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Inhibition of Site I mitochondrial electron transport by an extract of the seeds of *Millettia thonningii*: a potential mechanism for the plant's molluscicidal and schistosome larvicidal activity

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Abstract

A dichloromethane extract of the seeds of *Millettia thonningii* (Leguminosae) which contains a mixture of isoflavonoids (predominately robustic acid, alpinumisoflavone and dimethylalpinumisoflavone) is known to have larvicidal activity towards the miracidia and cercariae of schistosomes and to possess significant molluscicidal activity. The present investigation has assayed the effects of this extract on the electron transport systems of isolated rat liver mitochondria. The extract was found to inhibit mitochondrial electron transport at Site I (NADH dehydrogenase) at concentrations of 30–159 mg l^{-1}. Although the extract is not as potent an inhibitor at Site I as rotenone, a known inhibitor of NADH dehydrogenase, such observations could explain the molluscicidal and schistosomicidal activity of dichloromethane extracts of the seeds of *M. thonningii*.

Introduction

In searching for new methods to augment current control methods for schistosomiasis, plant based molluscicides have been investigated for the potential control of intermediate snail hosts (Kloos & McCullough, 1982).

The mature seeds of the West African legume *Millettia thonningii* contain bioactive compounds of potential relevance in the control of schistosomiasis. Seed extracts have demonstrated molluscicidal activity toward schistosome-transmitting snails (Evans *et al.*, 1986) and their eggs (Tang, 1994; Tang *et al.*, 1995), as well as possessing schistosomicidal activity towards *Schistosoma mansoni* adult worms, cercariae and miracidia *in vitro* (Squire & Whitfield, 1989; Perrett, 1994; Perrett *et al.*, 1994; Lyddiard, 1997; Lyddiard *et al.*, 1998). Such studies suggest that *M. thonningii* seed extracts could have a use as cheap, locally produced larvicides and molluscicides in countries where schistosomiasis is endemic.

Lyddiard *et al.* (1998) described a video-imaging method which directly measured miracidial motility, demonstrating a sub-lethal reduction of swimming velocity in response to a dichloromethane extract of *M. thonningii* seeds. Inhibition of energy metabolism by the extract might cause the observed reduction in motility. Such inhibition could also cause disrupted osmoregulation which might explain the secondary observation of miracidial swelling (approximately a 10% increase in volume after a 10 min exposure to 50 mg l^{-1} of the extract) (Lyddiard *et al.*, 1998).

NMR characterization of the compounds present in a deuterochloroform extract of hexane defatted seeds of *M. thonningii* demonstrated three major components in an approximate ratio of 1:2:1 (Lyddiard, 1997). These compounds were identified as the isoflavonoids robustic acid, alpinumisoflavone and dimethylalpinumisoflavone (fig. 1a–c) by comparison with the data of Olivares *et al.* (1982) and Khalid & Waterman (1983). These compounds

