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Bioactivity guided isolation and characterization of the phytoconstituents from the *Tridax procumbens*

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Abstract: *Tridax procumbens* L., Asteraceae, has been extensively used in Ayurvedic system of medicine for various ailments. Previous studies on the extracts of *T. procumbens* revealed remarkable immunomodulatory activity of TPEIF (*T. procumbens* ethanol insoluble fraction) extract. The dried methanol extract of *T. procumbens* was dissolved in distilled water, and then fractioned by re-extracting with chloroform, ethyl acetate and *n*-butanol subsequently. Immunomodulatory activities of these fractions were determined *in vivo*. The amounts of total phenolic compounds were also determined. Ethyl acetate and n-butanol fractions showed the significant immunomodulary activity. However, the ethyl acetate fraction exhibited the highest total phenolic content. Therefore, ethyl acetate fraction was subjected to further separation by chromatographic methods. Two phytochemicals SA-3 and SA-4 were obtained by repeated purification in sufficient amount to screen them for the immunomodulatory activity by the *in vivo* models *i.e.* neutrophil adhesion and delayed type hypersensitivity. In addition, the *n*-butanol fraction was subjected to silica gel column chromatography (CC); SA-6 was isolated from it. Mice were treated with two doses of SA-3, SA-4 and SA-6 (2 and 4 mg/kg) for fifteen days. Immune responses to T-dependent antigen SRBCs were observed using parameters like DTH and Neutrophil adhesion. Overall, SA-4 and SA-6 showed dose relative immunostimulatory effect on *in vivo* immune functions in mice. From these results, it can be suggested that these compounds may be used as potential immunostimulators. The structures of isolated phytochemicals were determined by UV, IR, NMR, and MS spectroscopic methods.

Introduction

The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The world Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe (GOI, 2001). In the developing countries, vast number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have no alternative for primary health care (GOI, 2001). Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of primary health care cannot be over-looked. Additionally, herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly (Fazly-Bazzaz et al., 2005). They are also economic, easily available and affordable. Therefore, there is need to look inwards to search for herbal medicinal plants with the aim of validating the ethno medicinal use and subsequently an isolation and characterization of compounds which will be added to the potential lists of drugs.

The immune system is involved in the etiology as well as in the pathophysiologic mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Sharma, 1983). Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (Sainis et al., 1997). Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health - a concept of strengthening host defenses against different diseases (Thatte & Dahanukar, 1986). These plants, labelled as 'Rasayana', have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the

psycho-neuro-immune axis (Katiyar et al., 1997). The isolation, purification and chemical characterisation of the immunoactive moieties have been carried out in some of these plants (Wagner, 1984).

Tridax procumbens L. (TP) is commonly known as Coat Button or Kansari (in Hindi) or Ghamara (in local language) and belongs to family Asteraceae. It is extensively used in Ayurvedic system of medicine for various ailments and is shown to possess a number of pharmacological activities like hypotensive (Salahdeen et al., 2004), insecticidal, leishmanicidal (Peraza-sanchez et al., 2007), hair growth promoting (Saraf et al., 1991), wound healing, anti-inflammatory (Margaret et al., 1998), hepatoprotective (Devaki et al., 2005), antiviral (Chien et al., 2001) immunomodulatory (Vyas et al., 2004) and antioxidant activity (Agrawal et al., 2009) due to the presence of phenolics, tannins, saponins, and glycosides. The aim of this study was to isolate, characterize the chemical constituents of *T. procumbens* and to screen their potential as an immunomodulator.

Materials and Methods

Plant material

The whole aerial parts of the plant *Tridax procumbens* L., Asteraceae, were collected in the months of August -December from the locality in Maharashtra state, India, and authenticated by the Dr. DA Patil, HOD Botany Department, SSVPS College, Dhule, North Maharashtra University, Jalgaon, Maharashtra, India.

