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Immunomodulatory active compounds from *Tinospora cordifolia*

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ABSTRACT

Ethnopharmacological relevance: Tinospora cordifolia mentioned as "Rasayana" is extensively used in various herbal preparations for the treatment of different ailments for its general tonic, antiperiodic, antispasmodic, antiinflammatory, antiarthritic, antiallergic and antidiabetic properties. It is extensively used in Ayurveda due to its potential in improving the immune system and the body resistance against infections.

Aim of the study: The aim of the study was to isolate and characterise the immunomodulatory active compounds of *Tinospora cordifolia*.

Materials and methods: The immunomodulatory activity of different extracts, fractions and isolated compounds in relation to phagocytosis and reactive oxygen species production in human neutrophil cells have been investigated using the PMN phagocytic function studies, NBT, NO and chemiluminescence assay.

Results: The results obtained indicate that ethyl acetate, water fractions and hot water extract exhibited significant immunomodulatory activity with an increase in percentage phagocytosis. Chromatographic purification of these fraction led to the isolation of a mixture of two compounds **2**, **3** isolated for the first time from natural source and five known compounds **1**, **4**–**7** which were characterized as 11-hydroxymustakone (**2**), *N*-methyl-2-pyrrolidone (**3**), *N*-formylannonain (**1**), cordifolioside A (**4**), magnoflorine (**5**), tinocordiside (**6**), syringin (**7**) by nuclear magnetic resonance (NMR) and mass spectrometry (MS) and comparing the spectral data with reported one. Cordifolioside A and syringin have been reported to possess immunomodulatory activity. Other five compounds showed significant enhancement in phagocytic activity and increase in nitric oxide and reactive oxygen species generation at concentration 0.1–2.5 μg/ml.

Conclusions: Seven immunomodulatory active compounds belonging to different classes have been isolated and characterised indicating that the immunomodulatory activity of *Tinospora cordifolia* may be attributed to the synergistic effect of group of compounds.

1. Introduction

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases (Patwardhan et al., 1990). Chemotherapeutic agents available today have mainly immunosuppressive activity and most of them are cytotoxic and exerts a variety of side effects. Due to which plant products as immunomodulator are getting more importance. Although cytokines like interleukins and interferons are used as immunostimulants, these are not very effective in the long term both because of their cost and adverse effects. Thus, medicinal plants and their active components as a source of immunomodulatory agents are gaining importance. Uses of plant products to enhance the phagocytic ability of macrophages and increase the antibody production by B cells have been well documented by several researchers (Chopra et al., 1956; Kirtikar and Basu, 1975; Nadkarni and Nadkarni, 1976; Chopra et al., 1982; Zhao et al., 1991). Many herbs such as *Centella asiatica* (Jayathirtha and Mishra, 2004), *Azadirachta indica* (van der Nat et al., 1987), *Phyllanthus debelis* (Diwanay et al., 2004), *Asparagus racemosus* (Gautam et al., 2004) and *Chenopodium ambrosioides*

Abbreviations: DEPT, distortionless enhancement by polarization transfer; HMQC, heteronuclear multiple quantum correlation; HMBC, heteronuclear multiple mond correlation; ESI-QTOF-MS, electrospray ionization-quadrupole time of flight-mass spectrum; MEM, minimum essential medium; MS, mass spectrometry; NMR, nuclear magnetic resonance; NBT, nitroblue tetrazolium; NO, nitrous oxide; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species.

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(Cruz et al., 2007) have been shown to alter the immune function and to possess a wide array of immunomodulatory effects. For example, hydroalcoholic extract of *Chenopodium ambrosioides* leaves was reported to enhance phagocytic ability and nitric oxide (NO) production. Similarly, the hemicellulose preparation Natramune (PDS-2865), increases macrophage phagocytosis and NO production (Weeks and Perez, 2009).

Tinospora cordifolia (Willd.) Miers ex Hook. f. & Thomas. (Menispermaceae) is one of these important herbs with known immunomodulatory activities (Sonel and Kuttan, 1999). The task force on conservation and sustainable use of medicinal plants identified the species as one of the most commercially exploited plants in pharmaceuticals. The estimated annual demand of this species used in the preparation of crude herbal drugs in the Indian System of Medicines is 10,000 tonnes (Singh and Warrier, 2004). Extracts of this plant has been shown to possess many therapeutic properties including general tonic, antiinflammatory, antiarthritic, antimalarial, aphrodisiac (Rao et al., 2008), antiallergic (Nayampalli et al., 1986), antidiabetic (Wadood et al., 1992), antihepatotoxic (Bhupindu et al., 1981; Rege et al., 1984) and antipyretic (Kumar and Shrivastav, 1995). However, several efforts have been made, the phytochemical constituents in the individual extract have not been specified, it may be possible that multiple constituents of *Tinospora cordifolia* exhibit similar pharmacological properties irrespective of their nature. Although the active components responsible for therapeutic effects of *Tinospora cordifolia* are not well defined, phenyl propanoid glycosides such as cordifolioside A, cordifolioside B and syringin, have been reported to be main immunomodulatory active components (Maurya et al., 1996; Kapil and Sharma, 1997; Cho et al., 2001). In addition α-D-glucan, isolated from the stem of the plant has been shown to stimulate the immune system (Nair et al., 2004, 2006).

Phagocytosis is an essential cell defence mechanism against foreign, non-self materials and has been used as an important non-specific immunological parameter to evaluate the immune function (Galloway and Depledge, 2001). Phagocytes also kill the microbes by oxygen-independent mechanism, although, not as effectively as oxygen-dependent. The main mediators of these mechanisms are electrically charged proteins in bacterial cell membranes, lactroferrins, lysozymes and proteases. The measurement of these reactive oxygen species (ROS) produced during an inflammatory response is useful in determining the oxygen-dependent immunological activities of phagocytic cells.

In the present study, immunomodulatory activity of different fractions and extracts of stem of *Tinospora cordifolia* were evaluated using the polymorphonuclear neutrophil (PMN) phagocytic function studies. Active fractions were subjected to chromatographic purification that led to the isolation of seven compounds. The immunomodulatory potential of isolated compounds was also estimated using the PMN phagocytic function test. Activation of the ROS *in vitro* by the isolated compounds was estimated using three different assays *viz*. the nitroblue tetrazolium (NBT), nitrous oxide (NO) and chemiluminescence assays.