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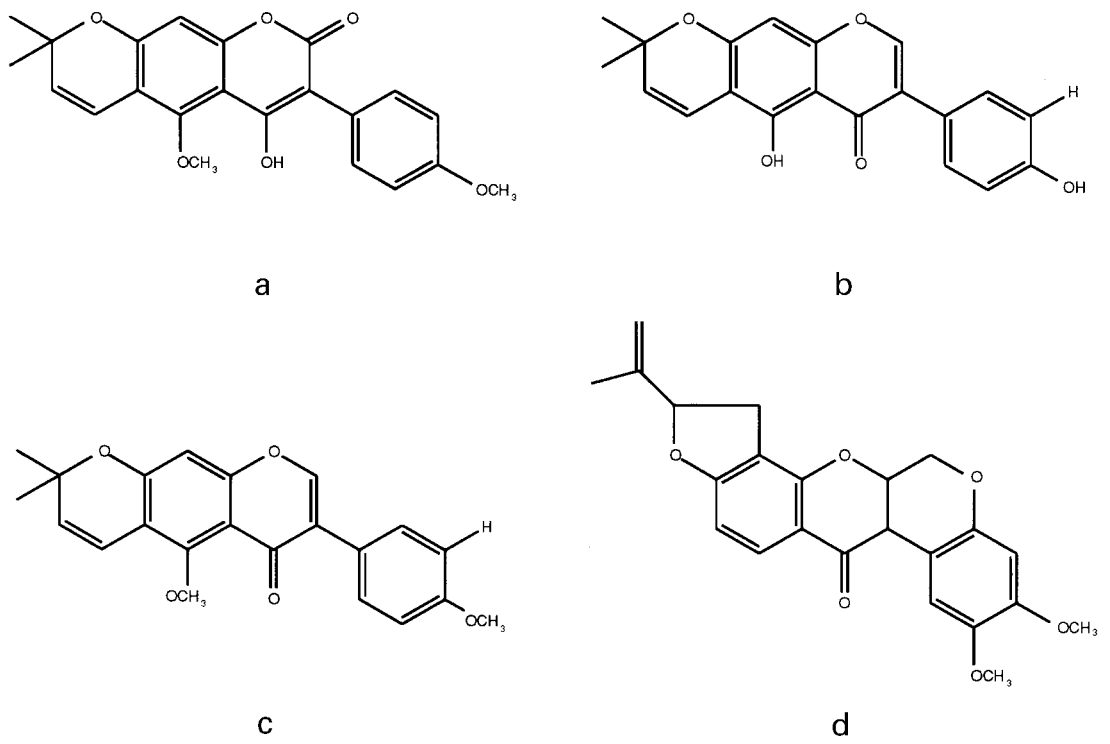

Fig. 1. Structures of the three major isoflavonoid components of the seeds of *Millettia thonningii*; a, robustic acid; b, alpinumisoflavone; c, dimethylalpinumisoflavone; and the insecticide rotenone (d).

were also identified as the three major components of dichloromethane extracts of the defatted seeds (Lyddiard, 1997).

Rotenone (a rotenoid isoflavonoid, fig. 1d), a potent insecticide and piscicide, was first isolated from the roots of *Derris* sp. (Shepard, 1951). Rotenone acts as an inhibitor of mitochondrial electron transport at Site I (NADH dehydrogenase) (Burgos & Redfearn, 1965; Horgan & Singer, 1968; Bois & Estabrook, 1969). The main mode of action of many molluscicides is also inhibition of energy metabolism (for example Phebrol (Furushima *et al.*, 1991) and niclosamide (Andrews *et al.*, 1983)) and the disruption of osmoregulation (caused by inhibition of energy metabolism) (Duncan, 1987).

Such observations and the similarity of the structure of rotenone with the component isoflavonoids of the dichloromethane extract of *M. thonningii* indirectly support the hypothesis that one or more of the compounds in dichloromethane extracts of *M. thonningii* may inhibit energy metabolism, accounting for both the larvicidal and molluscicidal activity observed. The current study investigates the inhibitory action of the dichloromethane extract of the seeds of *M. thonningii* on electron transport in rat liver mitochondria.

Materials and methods

All chemicals, unless otherwise stated, were purchased from BDH, Merck.

Mature seeds of *M. thonningii* (Schum. et Thonn.) Bak. (Leguminosae), were collected from the Dodowa Plains, Ghana by Kwame Boateng Botanicals (Accra, Ghana), and their authenticity was verified by the Royal Botanical Gardens (Kew, London, Specimen: GC 6490 J.K. Morton). The ground air-dried seeds were initially extracted for 4 h in a Soxhlet apparatus with hexane to remove non-polar compounds, and subsequently extracted with dichloromethane for a further 4 h. The extract was dried by rotary evaporation and stored at −20°C before use.

Isolation of fresh rat liver mitochondria

The liver from an adult Wistar rat killed by cervical dislocation was macerated with scissors in ice-cold 0.9% saline solution. The washed and chopped liver was then homogenized in a pre-cooled glass-Teflon homogenizer, using six up and down strokes of the pestle rotating at approximately 500 rpm, in an excess of ice-cold homogenization medium (75 mM sucrose; 225 mM mannitol; 10 mM MOPS; 1 mM EDTA; 5 mg ml^{-1} BSA-fatty acid free (Albumin factor V); pH 7.4). The homogenate was then centrifuged at 4°C for 10 min at 1000 *g* and the supernatant decanted and centrifuged at 10 000 *g* for 10 min at 4°C. The mitochondrial pellet was re-suspended in fresh homogenization medium and re-centrifuged at 10 000 *g* for 10 min at 4°C. The pellet was then re-suspended in 1 ml of homogenization medium and kept on ice for experimentation (Rickwood *et al.*, 1987).

