative control

Phytochemical tests

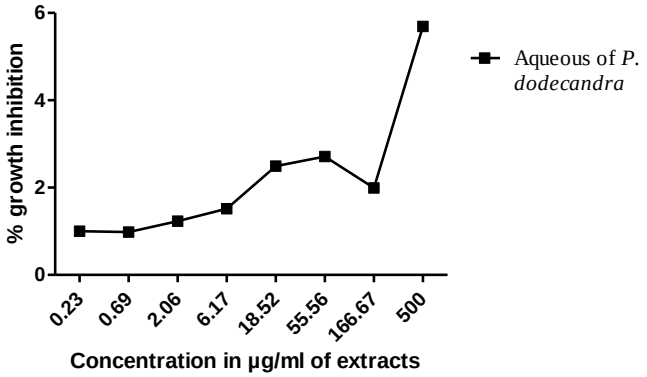
Results of phytochemical tests done on aqueous extract of *P. dodecandra* extract are shown in Table 2. Phytochemicals present were saponins, flavonoids, terpenoids and steroids.

Table 2: Results of phytochemical tests done on aqueous extract of *P.dodecandara*

Extract	**Results of Phytochemicals tested**						
	Saponin	**Tannins**	**Flavanoids**	**Phlobatanins**	**Steroids**	**Terpenoids**	**Cardiac glycosides**
Aqueous extract of *P.dodecandra*	+	-	+	-	+	+	-
Present + Absent-							

Cytotoxicity studies

Growth inhibition of vero cells by aqueous extract of *P. dodecandra*is presented in Figure 1. According to guidelines set by the National Cancer Institute (NCI) an extract is considered cytotoxic if the IC_{50}< 20 µg/ml (Geran*et al.,* 1972). In the present experiment, the extract was considered not cytotoxic since 50% growth inhibition could not be experienced even at the highest concentrationof 500 ug/ml.



IV. Discussion

The acaricidal activity observed in the aqueous extract of *P. dodecandra*is in agreement with previous studies on several species of genus *phytolaccaeae*, which have shown that aqueous extract of fruits and leaves of these plants carry bio-active compounds. These bio-active compounds in aqueous extracts of this plant, have been reported to have analgesic, antiinflammatory, bactericidal, fungicidal, mitogenic and molluscicide action (Quirogae*t al.,* 2001; FariasMagalhãese*t al.,* 2003; Delporte*et al.,* 2009). Saponins are the primary toxic constituents of *phytolaccaeae*(Armstrong, 2009) and has been isolated in many aqueous plant extracts of genus *phytolaccaeae* (Hernández1 *et al.,* 2013), thus giving a chemotaxonomic significance to the subfamily *phytolaccaeae* (Gattuso, 1996). Systemic screening of some 600 wild types of Endod plants indicated that berries of a *Phytolacca*species, type 44, in Ethiopia contained as much as 25% by weight of saponins, from which the molluscicides, lemmatoxins, have been isolated and purified with organic solvents (Lemma *et al.,* 1972).

Saponin isolated from *Phytolaccatetramera* fruits have been reported to cause inhibitory effect against human pathogenic fungi (Escalante *et al*., 2002). Oleanoglycotoxin-A a triterpenesaponin isolated from aqueous berry extracts of *P. decandra* has been attributed to the molluscicidal properties of this plant. Other saponins present in *P. dodecandra* are lemmatoxin A, B and C which have also been attributed for the molluscicidal activity of this plant (Harold *et al.,* 1993). Biological activities such as molluscicidal effect, antifertility, induction of immune interferon (INF-γ); enhancement of leukocyte phagocytosis and promotion of DNA transformation present in *Phytolaccaacinosa* has been attributed to saponins, (Ma *et al.,* 2010).

In the present study, the acaricidal activity observed in the aqueous extract of *Phytolaccadodecandra,* could be also be attributed to the presence of saponins in this extract. This is based on the abundance of this phytochemical in this family. Besides, saponins have also been attributed to the acaricidal efficacy of*Phytolaccadecandra*against *Tetranychuscinnabarinus* spider mite. (Ding *et al.,*2013). The results of the current cytotoxicity study are in agreement with previous toxicity studies, which reported that extracts of this plant wasnontoxic.A 28-day oral administration of extracts of unripe berries of *P. dodecandra* to rats in an acute mammalian test, showed the extract being nontoxic (Lambert *et al.,* 1991). Lemma and Ames, (1975) reported that extracts of *P. dodecandra* were neither mutagenic norcarcinogenic. Both human and guinea pigs have been reported to tolerate skin irritation of *P. dodecandra* (Mekonnen*et al.,* 2012).

Results of this study showed that aqueous extract of *P. dodecandra*has potential lead compounds for development of plant based acaricides based on its activity and safety.

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