Research Journal of Parasitology

ISSN 1816-4943

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Parasitology

ISSN 1816-4943
DOI: 10.3923/jp.2016.27.32

Research Article
Pheophorbide a Potential Source of Plant Molluscicide to Combat Against Neglected Tropical Disease Fasciolosis

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Abstract

To evaluate the molluscicidal activity of photodynamic product pheophorbide against *Lymnaea acuminata*. Fresh water snail *Lymnaea acuminata* is the vector of liver flukes *Fasciola gigantica*, which cause endemic fasciolosis in cattle population as well as in human beings. Ten snails *Lymnaea acuminata* were placed in a glass aquarium containing 3 L of dechlorinated tap water. These snails were treated with different concentrations of pheophorbide. Pheophorbide is derivative of chlorophyll was extracted from fresh leaves of spinach and kept for 2 h in 100% ethanol at 55°C. Photodynamic product pheophorbide was concentration and time dependent toxic against snail *Lymnaea acuminata.* Toxicity of extracted pheophorbide when *L. acuminata* kept in sunlight (summer 96 h LC_{50} 65.89 mg L^{-1}, winter 96 h 263.24 LC_{50} mg L^{-1}) was less than pure pheophorbide (summer 96 h LC_{50}13.39 mg L^{-1}, winter 96 h 26.02 LC_{50} mg L^{-1}). Pure pheophorbide was more toxic in summer sunlight in comparison with extracted pheophorbide. The production of chlorophyll derived pheophorbide is economical and unproblematic to environment and as a result a promising approach to control water/food-borne disease in developing countries.

Key words: Liver rot, *Lymnaea acuminata*, *Fasciola gigantica*, plant molluscicides, pheophorbide

Received: December 21, 2015 **Accepted:** February 08, 2016 **Published:** March 15, 2016

Citation: Kavita Singh and Vinay Kumar Singh, 2016. Pheophorbide a potential source of plant molluscicide to combat against neglected tropical disease fasciolosis. Res. J. Parasitol., 11: 27-32.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Water-borne diseases of cattle, fasciolosis (liver-rot) cause severe economic losses in all continents. It is caused by liver-flukes *Fasciola hepatica* and *F. gigantica* (Mas-Coma *et al*., 2009; Sunita *et al*., 2016). Lymnaeidae serves as an intermediate host of at least 71 trematode species, distributed among 13 families with the implication for cattle and human health (Correa *et al*., 2010). The Snail *Lymnaea acuminata* (Family, Lymnaeidae) is the vector of liver flukes *Fasciola gigantica* and *F. hepatica*, which cause endemic fasciolosis in cattle and livestock in Northern parts of India (Singh and Agarwal, 1981; Sunita *et al*., 2015). Fasciolosis affects the general immune system of the animal and there is no accurate method of early diagnosis, before the time of egg deposition adopted (Soliman, 2008). The various synthetic drugs and other control methods to combat against this disease are not successfully because of increase resistance and negative impacts to environment. Direct control of snail's populations by pesticides/drugs caused to development of drug resistance or residual effects in ecosystem. With growing awareness of chemical pollution, efforts are being made to interest in the use of plant product for the safe, effective and low cost to control the vectors of neglected tropical diseases. Plant products are biodegradable, no negative impact on environment and non-targeted organism. Plant derived products are rich in natural phytochemicals (Fan *et al*., 2011; Singh *et al*., 1996). Active ingredients from plant extracts are one of the possible approaches in control of snail population. A sure way to tackle the problem of fasciolosis is to destroy the carrier snails and remove an essential link in the life cycle of the flukes. This can be accomplished in a number of ways including the use of many plants as well as synthetic molluscicides (Singh *et al*., 1996). Pheophorbide is derivative of chlorophyll and chlorine based photosensitizer which is generally used for tumor treatment in Asia (Tang *et al*., 2009). It induces inhibitory effects on human hepatocellular carcinoma Hep3B cell as photosensitize with the approach of PDT (Chan *et al*., 2006; Tang *et al*., 2006). Chernomorsky *et al*. (1999) was observed toxicity of pheophorbide in mouse myeloma cell cultures. The aim of present study to evaluate the molluscicidal activity of photodynamically active product of spinach, pheophorbide against *Lymnaea acuminata* in winter and summer season.