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Protein analysis of mitochondria samples

Each mitochondrial suspension (100 μl) was diluted to 2 ml with distilled water and the absorbance at 540 nm measured after the mixture was incubated with 3 ml standard Biuret solution for 10 min at 37°C. Values for the protein content were determined from standards of BSA at concentrations between 1 and 10 mg ml^{-1} (which included 100 μl of homogenization buffer to account for the BSA in the mitochondrial suspension).

Mitochondrial assays

A Rank Brothers 3 ml oxygen electrode (anode-silver/silver chloride; cathode-platinum) was utilized and data were recorded on a BBC chart recorder (full scale deflection of 10 mV). To calibrate the electrode, a few crystals of sodium dithionite was added to 3 ml of electrode buffer (150 mM sucrose; 20 mM KCl; 1 mM KH_2PO_4; 5 mM $MgCl_2$; 10 mM Tris; pH 7.4) at 20°C and the chart recorder calibrated to zero. After wash-out the oxygen tension in 3 ml of electrode buffer was recorded, the chart recorder set to 90% full-scale deflection, corresponding to 260 μM dissolved oxygen. All solutions used were adjusted to pH 7.4.

For each experiment 100 μl of the fresh mitochondrial suspension was added to 3 ml of electrode buffer at 20°C. In each case sodium glutamate/malate was added to a final concentration of 2.5 mM each (or succinate (5 mM)) and the oxygen uptake monitored for 2 min. ADP (Sigma) was then added (180 μM) and the period of rapid oxygen utilization was monitored (state 3 respiration) followed by state 4 respiration (characterized by a reduced oxygen consumption). The P/O ratio (the ratio of the number of nanomoles of ADP added to the number of oxygen atoms (nanomoles) utilized during state 3 respiration) and the respiratory control ratio (RCR – the ratio of the rate of oxygen uptake in state 3 respiration to the rate of oxygen uptake in state 4 respiration) was then calculated.

For further experimental purposes, mitochondria were uncoupled by the inclusion of 2 μM FCCP (carbonyl cyanide-p-trifluoronethoxyphenyl-hydrazone, Sigma) in the reaction chamber. The properties of the dichloromethane extract of *M. thonningii* towards mitochondrial electron transport systems were investigated by the sequential stimulation of Site I, II and IV respiration with the inclusion of increasing concentrations (0–159 mg l^{-1}) of the plant extract (pH adjusted to 7.4). Control experiments were carried out by the additions of ethanol (the intermediary solvent) to the appropriate concentration (up to 2%). Any differences in the rate of oxygen utilization were monitored, experimental procedures being replicated eight times and controls five times.

Site I respiration was investigated by the inclusion of the substrates sodium glutamate/malate (2.5 mM each). A positive control was carried out by the addition of increasing concentrations of rotenone (15–110 nM). Site II respiration was investigated using 5 mM of sodium succinate as substrate with Site I respiration inhibited by an excess of rotenone (20 μM). For Site IV, 1.5 μg ml^{-1} Antimycin A (a Site II inhibitor, Sigma), 2 μM TMPD (N,N,N,N,-tetramethyl p-phenylenediamine; an electron donor, Sigma) and 5 mM of the substrate sodium ascorbate were added to the reaction chamber (Ernster *et al.*, 1963; Rickwood *et al.*, 1987, Pecci *et al.*, 1994; Colleoni *et al.*, 1996).

Mitochondria were also subjected to three cycles of freeze-thawing in rapid succession which resulted in the disruption of the mitochondrial membrane and release of mitochondrial enzymes (Greiff *et al.*, 1961; Lusena, 1965). Mitochondria, ruptured by this method, were then used for analysis in the oxygen electrode with 600 μM of NADH (Sigma) as substrate. The extract of *M. thonningii* seeds and rotenone were tested as before to investigate if the compounds inhibited transport of substrate into the mitochondrial matrix.