2. Experimental

2.1. General

Phorbol myristate acetate (PMA) was purchased from Sigma Aldrich and Sabouraud's agar, for maintaining *Candida albicans*, was purchased from HiMedia. Diaion HP-20 was procured from Kitten Enterprises Pvt. Ltd., Mumbai, India. Silica gel (60–120 mesh), TLC silica gel 60 F_{254} plates and all other chemicals used were purchased from Merck India Ltd. Mumbai. Mass spectra were recorded on QTOF-Micro of Waters Micromass. NMR experiments were performed on Bruker Avance-300 spectrometer.

2.2. Plant material

Plant material was collected in June 2007 from Mandi, Himachal Pradesh, India. A voucher specimen (PLP-12994) has been deposited in the herbarium of IHBT, Palampur, India.

2.3. Extraction and isolation of compounds

The air dried and powered stem of *Tinospora cordifolia* (3 kg) was extracted three times with 80% methanol in water at room temperature. The combined percolations were concentrated under reduced pressure to obtain 215 g of the dried brown extract. The residue remained after extraction was extracted with hot water and dried to obtain 109 g of hot water extract. The dried brown extract (215 g) was dissolved in H_2O and fractionated, sequentially, with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. Each fraction was dried to yield *n*-hexane (9.3 g), $CHCl_3$ (7.4 g), EtOAc (11.3 g), *n*-BuOH (22.1 g) and H_2O (135.9 g) fractions.

The EtOAc (11.3 g) fraction was subjected to column chromatography over silica gel (60–120 mesh) using a gradient elution with mixture of *n*-hexane and $CHCl_3$. Fraction No. 97-106 (each 50 ml) eluted with $CHCl_3$:*n*-hexane (2:8) yielded compound **1** (25 mg). Mixture of two compounds (**2** and **3**) was eluted with $CHCl_3$:*n*-hexane (9:1) in fractions No. 177-211.

The hot water extract (109 g) was chromatographed over Diaion HP-20 and eluted with water followed by mixture of ethanol:water in ratio 1:9, 2:8, 3:7, 4:6, 5:5, 7.5:2.5 and 10:0. The ethanol:water (2:8) fraction was dried and chromatographed over silica gel 60–120 mesh with elution in $CHCl_3$:CH_3OH:H_2O and increasing polarity with CH_3OH. Fraction No. 46-53 eluted with $CHCl_3$:CH_3OH:H_2O (6.5:1.5:1) gave compound **4** (51 mg). Fraction No. 62-73 eluted with $CHCl_3$:CH_3OH:H_2O (6.5:2.2:1) gave compound **5** (27 mg). The ethanol:water (3:7) fraction was chromatographed over silica gel 60–120 mesh and eluted with $CHCl_3$:CH_3OH:H_2O by increasing polarity with CH_3OH. Fraction No. 25-31 eluted with $CHCl_3$:CH_3OH:H_2O (6.5:1.2:1) gave compound **6** (29 mg). Fraction No. 44-59 eluted with $CHCl_3$:CH_3OH:H_2O (6.5:1.8:1) gave compound **7** (36 mg). Isolation of compounds from water fraction was also attempted that resulted in the isolation of compound **4**, **6** and **7**.

2.4. Assessment of immunomodulatory activity

Immunomodulatory activity of parent extract, five fractions (*n*-hexane, chloroform, ethyl acetate *n*-butanol and water), hot water extract and isolated compounds were evaluated using the PMN phagocytic function studies. The study was carried out in two parts (a) viability study, (b) PMN phagocytic function assessment.

2.4.1. Sample preparation

The methanol, *n*-butanol and chloroform extracts were dissolved in 0.1% ethanol while all other fractions and isolated compounds were dissolved in 0.1% DMSO. The final concentrations of all the stock solutions were 100 μg/ml.

2.4.2. Viability study

2.4.2.1. Separation of PMN. Venous blood (10 ml) was collected from each volunteer using aseptic precaution in a sterile heparinised tube. The blood was subjected to Ficoll hypaque density gradient centrifugation for the separation of PMN. The RBC-PMN pellet was separated and mixed with 1 ml autologous plasma (obtained earlier) and 1 ml of 5% dextran. The mixture was allowed to stand at 37 °C for 1 h for sedimentation of RBCs. The supernatant

layer, which contains more than 90% of PMN was collected. Cell density (count/ml) of PMN was calculated using Neubaeur's chamber.

2.4.2.2. Viability studies. The viability of these samples was assessed using the Trypan blue dye exclusion test over concentrations ranging from 5 to 100 μg/ml. Appropriate controls were also assessed for effects on cell viability, *viz.*, cell control, ethanol and DMSO. All sets were assessed in duplicates. The concentrations of the study agent showing a viability of 90% and more were selected for the actual assay.

2.4.3. Preparation of Candida albicans

Candida albicans was used as the test organism for the phagocytosis assays. *Candida albicans* was maintained on Sabouraud's agar (HiMedia). For the assay, 18 h old culture was washed using saline and the cells were counted using Neubaur's chamber. The count was adjusted to 1×10^6 cells/ml using minimum essential medium (MEM).

2.4.4. PMN phagocytic function studies

For the bioassay, the count of the isolated PMNs from individual volunteers was adjusted to 1×10^6 cells/ml using MEM. A method, described by Lehrer and Cline (1969) as "specific cell assay" applying *Candida albicans* for assessing phagocytic activity of neutrophils, was used with few modifications. In all, six sets were put for statistical significance.

In brief, 1×10^6 neutrophils (in MEM) were suspended with 1×10^6 cells of *Candida albicans* in absence or presence of varying concentrations of the samples. The concentrations tested were 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml 50 μg/ml 75 μg/ml and 100 μg/ml, of most of the samples. 5 μl of the test suspension prepared was added along with 245 μl of MEM to obtain the desired concentration of the test drug *in vitro*. As control, the PMN were incubated with MEM and the respective vehicle that was used to dissolve the test agents. Phorbol myristate acetate (PMA), a known immunostimulant, at two different concentrations *viz.*, 10^{-4} M and 10^{-6} M was used as standard. The tubes containing the systems were incubated at 37 °C for 1 h in 5% CO_2. Cytosmears were prepared after the incubation. They were fixed with methanol, stained with Giemsa stain and observed under 100× oil immersion objective to determine the phagocytic activity of neutrophils. 100 neutrophils were scanned and the number of cells with ingested organisms was counted and considered as the percentage phagocytois and the number of organisms engulfed by a single phagocytic neutrophil were counted and considered as the phagocytic Index.