MATERIALS AND METHODS

Pure compound: Pheophorbide is purchased from sigma chemical Co.USA.

Experimental animal: The adult snail *L. acuminata* of average size (2.25±0.30 cm in length) were collected locally from ponds, lakes and low-lying submerged fields of the district Gorakhpur, UP, India. Gorakhpur district lies between 26.5-27.9° N and 83.4-84.25° E at an altitude of 84 m above the sea level. The collected snails were kept in glass aquarium containing dechlorinated tap water for 72 h for acclimatization. The animals were kept in dechlorinated tap water at room temperature (22-25°C). The pH, dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 7.2-7.3, 6.4-7.3, 5.1-6.3 and 102-105 mg L^{-1}, respectively. Water was changed once every 24 h and dead animals were removed to prevent the water from being contaminated by decaying tissue.

Preparation of pheophorbide: Pheophorbide was prepared by the method of Wohllebe *et al*. (2011). Chlorophyll was extracted from spinach with the help of fresh leaves and kept for 2 h in 100% ethanol at 55°C in the incubator. The extract was subsequently filtered with the help of Whatmann filter paper and equal volume of petroleum benzene was added. After shaking the mixture in orbital shaking incubator the chlorophyll moved into the lipophillic benzene phase. The two phases were separated in separatory funnel and about 1 mL HCl was added to 50 mL of the benzene phase. The chlorophyll came into contact with the HCl and was transformed into water-soluble pheophorbide (Fig. 1).

Toxicity determination: Toxicity experiments were done according to the method of Singh and Agarwal (1984). Ten test snails *Lymnaea acuminata* were placed in a glass aquarium containing 3 L of dechlorinated tap water. These snails were treated with different concentrations of pheophorbide and kept for 3 h in darkness. Subsequently, they were kept in laboratory condition (light intensity

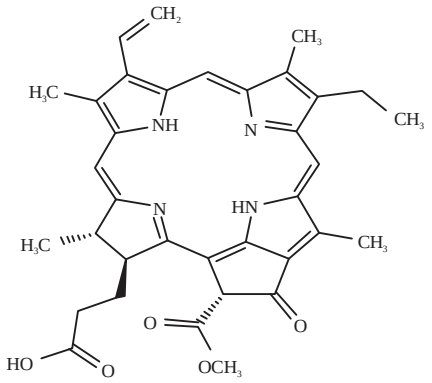

Fig. 1: Pheophorbide

130 W m^{-2}) sunlight (light intensity 900 W m^{-2} in winter season and 1200 W m^{-2} in summer season) upto 96 h. Light intensity was measure with the help of digital lux meter (Mextech LX-1010B). Mortality of snails was recorded at every 24 h upto 96 h. Each treatment was replicated six times. In control experiments snails were kept in dark condition after 3 h of dark incubation for 96 h (control 1), where as in another control except pheophorbide all treatments were same (control 2). In sunlight no treatment was given to the snail and kept for 96 h (control 3). Dead animals were removed instantly from the aquarium to avoid any contamination of the water. Snail mortality was confirmed by the contraction of the body within the shell and absence of any response to a needle probe.