Results

In all cases, control experiments (with up to 2% ethanol in-well) demonstrated little effect on mitochondrial oxygen utilization. Maximum reduction in utilization within any experiment was less than 10% of the initial rate.

Figure 2 demonstrates the action of the dichloromethane extract of *M. thonningii* on Site I respiration on rat liver mitochondria using glutamate/malate as substrates. There is a dose-dependent reduction in the oxygen utilization and at a concentration of approximately 160 mg l^{-1} the initial rate of oxygen utilization was reduced by nearly 90%. The concentration of the extract required to give 50% inhibition of oxygen utilization (IC_{50}) was approximately 50 mg l^{-1}. A Student's unpaired, two-tailed t-test demonstrated significant differences between experimental and control values ($t > 4.22$; $P < 0.01$; d.f. = 12).

The extract, at concentrations up to 160 mg l^{-1}, had no significant inhibitory effect on the rate of oxygen utilization when Site I was inhibited and succinate was tested as a substrate (Site II) and similarly when Site II was inhibited with antimycin A and ascorbate (in the presence of TMPD) was tested as a substrate (Site IV). A Student's unpaired, two-tailed t-test demonstrated no significant differences between experimental and control values ($t < 1.78$; $P > 0.1$; d.f. = 12).

The result of the positive control with rotenone, a known Site I inhibitor (fig. 3), demonstrated an IC_{50} of approximately 45 nM, and almost complete inhibition at 120 nM (Student's unpaired, two-tailed t-test; $t > 4$; $P < 0.01$; d.f. = 12). The results being in a similar concentration range to those observed by Lindahl & Öberg (1961) and Ernster *et al.* (1963).

Using NADH and disrupted mitochondria, the initial rate of respiration of the mitochondria was almost four times greater than when testing intact mitochondria and using malate/glutamate as substrates. Similar trends were observed for the inhibition of oxygen utilization for both the extract of *M. thonningii* and for rotenone (figs 4 and 5) both demonstrating significant inhibition of the rate of oxygen utilization (Student's unpaired, two-tailed t-test; $t > 4$; $P < 0.01$; d.f. = 12).

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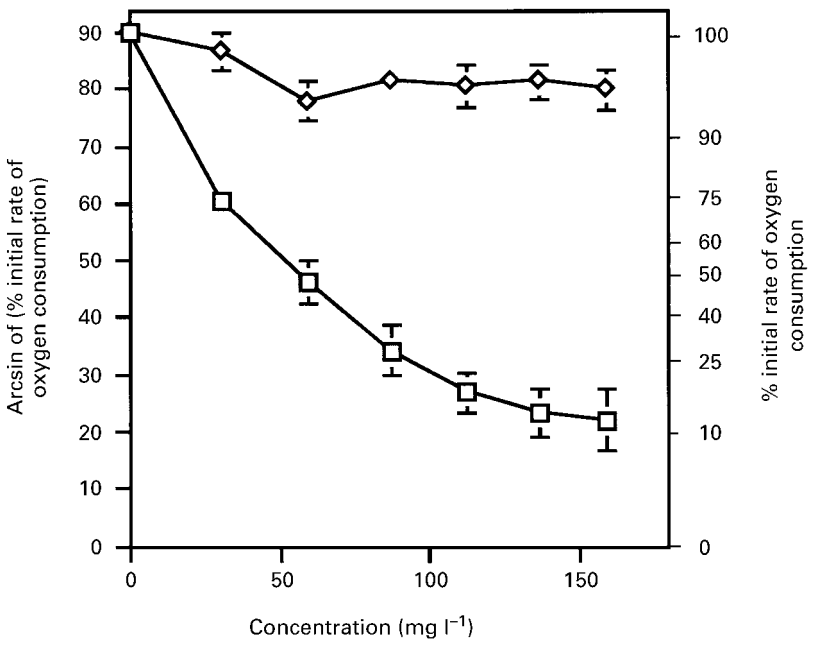

Fig. 2. Inhibition of oxygen utilization of rat liver mitochondria (glutamate/malate as substrate – Site I) by a dichloromethane extract of the seeds of *Millettia thonningii* (□); ethanol control (◇). RCR = 15; P/O = 2.4; uncoupling constant = 3.1; protein content = 1.83 mg ml^{-1}. Error bars represent standard errors.

