

in the patients, clearly denoting extensive damage caused by reperfusion of ischaemic myocardium.

To summarize, the present study shows that during reperfusion of ischaemic myocardium, there is copious generation of ROS, depletion of antioxidants and inhibition of enzymatic free radical scavenging system. These changes are conducive to post-reperfusion injury of myocardial tissues and may perhaps be prevented or minimized by administration of antioxidants such as vitamin C, the safest one, along with routine post-reperfusion therapy.

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Phototoxic effect of some porphyrin derivatives against the larvae of *Aedes aegypti*, a major vector of dengue fever

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Porphyrins when photosensitized, transfer their energy/electron to the triplet ground state oxygen, producing cell-lethal reactive oxygen species. Dengue is a tropical disease and the causative viruses are maintained in a cycle involving humans and the principal urban mosquito vector, *Aedes aegypti*. Infection with dengue virus causes a spectrum of clinical illnesses, ranging from a nonspecific viral syndrome to severe and fatal haemorrhagic disease. In this communication we report on the promising photodynamic effect of porphyrin-generated singlet oxygen on the larvae of *A. aegypti*, with possible applications in controlling the dengue vector.

THERAPEUTIC effects of a chemical agent in the presence of light have long been recognized by mankind. In India, vertigo was treated with plant extracts rich in furanocou-

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marins administered orally, followed by exposure to sunlight^{1,2}. Photodynamic therapy is an emerging treatment for a variety of conditions such as cancer and neovascular age-related macular degeneration. It requires a combination of oxygen, visible light and a photosensitizer (drug) which causes damage to living tissue. Two oxidative mechanisms are principally involved in the photodestruction of tissue³, namely Type I and Type II. In Type I mechanism, the photosensitizer interacts with oxygen, resulting in electron transfer to produce superoxide radicals. In Type II mechanism, singlet oxygen is generated as a result of energy transfer from triplet excited state of the photosensitizer to the triplet ground state of oxygen. Biomolecules such as unsaturated lipids, cholesterol, amino acid side chains such as tryptophanyl, histidyl, and methionyl react readily with singlet oxygen. Therefore, it is likely that membrane damage is an important process leading to both photonecrosis and vascular shutdown³.

Dengue and dengue haemorrhagic fever (DHF) are caused by one of the four closely related, but antigenically distinct virus serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) of the genus *flavivirus*. Infection with one of these serotypes does not give cross-protective immunity.

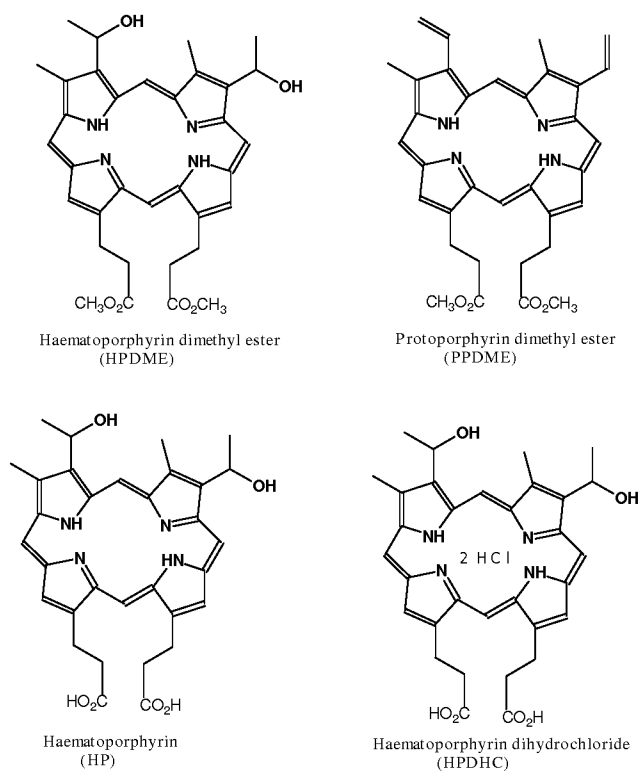
In the 1980s, DHF began a second expansion into Asia when Sri Lanka, India and the Maldiv Islands had their first major epidemics⁴. In 1997, dengue was the most important mosquito-borne viral disease affecting humans; its global distribution is comparable to that of malaria and an estimated 2.5 billion people live in areas at risk for epidemic trans-

mission. The case fatality rate of DHF in most countries is about 5% and most fatal cases are among children and young adults⁴.

