

# Artemisinin Inhibits Chloroplast Electron Transport Activity: Mode of Action

Adyasha Bharati<sup>1</sup>, Monaranjan Kar<sup>2</sup>, Surendra Chandra Sabat<sup>1\*</sup>

**1** Gene Function and Regulation, Stress Biology Laboratory, Institute of Life Sciences, Bhubaneswar, Odisha, India, **2** Department of Botany, Utkal University, Vani Vihar, Bhubaneswar, Odisha, India

## Abstract

Artemisinin, a secondary metabolite produced in *Artemisia* plant species, besides having antimalarial properties is also phytotoxic. Although, the phytotoxic activity of the compound has been long recognized, no information is available on the mechanism of action of the compound on photosynthetic activity of the plant. In this report, we have evaluated the effect of artemisinin on photoelectron transport activity of chloroplast thylakoid membrane. The inhibitory effect of the compound, under *in vitro* condition, was pronounced in loosely and fully coupled thylakoids; being strong in the former. The extent of inhibition was drastically reduced in the presence of uncouplers like ammonium chloride or gramicidin; a characteristic feature described for energy transfer inhibitors. The compound, on the other hand, when applied to plants (*in vivo*), behaved as a potent inhibitor of photosynthetic electron transport. The major site of its action was identified to be the  $Q_B$ ; the secondary quinone moiety of photosystemII complex. Analysis of photoreduction kinetics of *para*-benzoquinone and duroquinone suggest that the inhibition leads to formation of low pool of plastoquinol, which becomes limiting for electron flow through photosystemI. Further it was ascertained that the *in vivo* inhibitory effect appeared as a consequence of the formation of an unidentified artemisinin-metabolite rather than by the interaction of the compound *per se*. The putative metabolite of artemisinin is highly reactive in instituting the inhibition of photosynthetic electron flow eventually reducing the plant growth.

**Citation:** Bharati A, Kar M, Sabat SC (2012) Artemisinin Inhibits Chloroplast Electron Transport Activity: Mode of Action. PLoS ONE 7(6): e38942. doi:10.1371/journal.pone.0038942

**Editor:** Valdur Saks, Université Joseph Fourier, France

**Received:** January 25, 2012; **Accepted:** May 14, 2012; **Published:** June 13, 2012

**Copyright:** © 2012 Bharati et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** The financial support was institutional, obtained from Director, Institute of Life Sciences, which is intramural. No extramural funding was used in this study. The intramural funding in the authors' case had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: surendrachandra@gmail.com

## Introduction

Nature produces a large array of bioactive materials and compounds with unexploited properties. These products have been used more often as structural leads for the discovery and development of natural product based herbicides or pesticides. Often these products have novel sites of action, and even if unsuitable for commercial use, the identification of a new target site is valuable for designing synthetic herbicides [1].

An example of such a natural product is artemisinin, a sesquiterpenoid lactone with a highly reactive endoperoxide bridge from *Artemisia annua L.*, commonly used as a potential antimalarial drug [2]. The compound and several of its structural analogues like, artemisinic acid, arteannuin B, deoxyartemisinin, artesunic acid and sodium arteanninate have been reported to possess phytotoxic potential [3–8]. It is also shown to inhibit seedling growth in a number of mono and dicotyledonous plants [3]. However, its mode of action and the mechanistic details still remain undeciphered.

Some allelochemicals have been recognized to disrupt the electron transport activity both in chloroplast and mitochondria; including alteration in their membrane architecture [9]. The mitochondrial electron transport chain in yeast has been identified as the major target of this compound as antimalarial agent [10]. Artemisinin, as argued by Li et al. [10] can disrupt the normal electron flow in yeast mitochondria by depolarizing its membrane

potential and interacts with NADH-dehydrogenase complex (complex-I).

The chloroplast and mitochondria perform largely a similar kind of function; producing high energy compounds for use in other cellular metabolic activities. Many of the electron transport components between these two organelles are structurally and functionally comparable, like cytochrome system, quinone moieties, ATP-synthesizing complex etc. Further, the chloroplast lamellar system also harbors a mitochondrial homologous NADH-dehydrogenase [11], the chloroplast NADH-dehydrogenase that is engaged in respiratory function of the chloroplast (chloro-respiration).

The discovery of artemisinin interaction with the electron transport chain in yeast and malarial mitochondria systems [10,12], and considering that the process of photosynthesis, particularly the thylakoid photofunction, being highly susceptible for a large range of herbicides [13], we examined the phytotoxic effect of artemisinin on the chloroplast photoelectron transport activity both under *in vitro* and *in vivo* experimental conditions.

The results from our *in vitro* and *in vivo* studies suggest the presence of two different modes of action of the compound on photosynthetic electron transport. Artemisinin is primarily an energy transfer inhibitor of isolated thylakoid membranes. However, the *in vivo* studies indicate that the compound impairs the thylakoid electron flow as an inhibitor of secondary quinone

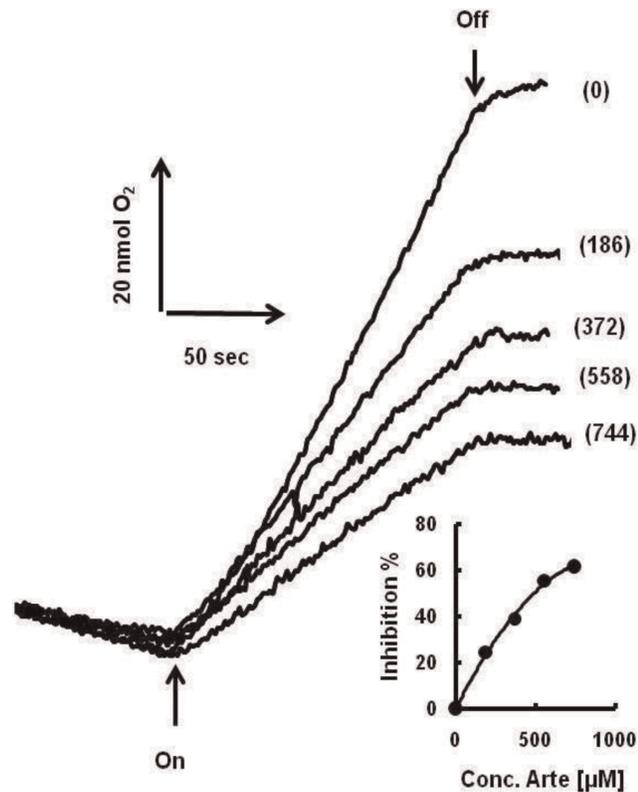
( $Q_B$ ) of PSII (Photosystem II). The inhibition was determined to be caused by a yet uncharacterized artemisinin- metabolite rather than its direct interaction with thylakoid membrane. Thus, artemisinin may act as a natural prophytoxin.

## Results

### Inhibitory effect of artemisinin on electron transport activity: an *in vitro* study

To find out the potency of artemisinin as an inhibitor of thylakoid photofunction, the effect of artemisinin on electron transport rate supported by the transfer of electrons from water to FeCN (potassium ferricyanide) was examined. The extent of inhibition was assessed relative to DMSO (dimethyl sulfoxide) supplemented reaction rates as a control (Fig. 1; trace '0'), which was identical to the rate obtained without addition of DMSO. The degree of inhibition was concentration dependent and showed the onset of a saturating tendency around 744  $\mu\text{M}$ ; the highest concentration tested in this report. About 65–70% inhibition of control activity was observed at this concentration of artemisinin (inset fig. 1).