Discussion

The dichloromethane extract of the seeds of *M. thonningii* demonstrated an inhibition of mitochondrial electron transport by about 90% at a concentration of 159 mg l^{-1}. Such inhibition is only observed when the substrates glutamate/malate are used and not when Site I or Site II are inhibited and succinate or ascorbate (in the presence of TMPD) are used as substrates. The extract therefore appears to inhibit electron transport in a classic,

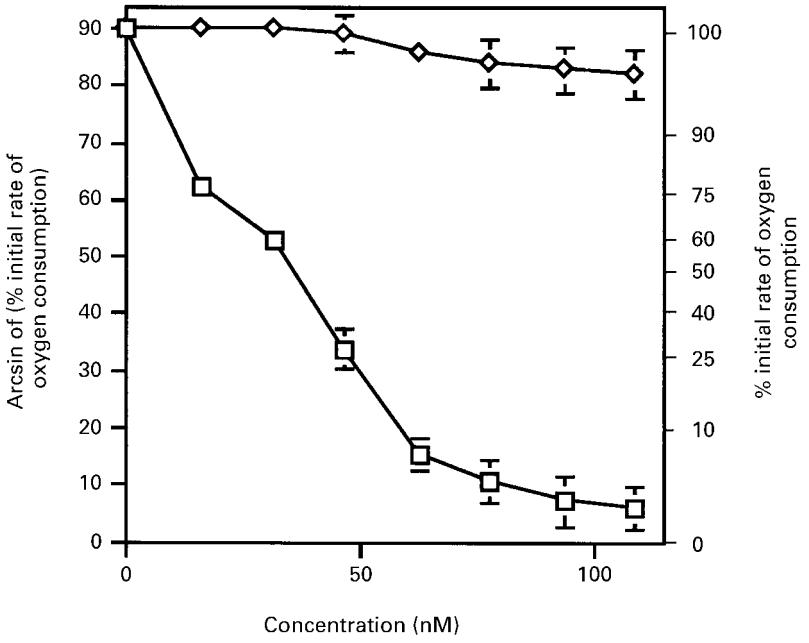

Fig. 3. Inhibition of oxygen utilization of rat liver mitochondria (glutamate/malate as substrate – Site I) by rotenone (□); ethanol control (◇). RCR = 15; P/O = 2.4; uncoupling constant = 3.1; protein content = 1.83 mg ml^{-1}. Error bars represent standard errors.

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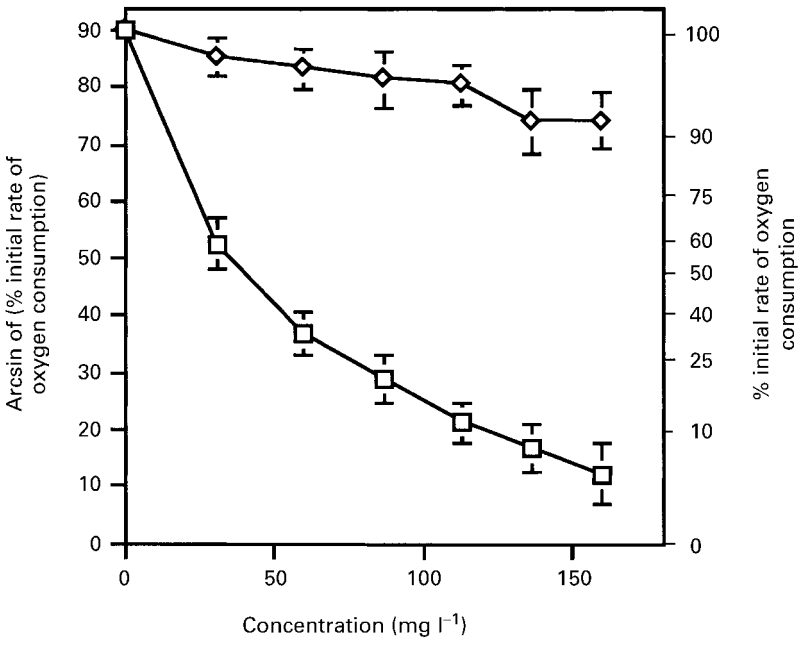