2.4.5. Effect on formation of ROS

2.4.5.1. NBT assay. The neutrophils after separation were seeded in 96 well plate with count adjusted to 1×10^6 cells. The plate was then incubated for 30 min in humid conditions for the adherence of the cells to the bottom of the well. The adherence of the cells was observed under an inverted microscope. The supernatant was then discarded and replaced by fresh MEM medium. PMA or test drugs were added to the standard and test control wells, respectively. Wells containing only neutrophils served as cell control wells. 50 μl of the 1:1 diluted NBT solution (stock: 10 mg/ml) was then added to the wells and the plate was incubated for 2 h in the laminar air-flow unit in dark conditions. The supernatant was then removed and the neutrophils were fixed by treatment with absolute methanol. The cells were then washed twice with 70% methanol and air dried. The formazan deposits obtained by the reaction were then solubilized in 2 M potassium hydroxide and DMSO. The contents of all the wells were then mixed and the plate was read at 620 nm in an ELISA reader.

2.4.5.2. NO assay. The neutrophils after separation were seeded in 96 well plate with count adjusted to 1×10^6 cells. The plate was then incubated for 30 min in humid conditions for the adherence of the cells to the bottom of the well. The adherence of the cells was observed under an inverted microscope. The supernatant was then discarded and replaced by fresh MEM. Test drugs were added to the test control wells. Wells containing only neutrophils served as cell control wells. The plate was then incubated for 1 h in laminar air flow in dark conditions. After 1 h, 2 μl of PMA and 100 μl of Griess solution (1 mg/2.5 ml in de-ionized water) was added to the wells. The plate was then immediately read at 550 nm on an ELISA reader.

2.4.5.3. Chemiluminescence assay. The neutrophils after separation were seeded in 96 well plate with count adjusted to 1×10^6 cells. Test drugs were added to the test control wells and volume in the wells was adjusted using MEM. Wells containing only neutrophils served as cell control wells. 10 μl luminol was added to all the wells. PMA was also added to all the wells except the cell control wells. The plate was then loaded in the chemiluminiscence counter and readings were recorded at 0 min (just after addition of luminol) and after 40 min of exposure.

2.4.6. Statistical analysis

The results obtained of percent phagocytosis and phagocytic index for the samples were compared with that of the control and the standard used in each test. In cases, where a vehicle (either ethanol or DMSO) was used, the results were also compared with that of the vehicle control. The results were mentioned as mean ± SD. The results were analyzed using one-way ANOVA followed by Tukey's post hoc test. The level of significance for all analysis was taken as $p < 0.05$.

3. Results

3.1. Assessment of immunomodulatory activity

The viability of all the samples was assessed using the Trypan blue dye exclusion test over a concentration range of 5–100 μg/ml except for compound **1** for which the testing range was 0.5–5 μg/ml (results are not given here). Only those concentrations, that demonstrated a viability of at least 90%, were selected for the actual assay. From the viability results it was observed that the viability was affected at higher concentrations, *i.e.* from 75 μg/ml onwards in case of the methanol extract and ethyl acetate, *n*-hexane and water fractions, while it was affected at 50 μg/ml for the *n*-butanol and chloroform fractions. Hence, a concentration range of 1–50 μg/ml was selected for all these test samples. It was observed that viability was marginally affected at 5 μg/ml for compound **1**. Hence, all the studied concentrations were selected. For hot water extract and compounds (**2**, **3**, **5**, **6**) the viability was affected at all the tested concentrations. Hence, a concentration range lower than that selected for viability, *i.e.* 0.01–2.5 μg/ml was selected for testing the activity.

3.2. PMN phagocytic function assessment

3.2.1. Methanol extract

A dose dependent decrease in the percent phagocytosis was observed with the maximal effect at 0.1 μg/ml which was not significant as compared to its vehicle control (Table 1). With regard to phagocytic index a dose dependent increase was observed with a maximal response observed at 0.5 μg/ml (Table 2).

3.2.2. n-Hexane fraction

An increase in the percent phagocytosis was seen with the *n*-hexane fraction as compared to the vehicle control with maximal effect seen at 0.1 μg/ml which was comparable to PMA (Table 1).

Table 1
In vitro effect of parent extract and different fractions on % phagocytosis ($n = 6$).

Concentration (μg/ml)	% Phagocytosis					
Vehicle used	Ethyl acetate fraction 0.1% DMSO	*n*-Hexane fraction 0.1% DMSO	Water fraction 0.1% DMSO	Chloroform fraction 0.1% ethanol	*n*-Butanol fraction 0.1% ethanol	Methanol extract 0.1% ethanol
Control	27.83 ± 1.46	27.85 ± 1.46	27.85 ± 1.48	27.83 ± 1.60	27.85 ± 1.46	27.83 ± 1.60
Vehicle control	23.14 ± 4.09	23.14 ± 4.09	23.14 ± 4.09	25 ± 3.78	25 ± 3.78	25 ± 3.78
PMA 10^{-6} M	33.42 ± 3.21	33.42 ± 3.21	33.42 ± 3.21	35.28 ± 2.36	35.28 ± 2.36	35.28 ± 2.36
0.1	30.66 ± 1.52	**32.66 ± 7.57**	35.66 ± 2.51	24.66 ± 5.46	**37.33 ± 1.15**	**37.66 ± 3.05**
0.5	38 ± 3.00**	29 ± 2.64	**39.33 ± 5.13****	30.66 ± 5.12**	35 ± 1.73	29.66 ± 0.58
1	36 ± 3.94**	26.16 ± 4.11	37.33 ± 3.01***	**37.16 ± 3.48*****	27.5 ± 3.27	24.66 ± 5.46
5	**39.66 ± 3.14*****	27.83 ± 3.37	36 ± 3.74**	33.33 ± 3.72	31 ± 3.22	30.66 ± 5.12
10	38.16 ± 4.21***	26.66 ± 3.77	38 ± 5.4***	30.66 ± 2.42	26 ± 6.60	30.83 ± 2.23
25	33 ± 4.69	28 ± 0.63	37.5 ± 5.24***	33 ± 2.19	26 ± 2.28	28.5 ± 3.98
50	32.16 ± 2.04	23.16 ± 2.71	32.66 ± 2.06	28 ± 4.33	28.33 ± 1.96	28 ± 5.76

** $p < 0.01$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

In case of phagocytic index, the maximal response was observed at 10 μg/ml (Table 2).