For the control of DHF, emphasis placed on insecticide sprays for the adult mosquito, has led to environmental problems and resistant mosquito populations. With no new mosquito control technology available, in recent years public health authorities have emphasized on disease prevention and mosquito control through community efforts to reduce larval breeding.

During our screening programme for biopesticides, we observed the photodynamic effect of protoporphyrin dimethyl ester (PPDME) and haematoporphyrin dimethyl ester (HPDME; Scheme 1) against the 2nd instar larvae of *Aedes aegypti* L. (Diptera: Culicidae). Thus, standard solutions of compounds were made by dissolving the porphyrin derivatives in acetone (1 ml) and making up to 250 ml with distilled water. Two sets of dilution series ranging from 2.5 to 0.62 ppm were made from the standard solutions and 30 ml each was placed in pyrex glass beakers (50 ml). Mosquito larvae (2nd instar; ten per solution) were introduced into each beaker; one set was kept under diffused sunlight and the other set was kept in dark. Control larvae were exposed to sunlight without the photosensitizer and only those experiments which had less than 20% control mortality were considered. Mortality was counted after 24 and 48 h (Table 1) and adjusted against control mortality using Abbott's formula⁵.

Results showed that both HPDME and PPDME exhibited potent photodynamic effect against the 2nd instar larvae of *A. aegypti*. The mean mortalities at 2.5 ppm HPDME and PPDME were 90 and 85% in 24 h, respectively, with both reaching 100% in 48 h (LC₅₀ value for HPDME = 1.00 ppm and for PPDME = 0.89 ppm in 24 h). When the same experiment was carried out against the late 3rd instar larvae (PPDME only) at 2.5 ppm the mortality reached 100% in four days, while the same result was obtained at 10 ppm in two days (LC₅₀ = 2.11 ppm; Table 2). Importantly, there was no significant dark toxicity from both porphyrins at any of the above concentrations. The late 3rd instar larvae possess a thicker cuticle and therefore, are probably less susceptible to phototoxicity compared to the 2nd instar larvae. As such,



Scheme 1.

Table 1. Photodynamic effect (% mortality) of HPDME and PPDME against 2nd instar larvae of *A. aegypti*

Concentration (ppm)	HPDME ¹ under sunlight	PPDME ² under sunlight
2.50	90.0* (100) [#]	84.0 (100)
1.25	64.0 (100)	73.7 (100)
0.62	40.0 (90)	52.6 (84.2)

*Mean of four replicates (ten larvae each) was used in all experiments.

[#]Mortality after 48 h is given in parenthesis (control mortalities adjusted using Abbot's formula).

¹LC₅₀ = 1.00 ppm; ²LC₅₀ = 0.89 ppm (dark toxicity for PPDME was 0% at all concentrations); HPDME showed 5% mortality at 2.5 ppm in 24 h.

late 3rd instar larvae of *A. aegypti* were used in all subsequent experiments.

Porphyrin derivatives from Aldrich were used as received. The purity of porphyrins was checked by TLC and characterized by UV-visible spectroscopy (highly intense Soret band owing to a strong transition to the second excited state ($S_0 \rightarrow S_2$) at about 400 nm and four weak transitions (Q -band) to the first excited state ($S_0 \rightarrow S_1$) in the range of 500 to 700 nm.

The study was extended to include haematoporphyrin (HP) and haematoporphyrin dihydrochloride (HPDHC; Scheme 1) as photosensitizers. Experiments (procedure identical to that described above) where HP and HPDHC were used, showed a larval mortality of 100 and 80% respectively at 1.25 ppm, which is a remarkable increase compared to PPDME (Table 3).