The slope of the probit line was also estimated. This program ran chi-square tests for goodness of fit of the data to the probit model. If the model fits, the calculated value of chi-square is less than the chi-square table value for appropriate degree of freedom. If the model does not fit, the LC_{50} value for the particular population may not be reliably estimated and is adjusted with the heterogeneity factor as correction factor when the value of Pearson's chi-square statistics is significant ($p = 0.05$). The index of significance for potency estimation (g-value) was used to calculate 95% confidence intervals for potency (relative potency is equivalent to tolerance ratio). Parallelism of the probit regression lines implies a constant relative potency at all levels of response. Polo-PC was used to test equality and parallelism of the slope of the probit lines was calculated by using the probit analysis programme, POLO-PC (LeOra software) of Robertson *et al*. (2007). The regression co-efficient between exposure time and different values of LC_{50} was determined by the method of Sokal and Rohlf (1995).

Thin layer chromatography: Thin Layer Chromatography (TLC) was performed according to the method of Barone and Tansey (1977) as modified by Upadhyay and Singh (2011) to identify the active molluscicidal component present in spinach extracted Pheophorbide (Pa). The TLC was done on 20×20 cm precoated silica gel (Merck Specialities Private Limited, Mumbai, India) using benzene/ethyl acetate (9:1, V:V) as the mobile phase. Spots of extracted pheophorbide along with their respective synthetic pheophorbide were applied on TLC plates with a micropipette. Furthers, the TLC plates were developed I_2 vapour. Copies of chromatogram were made by tracing the plates immediately and Retardation Factors (Rf) were calculated.

RESULTS

The molluscicidal activity of pheophorbide in summer season (Sunlight/laboratory condition) and in winter season (Sunlight/laboratory condition) against *L. acuminata* was concentration and time-dependent. The LC_{50} of extracted pheophorbide in summer season (Sunlight/laboratory condition) and in winter season (Sunlight/laboratory condition) at 24 h were (170.24, 673.80, 545.07 and 1402.64 mg L^{-1}) and pure pheophorbide were (25.11, 48.11, 79.26 and 89.99 mg L^{-1}), respectively (Table 1 and 2). Among extracted and pure pheophorbide, pure pheophorbide was more toxic in summer season (sunlight condition) at 96 h. The 96 h LC_{50} of extracted and pure pheophorbide in summer season (sunlight condition) were (65.89 and 13.39 mg L^{-1}) (Table 2). There was no snail's mortality in control group 1, 2 and 3.

The thin layer chromatography analysis demonstrated that the Rf values of extracted pheophorbide (0.32) were equivalent to pure pheophorbide (0.32).

The slope values were steep and separate estimations of LC_{50} based on each of the six replicates were found within the 95% confidence limits of LC_{50}. The t-ratio values were greater than 1.96 indicating a significant regression of each dose response line. The heterogeneity factor was less than 1.0, demonstrating the log-dose-probit lines are within the 95% confidence limits and thus the model fitted our data. Value of g less than 0.5 indicated that mean was within the limit at all probability levels of 90, 95 and 95%.

DISCUSSION

It is evident from the results that pheophorbide extracted from spinach has much potential to kill the fresh water snail *Lymnaea acuminata*. Pheophorbide is a derivative of chlorophyll is the most active compound which was firstly isolated from *Scutellaria barbata* (Chan *et al*., 2006). Pheophorbide is synthesized by the removal of Mg^{2+} and phytol tail from chlorophyll (Wohllebe *et al*., 2011). Loss of phytol tail pheophorbide becomes more soluble in water. Both extracted and pure pheophorbide are more effective in summer than winter. In summer water temperature is higher and dissolved oxygen concentration is low, which pose higher mortality of snails and one of the main reasons in increasing the mortality of snail. Contrarily, in winter season the cause of less mortality of snail during this period is that in winter, water holds more oxygen (WANTM., 2002). Dissolved oxygen is one of the major components which are used by snails during metabolic activity (Ishak and Mohamed, 1975). Consequently,

Table 1: Toxicity of pheophorbide in laboratory condition against *Lymnaea acuminata* in winter and summer season