3.2.3. Chloroform fraction

A dose dependent increase in the percent phagocytosis was observed with maximal effect at 1 μg/ml and this was significantly higher than that of its vehicle control ($p < 0.001$) (Table 1). Although a dose dependent increase was also observed with regard to the phagocytic index with a maximal response at 1 μg/ml, the results were not significant as compared to control. The results observed at these concentrations were similar to that noted with the PMA (Table 2).

3.2.4. Ethyl acetate fraction

A dose dependent increase in the percent phagocytosis was observed with maximal effect at 5 μg/ml. This effect was significantly higher than that of its vehicle control ($p < 0.001$) (Table 1). A dose dependent increase was also observed with regard to the phagocytic index with a maximal response observed at 10 μg/ml and this was significantly higher than that of its vehicle control. The results observed at these concentrations were similar to that noted with PMA (Table 2).

3.2.5. n-Butanol fraction

A dose dependent decrease in the percent phagocytosis was observed with the maximal effect at 0.1 μg/ml which was not significant than that of both the control and its vehicle control (Table 1). With regard to phagocytic index, a dose dependent increase was observed with a maximal response observed at 10 μg/ml and the results were significant as compared to vehicle control ($p < 0.05$) (Table 2).

3.2.6. Water fraction

A fluctuating response was observed in the percent phagocytosis with maximal effect at 0.5 μg/ml and this was significantly higher than that of its vehicle control ($p < 0.001$). However, a decrease in response was noted with higher doses (Table 1). Similar results were obtained in the Phagocytic index, with the maximal response observed at 10 μg/ml and this was significant as compared to vehicle control ($p < 0.05$). The results observed at these concentrations were similar to PMA (Table 2).

3.2.7. Hot water extract

The maximum response in case of percent phagocytosis was observed at 0.25 μg/ml which was significantly higher than the control ($p < 0.001$) and its vehicle control ($p < 0.001$). With regard to phagocytic index, the effect was maximal at 0.5 μg/ml and was significantly higher as compared to control ($p < 0.001$) and its vehicle control ($p < 0.01$). The results observed at these concentrations were higher than observed with the PMA (Table 3).

3.2.8. N-formylannonain (**1**)

In case of percent phagocytosis, maximal effect was observed at 1 μg/ml which was significantly higher than the control ($p < 0.001$) as well as vehicle control ($p < 0.01$). For phagocytic index, an increase in response was observed at the same concentration but it was not statistically significant (Table 4).

3.2.9. Mixture of N-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**)

The maximal effect on percent phagocytosis was observed at 1 μg/ml and was significantly higher than the control ($p < 0.01$) and vehicle control ($p < 0.05$). The phagocytic index was same at

Table 2
In vitro effect of parent extract and different fractions on phagocytic index ($n = 6$).

Concentration (μg/ml)	Phagocytic index					
Vehicle used	Ethyl acetate fraction 0.1% DMSO	*n*-Hexane fraction 0.1% DMSO	Water fraction 0.1% DMSO	Chloroform fraction 0.1% ethanol	*n*-Butanol fraction 0.1% ethanol	Methanol extract 0.1% ethanol
Control	1.59 ± 0.13	1.59 ± 0.14	1.61 ± 0.15	1.59 ± 0.13	1.656 ± 0.06	1.65 ± 0.06
Vehicle control	1.61 ± 0.12	1.61 ± 0.12	1.61 ± 0.12	1.57 ± 0.07	1.57 ± 0.07	1.57 ± 0.07
PMA 10^{-6} M	1.77 ± 0.12	1.77 ± 0.12	1.77 ± 0.11	1.77 ± 0.11	1.77 ± 0.11	1.77 ± 0.11
0.1	1.67 ± 0.15*	1.61 ± 0.12	1.74 ± 0.08	1.57 ± 0.16	1.56 ± 0.21	1.69 ± 0.11
0.5	1.65 ± 0.05	1.7 ± 0.06	1.64 ± 0.08	1.73 ± 0.11	1.60 ± 0.14	**1.77 ± 0.09**
1	1.88 ± 0.17*	1.66 ± 0.11	1.91 ± 0.15*	**1.83 ± 0.17**	1.60 ± 0.18	1.46 ± 0.11
5	1.82 ± 0.07	1.84 ± 0.12	1.82 ± 0.11	1.75 ± 0.08	1.75 ± 0.17	1.55 ± 0.26
10	**2.04 ± 0.28*****	**1.88 ± 0.18**	**1.92 ± 0.08***	1.75 ± 0.21	**1.76 ± 0.18***	1.63 ± 0.24
25	1.66 ± 0.17	1.54 ± 0.29	1.82 ± 0.33	1.65 ± 0.13	1.63 ± 0.18	1.52 ± 0.11
50	1.87 ± 0.15	1.57 ± 0.31	1.92 ± 0.13*	1.67 ± 0.08	1.67 ± 0.14	1.72 ± 0.35

* $p < 0.05$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

Table 3
In vitro effect of hot water extract on phagocytic function of neutrophils ($n = 6$).

Concentration (μg/ml)	% Phagocytosis	Phagocytic index
Control	29.5 ± 2.66	1.65 ± 0.06
Vehicle control	27 ± 2.58	1.65 ± 0.08
PMA 10^{-6} M	34.16 ± 2.40$^{@@}$	1.74 ± 0.21
0.1	37.5 ± 2.58$^{***,@@@}$	1.74 ± 0.13
0.25	41.16 ± 3.92$^{***,@@@}$	1.80 ± 0.11*
0.5	35.66 ± 3.66$^{**,@@}$	**1.95 ± 0.12**$^{***,@@}$
1	35.5 ± 3.14$^{**,@@}$	1.71 ± 0.13
2.5	33.5 ± 3.78$^{@}$	1.65 ± 0.06

* $p < 0.05$ as compared to control.
** $p < 0.01$ as compared to control.
*** $p < 0.001$ as compared to control.
$^{@}$ $p < 0.05$ as compared to vehicle control.
$^{@@}$ $p < 0.01$ as compared to vehicle control.
$^{@@@}$ $p < 0.001$ as compared to vehicle control.