Analytical material and methods

NMR spectra were recorded on a Varian 200 MHz spectrometer, operating at 200 and 50 MHz for ^{1}H and ^{13}C, respectively, using deuterochloroform ($CDCl_3$), dimethylsulfoxide-$_{d6}$ (DMSO-$_{d6}$), and deuteromethanol (CD_3OD). Chemical shifts are expressed in δ downfield from tetramethylsilane (TMS) as an internal standard and coupling constants reported in Hz. The IR spectra were determined on a Shimadzu FT-IR 8000 spectrophotometer. Electron impact-mass spectroscopy (EI-MS) was recorded on Micromass Quattro II triple quadrupole mass spectrometer. Column chromatography (CC) was carried out using silica gel 60 (70-230 mesh), thin layer chromatography (TLC) and prep. TLC on silica gel 60 precoated plates, F_{254} (Merck). The spots on TLC were visualized by spraying with 1% vanilin-H_2SO_4 followed by heating and hold to vapour of NH_3.

Extraction procedures

The dried aerial parts of *T. procumbens* (800 g) were extracted with methanol (MeOH) (3x 4 l). The MeOH extract was evaporated under vacuum to dryness as a dark brown mass (85.4 g) and then the concentrated MeOH extract was dissolved in distilled water. The solution successively partitioned with $CHCl_3$, ethyl acetate (EtOAc) and *n*-butanol, yielded 6.9, 2.9, and 12.5 g, respectively. Water fraction was lyophilized to obtain dried powder.

Isolation procedures

The concentrated EtOAc fraction (6.9 g) was fractioned on silica gel CC (200 g) using petroleum ether-EtOAc (1:1, 1:3, and 0:1) and EtOAc-MeOH (3:1, 1:1, 1:3, and 0:1 v/v) giving two major fractions (A and B). Fraction A on repeated CC and purification was determined to contain only compound SA-4 (106 mg) and monitored by TLC. Fraction B (800 mg), eluted with EtOAc-petroleum ether (3:1), was further subjected to silica gel CC (50 g) with EtOAc-formic acid-acetic acid-MeOH (100:1:1:5) and on repeated CC compound SA-3 (158 mg) was purified. The butanol fraction (10.0 g) was separated over silica gel column with a solvent system of $CHCl_3$:MeOH: H_2O (9:4:0.5-3:5:0.5) as the eluent to give five fractions, among them the major fraction was purified by repeated CC using methanol as eluent, to give SA-6. Characterization of isolated compounds was done by comparing the spectral data in the literature (Carotenuto et al., 1996; Ali et al., 2007; Seebacher et al., 2003).

Animals

Swiss Albino Mice, strain C57BL6 weighing between 20-40 g were used in the study with prior approval and scrutinization from the Institutional Animal Ethical Committee (RCPIPER/IAEC/2008-09/30). The animals were housed in clean and spacious cages provided with net and feeding bottle, at ambient temperature of 25±2 °C with 12 h light and 12 h dark cycles and provided free access to standard laboratory chow mixture provided water *ad libitum* for fixed period so as to acclimatize all animals and to achieve normal constant basal food intake in all.

Antigen

Fresh blood was collected from sheep's sacrificed in the local slaughter house. Sheep red blood cells (SRBC) were washed three times in normal saline and adjusted to a concentration of 0.1 mL containing $1x10^8$ cells for immunization and challenge.

Treatment

The animals were divided into six groups consisting of six animals each. A group of six untreated

rats were taken as control (Group I). The isolated phytoconstituents were dissolved in water and fed orally for 14 days at a dose of 2 mg/kg/day (Groups II and IV), 4 mg/kg/day (Groups III and V), for assessment of immunomodulatory effect.

Delayed-type hypersensitivity (DTH) response (footpad swelling)

Six animals per group (control and treated) were immunized on day 0 by *i.p.* administration of $0.5x10^9$ SRBC/rat and challenged by a subcutaneous administration of $0.025x10^9$ SRBC/mL into right hind footpad on day +14. The isolated constituents were administered orally from day -14 until day +13. DTH response was measured at 24 h after SRBC challenge on day +14 and expressed as mean increase in paw edema measured by Baker's pocket thickness gauge (Puri et al., 1993).

Neutrophil adhesion test

The method described by Wilkinson (1978) was used for evaluating the effect of extracts on neutrophil adhesion. After 14 days of treatment of all the three groups, blood samples were collected by retroorbital puncture in heparinized vials and subjected to total as well as differential leukocyte count. After initial counts the blood samples were incubated with 80 mg/ml of nylon fibers at 37 °C for 15 min. The incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and % neutrophil known as neutrophil index was determined for each of the respective groups (Shinde et al., 1999; Fulzule et al., 2003). The % neutrophil adhesion for each of the test groups was determined as follows,

$$\% \text{ Neutrophil adhesion} = \frac{\text{difference of netrophil count in untreated and fibre treated blood}}{\text{neutrophil count of untreated blood}} \times 100$$

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Student's t-test. *p* values <0.05 were considered significant.

