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An immunomodulator from *Tinospora cordifolia* with antioxidant activity in cell-free systems

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Abstract. Several plant products are known to exhibit immense medicinal value against human diseases. Our earlier studies showed that dry stem crude extract (DSCE) of *Tinospora cordifolia* contained a polyclonal B cell mitogen, G1-4A. DSCE as well as G1-4A also enhanced immune response in mice. In order to explore the possibility of using G1-4A/PPI (partially purified immunomodulator) to modulate radiation induced immunosuppression, the antioxidant effect of PPI from this plant was examined against reactive oxygen and nitrogen species (ROS/RNS), generated by photosensitization/peroxynitrite. Levels of lipid peroxidation products, superoxide dismutase (SOD) and catalase in liver/spleen homogenate from mouse were monitored. Photosensitization induced significant increase in thiobarbituric acid reactive substances (TBARS) in liver. The activities of SOD and catalase were reduced considerably. PPI, present during photosensitisation, prevented lipid peroxidation and restored the activities of both the enzymes. Likewise, oxidative damage induced by peroxynitrite was inhibited by PPI. The degradation of proteins due to photosensitization as assessed by SDS-PAGE was effectively reduced by simultaneous treatment with PPI during photosensitization. Selective inhibitors of ROS like mannitol, SOD, sodium azide and antioxidants, GSH and vitamin C brought about significant inhibition of formation of TBARS suggesting possible involvement of $O_2^{-\bullet}$,$^{\bullet}OH$ and $^1O_2$. Photosensitization in deuterated buffer enhanced formation of TBARS thus indicating generation of $^1O_2$. Thus, the action of PPI may be against oxidative damage through Type I and II photosensitization mechanisms. Therefore, the immunomodulator from *Tinospora cordifolia* may also be beneficial as an antioxidant.

Keywords. Immunomodulator; *Tinospora cordifolia*; antioxidant; photosensitization.

1. Introduction

Several plant products have useful medicinal properties for the treatment of various ailments. One such plant selected in our study is *Tinospora cordifolia*, called Gulvel/ Guduchi/Gulancha, belonging to the family Menispermaceae. It has been reported to contain immunomodulatory constituents.[1–4] Earlier studies from our laboratory showed that the dry stem crude extract (DSCE) of this plant contained a polyclonal B cell mitogen which was polysaccharide in nature.[2,3] The active component G1-4A enhanced humoral immune response in mice and also protected them against lipopolysaccharide induced endotoxic shock.[4,5] G1-4A showed binding to macrophages and induced secretion of IL-1. It has also been observed that treatment of mice with DSCE prevented cyclophosphamide induced myelosuppression[1] as well as immunosuppression,[6] indicating

its possible use for prevention/treatment of radiation induced immunosuppression. Since radiation exposure as well as activation of macrophages can result in oxidative stress through reactive oxygen and nitrogen species (ROS/RNS),[7] the aim of the present study was to evaluate the effect of this immunomodulator on oxidative damage. ROS/RNS were induced by exposing mouse liver/spleen homogenate to peroxynitrite and photosensitization. The effect of partially purified immunomodulator (PPI) from *T. cordifolia* on oxidative damage was assessed using several parameters such as lipid peroxidation, activities of SOD, catalase, protein degradation etc.

2. Materials and methods

2.1 *Animals*

Eight- to twelve-week old C3H mice of either sex were used for experimental work.

2.2 *Isolation of PPI*

The PPI containing the polysaccharide G1-4A was obtained from dry stem of *T. cordifolia.* Powdered dry stems of *T. cordifolia* were extracted overnight in methanol to remove low molecular weight constituents. The residue was then boiled in water and the supernatant precipitated with acetone to get the polysaccharide rich fraction which was reprecipitated with 15% trichloroacetic acid to remove protein constituents. Precipitation with acetone was repeated and the precipitate obtained was dialysed and lyophilised to get PPI. It was screened for mitogenic activity towards C3H mouse spleen cells by using ^{3}H-thymidine incorporation assay.[2]

2.3 *Preparation of homogenates*

10% tissue homogenates from liver and spleen of mice were prepared in 50 mM phosphate buffer, *p*H 7·4. Protein concentration was estimated by Lowry's method.[8]

2.4 *Photosensitization*

Liver homogenate was exposed to 100 W tungsten lamp, a source of visible light, placed 15 cm away from the sample in presence of methylene blue (50 *m*M) with constant bubbling of oxygen at 37°C.[9]

