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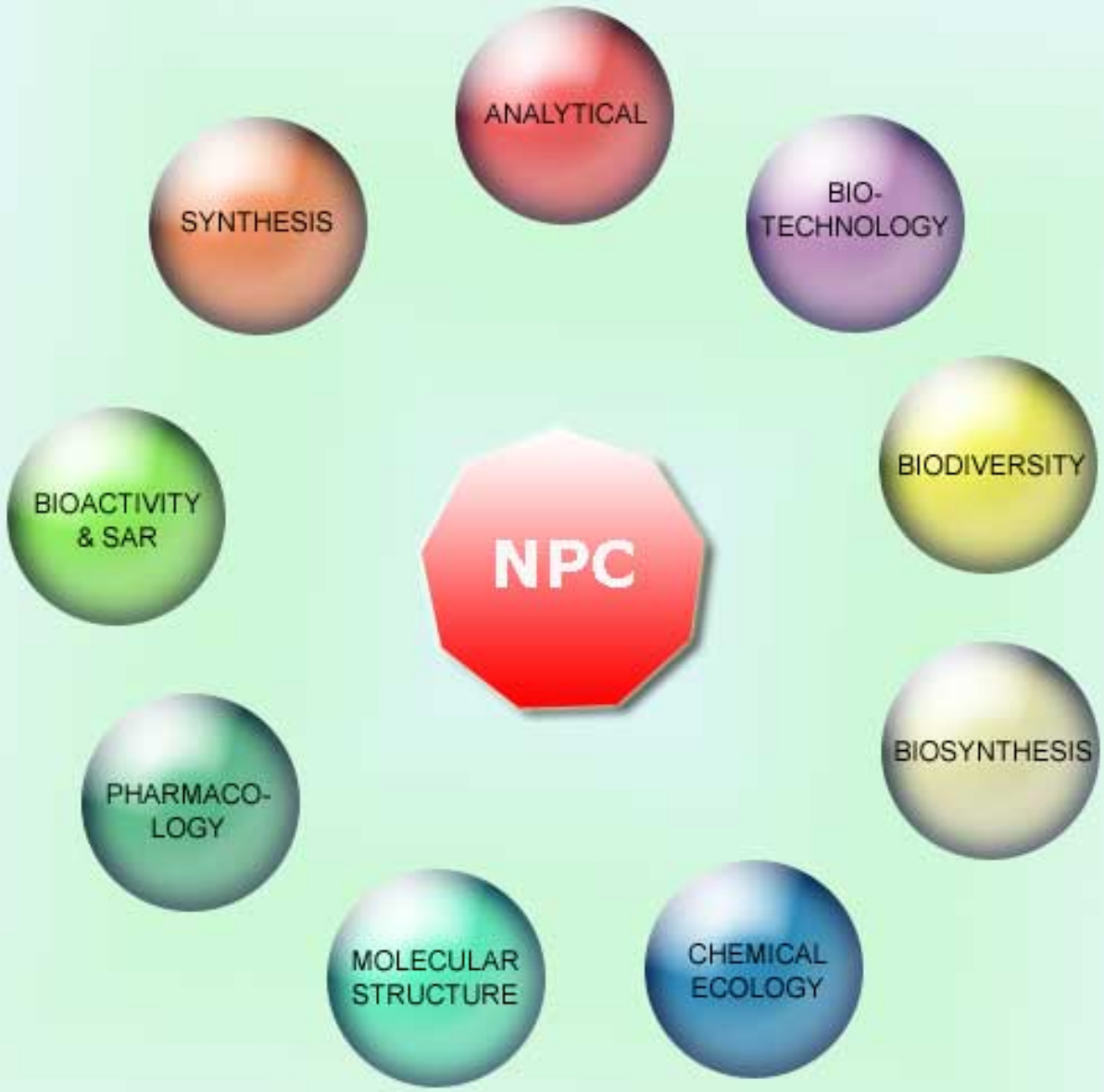

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An Immunomodulator from *Terminalia arjuna* and Biological Evaluation of its Derivatives