The inhibitory effect of the compound was further evaluated under phosphorylating and uncoupled conditions of electron flow. Two different uncouplers known to dissipate the formation of high-energy state through different means were used in this study. The  $\text{NH}_4\text{Cl}$  (ammonium chloride), an amine type of uncoupler is known to arrest the acidification (chemical potential,  $\Delta\text{pH}$ ) of



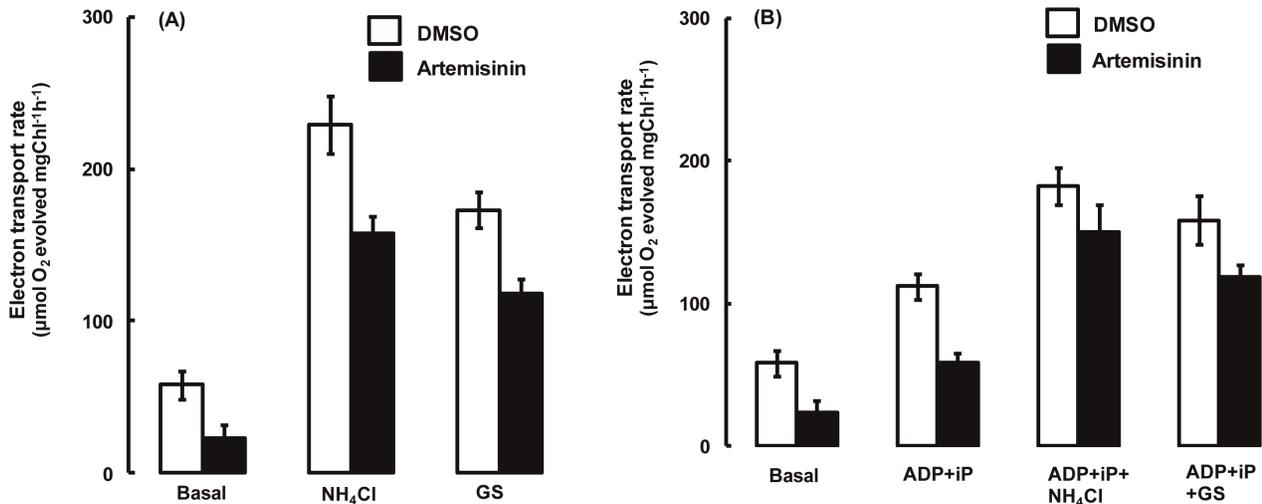
**Figure 1. Effect of increasing concentration of artemisinin on FeCN supported  $\text{O}_2$  evolution activity of spinach thylakoids.** The concentration of artemisinin used in  $\mu\text{M}$  is shown in parenthesis. The inset depicts the increase in percent inhibition of electron transfer rate relative to control with increase in artemisinin concentration. Arrow up, light on; arrow down, light off.  
doi:10.1371/journal.pone.0038942.g001

thylakoid lumen [14] and GS (gramicidin-S) is a pore forming ionophore [15] known to collapse the electrical potential ( $\Delta\Psi$ ) of high energy state of the membrane. In presence of  $\text{NH}_4\text{Cl}$  or GS, the extent of artemisinin-mediated (744  $\mu\text{M}$ ) inhibition of basal electron transport was reduced by nearly 50% (Fig. 2, A). Addition of ADP (adenosine diphosphate) and iP (inorganic phosphate), as expected, stimulated the basal electron transport rate. However, a decline in the artemisinin-mediated inhibition to 45–50% as against 65–70% in basal conditions was observed under phosphorylating condition (Fig. 2, B). Although the phosphorylating electron transport rate was further stimulated in presence of uncouplers, the toxicity of artemisinin, however drastically reduced it to the tune of 15–20% in presence of  $\text{NH}_4\text{Cl}$  and about 20–25% with GS (Fig. 2, B).

### Fast chlorophyll (Chl.) *a* (*O-J-I-P*) fluorescence transient and effect of artemisinin: an *in vivo* study

To gain insight about the effect of artemisinin *in vivo*, rice plants grown under greenhouse conditions were sprayed with artemisinin as described in materials and methods. Dark adapted leaves when exposed to a strong saturating light (3000  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ , red light of 650 nm) for 1 ms, the Chl. *a* fluorescence emission rapidly increases from an initial *O* level to a maximum *P* through transient steps, the *J* and *I*. The *O-J-I-P* uphill rise in fluorescence ( $F_O$ ,  $F_J$ ,  $F_I$  and  $F_P$ ) sequence represents the successive reduction of the electron acceptor pools of PSII [16–18]. We followed the notations as defined by Strasser et al. [17] to explain the redox significance of these transients. Artemisinin administration induced a small but discernible higher  $F_J$  and a higher  $F_I$ . The major difference noted due to artemisinin treatment, was the disappearance of the  $F_I - F_P$  transient (Fig. 3, A). It is known that  $F_J$  is very sensitive to changes in the redox state of the  $\text{PQ}$ -pool. The  $F_I - F_P$  phase is correlated to the transfer of electrons through PSI as shown by DBMIB (dibromothymoquinone) treatment [19], primarily an antagonist to cytochrome  $b_{563}$  but having limited effect on  $Q_B$  site as well [20,21]. These alternations in fluorescence characteristics may have arisen due to reduced level of electron flow through PSI, accompanied with changes in the redox state of  $\text{PQ}$ .

A small but discernible rise in  $F_O - F_J$  fluorescence intensity suggests the existence of a small effect on  $Q_A$  reduction in dark adapted leaves. However, a faster rise in Chl. fluorescence from  $F_I$  to  $F_P$  level in artemisinin treated leaves suggest a slow reduction of  $Q_B$  resulting in formation of lower pool of  $\text{PQH}_2$  (plastoquinol), which in turn alters the overall redox chemistry of  $Q_A$  to  $Q_B$  to  $\text{PQH}_2$  in PSII complex. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) treatment that inhibits the electron flow from  $Q_A$  to  $Q_B$  by binding to  $Q_B$ , results in the transformation of three step fluorescence rise *O-J-I-P* into an *O-J* rise only due to the accumulation of  $Q_A^-$ . Comparison of the fluorescence induction curves of control and artemisinin treated leaves showed similar sensitivity to DCMU (Fig. 3, B). These results indicate that the electron flow from water to  $Q_A$  is insensitive to artemisinin toxicity and the compound renders its toxic affect in the span of electron flow beyond  $Q_A$ . The room temperature (25°C) and 77 K fluorescence emission spectral analysis (See Fig. S1, Supporting Information) also suggest that the compound has no effect on the energy transfer from core antenna to PSII reaction centre Chl. [22]. Therefore, to decipher the effect of the compound on the span of electron flow beyond  $Q_A$ , we studied the slow Chl. *a* fluorescence transient, which gives the signature for various photosynthetic events [23].



**Figure 2. Effect of artemisinin on FeCN supported electron transport in thylakoids under basal, phosphorylating and uncoupled conditions.** Artemisinin (744 μM) was added under basal (minus ADP and iP), coupled (plus ADP and iP) and uncoupled (plus NH<sub>4</sub>Cl or GS) conditions with FeCN as terminal electron acceptor. (A) shows electron transport rate as measured in absence of phosphorylating conditions (minus ADP and iP) and (B) shows electron transport rate under phosphorylating conditions (plus ADP and iP). Open bars denote the control (DMSO) and closed bars denote treated (Artemisinin supplemented) rates. Measuring cuvette contained 20 μg equivalent of Chl. thylakoids suspension in 1 ml of the reaction mixture. The error bars show ± SD of electron transport from 5 independent experiments. doi:10.1371/journal.pone.0038942.g002

### Chl. *a* slow fluorescence kinetics

The control rice leaves showed multiphasic Chl. *a* fluorescence induction. Artemisinin treatment to leaves, however, induced an alteration in the relative amplitude of these transients (Fig. 4). A major difference was marked in the quenching of fluorescence from  $F_p$  to  $F_t$  level. The rise in fluorescence at  $F_p$ , in artemisinin treated leaves is followed by a decline to the  $t'$  level but having a much higher amplitude than the control leaf sample. This characteristic phenomenon of alteration in Chl. *a* transient by artemisinin was also observed in spinach and pea (data not shown).