Table 4
In vitro effect of *N*-formylannonain (**1**) on phagocytic function of neutrophils ($n = 6$).

Concentration (μg/ml)	% Phagocytosis	Phagocytic index
Control	28.67 ± 2.50	1.47 ± 0.22
Vehicle control	29.83 ± 1.33	1.36 ± 0.21
PMA 10^{-6} M	36.5 ± 3.21$^{**,@}$	1.62 ± 0.18
0.5	34.66 ± 5.89	1.57 ± 0.35
1	**38.5 ± 4.56**$^{***,@@}$	**1.59 ± 0.29**
2	34.67 ± 3.01	1.53 ± 0.32
5	33.5 ± 2.35	1.51 ± 0.20

** $p < 0.01$ as compared to control.
*** $p < 0.001$ as compared to control.
$^{@}$ $p < 0.05$ as compared to vehicle control.
$^{@@}$ $p < 0.01$ as compared to vehicle control.

Table 5
In vitro effect of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**) on phagocytic function of neutrophils ($n = 6$).

Concentration (μg/ml)	% Phagocytosis	Phagocytic index
Control	30.16 ± 1.47	1.56 ± 0.18
Vehicle control	31 ± 1.26	1.74 ± 0.24
PMA 10^{-6} M	37.33 ± 1.96$^{**,@}$	1.80 ± 0.18
0.01	35.67 ± 3.26	1.76 ± 0.29
0.05	34.5 ± 4.04	1.78 ± 0.18
0.1	36.67 ± 2.94*	1.88 ± 0.29
0.5	33.33 ± 3.20	1.83 ± 0.14
1.0	**37.17 ± 4.57**$^{**,@}$	**1.87 ± 0.15**
2.5	33.4 ± 3.28	1.77 ± 0.25

* $p < 0.05$ as compared to control.
** $p < 0.01$ as compared to control.
$^{@}$ $p < 0.05$ as compared to vehicle control.

all concentrations of the drug. The results observed at these concentrations were comparable to that noted with the standard PMA (Table 5).

3.2.10. Magnoflorine (**5**)

A dose dependant increase in the percent phagocytosis and phagocytic index was observed at 0.5 μg/ml. However, statistical significance was seen only for percent phagocytosis as compared to cell control and vehicle control ($p < 0.001$). The results observed at these concentrations were similar to that noted with the standard PMA (Table 6).

3.2.11. Tinocordiside(**6**)

The maximal effect was observed at 0.5 μg/ml in case of percent phagocytosis as well as phagocytic index and the results were significantly higher as compared to the control and vehicle control (% phagocytosis: $p < 0.001$, phagocytic index: $p < 0.05$). Although the results noted at this concentration was higher than of the standard PMA; the difference was not statistically significant (Table 7).

Table 6
In vitro effect of magnoflorine on phagocytic function of neutrophils ($n = 6$).

Concentration (μg/ml)	% Phagocytosis	Phagocytic index
Control	30.44 ± 1.42	1.60 ± 0.09
Vehicle control	30.00 ± 2.50	1.60 ± 0.10
PMA 10^{-6} M	37.89 ± 1.83$^{**,@}$	1.78 ± 0.16
0.01	35.33 ± 2.66	1.68 ± 0.21
0.05	34.83 ± 1.83	1.67 ± 0.19
0.1	38.33 ± 4.23$^{***,@}$	1.73 ± 0.26
0.5	39.00 ± 3.74$^{***,@}$	1.8 ± 0.10
1.0	33.67 ± 2.73	1.81 ± 0.13
2.5	34.00 ± 3.52	1.71 ± 0.11

** $p < 0.01$ as compared to control.
*** $p < 0.001$ as compared to control.
$^{@}$ $p < 0.001$ as compared to vehicle control.

Table 7
In vitro effect of tinocordiside on phagocytic function of neutrophils ($n = 6$).

Concentration (μg/ml)	% Phagocytosis	Phagocytic index
Control	30.83 ± 3.06	1.64 ± 0.11
Vehicle control	32.16 ± 3.37	1.75 ± 0.26
PMA 10^{-6} M	36.00 ± 3.03*	1.79 ± 0.11
0.01	29.83 ± 1.16	1.795 ± 0.10
0.05	36.00 ± 1.41*	1.75 ± 0.16
0.1	36.00 ± 2.00*	1.58 ± 0.10
0.5	**42.17 ± 2.86**$^{***,@@}$	**1.95 ± 0.19**$^{*,@}$
1.0	32.17 ± 1.94	1.69 ± 0.14
2.5	37.75 ± 1.71$^{***,@}$	1.76 ± 0.21

* $p < 0.05$ as compared to control.
*** $p < 0.001$ as compared to control.
$^{@}$ $p < 0.05$ as compared to vehicle control.
$^{@@}$ $p < 0.001$ as compared to vehicle control.

Table 8
Effect of ethyl acetate fraction on release of ROS (using NBT assay) ($n = 6$).

Concentration (μg/ml)	Absorbance
Cell control	0.277 ± 0.034
Vehicle control	0.2358 ± 0.068
PMA 10^{-6} M	0.364 ± 0.098**
0.1	0.301 ± 0.046
0.5	0.396 ± 0.071$^{@,***}$
1	0.356 ± 0.051*
5	0.308 ± 0.050*
10	0.281 ± 0.033
25	0.257 ± 0.026
50	0.220 ± 0.026

$^{@}$ $p < 0.05$ as compared to cell control.
* $p < 0.05$ as compared to vehicle control.
** $p < 0.01$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

3.3. Effect on formation of ROS

PMN phagocytic function study showed that ethyl acetate fraction is most active and the activity is comparable to the standard immunomodulator used. All the tested molecules showed significant activity. Hence to further confirm the activity of ethyl acetate fraction and isolated molecules, more sensitive assay such as the NBT, NO and chemiluminescence assays were applied.

3.3.1. NBT assay

Ethyl acetate fraction was evaluated over a concentration range of 0.1–50 μg/ml. All the concentrations demonstrated increase in the formation of formazan crystals indicating superoxide generation as compared to the vehicle control. However, the maximal effect was seen at 0.5 and 1 μg/ml, with a dose dependent decrease in activity observed at higher concentrations (Table 8).