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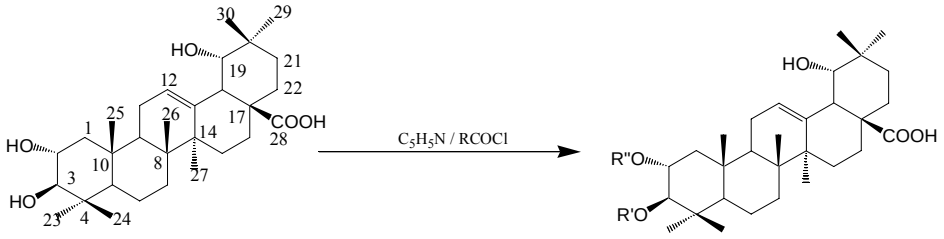
Detailed chemical investigation of *Terminalia arjuna* bark resulted in the isolation and identification of an immunomodulatory constituent, arjunic acid (**1**), which was converted into seven semi-synthetic derivatives (**2-8**). All the test compounds in 0.1, 1 and 10 µg/mL concentrations exhibited immunomodulatory effects in a dose dependant manner. 2-*O*-Lauroyl arjunic acid (**3**) and 2-*O*-palmitoyl arjunic acid (**4**) exhibited immunostimulatory response at all the tested concentrations, while arjunic acid (**1**), 2,3-di-*O*-acetyl arjunic acid (**2**), 2,3-di-*O*-benzoyl arjunic acid (**6**), and 2-*O*-*p*-anisoyl arjunic acid (**7**) exhibited immunostimulatory responses at lower doses, whereas at higher concentrations they exhibited immunosuppressive activity. Only 2,3-di-*O*-palmitoyl arjunic acid (**5**) showed immunosuppressive activity at all the concentrations; this is being reported for the first time.

Keywords: *Terminalia arjuna*, arjunic acid derivatives, immunomodulatory, immunostimulatory, immunosuppressive.

Use of plant products as immunomodulators is becoming more and more important. Some of the species with known immunomodulatory activities are *Tinospora cordifolia* [1], *Withania somnifera* [2] and *Piper longum* [3]. *Terminalia arjuna* (Roxb.) Weigh & Arn. is a well known medicinal plant whose bark is extensively used in Ayurvedic medicine, particularly as a cardiac tonic [4]. Considerable work has been carried out on the chemical constituents of different parts of *T. arjuna*, which has revealed the presence of a number of tannins, sugars, flavanoids, triterpenoid acids and their glycosides [5]. In an activity guided separation [6], gallic acid, ethyl gallate flavone, and luteolin were isolated as cytotoxic compounds from *T. arjuna*. Later on, a new ellagitannin named arjunin was isolated, which showed moderate cytotoxic activity against BT-20 human breast carcinoma cells [7]. As a part of our drug discovery program for cytotoxic agents from Indian medicinal plants [8], we isolated from the bark of *T. arjuna* and identified four novel cytotoxic agents, arjunic acid (**1**), arjungenin, arjunetin and arjunoglucoside I. Out of the four compounds, arjunic acid (**1**) was significantly active against the human oral (KB), ovarian (PA 1) and liver (HepG-2 & WRL-68) cancer cell lines [9]. In this current study, the immunomodulatory activity of the cytotoxic agent arjunic acid and its derivatives have been demonstrated for the first time.

From the bark of *T. arjuna*, arjunic acid was isolated by the process earlier developed by us [9] and characterized by its spectral data [10]. Arjunic acid (**1**) was quantitatively converted into four aliphatic (**2**-**5**) and three aryl (**6**-**8**) ester derivatives, as shown in scheme 1. Structures of these derivatives were deduced on the basis of their spectroscopic data (supplementary data). Immunomodulatory activity of arjunic acid (**1**) and its ester derivatives (**2-8**) was studied in murine spleenocytes using the MTT assay [11] and the results are presented in Table 1. The test compounds **1**-**8** in 0.1, 1 and 10 µg/mL concentrations exhibited an immunomodulatory effect, specifically; compounds **2**,**3**,**5**,**6** and **7** exhibited a dose dependant immunomodulation. To



Arjunic acid (**1**)

Acyl / aryl derivatives of arjunic acid

R" & R' = CH_3CO- [2,3-di-*O*-acetyl arjunic acid (**2**)], R" = $CH_3(CH_2)_{10}CO$- & R' = H [2-*O*-lauroyl arjunic acid (**3**)], R" = $CH_3(CH_2)_{14}CO$- & R' = H [2-*O*-palmitoyl arjunic acid (**4**)], R" & R' = $CH_3(CH_2)_{14}CO$- [2,3-di-*O*-palmitoyl arjunic acid **(5)**], R" & R' = C_6H_5CO-[2,3-di-*O*-benzoyl arjunic acid **(6)**], R" = OMe-*p*-C_6H_4CO-, R' =H[2-*O*-*p*-anisoyl arjunic acid **(7)**], R" & R' = NO_2-*p*-C_6H_4CO- [2,3-di-*O*-*p*-nitrobenzoyl arjunic acid **(8)**].

Scheme 1: Chemical derivatization of arjunic acid (**1**).