The  $F_p$  to  $F_t$  decay is manifestly far more complex because of the participation of multiple reactions to quench the fluorescence. The principal causes of this decay relate to gradual re-oxidation of  $Q_A$  by PSI (photochemical quenching, the qP), the energization of the thylakoid membrane due to proton translocation (non-photochemical or energy dependent quenching, the qE) and possibly the state transition (qT) [24]. The normal deviation of fluorescence decay from  $F_p$  to  $F_t$  in artemisinin treated leaves as compared to control leaf suggest an interruption of electron flow in a site(s) beyond the  $Q_A$  site of electron acceptance.

The estimated Chl. concentration in both control and artemisinin treated leaves were comparable suggesting that artemisinin treatment had no effect on the Chl. pigment synthesis (See Fig. S2, Supporting Information). We, therefore, further analyzed the electron flow in thylakoid membranes, isolated from artemisinin treated leaves by dissecting the electron transport chain into smaller segments with the use of site specific artificial electron acceptors, donors and inhibitors [25,26].

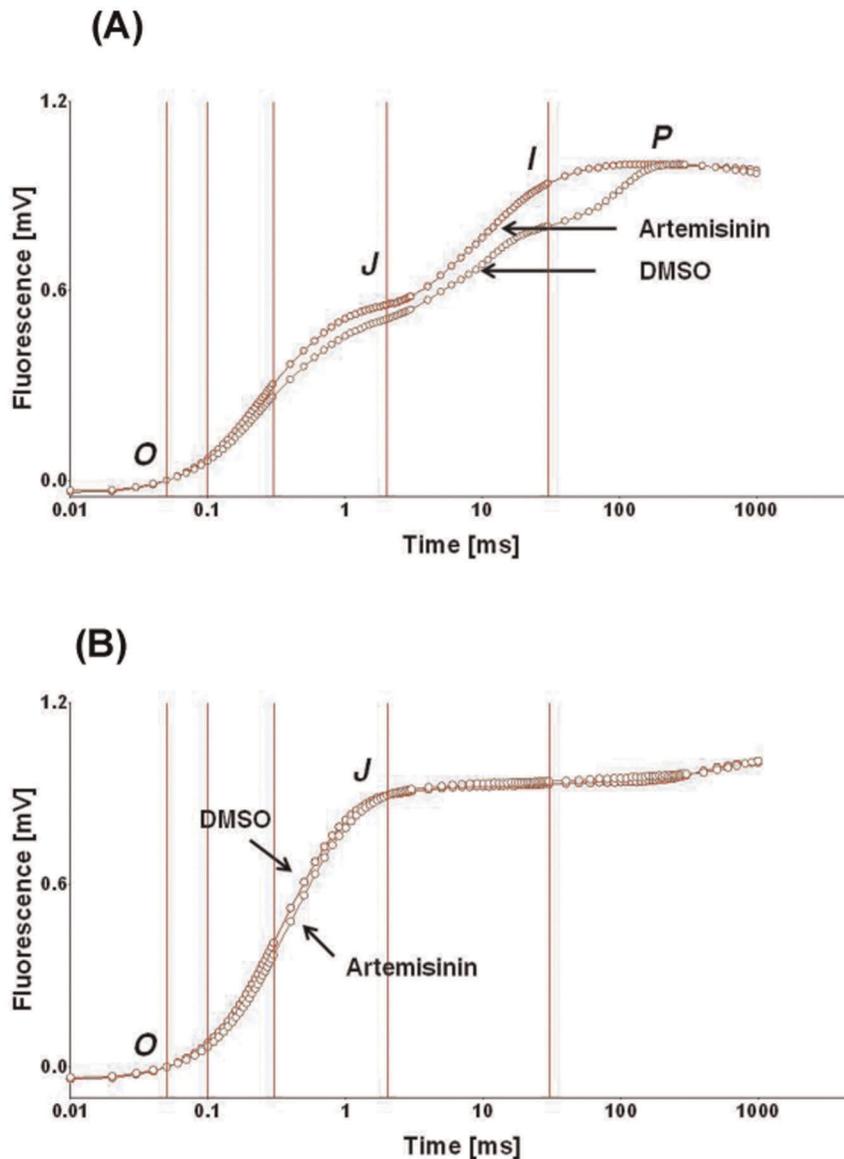
### Identification of the target site of artemisinin in the electron transport chain

Thylakoids were isolated from leaves of control and artemisinin sprayed rice plants as described in materials and methods. The FeCN supported whole chain electron transport rate in chloroplasts of artemisinin treated leaves showed a substantially low activity as compared to the rate obtained in control leaf chloroplasts. From various batches of thylakoid preparations, it

was inferred that the electron transport rate in chloroplast of treated leaves was reduced by 80–85% as compared to control (Fig. 5, A). Similar extent of inhibition was noted, when FeCN was replaced with MV (methylviologen), another end terminal electron acceptor, which also intercepts electron from the reducing side of PSI (data not shown). Of the total reduction in full chain electron transport, a major reduction in rate of about 65–70% was accounted for  $pBQ$  (*para*-benzoquinone) supported PSII catalyzed electron transport reaction (Fig. 5, B). The PSI catalyzed electron flow from DCIPH<sub>2</sub> (reduced-dichlorophenolindophenol) to MV was reduced by 10–15% in artemisinin treated leaves as compared to control rate (Fig. 5, C).

Unlike the *in vitro* situation, the inhibitory affect was persistent to a comparable extent both in loosely coupled (basal) and chemically uncoupled thylakoids (Fig. 6), which specifically suggests that, the compound acts primarily as an electron transport inhibitor rather than as an energy transfer inhibitor.

The lipophilic electron acceptor  $pBQ$  is known to intercept electrons after  $Q_A$  in a DCMU sensitive manner, mostly from the plastoquinol pool ( $PQH_2$ ) and also from  $Q_B$  (bound form) site of PSII complex [27,28]. These sites can be resolved by analyzing the kinetic parameters of electron transport reaction ( $K_m$  and  $V_{max}$ ), the reciprocal plot of rate ( $O_2$ -evolving activity) against increasing concentrations of  $pBQ$  (Fig. 7 A, B and C). Similar to an earlier report [27], the plastoquinol pool ( $PQH_2$ ) with  $pBQ$  maintained a high affinity (low  $K_m$ ) with low  $V_{max}$  against the  $Q_B$  that possess a low affinity (high  $K_m$ ) with high  $V_{max}$  value in thylakoids prepared from control leaf (Fig. 7, B). However, artemisinin treatment alters the relative  $pBQ$  binding affinity ( $K_m$ ) and the  $V_{max}$  of both these sites in an opposite manner (Fig. 7, C) with a significant reduction in their respective  $V_{max}$  amplitude (see the inset table in Fig. 7). The electron transport reaction was further examined as a function of increasing concentration of DQ (duroquinone) that supports PSII catalyzed reaction by intercepting electron only from  $PQH_2$  pool [27,29]. Thylakoids prepared from artemisinin treated leaves is found to support a substantially low rate of electron flow from water oxidation complex to DQ as