2.5 *Treatment with peroxynitrite*

Peroxynitrite was synthesised by passing the gas stream from an ozonator (5% ozone in oxygen) in sodium azide solution (0·1 M in water, *p*H 12·0) at 0–4°C. The liver homogenate (0·1 mg protein/ml) was incubated with 100 *m*M peroxynitrite at 37°C for 15 min.[10]

2.6 *Assessment of oxidative damage*

Oxidative damage was studied by measuring lipid peroxidation products such as TBARS[9,11] and lipid hydroperoxides (LOOH), activities of SOD and catalase and protein degradation. LOOH formed were measured by iodometric method.[12] Assay of SOD

activity was based on the spectrophotometric detection of adrenochrome at 320 nm formed by autoxidation of epinephrine at *p*H 10·2.[13] Catalase activity was measured in terms of the disappearance of peroxide, which was followed spectrophotometrically at 240 nm.[14] Degradation of proteins was assessed by SDS-PAGE.[15]

To understand the mechanism involved in prevention of oxidative damage by PPI, deuterated buffer was used during photosensitization and selective inhibitors of ROS such as mannitol, SOD and established antioxidants like GSH and vitamin C were added during photosensitization to examine the types of ROS involved.

3. Results and discussion

3.1 *Inhibition of photosensitisation induced oxidative damage by PPI*

The formation of TBARS in the photosensitised liver homogenate was inhibited in a concentration dependent manner in presence of PPI of *T. cordifolia*. Control value for TBARS produced by the homogenate without photosensitization was 0·58 ± 0·07 nmoles/mg protein. Maximum inhibition (55%) was seen at 500 *m*g/ml of PPI (figure 1a). The amount of LOOH, was significantly increased following photosensitization. Control

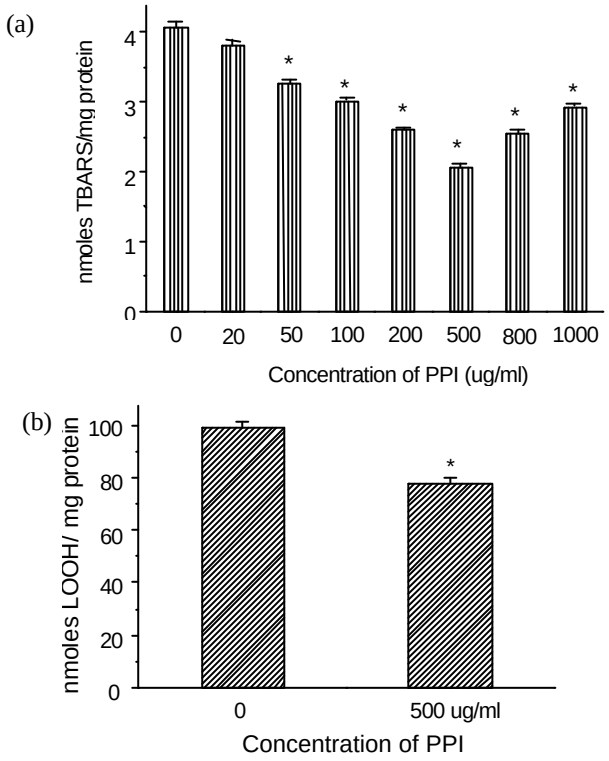

Figure 1. Effect of PPI on photosensitization (PS) induced lipid peroxidation. Liver homogenate was exposed to photosensitization for 30 min with and without PPI and estimation of TBARS (**a**) and LOOH (**b**) was carried out. The values are mean ± SEM for 3 experiments. **P* < 0·001 compared to damage produced by PS in absence of PPI.

value for LOOH produced by the homogenate without photosensitization was 68·9 ± 3·73 nmoles/mg protein. PPI at a concentration of 500 *m*g/ml reduced the levels of lipid hydroperoxides almost to the control levels (figure 1b). PPI thus inhibited both the products of lipid peroxidation.

Photosensitisation depleted the activities of antioxidant enzymes, SOD and catalase, in the liver homogenate. PPI effectively (65–80%) inhibited the depletion in the activities of SOD and catalase.

Photosensitization is known to induce remarkable increase in the levels of protein carbonyls, an indicator of protein oxidation[11] which would lead to protein degradation. The effect of PPI on photosensitization induced degradation of proteins in liver homogenates was assessed by SDS-PAGE. The intensity of the protein bands was less in the lanes containing the photosensitised samples. Photosensitization of the homogenate in presence of PPI (500 *m*g/ml) prevented protein degradation as seen from the higher intensity of bands (figure 2).