Table 1: Immunomodulatory activity of arjunic acid (**1**) its derivatives (**2-8**).

Treatment	% Stimulation			% Modulation		
	0.1(µg/mL)	1(µg/mL)	10(µg/mL)	0.1(µg/mL)	1(µg/mL)	**10(µg/mL)**
Vehicle Control		41.9			0.0	
Compound **1**	42.4	34.3	37.6	1.3	-18.1	-10.3
Compound **2**	45.7	39.5	38.9	9.0	-5.8	-7.1
Compound **3**	51.3	46.2	45.4	22.6	10.3	8.4
Compound **4**	46.2	46.2	44.0	10.3	10.3	5.2
Compound **5**	37.8	40.8	41.1	-9.7	-2.6	-1.9
Compound **6**	48.1	44.3	32.7	14.8	5.8	-21.9
Compound **7**	42.4	42.2	38.4	1.3	0.6	-8.4
Compound **8**	42.7	39.2	48.1	1.9	-6.4	14.8
Levamisole HCl		53.0			26.4	

arrive at the percentage modulation, we considered the percentage stimulation of vehicle control cells, along with mitogen as the base value for modulation (zero %). The concentrations exhibiting percentage values above this were considered to be immunostimulators and those with lower values were considered as immunosuppressants. From Table 1 it is evident that at lower concentration (0.1 µg/mL), arjunic acid (AA, **1**) acts as immunostimulator, but at higher concentrations (1 and 10 µg/mL), it behaves like an immunosuppressant. Further, converting arjunic acid into its 2,3-di-*O*-acetyl derivative (**2**), increases its immunostimulatory activity seven times at a lower concentration (0.1 µg/mL), but at higher concentrations (1 and 10 µg/mL) compound **2** also behaved like an immunosuppressant. However, careful observation of the data revealed that when the aliphatic chain length in arjunic acid derivatives was increased from a two carbon derivative (2,3-di-*O*-acetyl AA **2**) to a twelve carbon derivative ((2-*O*-lauroyl AA **3**), the immunostimulant activity dramatically increased 17.5 fold with respect to the starting material (AA **1**) and 2.5 fold with respect to compound **2**. Furthermore, it was also interesting to note that unlike compounds **1** and **2**, compound **3** showed immunostimulatory activity at all the tested concentrations, but was most active at lower concentration (0.1 µg/mL). Further increase in the aliphatic chain length from a twelve carbon AA derivative (2-*O*-lauroyl AA, **3**) to a sixteen carbon derivative (2-*O*-palmitoyl AA, **4**) resulted in a two fold reduction in its immunostimulatory activity at the lower concentration, but at higher concentrations, the activity was almost unchanged. It was interesting to note that when the monopalmitoyl derivative **4** was converted into dipalmitoyl AA **5**, the activity took a "U" turn from immunostimulatory to immunosuppressant. Compound **5** showed immunosuppressive activity at all the three tested concentrations, but was most active at the lowest concentration (0.1 µg/mL). Arjunic acid (**1**) was also converted into three aryl ester derivatives (**6**–**8**). It was interesting to note that 2,3-di-*O*-benzoyl AA (**6**) showed 11.5 times enhancement in its immunostimulatory activity at the lowest concentration used (0.1 µg/mL), while at moderate concentration (1 µg/mL), the immunostimulatory activity decreased 2.5 fold, and at the highest concentration (10 µg/mL), it showed potential immunosuppressant activity. On the other hand, 2-*O*-*p*-anisoyl AA (**7**) and 2,3-di-*O*-*p*-nitrobenzoyl AA (**8**) did not show any marked enhancement of their immunostimulatory activity with respect to AA (**1**).

From the above discussion it is evident that compounds **3** and **4** exhibited immunostimulatory response at all the concentrations tested. On the other hand, compounds **1**, **2**, **6** and **7** exhibited

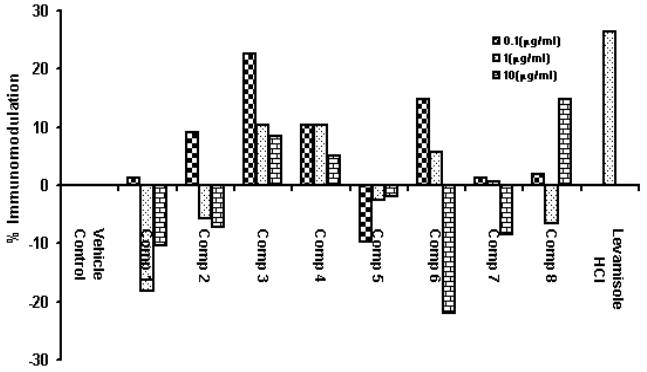


Figure 1: % modulation of spleenic lymphocytes by test compounds.