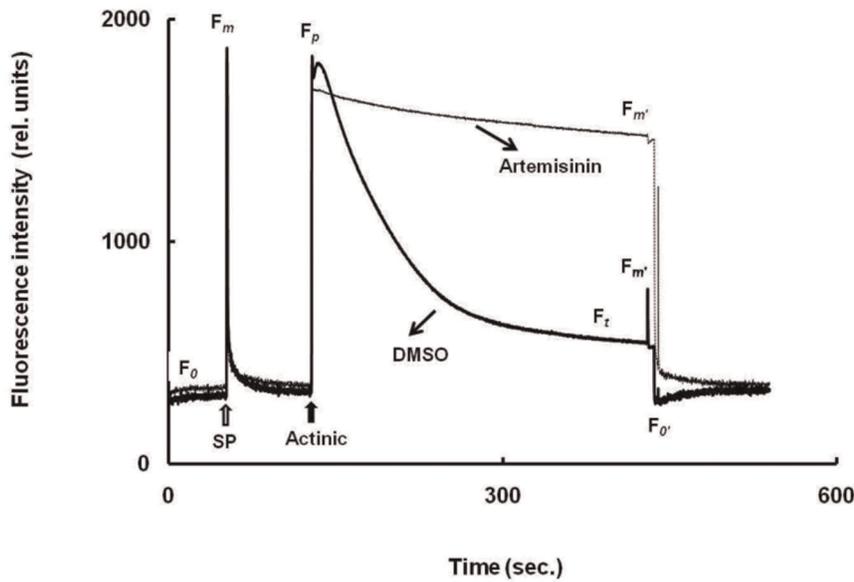


**Figure 3. The room temperature Chl. *a* O-J-I-P fluorescence transient of control (DMSO) and artemisinin-treated (Artemisinin) leaves (A), and the effect of DCMU (B).** Leaves were floated in DCMU (20 μM) solution for 1 h in complete dark to evaluate its effect in DMSO and artemisinin sprayed leaves. The minor difference in  $F_o$  (O) and  $F_p$  (P) obtained was double normalized at  $F_o$  and  $F_m$  level using biolyzerhp3 software. Each tracing is average plot of nine individual readings. The SD for  $F_o$  and  $F_m$  in control leaves was  $358 \pm 9$  and  $1784 \pm 13$  and for treated leaves was  $368 \pm 11$  and  $1757 \pm 19$ . doi:10.1371/journal.pone.0038942.g003

compared to thylakoids of control leaf (Fig. 8). The affinity ( $K_m$ ) of DQ for reduced PQ did not change in the thylakoids from the artemisinin treated plants (104–106 μM) despite that, the electron transport rate was 5 times higher in the absence of the sesquiterpenoid lactone (Fig. 8 inset). This indicates that the available pool size of the substrate (PQH<sub>2</sub>) in artemisinin treated leaves is significantly low as compared to control; a condition that might have arisen as or due to inability of conversion of bound quinone to free pool of reduced PQ.

Based on the *in vitro* and *in vivo* experimental conditions, two contrasting situations about the interaction of artemisinin with thylakoid photofunction were observed. These two conditions also differed significantly on the required concentration of the compound to exhibit its maximum inhibitory affect. The effective concentration under *in vivo* condition (see materials and methods)

may still be at a lower side considering the penetration efficiency of the compound, spray drift and also the leaf density per plant. However, all our explanations are based on the external sprayed quantity of the compound. Such large discrepancies in artemisinin concentration dependency and differential mode of action under *in vitro* (energy transfer inhibitor) and *in vivo* (electron transport inhibitor) experimental conditions suggest that the inhibition of electron flow under *in vivo* experimental condition may not be a direct consequence of interaction of artemisinin as was the case for *in vitro* situation. Therefore, it is presumed at this stage, that the *in vivo* damage to thylakoid function is possibly routed through some artemisinin-metabolite, rather than direct interaction of the compound *per se*.



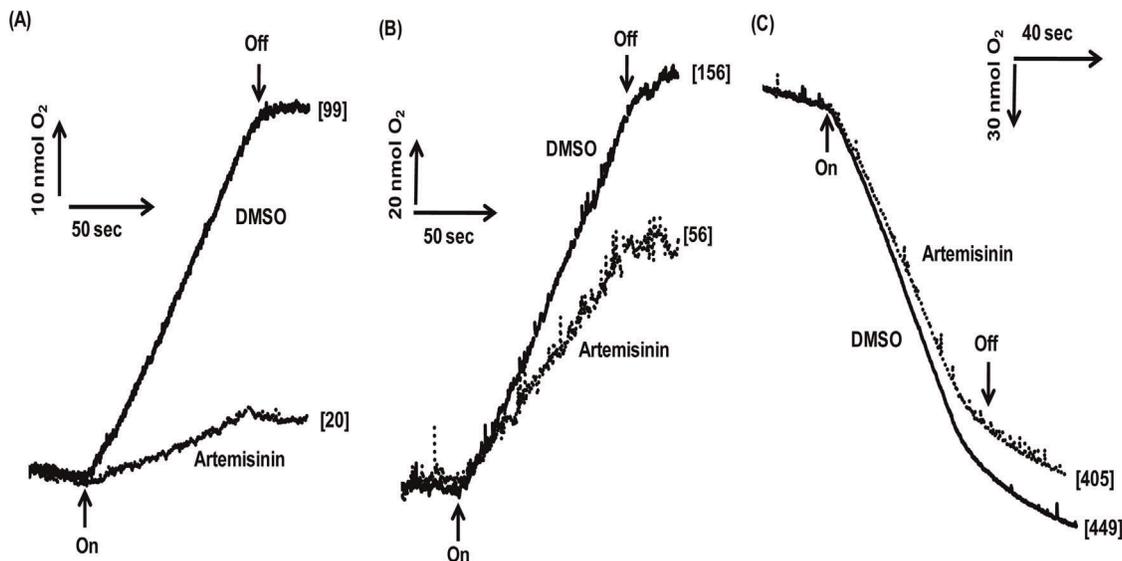
**Figure 4. The characteristic room temperature Chl. *a* fluorescence transient of control (DMSO) and artemisinin-treated (Artemisinin) rice leaves.** The leaves were washed and dark adapted for 20 min. before subjected for fluorescence measurements. The sequence of light used to evoke the various fluorescence transients are as follows. Minimal fluorescence ( $F_0$ ) was obtained with low intensity modulated light. Saturating pulse (SP,  $\leq 1$  s duration,  $18,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was used to give  $F_m$  level of fluorescence in darkness and  $F_{m'}$  in light. Actinic light (300 min duration,  $615 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was applied to drive photosynthesis and gives the transient  $F_p$ , and  $F_t$  level of fluorescence. A short pulse of far-red light was used to get  $F_{0'}$  fluorescence. Each tracing is the average plot of five individual readings. The  $R_{fd}$  (fluorescence decline ratio from 'p' to 't' level) value for control and treated sample was noted to be  $1.98 \pm 0.04$  and  $0.08 \pm 0.006$  respectively.  
doi:10.1371/journal.pone.0038942.g004

#### Evidences for the formation of a putative artemisinin-metabolite

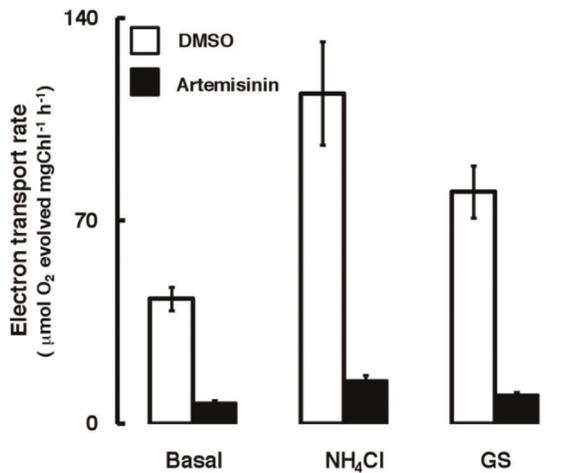
In search of an artemisinin-metabolite that could be of importance in instituting the *in vivo* inhibitory affect, we examined the electron transport activity in freshly prepared thylakoids

following its incubation with the supernatant prepared from artemisinin treated leaves (see materials and methods).

An inhibition in FeCN supported electron flow was clearly evident in thylakoids incubated with the supernatant obtained from artemisinin treated leaves compared to its counterpart following incubation (See Fig. S3, Supporting Information).