Similar antioxidant effect of PPI was observed in spleen cell homogenate. PPI (500 *m*g/ml) also inhibited the formation of TBARS and protected the activities of SOD and catalase (table 1).

3.2 *Inhibition of oxidative damage induced by peroxynitrite*

Peroxynitrite (PXN), which is a reactive nitrogen species, also induces oxidative damage in biological systems. Formation of TBARS in liver homogenate, which was enhanced significantly following treatment with peroxynitrite (100 *m*M) was inhibited in presence of PPI (200 *m*g/ml). Likewise, the reduction in activities of SOD and catalase was prevented in presence of PPI albeit at a higher concentration (500 *m*g/ml; table 1).

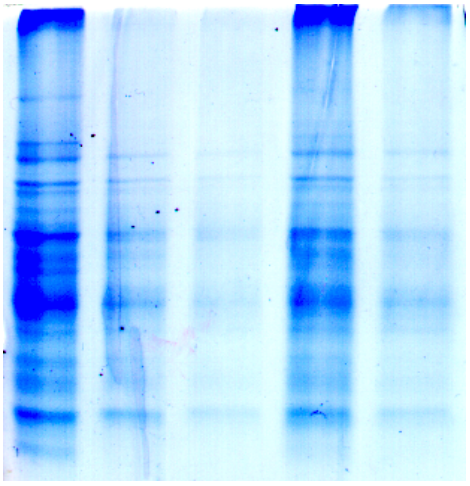

Figure 2. Effect of PPI on protein degradation due to photosensitization in liver homogenate. Liver homogenate was exposed to photosensitization for 30 and 60 min with (500 *m*g/ml) and without PPI and was subjected to SDS-PAGE. Lane 1: No PS; Lane 2: 30 min PS; Lane 3: 1 h PS; Lane 4: 30 min PS + 500 *m*g/ml PPI; Lane 5: 1 h PS + 500 *m*g/ml PPI.

Table 1. Antioxidant effect of PPI on oxidative stress induced by photosensitization and peroxynitrite in liver and spleen homogenates.

Tissue	Oxidative stress inducer	Treatment	LOOH (nmoles/mg protein)	TBARS (nmoles/mg protein)	SOD activity (% of control)	Catalase activity (% of control)
Liver	None	–	68·93 ± 3·73	0·58 ± 0·07	100	100
	PS	–	99·44 ± 1·8	4·07 ± 0·096	2·18 ± 2·08	46·25 ± 6
	PS	PPI	77·97 ± 2·15*[a]	2·1 ± 0·096*[a]	76·3 ± 3·92*[b]	82·5 ± 10·2 [#b]
	None	–	–	0·14 ± 0·04	100	100
	PXN	–	–	0·78 ± 0·05	26·1 ± 11·3	19·8 ± 3·3
	(100 *m*M)	PPI	–	0·61 ± 0·04 [+b]	60·9 ± 4·5 [#b]	56·9 ± 13·6 [#a]
Spleen	None	–	–	0·92 ± 0·017	100	100
	PS	–	–	1·35 ± 0·031	10·0 ± 3·3	46·2 ± 2·1
	PS	PPI	–	0·97 ± 0·04 *[a]	82·6 ± 10·5 *[a]	65·4 ± 1·4 [#a]

The values are mean ± SEM for 3 experiments. *$P < 0{\cdot}001$, [#]$P < 0{\cdot}01$, [+]$P < 0{\cdot}05$ as compared to damage induced by PS/PXN in absence of PPI; [a]Concentration of PPI 500 *m*g/ml; [b]Concentration of PPI 200 *m*g/ml

3.3 *Possible mechanism of prevention of oxidative damage by PPI*

Photosensitization is known to induce various ROS including singlet oxygen, superoxide and hydroxide radicals. Different inhibitors of ROS induced damage were used to indicate the contribution of the specific species to photosensitization induced damage.[9] These experiments were carried out in deuterated buffer that increases the lifetime of $^1O_2$.[16] The results with selective inhibitors of ROS, sodium azide (for $^1O_2$) SOD (for $O_2^{-\bullet}$) and GSH and vitamin C (for $^\bullet OH$) clearly indicated that all three species were the major contributors to photosensitization induced lipid peroxidation (figure 3a). When equal amounts of PPI, mannitol and vitamin C (500 *m*g/ml) were used it was observed that inhibition of the formation of TBARS by PPI was more than that shown in presence of mannitol and vitamin C (figure 3b). This did not mean that PPI was a better antioxidant than vitamin C since at high concentration the latter could have undergone autooxidation. Likewise, mannitol may not be maximally active at 500 *m*g/ml.

