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J. Nat. Prod., **1993**, 56 (7), 995-999• DOI: 10.1021/np50097a002 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 7, pp. 995-999, July 1993

IMMUNOSTIMULANT AGENTS FROM *ANDROGRAPHIS PANICULATA*[1]

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ABSTRACT.—EtOH extract and purified diterpene andrographolides of *Andrographis paniculata* (Acanthaceae) induced significant stimulation of antibody and delayed type hypersensitivity (DTH) response to sheep red blood cells (SRBC) in mice. The plant preparations also stimulated nonspecific immune response of the animals measured in terms of macrophage migration index (MMI) phagocytosis of ^{14}C-leucine labelled *Escherichia coli* and proliferation of splenic lymphocytes. The stimulation of both antigen specific and nonspecific immune response was, however, of lower order with andrographolide than with the EtOH extract, suggesting thereby that substance(s) other than andrographolide present in the extract may also be contributing towards immunostimulation.

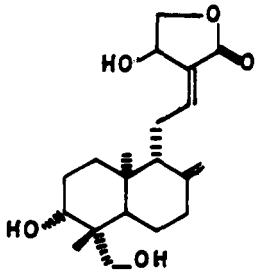
In our research program for development of chemotherapeutic agents from Indian medicinal plants, we have encountered promising immunostimulant activity in *Andrographis paniculata* Nees (Acanthaceae), a plant widely used in traditional remedies and folkloric medicines for liver disorders, bowel complaints of children, colic pain, cases of general debility, and convalescence after fevers. It is also used as a stomachic, anthelmintic, antiperistaltic, and antispasmodic (1–3). Bioassay-linked extraction and isolation of compounds revealed the presence of immunostimulant activity in the EtOH extract and pure diterpenes andrographolide [**1**] and neoandrographolide [**2**] (3).

MATERIALS AND METHODS

PLANT MATERIAL.—Fresh plant material was collected in October 1986 from the campus of CDRI, Lucknow, India. A voucher specimen (no. 11783) is deposited in the herbarium of CDRI.

EXTRACTION AND INITIAL FRACTIONATION.—Fresh plant material (10 kg) was crushed and percolated with EtOH (3×15 liters). The EtOH extract was concentrated in vacuo at a temperature below 50° to a concentrated green mass (700 g).

ISOLATION OF COMPOUNDS AND PHYSICAL DATA.—The EtOH-soluble fraction (125 g) was



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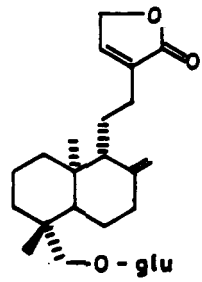

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[1]CD Communication No. 5030.

chromatographed on a column of Si gel (2 kg). Elution of the column was carried out successively with hexane, $CHCl_3$/EtOAc, EtOAc (saturated with H_2O), EtOAc (saturated with H_2O)/MeOH, and MeOH. Fractions of 250 ml were collected and mixed according to a similar pattern. Pure compounds were obtained only after repeated cc of various fractions. The system used was $CHCl_3$-MeOH (9:1), and R_f's of andrographolide [**1**] and neoandrographolide [**2**] were 0.58 and 0.5, respectively. The compounds were identified by comparison of their data with that reported in literature (4–6).

ANIMALS.—BALB/c albino mice of either sex (20–22 g) from the CDRI colony were used for this study. The animals had access to the standard diet and H_2O ad libitum.

TREATMENT.—The EtOH extract was fed orally at the dose of 25 mg/kg for 7 consecutive days prior to immunization with sheep red blood cells (SRBC) and determination of nonspecific immune response. Purified crystalline compounds dissolved in 0.05 M phosphate buffer pH 7.2 containing 0.1 M NaCl (PBS) (0.5 ml) were administered orally at a dose of 1 mg/kg for 7 days or ip at a dose of 4 mg/kg on days −7 and −3 prior to the immunization or determination of nonspecific immune response.

SPECIFIC IMMUNE RESPONSE TO SHEEP RED BLOOD CELLS (SRBC).—Sheep blood was collected from the jugular vein in Alsever's solution (pH 6.4). The erythrocytes were collected by centrifugation and washed three times with PBS and dispensed in the same medium to give a suspension of 1×10^8 cells/ml.