**Figure 5. Oxygen exchange activity in whole chain ( $\text{H}_2\text{O}$  to FeCN, A), PSII ( $\text{H}_2\text{O}$  to  $p\text{BQ}$ , B) and PSI ( $\text{DCIPH}_2$  to MV, C) catalyzed electron flow in thylakoids isolated from leaves of control (DMSO) and artemisinin-treated (Artemisinin) rice plants.** The measuring cuvette contained  $20 \mu\text{g}$  Chl. equivalent thylakoids suspension in 1 ml of the reaction medium. The numbers in parenthesis denote the electron transport rate, expressed as  $\mu\text{mol O}_2$  evolved (A and B) or consumed (C)  $\text{mg Chl.}^{-1} \text{h}^{-1}$ . Arrow up, light on; arrow down, light off.  
doi:10.1371/journal.pone.0038942.g005



**Figure 6. Alteration in the electron transport activity in thylakoids prepared from control (DMSO) and artemisinin-treated (Artemisinin) rice leaves under basal and uncoupled conditions.** Thylakoids isolated from leaves of control and treated plants were assayed for alteration in basal and uncoupled (NH<sub>4</sub>Cl or GS) electron transport activities with FeCN as terminal electron acceptor. Measuring cuvette contained 20 μg Chl. equivalent thylakoids suspension in 1 ml of the reaction medium. The number in parenthesis denotes the electron transport rates in μmol O<sub>2</sub> evolved mg Chl<sup>-1</sup> h<sup>-1</sup>. Arrow up, light on; arrow down, light off. The error bars indicate ±SD of electron transport (n = 3). doi:10.1371/journal.pone.0038942.g006

The biological activity of the supernatant was checked by fold diluting the supernatant. It was marked that the extent of inhibition was reduced upon dilution of the extract (See Fig. S4, Supporting Information). Further, the supernatant from the 5000×g centrifugation was ultracentrifuged as described in materials and methods section. Electron transport rate was analysed by incubating control chloroplasts with both fractions. Samples incubated with the supernatant showed no change in the rates (Fig. 9, A), while about 40–45% decline in electron transport rate was evident in samples incubated with the re-suspended pellet obtained from artemisinin treated leaves (Fig. 9, B).

## Discussion

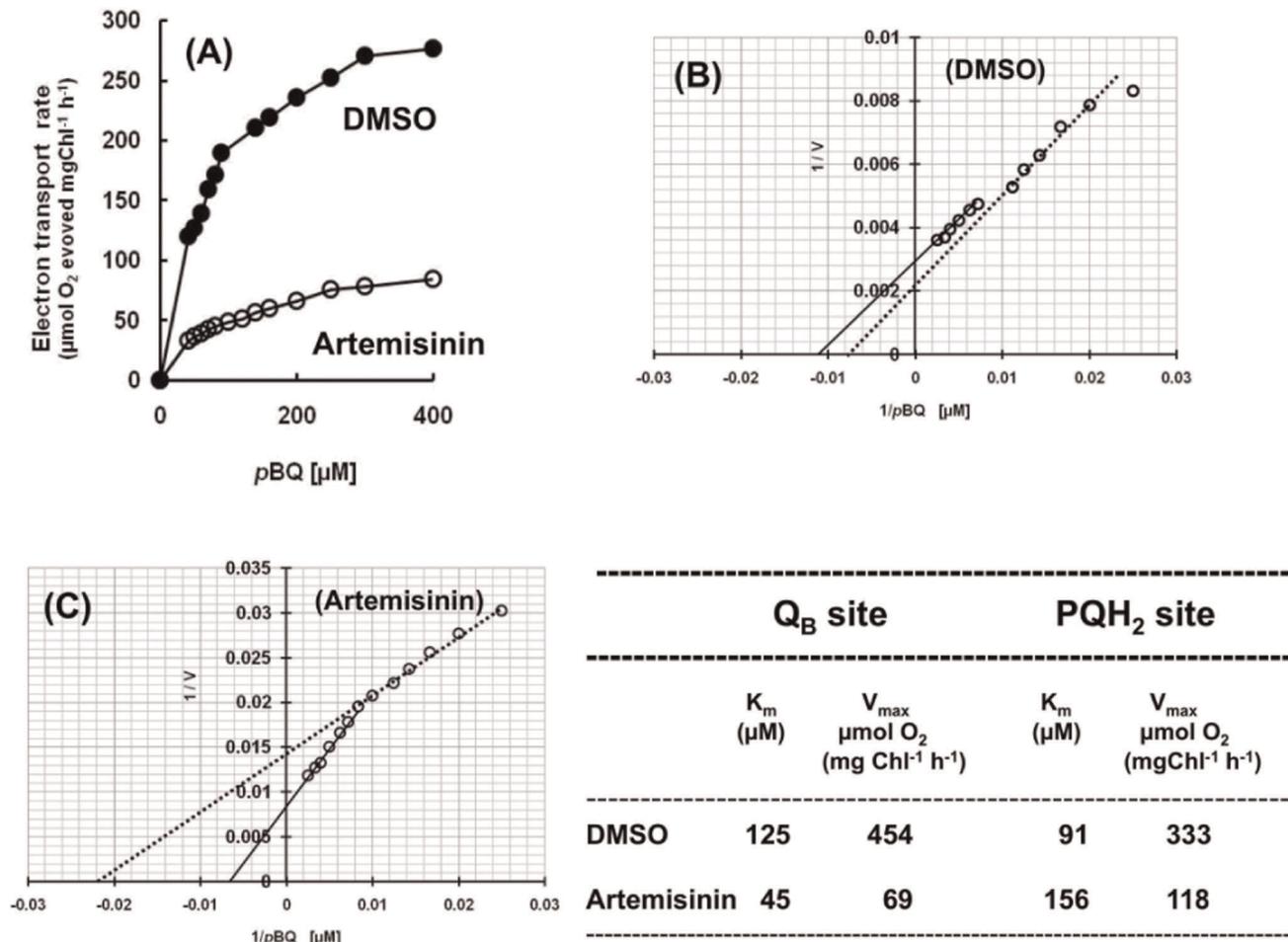
Much interest is drawn towards the compound, artemisinin, due to its herbicidal activity [3, 4, 30 and 31] in addition to its medicinal use. Although the compound has earlier been tested for growth regulating activity, there is no report on the structural moieties essential for its bioactivity and mechanism of action. We report here the biological activity of artemisinin tested both under *in vitro* and *in vivo* conditions using thylakoid/chloroplast photo-function as assay system. Artemisinin inhibited electron transport rate when added to isolated thylakoids. The assessment of *in vitro* experimental results suggest that artemisinin is effective in developing its maximal inhibitory affect in loosely coupled (basal) followed by fully coupled (phosphorylating) thylakoid electron transport reactions and has a significant low reactivity in chemically uncoupled thylakoids. Therefore, under *in vitro* system the compound behaves more as an energy transfer inhibitor [25] than as a potent inhibitor of electron transport. However, the apparent requirement of a high concentration of artemisinin (744 μM) does not explain the difference in inhibition (of about 65–70%) as shown in this report with that of previous reports on growth regulatory effects in plants as well as in yeast cells. At 33 μM artemisinin, inhibited the germination of lettuce and

annual wormwood, and growth of roots and shoots in lettuce, redroot pigweed, pitted morning glory, annual wormwood and common purslane [6]. Also at a concentration of 80 μM, artemisinin arrests the growth of yeast cells [10]. An attempt was, therefore, made here to reassess the effect of artemisinin on the photoelectron transport activity in thylakoids isolated from leaves of plants externally treated with the compound.

In order to elucidate the relationship between the concentration and biological activity of the compound, it was necessary to use a test system that required minimal amount of the compound and still showed biological activity. Therefore, we sprayed rice plants with artemisinin at concentrations 1.86 μmol of artemisinin/plant (see materials and methods) and reassessed its toxicity. Chl. *a* fluorescence transient was measured to evaluate the effect of artemisinin on the photochemical efficiency of PSII. The *O-j-I-P* transient depicts the rate of reduction kinetics of various components of PSII. Addition of artemisinin resulted in the disappearance of *F<sub>F</sub>-F<sub>P</sub>* transient which has been interpreted as the result of slower reduction of *Q<sub>B</sub>* due to presence of a smaller plastoquinol pool in comparison to the control. A small but relative increase in variable fluorescence as a function of artemisinin was indicative of a loss in *Q<sub>A</sub><sup>-</sup>* re-oxidation capacity. Further, the presence of comparable DCMU mediated induction of fluorescence emission suggest that artemisinin has no affect on the span of electron flow from water to *Q<sub>A</sub>*. The above conclusion was further supported from the slow fluorescence transient obtained following *in vivo* exposure of rice leaves to artemisinin. The ground level fluorescence (*F<sub>0</sub>*) and the maximal fluorescence (*F<sub>m</sub>*) yield did not change appreciably, but there were pronounced changes in the relative decay from *F<sub>P</sub>* to *F<sub>t</sub>*. This deviation represents the slow rate of electron flow from *Q<sub>B</sub>* to intersystem electron transport components.