Results and Discussion

Immunomodulatory agents of plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis is, nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing. In the present study phytochemicals isolated from both the plants evoked a significant increase in percent neutrophil. This may help in increasing immunity of the body against microbial infections. In the present study isolated phytochemicals, significantly increased the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels (Table 1). The neutrophil adhesion was significantly increased with the dose of 4 mg/kg/day when compared to untreated control indicating possible immunostimulant effect.

The DTH response to SRBCs, which corresponds with cell-mediated immunity, showed a dose-dependent increase on treatment with isolated phytoconstituents. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive (inflammatory) reaction. In our studies, foot volume was enhanced after phytochemical treatment suggesting cell-mediated immune enhancement (Gupta et al., 2006). Oral doses of 2 and 4 mg/kg/day of SA-6, the DTH response was 0.58±2.31 and 1.11±1.33, respectively, and the response for SA-4 with doses of 2 and 4 mg/kg/day was 0.34±2.11 and 1.41±1.14 respectively, in comparison with corresponding value of 0.25±0.06 for untreated control group. The differences in DTH response were statistically significant (Table 1). Thus, the treatment induced marked enhancement of DTH response to SRBCs in the animals. Increase in the DTH response indicates stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction (Mitra et al., 1999). On the basis of the results obtained in the current study, these phytochemicals can be potential therapeutic candidates in several immunosuppressed clinical conditions

The immunoactive phytochemicals have been analysed by spectroscopic method and then interpreted by comparing the IR, ^{1}H- and ^{13}C-NMR and MS data with previous literature. Compounds SA-3 and SA-4 were isolated using silica gel CC from the ethyl acetate fraction. These compounds were identified as kaempferol 3-*O*-α-L-rhamnopyranosyl-(3→6)-β-D-glucopyranoside (Carotenuto et al., 1996) and lupeol (Ali et al., 2007) respectively. SA-6 was isolated from the *n*-butanol fraction and was identified as 18-α-oleanolic acid based on the spectral information (Seebacher et al., 2003).

Conclusion

We contend that patient preferences for therapies are guided by cultural heritage and by the natural environment of the region they live in. The results of this study provide the necessary data for clinical trials in

Table 1. Effect of phytoconstituents on SRBC induced delayed type hypersensitivity and % neutrophil adhesion.

Groups	Treatment	Dose mg/kg *p.o.* 7 days	TLC (10^3/mm^3)		Neutrophil (%)		Neutrophil index		% Neutrophil adhesion (Mean ± S.D.)	DTH response (mm) paw
			UB	NFTB	UB	NFTB	UB	NFTB		
I	Control	-----	6.34±2.02	5.56±1.18	25.86±3.18	21.88±1.02	163.95±3.52	121.65±2.45	34.77±3.49	0.25±0.63
II	SA-3	2	7.10±2.1	6.81±1.32	28.32±2.23	17.97±0.99	201.07±1.47	122.37±0.94	39.13±4.6**	0.12±2.17**
III	SA-3	4	7.42±1.95	6.96±2.48	30.72±2.7	18.17±2.84	227.94±3.92	126.46±2.35	44.51±2.9**	0.60±1.19**
IV	SA-4	2	6.8±1.65	6.11±3.2	26.69±1.84	18.94±3.74	181.49±2.29	115.72±1.96	36.23±3.92	0.34±2.11
V	SA-4	4	6.92±2.64	6.2±1.52	26.93±0.88	19.12±0.96	186.36±0.86	118.54±0.68	36.39±2.75	1.41±1.14*
VI	SA-6	2	8.84±1.43	7.90±2.42	34.48±3.04	26.61±1.25	304.80±1.28	210.21±1.45	44.99±2.20**	0.58±2.31**

immunocompromised patients with *Tridax procumbens* extract, fractions and isolated compounds. These can also be developed as an 'add on' therapy to established oral immunostimulants specially in immunocompromised patients. Further studies are needed towards a better understanding the exact mechanism responsible for the action.

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