Fig. 4. Inhibition of oxygen utilization of disrupted rat liver mitochondria (NADH as substrate – Site I) by a dichloromethane extract of the seeds of *Millettia thonningii* (□); ethanol control (◇). Protein content = 1.92 mg ml^{-1}. Error bars represent standard errors.

rotenone-like manner, acting as a Site I inhibitor. Rotenone demonstrated similar activity, but showed a higher specific activity than the extract. This difference in activity could possibly be due to one or more causes. Firstly, the active component(s) in the extract may not be as active as rotenone. Secondly, the active component(s) may only be present in relatively small amounts and therefore, if purified, may have a higher activity. Lastly, the active component(s) may not be transported across the mitochondrial membrane as efficiently as rotenone and so exert less influence on electron transport.

NADH cannot be used with intact mitochondria as this substrate is not transported across the mitochondrial membranes (Rickwood *et al.*, 1987) and is active only

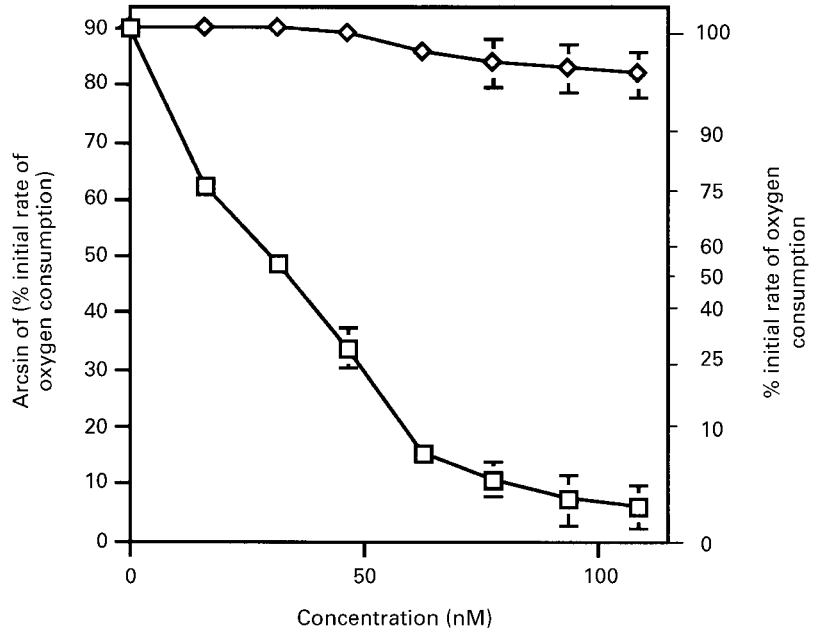

Fig. 5. Inhibition of oxygen utilization of disrupted rat liver mitochondria (NADH as substrate – Site I) by rotenone (□); ethanol control (◇). Protein content = 1.92 mg ml^{-1}. Error bars represent standard errors.

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when the structural integrity of the mitochondria is breached. In fact, both the extract and rotenone demonstrated a similar pattern of inhibitory activity with disrupted mitochondria, the extract showing no more activity than observed with the intact mitochondria. It is not, therefore, the inability of the active component(s) from the extract to enter the mitochondria which accounts for the lower activity of the extract compared with rotenone. Unfortunately, it has not proved possible, to date, to isolate all the bioactive compounds in the dichloromethane extract in quantities sufficient for biological screening which would allow these hypotheses to be tested directly.

The dichloromethane extract of the seeds of *M. thonningii* therefore demonstrates inhibitory activity towards Site I of mitochondrial electron transport (NADH deyhdrogenase) in a rotenone-like manner when tested against rat liver mitochondria. This inhibition may well account for both the molluscicidal and schistosomicidal activity of the extract. It is not yet apparent, though, why the active components appear to have higher activity towards these two invertebrate targets than towards fish (Squire *et al.*, 1989). Perhaps differential uptake dynamics in snails, schistosomes and fish account for the differences. The results of the present investigation do not identify which, if any, of the major isoflavonoid components of dichloromethane extracts of *M. thonningii* seeds, have inhibitory effects on mitochondrial electron transport. Further experiments are required on purified compounds to answer this question.

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