To investigate the contribution of artemisinin towards the inhibition of electron flow, thylakoids were isolated from leaves of control and artemisinin sprayed plants and evaluated for their respective electron transport rate in the presence of site specific electron donors and acceptors, and also uncouplers. This sesquiterpene lactone was found to behave as a strong inhibitor of electron flow, potentially a specific inhibitor to *Q<sub>B</sub>* functioning. The *Q<sub>B</sub>* quinone moiety in thylakoid membrane remains housed in a 32 kDa peptide (D1 polypeptide) of PSII core complex, which otherwise is also known as the herbicidal binding protein. Many synthetic products like DCMU, atrazine and also natural compounds such as fisherellin, grandinol, sorgoleone etc. [32,1] have been shown to interact with this protein impairing the electron flow beyond *Q<sub>A</sub>*. The detailed mechanism of interaction at molecular level, either by forming an inhibitory complex with the protein (chemical modification) or with the quinone moiety (steric hindrance) for some of these compounds has also been deciphered [32].

Both the *in vitro* and *in vivo* scenarios presented different outcomes in our study, thus emphasizing that the mode of action of the compound in plant system (*in vivo*) is not a direct interaction of the compound with the thylakoid electron transport event, but illustrates the presence of a putative artemisinin-inhibitory-metabolite. The metabolite on homogenization comes out of the cellular component and is present in the supernatant. The supernatant was tested for inhibition in normal chloroplasts. Results from incubation studies show that the supernatant obtained from homogenization of artemisinin treated leaves is capable of inhibiting electron flow to about 65–70% (See Fig. S3, Supporting Information). Further, the metabolite is confirmed to be particulate in nature as ultracentrifugation data reveal its presence in the pellet. The pelleted fraction is mostly comprised of



**Figure 7. Kinetics of pBQ concentration dependent electron transport rate in thylakoids prepared from control (DMSO) and Artemisinin-treated (Artemisinin) leaves.** The electron transport rate obtained from the control and treated samples (A) was further analysed for Michaelis-Menten type enzymatic reaction kinetics (B and C). The intersection of the line with the ordinate and the abscissa denotes the inverse values of  $V_{max}$  and  $K_m$  respectively. The values calculated for the respective  $K_m$  and  $V_{max}$  has been shown in the inset table. The pBQ titration experiments were carried out in thylakoids isolated both from spinach and rice leaves following similar *in vivo* treatments with artemisinin and the results shown here are from spinach. doi:10.1371/journal.pone.0038942.g007

cell organelles like mitochondria, golgi and other membrane structures, but less of nuclei and plastids. Here, further experiments are needed to exactly decipher the organelle specific location and association of the artemisinin-metabolite. However, the compound does get degraded in the cell free extract, as storage of the supernatant leads to decline in its inhibitory effect (chloroplasts incubated with supernatant stored for 24 h at 4°C does not show any inhibition). We also observed that long term administration of artemisinin leads to the death of plant (See Fig. S5, Supporting Information).

For artemisinin, as described in this report, we have so far been successful in only identifying the mode (site) of its action on the photosynthetic electron transport. The significance of addressing the action of a natural phytotoxin often lies in the novelty of their site and mechanism of action. Although, as mentioned earlier their use may not be commercially viable, the identification of a new target site is always valuable in the design of new synthetic herbicides. Therefore, artemisinin could be selected as a suitable lead for development as an effective herbicide and/or for the design of new biodegradable agent with agrochemical applications [1]. However in absence of the structural features of the proposed

putative artemisinin-inhibitory-metabolite, the intrinsic mechanism of its interaction either with the protein or with the Q<sub>B</sub> moiety of PSII reaction centre remain to be worked out to fully understand the mechanism of action of artemisinin.

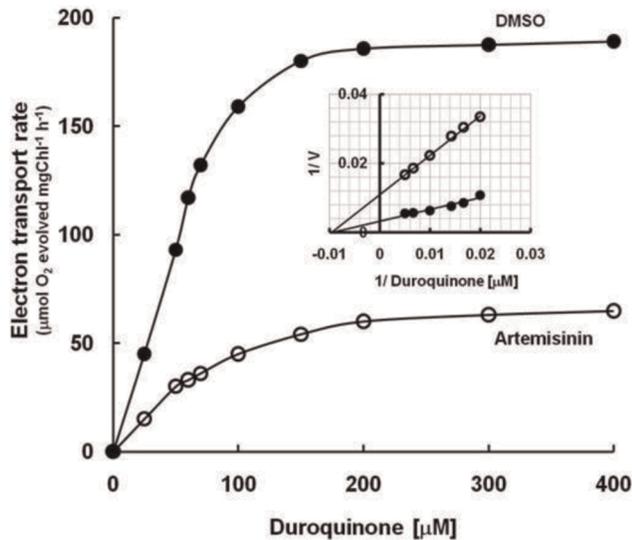
## Materials and Methods

### Materials

Purified artemisinin was obtained as gift from Ipca Laboratories Ltd., (India). DMSO was used to make stock solution of artemisinin. The electron acceptors like FeCN, DCIP, MV, DQ, pBQ were obtained from Sigma. The electron transport inhibitor DCMU from Sigma was recrystallized from 95% ethanolic solution. The buffers used were also procured from Sigma. Other chemicals used in the experiment were of analytical grade.

### Plant materials

Fresh spinach (*Beta palonga*) was obtained from local market. Rice (*Oryza sativa* var. Safari) plant was grown in soil under greenhouse conditions of light, temperature and humidity. The



**Figure 8. Kinetics of DQ supported electron transport rate in thylakoids prepared from control (DMSO) and Artemisinin-treated (Artemisinin) leaves.** The reciprocal plot of the velocity-versus-concentration is shown as inset. The intersection lines with ordinate and abscissa respectively represents the inverse of  $V_{max}$  and  $K_m$ .  
doi:10.1371/journal.pone.0038942.g008

leaves were thoroughly washed in distilled water before using them for experimental purpose.

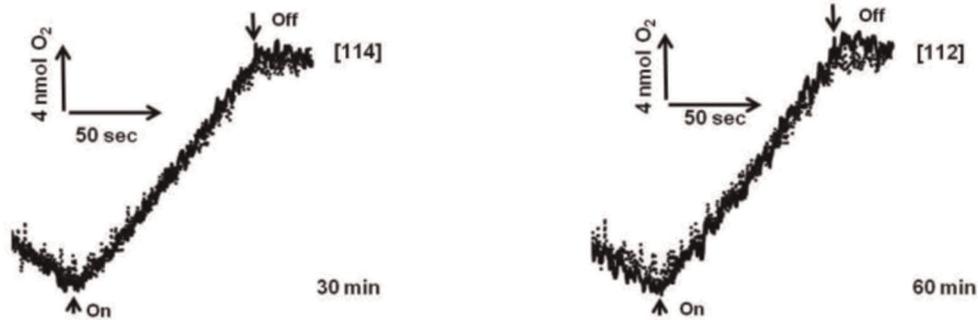
#### Plant growth and artemisinin treatment

Twenty one day old rice plants were sprayed with artemisinin twice at a 24 h interval. One milliliter of 74.4 mM artemisinin in 1% (V/V) DMSO, 0.1% (V/V) TWEEN-20 was diluted 100 fold with water and used to treat 40 plants ( $\sim 1.86 \mu\text{mol}$  of artemisinin/plant). Control plants were sprayed with the same solution without artemisinin. The 4<sup>th</sup> (quaternary) leaf was harvested 20 h after the 2<sup>nd</sup> spray and used for all experiments. The individual effect of the surfactant and DMSO-plus-surfactant was first evaluated against water control. Since no effect was obtained with these variations, we have routinely used the DMSO plus the surfactant sprayed leaves as control against artemisinin treated leaves and in figures the same has been depicted as DMSO (control).