Exposure time	Treatments	Winter $LC_{50\ (LCL-UCL)}$ Nov, 21°C Dec,18°C Jan, 12°C Feb, 20°C	Slope value	t-ratio	g-value	Heterogeneity	Summer $LC_{50\ (LCL-UCL)}$ March, 30°C Apr, 39°C May, 33°C Jun, 38°C	Slope-value	t-ratio	g-value	Heterogeneity
24 h	Ext Pa	1402.64 (1245.34-1910.56)	6.74±1.64	4.11	0.22	0.33	673.80 (659.56-705.85)	22.82±5.25	4.34	0.20	0.21
	Pure Pa	89.99 (85.52-102.38)	13.11±3.18	4.11	0.22	0.21	48.11 (45.61-52.71)	8.10±1.57	5.13	0.14	0.17
48 h	Ext Pa	1393.04 (1206.18-2276.38)	4.76±1.41	3.38	0.33	0.13	658.79 (644.98-688.14)	18.29±4.81	3.79	0.26	0.18
	Pure Pa	86.75 (82.61-99.28)	10.26±2.80	3.65	0.28	0.13	43.93 (41.98-46.43)	8.42±1.51	5.55	0.12	0.26
72 h	Ext Pa	1227.64 (1102.79-1685.97)	4.62±1.32	3.48	0.31	0.18	637.16 (627.23-647.16)	26.07±4.88	5.33	0.13	0.15
	Pure Pa	82.35 (78.82-91.93)	8.75±2.66	3.28	0.35	0.11	40.28 (38.46-41.88)	10.00±1.55	6.42	0.09	0.22
96 h	Ext Pa	931.59 (886.25-974.43)	8.71±1.40	6.20	0.10	0.28	617.29 (604.05-626.28)	28.15±5.01	5.61	0.12	0.56
	Pure Pa	74.04 (72.15-75.55)	20.02±2.99	6.69	0.08	0.50	38.42 (36.61-39.85)	11.40±1.65	6.89	0.08	0.34

Each experiment was replicated six times. Toxicity measured at intervals of 24 h up to 96 h. Concentrations given is the final concentration (w/v) in the glass aquarium water. Ts- testing significant of the regression coefficient, Ext Pa-in winter, 2425^{++}, Pure Pa-in winter -0.06981^{+}, Ext Pa in summer -0.6225^{+}, Pure Pa in summer -0.06865^{+}, $^{+}$Linear regression between x and y, $^{++}$Non linear regression between log x and log y, Ext Pa: Extracted pheophorbide, Pure Pa: Pure pheophorbide, LCL: Lower confidence limit, UCL: Upper confidence limit

Table 2: Toxicity of pheophorbide in sunlight condition against *Lymnaea acuminata* in winter and summer season

Exposure time	Treatments	Winter $LC_{50\ (LCL-UCL)}$ Nov, 21°C Dec, 18°C Jan, 12°C Feb, 20°C	Slope value	t-ratio	g-value	Heterogeneity	Summer $LC_{50\ (LCL-UCL)}$ March, 30°C Apr, 39°C May, 33°C Jun, 38°C	Slope-value	t-ratio	g-value	Heterogeneity
24 h	Ext Pa	545.07 (437.33±808.82)	2.54±0.46	5.50	0.12	0.28	170.24 (118.45±519.54)	2.22±0.61	3.64	0.28	0.30
	Pure Pa	79.26 (73.84±129.24)	1.26±0.43	2.91	0.45	0.19	25.11 (21.63±33.20)	3.16±0.64	4.93	0.15	0.17
48 h	Ext Pa	549.76 (413.82±974.25)	1.83±0.38	4.78	0.16	0.30	137.03 (103.55±280.92)	2.33±0.57	4.06	0.23	0.35
	Pure Pa	55.01 (44.16±81.49)	1.71±0.41	4.29	0.20	0.20	21.06 (18.69±25.14)	3.33±0.61	5.45	0.12	0.19
72 h	Ext Pa	411.66 (317.85±660.85)	1.61±0.34	4.62	0.17	0.24	102.92 (82.18±173.16)	2.07±0.51	4.05	0.23	0.24
	Pure Pa	36.61 (29.51±44.79)	1.98±0.40	4.84	0.16	0.20	17.80 (15.89±20.24)	3.41±0.59	5.77	0.11	0.41
96 h	Ext Pa	263.34 (214.87±330.62)	1.92±0.34	5.56	0.12	0.26	65.89 (58.06±76.05)	3.07±0.51	6.01	0.10	0.40
	Pure Pa	26.02 (20.91±30.30)	2.73±0.44	6.12	0.10	0.52	13.39 (11.45±14.99)	3.51±0.59	5.89	0.11	0.43