Our preliminary field experiments carried out (in Nagahattanne, Kandy district, Sri Lanka) inside and outside houses (10) using clear plastic containers also showed high larvicidal activity with 2.5 ppm HP (100% mortality on day-one) in the presence of light (outside). Conversely, the mortality was less inside houses (100% mortality on day-two).

Experiments set up in coconut shells (outside houses) with 2.5 ppm HPDME showed 60% mortality on day-six. Although the photodynamic effect is reduced by the limited amount of light penetrating the walls of coconut shells, the level achieved on day-six is significant at the low concentration of 2.5 ppm of porphyrin.

The different photodynamic activity of the three porphyrins on day-one (HP > HPDHC > PPDME) can be accounted for by the structural properties of porphyrins. Depending on the polar character of the peripheral propanoic substituents of HP (the mono anionic and dianionic forms), it has been shown to interact with polar groups of the lipid layer where the hydrophobic porphyrin core is embedded into the lipid region of the bilayer⁶. This behaviour is important, as the sensitizer must be able to get into cells through lipid membranes of the larvae. Furthermore, porphyrins localized on the lipid phase which exist predominantly in monomer forms⁷, exhibit high photosensitivity in comparison to aggregated forms in aqueous media. Positive charges at the centre of the HPDHC molecule would impart less affinity

to the lipid layer, hence showing a lower phototoxicity. HPDHC, being a protonated form is more water-soluble than HP, and therefore, the former is easy to administer into the larvae. However, for efficient larvicidal activity, the ability of the compound to penetrate the target cell membrane is perhaps more important than water-solubility. The low larvicidal activity of PPDME could be explained in part due to its hydrophobic nature, which facilitates dimerization in aqueous media due to additional stabilization from π - π interactions involving the vinyl group⁸.

Photosensitized generation of singlet oxygen in aqueous media has been implicated in photodynamic killing of microorganisms. For several porphyrins, high quantum yields of singlet oxygen production have been measured in aqueous media using both steady-state and time-resolved techniques^{9,10}. In order to assess the possible involvement of singlet oxygen in phototoxicity to the larvae, experiments were carried out in the presence of sodium azide, a known quencher of singlet oxygen¹¹⁻¹³. First, to ascertain the toxicity of azide ions on the late 3rd instar larvae, mortality was observed under different concentration levels of azide ions in distilled water (from 6 to 60 ppm) after exposure to diffused sunlight.

Interestingly, azide ions (even 60 ppm) were not toxic on larvae at 6 h. At 12 h, mortality at 60, 40, 20 and 6.0 ppm solutions was 30, 10, 10 and 0% respectively. Action of light on azide ions results in the formation of toxic nitrate ions, but the quantum yield for nitrate formation is negligible¹⁴. Based on the results, it was concluded that up to 12 h, 40 ppm azide solution shows little toxicity towards larvae, and hence

Table 3. Photodynamic effect (% mortality) of HP and HPDHC in distilled water on 3rd instar larvae of *A. aegypti*

Days	Concentration of HP and HPDHC/ppm				
	1.25	2.5	5.0	10	20
1	100 (80) [#]	100 (100)	100 (100)	100 (100)	100 (100)
2	100 (88)	100 (100)	100 (100)	100 (100)	100 (100)

n = 20 (each with 5 larvae/20 ml of HP; 5 × 4 replicates; control mortalities adjusted using Abbot's formula).

[#]Mortality with HPDHC is given in parenthesis.

n = 25 (5 × 5) per treatment, at one larva/4 ml of sensitizer per container.

Table 2. Photodynamic effect (% mortality) of PPDME in distilled water on late 3rd instar larvae of *A. aegypti*

Days	Concentration of PPDME/ppm		
	2.5	5.0	10
1	10	10	70
2	80	80	100
3	90	100	100

n = 20 (each with 5 larvae/20 ml of PPDME; 5 × 4 replicates); ¹LC₅₀ = 2.11 ppm; (Control mortalities adjusted using Abbot's formula).