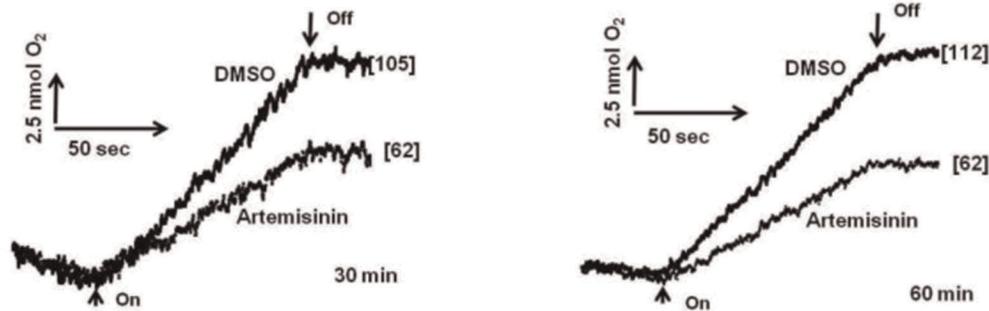
#### Chlorophyll *a* fluorescence measurement in intact leaves

The room temperature Chl. *a* fluorescence technique was used to study the photosynthetic activity in intact leaf samples. The fast Chl. *a* transients (*O-J-I-P*) in millisecond (ms) time span was studied with plant efficiency analyzer (Handy PEA fluorimeter) following Strasser et al. [17], while the relative thylakoid electron transport, membrane energization and carbon fixation activities were evaluated from slow fluorescence transients, obtained using FMS2 instrument (Hansatech) as per the detail outlined in Baker [23]. The readouts from Handy PEA were analyzed using biolyzerhp3 software.

(A)



(B)



**Figure 9. Inhibition of FeCN supported electron transport activity of spinach thylakoid incubated with the supernatant (A) and resuspended pellet (B) obtained following ultracentrifugation.** Hundred  $\mu\text{g}$  Chl. equivalent control thylakoids suspension were incubated with both the fractions (A, supernatant; B, pelleted fraction) for two different time frames of 30 and 60 min (mentioned below the tracings). FeCN supported electron transport activity was assayed in 1 ml of reaction mixture containing thylakoid suspension equivalent to 20  $\mu\text{g}$  Chl. The number in parenthesis denotes the electron transport rates in  $\mu\text{mol O}_2$  evolved  $\text{mg Chl}^{-1} \text{h}^{-1}$ . Arrow up, light on; arrow down, light off.  
doi:10.1371/journal.pone.0038942.g009

### Isolation of thylakoid membrane

Thylakoids were isolated from spinach and rice leaves following [33] with some modifications. Leaves were homogenized in cold grinding buffer [50 mM tricine-NaOH (pH 7.5), 400 mM sucrose, 5 mM MgCl<sub>2</sub> and 5 mM KCl]. The homogenate was filtered through Miracloth and the filtrate was centrifuged at 5000×g for 5 min. The chloroplast pellet was washed in 5 mM tricine-NaOH (pH 7.5) buffer containing, 400 mM sucrose and 2 mM MgCl<sub>2</sub> and centrifuged at 300×g for 1 min. The supernatant was re-centrifuged at 5000×g for 5 min. The pellet (thylakoids) was finally taken up in a minimal volume of suspending buffer [20 mM tricine-NaOH (pH 7.5), 100 mM sucrose and 5 mM MgCl<sub>2</sub>]. Chlorophyll concentration in methanolic extract was measured spectrophotometrically [34].

### Electron transport measurement

Photosystem II, PSI and whole chain (PSII plus PSI) catalyzed electron transport activities were measured as oxygen (O<sub>2</sub>) evolution or uptake in a Clarke type O<sub>2</sub> electrode assembly (Hansatech) at 22±1°C using site-specific artificial electron acceptors, donors, and inhibitors [25,26]. The light was rate-saturating (500 μE m<sup>-2</sup> s<sup>-1</sup>). Phosphorylating electron transport was measured with addition of 1 mM ADP and 5 mM K<sub>2</sub>HPO<sub>4</sub> (iP) to basal electron transport components (25 mM hepes-NaOH (pH-7.5), 100 mM sucrose, 5 mM MgCl<sub>2</sub> and 20 μg Chl. ml<sup>-1</sup>). NH<sub>4</sub>Cl and GS were used at a final concentration of 5 mM and 10 μM respectively as and when required. The whole chain electron flow was assayed in presence of 2 mM FeCN as terminal electron acceptor of PSI. *p*BQ and DQ (400 μM) were used to measure PSII catalyzed electron flow. To measure PSI catalyzed electron flow, DCIPH<sub>2</sub> (1 mM DCIP plus 2 mM ascorbate) was used as electron donor to cytochrome *b<sub>6</sub>/f* region and 1 mM MV as transient electron acceptor. The PSI reaction mixture additionally contained 2 mM sodium azide (catalase inhibitor) and 10 μM DCMU (PSII inhibitor).

All experiments were repeated with 3 to 4 individual batches of thylakoid preparations. Experiments in intact plant samples were also made from different batches of plants. Although the absolute values of electron transport activity among various batches varied between 10–12%, the phenomenological observations were consistent.

### Putative artemisinin-metabolite analysis

Leaf tissue (700 mg) each from control and artemisinin treated rice leaves were homogenized in 6 ml of the grinding buffer, filtered and the filtrate was centrifuged at 5000×g for 5 min. Supernatant was kept in ice for further experimentation. Freshly isolated thylakoids (100 μg Chl. equivalent) from spinach were incubated with 1 ml of the supernatant obtained from artemisinin treated leaves for different time intervals (supernatant obtained from homogenized tissue sprayed with DMSO were taken as control for all experiments). The thylakoids were then spun down (5000×g for 5 min), washed once with washing buffer, resuspended in suspension buffer and assayed for their respective electron transport activities. In order to further characterize the nature of the inhibitory-metabolite, the supernatant from 5000×g centrifugation was ultracentrifuged at 1,81,700×g (50,000 rpm in the Sorvall TFT 80.4 rotor) for 1 h at 4°C. The supernatant was collected carefully and the pellets were resuspended in 1 ml of the suspending buffer. Isolated chloroplasts (100 μg Chl. equivalent) from spinach were incubated in the fractions for 30 and 60 min.

The pelleted thylakoids, following the treatment, were assayed for whole chain electron transport activity with FeCN as electron acceptor.

### Supporting Information

**Figure S1** Room temperature (25°C) and 77 K fluorescence spectra of isolated thylakoid from control (DMSO) and artemisinin treated (Artemisinin) leaves. The Chl. concentration was 3 μg ml<sup>-1</sup>. 40% glycerol was used in the chloroplast suspension medium for low temperature (77 K) spectral measurements. (DOC)

**Figure S2** Chlorophyll quantification in plants sprayed with DMSO and artemisinin. Leaves (20 mg fresh weight) were cut into small pieces and homogenized in cold methanol. The methanolic extract was used for estimation of Chl. Open bars denote Chl. content of control (DMSO) and closed bars denote Chl. content of treated (Artemisinin) plants. The graph shows an average of 5 individual readings with ± SD as error bars. Inset depicts the Chl. (*a/b*) ratio in both control and artemisinin sprayed leaves. (DOC)