It is well established that $^1O_2$ can be generated in cells under oxidative stress[17] from decomposition of lipid peroxides or by spontaneous dismutation of superoxides. In addition, naturally occurring pigments such as riboflavin, cytochromes, and several other xenobiotics can generate $^1O_2$ inside the cells when irradiated by visible light.[18] Photosensitisation with methylene blue occurs through an excited triplet state that can act either by hydrogen atom or electron transfer reaction (Type I mechanism) or by transferring the excited energy forming $^1O_2$ (Type II mechanism).[19] Type I and Type II species cause widespread damage to biological macromolecules such as lipid, protein and DNA.[9,19,20] Various plant derived antioxidants and vitamins are known to exhibit profound effects against oxidative damage.[21] Inhibition of oxidative damage by PPI thus indicates possible scavenging of Type I and Type II species.

Peroxynitrite is another potent oxidant formed by the interaction of superoxide with nitric oxide under various pathophysiological conditions. Peroxynitrite is known to induce oxidative damage to cellular molecules which is implicated in various diseases.[22] PPI protected against peroxynitrite induced damage *in vitro.*

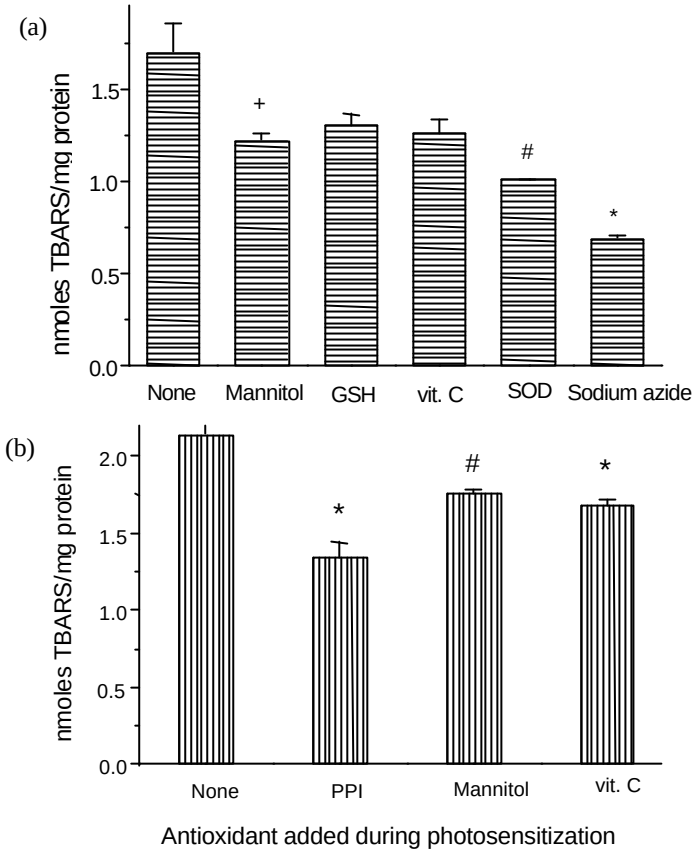

Figure 3. Effect of inhibitors of ROS on the formation of TBARS (**a**) and comparative antioxidant potential of PPI, mannitol and vitamin C (**b**) in liver homogenate. Inhibitors such as mannitol (100 mM), SOD (400 units/ml), GSH (10 mM), vitamin C (10 mM) and sodium azide (10 mM) were added during photosensitization (**a**). Data on TBARS produced in presence of equal concentration (500 mg/ml) of PPI, mannitol and vitamin C added during photosensitization are shown in (**b**). The values are mean ± SEM for 3 experiments. $^{*}P < 0{\cdot}001$, $^{\#}P < 0{\cdot}01$, $^{+}P < 0{\cdot}05$ compared to damage produced by PS in absence of any antioxidant.

PPI is rich in polysaccharide. Recently, a protein bound polysaccharide from the fungus *Cordyceps sinensis* was shown to possess antioxidant activity. This activity increased 10 to 30 fold in partially purified polysaccharide.[23,24] Antioxidant activity has also been reported in the polysaccharide derived from *Asparagus racemosus.*[25] Our results too demonstrate significant antioxidant properties in the polysaccharide immunomodulator obtained from *T. cordifolia* against peroxynitrite and photosensitization induced damage.

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