Each experiment was replicated six times. Toxicity measured at intervals of 24 h up to 96 h. Concentrations given is the final concentration (w/v) in the glass aquarium water. Ts-testing significant of the regression coefficient, Ext Pa- in winter 1166^{++}, Pure Pa-in winter 296.3^{++}, Ext Pa-in summer -1.335^{+}, Pure Pa-in summer -0.1335^{+}, $^{+}$Linear regression between x and y, $^{++}$Non linear regression between log x and log y, Ext Pa: Extracted pheophorbide, Pure Pa: Pure pheophorbide, LCL: Lower confidence limit, UCL: Upper confidence limit

at higher temperatures, the rate of metabolism in the snail body becomes increase (Toews *et al*., 1995; Berge *et al*., 2006). Pheophorbide is used as photosensitizers and these are the molecules which are excited by light (Wilson *et al*., 1986; Tang *et al*., 2009). Reactive oxygen species will be produced after photosensitizer receives light energy during illumination in an oxygen-rich environment, which eventually will initiate apoptosis or necrosis in the treated cells (Via and Magno, 2001). These species induces cellular apoptosis and damage the cellular component such as lipids, proteins, DNA and one of the best targets for photosensitizer is suggested to be mitochondria as it will initiate damage and finally trigger cell death (Pervaiz, 2001; Dolmans *et al*., 2003; Fantin and Leder, 2006). Wohllebe *et al*. (2009) reported that water soluble pheophorbide produced from chlorophyllin by acidification, when used as low concentrations and added to the water body, were able to kill mosquito larvae and other small organisms within a few hours under exposure of solar radiation. Wohllebe *et al*. (2009) determined EC_{50} in chaoborus at pheophorbide concentration was less than 2 mg L^{-1}. Dondji *et al*. (2005) determined the effect of different photosensitizer on adese and culex larvae. Higher toxicity of pheophorbide in summer (sunlight) in comparison to winter (sunlight) is due to the production of toxic singlet oxygen by pheophorbide exposed to light. Toxicity of pheophorbide is time and concentration dependent, as evident from negative regression between exposure period and LC_{50} of pheophorbide.

Thin layer chromatography study indicates the preliminary identification of the active components which is present in extracted and pure pheophorbide by showing same Rfvalue. Evidence from the steep slope shows values indicate that a small increase in the concentration of the different treatments causes a marked mortality in snails. A t-ratio value greater than 1.96 indicates that the regression is significant. Values of heterogeneity factor less than 1.0 denote that in the replicate test of random samples, the concentration response lines would fall within 95% confidence limits and thus the model fits the data adequately. The index of significance of potency estimation g-value indicates that the value of the mean is within the limits at all probability levels (90, 95 and 99) less than 0.5.

CONCLUSION

This study demonstrates the potential of pheophorbide on the snails with inhibitory effect on treated snail. It can be used in aquatic medium very easily and it may be used as effective molluscicides. The mechanism of photodynamic remedy of pheophorbide cause snail death is not exactly known and well requires further studies for elucidation.

ACKNOWLEDGMENT

One of the authors Kavita Singh is thankful to Department of Science and Technology, (DST) New Delhi for financial assistance (Inspire Fellowship Number-IF 140959).

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