Table 4. Effect of 40 ppm azide ions on mortality of late 3rd instar larvae of *A. aegypti* upon exposure to direct sunlight in presence of 2.5 ppm HP in distilled water

Control	Mean mortality (%)		
	N ₃	HP and N ₃	HP
0	10	40	90

n = 40 (10 × 4) per treatment; one larva/4 ml of solution per container.

this concentration was used in subsequent singlet oxygen quenching experiments.

Singlet oxygen quenching experiments were carried out in the presence of a mixture of HP (2.5 ppm) and NaN_3 (40 ppm) in distilled water. Control experiments with HP and azide alone were also set-up. Each solution was exposed to direct sunlight. Mortality of late 3rd instar larvae of *A. aegypti* under different conditions is summarized in Table 4.

In HP alone, the larval mortality was 90% in 3 h, and the value dropped to 40% in the mixture containing NaN_3 /HP. This suggests that azide ions cause significant lowering of photodynamic effect of HP by quenching singlet oxygen.

Our results show that porphyrin derivatives, in the presence of light, exhibit a strong photodynamic effect on the 2nd and 3rd instar larvae of *A. aegypti* under laboratory conditions. In order to assess the toxicity effects of porphyrins against non-target, ecologically important aquatic fauna, the following were tested under laboratory conditions in glass tanks (12" × 14"): Mayfly larvae (Ephemeroptera: Leptophlebiidae; 10 larvae/50 ml); tadpoles (*Bufo melanostictus*; 10 tadpoles/100 ml) and guppies (*Poecilia reticulata*; 10 guppies/1000 ml). Mayfly larvae and tadpoles did not show mortality even at 100 ppm levels, which is forty times higher than the concentration where mosquito larvae showed 100% mortality (2.5 ppm). Guppies survived for 30 days at 100 ppm in the presence of porphyrins. This apparent non-toxicity of porphyrins towards non-target freshwater fauna may be accounted for by the hard body cover of the organisms, which blocks the penetration of light. Thus, even if the particles are ingested in large quantities, they would remain inactive due to lack of light reaching them. Porphyrins show a high level of safety to humans and certain mammalian species. (The acute LC_{50} for systematically injected HPDHC is around 300–400 mg/kg body wt)¹⁵.

Both HP and HPDHC are efficient candidates in photodynamic killing of *A. aegypti* larvae, which are the major vectors of dengue fever. The undetectable phototoxic effect against other aquatic fauna even at high concentration of porphyrins (100 ppm), reveals that the photodynamic approach for control of dengue mosquito would have no apparent impact on other non-target aquatic fauna.

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Studies on reproductive biology of a threatened tree fern, *Cyathea spinulosa* Wall. ex Hook

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Cyathea spinulosa Wall. ex Hook. is a highly-prized ornamental and economic tree fern and a significant component of tropical forests in southern, central and northern India. Currently, it is under threatened status and listed in the Red Data Books. Since the successful colonization of a fern in new habitats is dependent on gametophyte generation, the present study was conducted to observe the reproductive biology of the species. Sex ontogeny showed male gametophytes to hermaphrodite condition and the gametophytes remained bisexual for considerable period of time. Regeneration of gametophyte was common; the regenerated portions bore both sexes and produced sporophytes extensively. The reproductive behaviour revealed considerable success in sporophyte production through intragametophytic selfing. This shows that the species is of lesser genetic diversity and the gene pool is charged with lesser amount of genetic load, and is a good colonizer. In contrast, the taxon is under threatened status. The cause of this and the probable mode of conservation are also discussed here.

ACCORDING to the literature, among the 11 species of *Cyathea* in India, *C. spinulosa* and *C. contaminans* are widely distributed and reported throughout mountainous regions¹. *C. spinulosa* is a highly-prized ornamental and economic fern with an arborescent growth habit. This species is distinguished from other *Cyathea* species in having conspicuous brittle spines on the frond bases with shiny brown scales. Fronds are dark green and finely divided. This terrestrial

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