immunostimulatory response at lower doses, but at higher concentrations, they exhibited immunosuppressive activity. Only compound **5** showed immunosuppressive activity at all the concentrations. However, it may be observed that all the values fall within the range of ConA and Levamisole, the concentrations of which have been standardized previously in our laboratory. Although it is desirable that a compound exhibits either a stimulatory or suppressant activity at all concentrations, our experience of immunomodulators has shown that most immunomodulatory agents exhibit an immunostimulatory effect at low concentrations and an immunosuppressive effect at higher concentrations. Similar results were obtained with our arjunic acid derivatives (Table 1).

From the above it may be concluded that arjunic acid derivatives **3** and **4** exhibit an immunostimulatory activity at low concentration so could be used in patients suffering from immunosuppressive conditions like TB, hepatitis, AIDS and SARS, while compound **5**, exhibiting an immunosuppressive activity at all the concentrations tested, could be used in the field of transplantation immunology and autoimmune disorders where suppression of the immune system is a desired factor.

Experimental

General: ^{1}H / ^{13}C NMR spectra were measured at 300/75 MHz in $CDCl_3$ solution at 25°C. Silica gel G was used for TLC. Compounds on TLC plates were visualized first in UV light (254 & 366 nm, CAMAG UV lamps) and then by spraying the plates with vanillin-ethanol-sulfuric acid reagent (1g: 95 mL: 5 mL)] followed by heating for 15 min at 110^0C. Reactions which required an inert atmosphere were carried out under N_2 using oven dried glassware.

Plant material: The bark of *T. arjuna* was collected from CIMAP's medicinal plants conservatory, during January, 1999, and identified in the Dept. of Botany and Pharmacognosy at CIMAP, where a voucher specimen (No. 5867) is maintained**.**

Isolation of arjunic acid: Isolation of arjunic acid from the air-dried bark of *T. arjuna* (4.5 kg) was carried out using the process developed by us [9], which gave arjunic acid in 0.04% yield.

Chemical derivatization: Arjunic acid (150 mg) was dissolved in pyridine (2.0 mL). To this solution the respective acyl / aryl chloride was separately added in a 1: 2.5 ratio. The airtight reaction mixture was kept overnight at room temperature and the progress of the reaction was monitored by TLC. After completion of the reaction, ice cold water was added and the mixture was extracted with chloroform (3 x 20 mL). The pooled chloroform extract was washed with water until neutral and dried over anhydrous Na_2SO_4 and evaporated under vacuum. This chloroform extract was further purified by column chromatography over silica gel to yield the respective derivatives in 85–95% yields.

In-vitro immunomodulatory study in murine spleenocytes using MTT assay

Isolation of murine spleenocytes: All procedures were conducted under aseptic conditions. Mice were euthanized by cervical dislocation under ether anesthesia and a single cell suspension was prepared by pressing the spleen between two sterile glass slides. The cell suspensions were passed through a 200 gauge stainless steel sieve and then allowed to stand for removal of tissue fragments. The cell suspensions were centrifuged (600 *g* for 10 min), and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum. The cell count was adjusted to 1 x 10^6 viable cells/mL. The viability of spleenocytes was determined by trypan blue dye. A 200 mL sample of the above concentration (1×10^6 cells/mL) was added to each well of a 96 well plate. After 24 h incubation in 5% humidified CO_2 at 37ºC, the test compounds were added to make final concentrations of 0.1, 1, and 10 µg/mL, in triplicate. After 48 h treatment at 37ºC, 10 µL/well MTT (50 mg/mL) was added and incubated for 4 h, after which the medium was removed by aspiration. DMSO (100 µL) was added to each well to dissolve the MTT formazan crystals. The plates were incubated for 20 min and read immediately in a micro plate reader (Versamax, Molecular Devices, USA) at

570 nm. Con A, in 5 µg/mL concentration, was added along with the test compounds as a mitogen for the proliferation of the spleenocytes [11]. The percentage stimulation of the spleenocytes was assessed using the formula:

% stimulation = [(OD of cells with mitogen – OD of cells without mitogen)/ OD of cells without mitogen] x 100.

The percent modulation was considered as the percent stimulation of vehicle control cells along with mitogen as the base value for modulation (zero %). The concentrations exhibiting values above this were considered to be immunostimulators and vice-versa as immunosuppressants. Levamisole at 0.1 µg/mL was used as the standard immunostimulant [12].

Supplementary data: Physical and spectral data for arjunic acid (**1**) and its ester derivatives (**2**-**8**).

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