**Figure S3** Analysis of inhibition of electron transport activity by putative artemisinin-metabolite. A, B, C and D respectively depicts the incubation times of 10, 20, 30, 60 min. The extent of inhibition was marked to be a time dependent phenomenon and a maximum inhibition of 65–70% was observed following 30 min of incubation. Increasing the incubation period to 60 min did not alter the extent of this inhibition. The numbers in parenthesis denote the electron transport rate (μmol O<sub>2</sub> evolved mg Chl.<sup>-1</sup> h<sup>-1</sup>) measured in presence of FeCN as electron acceptor. Using lesser amount of buffer during homogenization led to a much concentrated solution that failed to show any measurable O<sub>2</sub> exchange activity in thylakoids incubated with supernatant from leaves of artemisinin treated plants. Hence, we routinely used the mentioned ratio (see materials and methods) of tissue to grinding buffer for preparing the tissue supernatant. Arrow up, light on; arrow down, light off. (DOC)

**Figure S4** Analysis of biological activity of supernatant. The biological activity of supernatant was checked by fold dilution. With increase in dilution, the extent of inhibition was reduced, as measured against the undiluted supernatant. (DOC)

**Figure S5** Long term effect of artemisinin administration to rice plant. The plants were treated with artemisinin for 5 consecutive alternate day sprays. It resulted in complete death of the plants while the control plants (DMSO sprayed) continued to grow without symptoms of senescence. (DOC)

### Acknowledgments

The authors are thankful to the Director, Institute of Life Sciences for providing laboratory facilities. Technical help received from Mr. Suresh Martha is gratefully acknowledged.

### Author Contributions

Conceived and designed the experiments: AB SCS. Performed the experiments: AB SCS. Analyzed the data: AB SCS MK. Wrote the paper: AB SCS.

## References

- Duke SO, Dayan FE, Rimando AM, Schrader KK, Aliota G, et al. (2002) Chemicals from nature for weed management. *Weed Sci* 50: 138–151.
- Robert A, Benoit-Vical F, Meunier B (2005) Heme as trigger and target of the antimalarial peroxide artemisinin. In: *Medicinal Inorganic Chemistry*. ACS Symposium Series, Vol. 903, Chapter 16. pp. 281–294.
- Chen PK, Leather GR (1990) Plant growth regulatory activities of artemisinin and its related compounds. *J Chem Ecol* 16: 1867–1876.
- Duke SO, Vaughan KC, Croom EM, Elsohly HN (1987) Artemisinin, a constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. *Weed Sci* 35: 499–505.
- Bagchi GD, Jain DC, Kumar S (1997) Arteether: a potent plant growth inhibitor from *Artemisia annua*. *Phytochemistry* 45: 1131–1133.
- Bagchi GD, Jain DC (1998) The phytotoxic effects of the artemisinin related compounds of *Artemisia annua*. *J Med Aromat Plant Sci* 20: 5–11.
- Dayan FE, Hernandez A, Allen SN, Moraes RM, Vroman JA, et al. (1999) Comparative phytotoxicity of artemisinin and several sesquiterpene analogues. *Phytochemistry* 50: 607–614.
- Lydon J, Teasdale JR, Chen PK (1997) Allelopathic activity of annual wormwood (*Artemisia annua*) and the role of artemisinin. *Weed Sci* 45: 807–811.
- Dayan FE, Duke SO, Grossmann K (2010) Herbicides as probes on plant biology. *Weed Sci* 58: 340–350.
- Li W, Mo W, Shen D, Sun L, Wang J, et al. (2005) Yeast model uncovers dual roles of mitochondria in the action of artemisinin. *PLoS Genet* 1: 329–334.
- Nixon PJ (2000) Chlororespiration. *Phil Trans R Soc* 355:1541–1547.
- Wang J, Huang L, Li J, Fan Q, Long Y, L, et al. (2010) Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. *PLoS One* 5: e9582. doi:10.1371/journal.pone.0009582.
- Rutherford AW, Krieger-Liszka A (2001) Herbicide-induced oxidative stress in photosystem II. *Trends Biochem Sci* 26: 648–653.
- Deamer DW, Crofts AR, Packer L (1967) Mechanisms of light- induced structural changes in chloroplasts. I. Light- scattering increments and ultrastructural changes mediated by proton transport. *Biochim Biophys Acta* 131: 81–96.
- Nishio JN, Whitmarsh J (1991) Dissipation of the proton electrochemical potential in intact and lysed chloroplasts. I. The electrical potential. *Plant Physiol* 95: 522–528.
- Govindjee (1995) Sixty- three years since Kautsky: Chlorophyll *a* fluorescence. *Aust J Plant Physiol* 22: 131–160.
- Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Chlorophyll *a* fluorescence: a signature of photosynthesis. In: Papageorgiou, G C., Govindjee, editors. *Analysis of Chlorophyll a Fluorescence Transient*. Springer: Dordrecht. pp. 321–362.
- Stürbet A, Govindjee (2011) On the relation between the Kautsky effect (chlorophyll *a* fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. *J Photochem Photobiol* 104: 236–257.
- Schansker G, Toth SZ, Strasser R J (2005) Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl *a* fluorescence rise OJIP. *Biochim Biophys Acta* 1706: 250–261.
- Bowes JM, Crofts AR (1981) Effect of 2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone on the secondary electron acceptor B of photosystem II. *Arch Biochem Biophys* 209: 682–686.
- Satoh K, Kitatani Y, Ichimura T, Katoh S (1990) Interactions between various benzoquinones and the Q<sub>B</sub> site of oxygen-evolving photosystem II preparations from the thermophilic cyanobacterium *Synechococcus elongates*. In: Baltscheffsky M, editor. *Current Research in Photosynthesis*. Kluwer Academic Publishers: Dordrecht. pp. 583–586.
- Joshi MK, Mohanty P (1995) Probing photosynthetic performance by chlorophyll *a* fluorescence: Analysis and interpretation of fluorescence parameters. *J Sci Ind Res* 54: 115–174.
- Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annu Rev Plant Biol* 59:89–113.
- Papageorgiou GC, Tsimilli-Michael M, Stamatakis K (2007) The fast and slow kinetics of chlorophyll *a* fluorescence induction in plants, algae and cyanobacteria: a viewpoint. *Photosynth Res* 94: 275–290.
- Izawa S (1980) Acceptors and donors for chloroplast electron transport. *Methods Enzymol* 69: 413–434.
- Trebst A (2007) Inhibitors in the functional dissection of the photosynthetic electron transport system. *Photosynth Res* 92: 217–224.
- Kashino Y, Yamashita M, Okamoto Y, Koike H, Satoh K (1996) Mechanisms of electron flow through the Q<sub>B</sub> site in photosystem II. 3. Effects of the presence of membrane structure on the redox reactions at the Q<sub>B</sub> site. *Plant Cell Physiol* 37: 976–982.
- Dudekula S, Fragata M (2006) Investigation of the electron transfer site of *p*-benzoquinone in isolated photosystem II particles and thylakoid membranes using alpha- and beta-cyclodextrins. *J Photochem Photobiol B* 85: 177–83.
- Bukhov NG, Sridharan G, Egorova EA, Carpentier R (2003) Interaction of exogenous quinones with membranes of higher plant chloroplasts: modulation of quinone capacities as photochemical and non-photochemical quenchers of energy in Photosystem II during light–dark transitions. *Biochim Biophys Acta* 1604: 115–123.
- Chen PK, Leather GR, Klayman DL (1987) Allelopathic effect of artemisinin and its related compounds from *Artemisia annua*. *Plant Physiol* 83 (Suppl.): 68 (Abstr. 406).
- Duke S, Paul RN, Lee SM (1988) Terpenoids from the genus *Artemisia* as potential pesticides. In: Cutler HG, editor. *Biologically active natural products: potential use in agriculture*. American Chemical Society: Washington, DC. pp. 318–334.
- Van Rensen JJS (1989) Herbicides interacting with photosystem II. In: Dodge AD editor. *Herbicide and Plant Metabolism*. Cambridge University Press: New York. pp. 21–35.
- Saha S, Ouitrabul R, Izawa S, Good N (1971) Electron transport and phosphorylation in chloroplast as a function of the electron acceptor. *J Biol Chem* 246: 3204–3209.
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica Biophysica Acta* 975: 384–394.