Mixture of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**), magnoflorine (**5**) and tinocordiside

Table 9
Effect of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**), magnoflorine (**5**) and tinocordiside (**6**) on release of ROS (NBT assay) ($n = 6$).

Concentration (μg/ml)	Absorbance		
	N-Methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**)	Magnoflorine (**5**)	Tinocordiside (**6**)
Cell control	0.277 ± 0.034	0.277 ± 0.034	0.277 ± 0.034
Vehicle control	0.236 ± 0.068	0.236 ± 0.068	0.236 ± 0.068
PMA 10^{-6} M	0.364 ± 0.098**	0.364 ± 0.098**	0.364 ± 0.098**
0.01	0.238 ± 0.031	0.280 ± 0.028	0.257 ± 0.018
0.05	0.293 ± 0.021###,***	0.324 ± 0.070	0.281 ± 0.030
0.1	0.328 ± 0.018***	**0.410 ± 0.066**##,***	0.319 ± 0.047
0.5	0.353 ± 0.048**	0.3375 ± 0.035	0.358 ± 0.057*
1	**0.443 ± 0.055**###,***	0.278 ± 0.020	**0.396 ± 0.066**#,***
2.5	0.339 ± 0.032*	0.245 ± 0.009	0.302 ± 0.050

# $p < 0.05$ as compare to cell control.
$p < 0.01$ as compared to cell control.
$p < 0.001$ as compared to cell control.
* $p < 0.05$ as compared to vehicle control.
** $p < 0.01$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

Table 10
Effect of ethyl acetate fraction on NO release ($n = 6$).

Concentration (μg/ml)	Absorbance
Cell control	0.238 ± 0.008
Vehicle control	0.218 ± 0.008
PMA 10^{-6} M	0.355 ± 0.021###,***
0.1	0.242 ± 0.027
0.5	0.281 ± 0.048***
1	**0.385 ± 0.016**###,***
5	0.329 ± 0.028###,***
10	0.296 ± 0.013##,***
25	0.264 ± 0.013*
50	0.231 ± 0.009

$p < 0.01$ as compare to cell control.
$p < 0.001$ as compared to cell control.
* $p < 0.05$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

(**6**) were evaluated over a concentration range of 0.01–2.5 μg/ml. All the concentrations of tested molecules demonstrated an increase in the formation of formazan crystals indicating superoxide generation as compared to the vehicle control. Although the increase in superoxide generation was concentration dependent at lower concentrations with maximum activity observed at 1 μg/ml for mixture of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**), tinocordiside (**6**) and at 0.1 μg/ml for magnoflorine (**5**), a decrease in activity was observed at the higher concentration tested, *i.e.* 2.5 μg/ml for all compounds. In case of *N*-formylannonain (**1**) an increase in the formation of formazan crystals was observed as compared to control but not statically significant (results are not given here). The results are represented in Table 9.

3.3.2. NO assay

Ethyl acetate fraction was evaluated over a concentration range of 0.1–50 μg/ml. All the concentrations demonstrated increase in the formation of superoxide generation as compared to the vehicle control with maximum activity observed at 1 μg/ml. Although the increase in superoxide generation was concentration dependent at lower concentrations, a decrease in activity was observed at higher concentrations. The results are represented in Table 10.

Mixture of *N*-methyl-2-pyrrolidone (**3**) and 11-hydroxymustakone (**2**), magnoflorine (**5**) and tinocordiside (**6**) were evaluated over a concentration range of 0.01–2.5 μg/ml. All the concentrations of tested molecules demonstrated an increase in the superoxide generation compared to the vehicle control. The increase in superoxide generation was concentration dependent at lower concentrations with maximum activity observed at 0.5 μg/ml for *N*-methyl-2-pyrrolidone (**3**) and 11-hydroxymustakone (**2**), magnoflorine (**5**) and at 0.1 μg/ml for tinocordiside (**6**). Although the increase in superoxide generation was concentration dependent at lower concentrations, a decrease in activity was observed at higher concentrations, *i.e.* 1 and 2.5 μg/ml for *N*-methyl-2-pyrrolidone (**3**) and 11-hydroxymustakone (**2**), tinocordiside (**6**) and with no effect seen at higher concentration, *i.e.* 2.5 μg/ml for magnoflorine. The results are represented in Table 11.

3.3.3. Chemiluminescence assay

All the concentrations of ethyl acetate fraction showed a time dependent increase in luminescence indicating increased ROS generation when compared with vehicle as well as PMA treated cells. Maximum effect was observed with the intermediate concentration 5 μg/ml. The results are represented in Table 12.

Cells treated with *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**) showed a dose dependent increase in luminescence indicating increased ROS generation, which also increased over the time when compared with vehicle treated, and PMA treated cells. Maximum effect was obtained after 40 min exposure with 2.5 μg/ml concentration. The results are represented in Table 13. Cells treated with magnoflorine (**5**) at concentration 0.5 and 1 μg/ml initially showed an increase in luminescence at 0 min indicating increased ROS generation after which a decrease in activity was observed over time. At the 2.5 μg/ml concentration however, the effect of PMA which was augmented by the test drug at 0 min was further increased after 40 min of exposure. The results are represented in Table 14. All the concentrations of tinocordiside (**6**) showed a time dependent increase in luminescence indicating increased ROS generation when compared with vehicle treated cells as well as PMA treated cells. Maximum effect was observed at 0.1 μg/ml with a decrease observed with higher concentrations. The results are represented in Table 15.

3.4. Characterisation of isolated compounds

Compound **1**, **4**, **5**, **6** and **7** were identified as *N-formylannonain* (Pachaly et al., 1992), *cordifolioside A* (Maurya et al., 1996), *magnoflorine* (Barbosa-Filho et al., 1997), *tinocordiside* (Ghosal and Vishwakarma, 1997) and *syringin* (Sipahimalani et al., 1994), respectively by comparing the spectral data with reported values (Fig. 1).

Compound **2** and **3** were obtained as amorphous powder. The mixture gave positive test with Dragendorff's reagent for alkaloids. Its electrospray ionization-quadrupole time of flight-mass spectrum (ESI-QTOF-MS) in positive ion mode revealed two $[M+H]^+$

Table 11
Effect of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**), magnoflorine (**5**) and tinocordiside (**6**) on NO release ($n = 6$).