One set each of the treated and control animals (four animals in each group) were immunized by injecting ip with 1×10^8 SRBC (day zero). Five days later, hemagglutinating antibody (HA) titer, hemolytic plaque-forming cells (PFC) assay, and delayed type hypersensitivity (DTH) response to SRBC were determined.

HEMAGGLUTINATING ANTIBODY (HA) TITER.—Serum was collected from the blood samples of individual mice by retro-orbital puncture. Antibody levels were determined by the microtiter hemagglutination technique. Briefly, 50-μl aliquots of a twofold dilution of sera were prepared in 0.15 M PBS (pH 7.2) and dispensed in V shape bottom microtiter plates; 1% SRBC suspension (25 μl) in PBS was dispensed into each well and mixed thoroughly. After 1.5–2 h of incubation at room temperature, the reciprocal of the highest dilution of test sample giving 50% agglutination essentially according to the method of Haden (7) was expressed as the HA titer.

HEMOLYTIC PLAQUE-FORMING CELLS (PFC) ASSAY.—The assay was done according to the technique of Jerne and Nordin (8). The test was performed in duplicate for each individual mouse, and the mean values for the groups were expressed as mean PFC/10^5 spleen cells.

DELAYED-TYPE HYPERSENSITIVITY (DTH).—Delayed-type hypersensitivity response to SRBC antigen was induced in mice by the method of Doherty (9). The groups of SRBC-immunized mice were challenged by injecting 1×10^8 SRBC (50 μl) into the right foot pad. Twenty-four hours later the thickness of the left hind foot pad was measured with the help of Schnelltester (Kroplin, FRG). The foot pad reaction was expressed in mm as the difference in thickness between the right foot pad injected with SRBC and the other with PBS.

NONSPECIFIC IMMUNE RESPONSE.—*Macrophage migration index.*—The migration pattern of peritoneal macrophages in vitro was studied according to the method of Saxena *et al.* (10). Briefly, the PEC, packed in a microhematocrit capillary tube of uniform diameter, were allowed to migrate in a migration chamber (Laxbro India) for 18–24 h. The ratio of the area of migration of macrophages from the control and the experimental animals was expressed as macrophage migration index (MMI).

Phagocytic response.—The phagocytic activity of PEC macrophages was measured by in vitro incorporation of ^{14}C-leucine labelled (sp. act. 190 mCi/mmol, BRIT, India) *Escherichia coli* into PEC according to the method described elsewhere (11). Results were expressed in terms of percentage incorporation of ^{14}C-leucine.

Lymphocyte proliferation response.—Splenic lymphocytes from treated or untreated mice suspended (1.5×10^6 cells/ml) in complete RPMI-1640 medium containing fetal calf serum (GIBCO, Grand Island, NY) were cultured in triplicate at 37° for 3 days in 5% CO_2/air atmosphere, pulsed with 1 μCi ^{3}H-thymidine for another 18 h, and harvested. The radioactivity incorporated in lymphocytes was counted in a Liquid Scintillation Counter (LKB Wallace-1209, Rackbeta, Finland). Proliferation response was expressed as cpm±SD.

All the data were statistically analyzed using Student's *t*-test.

RESULTS

ANTIGEN SPECIFIC AND NONSPECIFIC RESPONSE IN MICE TREATED WITH EtOH

TABLE 1. Effect of EtOH Extract of *Andrographis paniculata* and Andrographolide [**1**] on Antigen-Specific and -Nonspecific Immune Response of BALB/c Mice.[a]

Parameter of immune response	Untreated	Treated[b]	
		EtOH extract	**1**
Antigen specific			
HA titer	204±31	1706±216[c]	1365.33±216[c]
PFC/10^5spleen cells	70±15	143.33±20[c]	128±8[c]
DTH response (mm)	2.1±0.1	2.95±0.07[c]	2.5±0.1[c]
Nonspecific			
MMI	1	2.52±0.42[c]	2.7±.26[c]
Phagocytosis of labelled *E. coli* (%)	35.5±2.28	60.66±1.76[c]	55.56±1.17[c]
^{3}H-thymidine uptake by lymphocytes (cpm)	6176±454	10192±1462[c]	6486±516[d]

[a]Data based on three separate experiments with four animals each. The values are mean ± SD excepting that for reciprocal HA titer which is geometrical mean ± SEM. HA titer=Hemagglutinating antibody titer (reciprocal); PFC=Plaque-forming cell; MMI=Macrophage migration index.