Concentration (μg/ml)	Absorbance		
	N-Methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**)	Magnoflorine (**5**)	Tinocordiside (**6**)
Cell control	0.238 ± 0.008	0.238 ± 0.008	0.238 ± 0.008
Vehicle control	0.218 ± 0.008	0.218 ± 0.008	0.218 ± 0.008
PMA 10^{-6} M	0.355 ± 0.021$^{###,***}$	0.355 ± 0.021$^{###,***}$	0.355 ± 0.021$^{###,***}$
0.01	0.195 ± 0.017	0.211 ± 0.009	0.236 ± 0.025
0.05	0.232 ± 0.009	0.238 ± 0.012	0.282 ± 0.009$^{##,***}$
0.1	0.295 ± 0.024**	0.277 ± 0.029$^{#,***}$	**0.371 ± 0.023$^{###,***}$**
0.5	**0.374 ± 0.019$^{###,***}$**	**0.373 ± 0.014$^{###,***}$**	0.325 ± 0.017$^{###,***}$
1	0.276 ± 0.014	0.252 ± 0.024	0.277 ± 0.012$^{##,***}$
2.5	0.242 ± 0.016	0.164 ± 0.037$^{###,**}$	0.232 ± 0.017

$^{#}$ $p < 0.05$ as compared to cell control.
$^{##}$ $p < 0.01$ as compared to cell control.
$^{###}$ $p < 0.001$ as compared to cell control.
** $p < 0.01$ as compared to vehicle control.
*** $p < 0.001$ as compared to vehicle control.

Table 12
Effect of ethyl acetate fraction on formation of ROS in presence of PMA ($n = 3$).

Time	Cell control	Vehicle control	PMA 10^{-4} M	Ethyl acetate fraction concentration (μg/ml)		
				1	5	10
0 min	1085.37 ± 822.39	1081.29 ± 983.23	2247.07 ± 650.16	9874.67 ± 10048.95	17598 ± 19992.08	13392.33 ± 1345067.24
40 min	1545.37 ± 581.09	1502.75 ± 633.39	4597.46 ± 2570.23	22953.67 ± 25677.64	36873.67 ± 42879.58	30538.33 ± 24066.90

Table 13
Effect of *N*-methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**) on formation of ROS in presence of PMA ($n = 3$).

Time	Cell control	Vehicle control	PMA 10^{-4} M	*N*-Methyl-2-pyrrolidone (**2**) and 11-hydroxymustakone (**3**) concentration (μg/ml)		
				0.5	1	2.5
0 min	1085.37 ± 822.39	1081.29 ± 983.23	2247.07 ± 650.16	4041.33 ± 2447.62	5518.33 ± 4483.62	6379.67 ± 3086.64
40 min	1545.37 ± 581.09	1502.75 ± 633.39	4597.46 ± 2570.23	25797.67 ± 17433.88	21895.00 ± 14361.17	31292.00 ± 37909.63

Table 14
Effect of magnoflorine (**5**) on formation of ROS in presence of PMA ($n = 3$).

Time	Cell control	Vehicle control	PMA 10^{-4} M	Magnoflorine (**5**) concentration (μg/ml)		
				0.5	1	2.5
0 min	1085.37 ± 822.39	1081.29 ± 983.23	2247.07 ± 650.16	28326.33 ± 47366.26	44359.00 ± 74167.49	2897.00 ± 2745.04
40 min	1545.37 ± 581.09	1502.75 ± 633.39	4597.46 ± 2570.23	19997.33 ± 27830.53	16377.00 ± 24493.28	20690.00 ± 33332.72

Table 15
Effect of tinocordiside (**6**) on formation of ROS in presence of PMA ($n = 3$).

Time	Cell control	Vehicle control	PMA 10^{-4} M	Tinocordiside (**6**) concentration (μg/ml)		
				0.1	0.5	1
0 min	1085.37 ± 822.39	1081.29 ± 983.23	2247.07 ± 650.16	10152.33 ± 8123.61	4849.67 ± 2936.03	3629.33 ± 3799.31
40 min	1545.37 ± 581.09	1502.75 ± 633.39	4597.46 ± 2570.23	17630.27 ± 21084.16	10883.67 ± 11372.93	5574.33 ± 2758.94

signals at m/z 100 and m/z 235.1679 corresponding to the molecular formula $C_5H_{10}NO$ and $C_{15}H_{23}O_2$. The presence of two sets of different intensities peaks in ^1H and ^{13}C NMR spectra of compounds **2** and **3** along with no correlation between these two sets indicated the possibility of mixture of two compounds. The high intensity set consists of five carbon signals (δ_C 17.6, 29.5, 30.6, 49.4 and 175.0) and four proton signals (δ_H 1.98–2.05, 2.85, 2.35–2.40, and 3.37–3.42) (Table 16). From distortionless enhancement by polarization transfer (DEPT) spectrum, it was observed that out of five signals three were methylene (δ_C 17.6, 30.6, 49.6), one was methyl (δ_C 29.5), and one was quaternary carbon. The presence of quaternary carbon signal at δ_C 175.0 indicated the presence of carbonyl group. From two-dimensional heteronuclear multiple quantum correlation (HMQC) experiment, it was observed that the methyl carbon signal at δ_C 29.5 correlated to proton signal at δ_H 2.85, indicating its linkage to nitrogen atom. Other carbon signals at δ_C 17.6, 30.6, 49.4 correlated to δ_H 1.98–2.05, 2.35–2.40, 3.37–3.41, respectively. In long range heteronuclear multiple bond correlation (HMBC) experiment it was observed that these five carbon atoms correlate to each other, demonstrating that these are in a ring system. On the basis of these observations the structure of the major compound in the mixture was elucidated as *N-methy-2-pyrrolidone* (**2**).

The ^1H and ^{13}C spectral data of other compound in the mixture showed the presence of one carbonyl group (δ_C 203.8), one β-trisubstituted olefinic double bond (δ_H 5.72, δ_C 121.2 and 171.0)

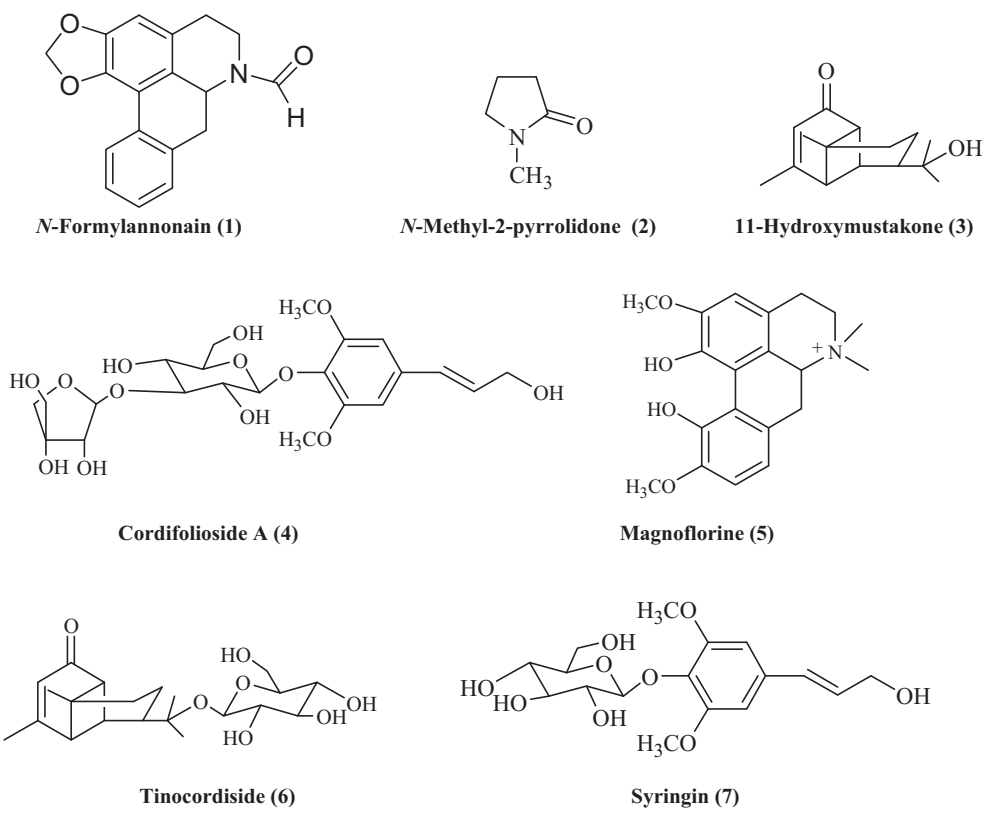

Fig. 1. Structure of isolated compounds.

attached to a carbonyl group that shifted the β-carbon atom downfield to δ_C 171.0 due to extended conjugation (Table 16). The ^{1}H and ^{13}C spectral data showed the presence of four methyl groups (δ_H 0.99, 1.13, 1.18, 2.01 and δ_C 20.1, 20.9, 23.6, 24.8). The connectivity of protons with carbons were determined by 2D HMQC and HMBC. The DEPT experiment showed five methines, two methylenes, four methyls and three quaternary carbons. When the spectral data was compared with reported NMR and other spectral data for the sesquiterpene mustakone (Nyasse et al., 1988), significant similarity was observed except that instead of the isopropyl at C-7 of mustakone, present compound contain isopropyl alcohol moiety at the same position which was further confirmed by quaternary carbon signal at δ_C 72.8. Hence, the structure of compound was deduced as *11-hydroxymustakone* (**3**).

4. Discussion and conclusion

The immune cells and mediators are directly involved in the processing of antigens, removal of microorganisms by phagocytosis, lysis of bacteria, viruses or tumour cells. Many malignant diseases are caused by a decreased number or function of immune competent cells (Wagner and Jurcic, 1991). Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating immune competent cells. However, there is a need to systematic studies on medicinal plants to substantiate the therapeutic claims made regarding their clinical utility. In the present study, the ethylacetate, water fraction and hot water extract of stem of *Tinospora cordifolia* significantly increased the phagocytic function of human neutrophils, indicating

Table 16
^{1}H and ^{13}C NMR data for compounds **2** and **3** (300.13 and 75.46 MHz, $CDCl_3$, *J* in Hz).

11-Hydroxymustakone (**3**)				*N*-Methyl-2-pyrrolidone (**2**)			
Position	Carbon type	δ_C	δ_H	Position	Carbon type	δ_C	δ_C
1	CH	54.6	3.87 (s)	1			
2	C	203.8		2	C	175.0	
3	CH	121.2	5.72 (s)	3	CH_2	30.6	2.35–2.40 (m)
4	C	171.0		4	CH_2	17.6	1.98–2.05 (m)
5	CH	57.0	1.98[a]	5	CH_2	49.4	3.37–3.42 (m)
6	CH	53.6	2.92 (s)		*N*-CH_3	29.5	2.85 (s)
7	CH	50.1	1.96[a]				
8	CH_2	20.5	1.76[a]				
9	CH_2	36.6	1.98[a]				
10	C	56.9					
11	CH	72.8					
12	CH_3	24.8	1.18 (s)				
13	CH_3	23.6	1.13 (s)				
14	CH_3	20.9	2.01[a]				
15	CH_3	20.1	0.99 (s)				

[a] Overlapped signal.

possible immunostimulating effect. Seven compounds of different classes such as alkaloids, phenylpropanoids and sesquiterpenes were isolated from the active fractions out of which two cordifolioside A and syringin were reported to have immunomodulatory activity (Maurya et al., 1996; Kapil and Sharma, 1997; Cho et al., 2001).

Two compounds isolated for the first time from natural sources were characterised in a mixture and were found active at concentration 1 μg/ml in the PMN phagocytic function tests and the activity was comparable to the standard PMA. This mixture also enhances ROS generation at concentration 0.5–1 μg/ml when evaluated using the NBT, NO and chemilunescence assays and was comparable to standard immunomodulator PMA. Three other compounds were also found active in PMN function study and enhance ROS generation at concentration 0.1–0.5 μg/ml.

Our study indicated that the immunomodulatory activity of *Tinospora cordifolia* may be attributed to the synergistic effect of groups of compounds. In Ayurvedic texts too, same indications have been described for polar, hydroalcohol and lipid dosage formulations of *Tinospora cordifolia* (Panchabhai et al., 2008). Since *Tinospora cordifolia* is already well explored for immunomodulatory effect involving stimulation of phagocytosis in *in vivo* models (Dahanukar et al., 1999; Rege et al., 1999; Panchabhai et al., 2008), the active compounds of the present study may show the same potential in *in vivo* models.

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