[b]EtOH extract was fed orally and purified andrographolide [**1**] was administered ip.

[c]$P<0.001$.

[d]Nonsignificant.

EXTRACT AND DITERPENES OF *AN. PANICULATA*—*Antigen specific immune response.*—Data on HA titer, PFC count, and DTH response to SRBC of mice treated with the EtOH extract and andrographolide [**1**] from *An. paniculata* is presented in Table 1. The treatment induced marked enhancement of humoral and DTH response to SRBC in the animals. The values for HA titer, PFC count, and DTH response in the extract-treated group of animals were 1706±216 (SEM), 143.33±20 (mean ± SD), and 2.95±0.07, respectively, in comparison to the corresponding figures of 204±31, 70±15, and 2.1±0.1, respectively, for the untreated control group. These differences were statistically significant ($p<0.001$).

Nonspecific immune response.—As is evident from the data presented in Table 1, *An. paniculata* was effective in inducing enhancement of nonspecific immune response also in the treated animals. The mean values of MMI, percent phagocytosis, and mitogen response were 2.52±0.42, 60.66±1.76, and 10192±1462, respectively, in the mice treated with EtOH extract as against the corresponding values of 1, 35.5±2.28, and 6176±454 for the control group of animals. The enhancement was statistically significant for all the three parameters. In the animals treated with andrographolide [**1**],

TABLE 2. Corresponding Values of MMI and HA titer in Mice Administered with the Pure Compounds **1** and **2** by ip and Oral Routes.[a]

Pure compound	Oral route		ip route	
	MMI (mean±SD)	HA titer (mean±SEM)	MMI (mean±SD)	HA titer (mean±SEM)
Andrographolide [**1**]	1.6±0.05	1280±256	2.84±1.30[b]	1536±295[c]
Neoandrographolide [**2**]	1.9±0.40	1365.33±341	2.51±0.14[b]	1638.4±240[c]

[a]In the case of oral route mice were administered each with 1 mg/kg of each preparation daily for 7 days and in the case of the ip route the animals were administered with 4/mg/kg on day −7 and −3 prior to immunization and determination of MMI. Data based on two separate experiments with four animals each.

[b]ip vs. oral route, $p<0.05$.

[c]ip vs. oral route, nonsignificant.

the values for MMI and percent phagocytosis were nearly the same as observed with the EtOH extract. The mitogenic response was significantly lower.

Effect of route of administration on immunostimulant activity.—Table 2 presents the data on HA titer (antigen specific) and MMI (nonspecific) of mice administered with andrographolide [**1**] and neoandrographolide [**2**] by oral and ip routes. These data show that stimulation of the two responses was higher by the ip route with both the purified compounds as compared to the oral route.

DISCUSSION

Results of the present study indicate that *An. paniculata* is a potent stimulator of the immune response and is capable of enhancing both antigen specific and nonspecific responses. Enhancement of antigen specific response was observed against humoral as well as cell-mediated immune response. The EtOH extract induced about a 3-fold increase in the HA titer and a more than 2-fold increase in the PFC in the treated animals as compared to the controls ($P<0.001$). The DTH response to SRBC, which is a correlate of CMI, was also found to be increased by 40%. The diterpenes andrographolide [**1**] and neoandrographolide [**2**] extracted from the crude extract also induced increase in all of the above parameters but of relatively lower order, which suggests that substances other than andrographolides present in the EtOH extract may also contribute toward the immunostimulant activity of the extract.

Evidence regarding stimulation of nonspecific immune response by *An. paniculata* is provided by the results on the effect of the EtOH extract and **1** on certain macrophage and lymphocyte functions. Both the EtOH extract and **1** induced significant increase in phagocytic activity and MMI (peritoneal macrophages) of the treated animals. It may be pointed out that MMI has been shown to be correlated with the status of macrophage activation and CMI (8). The mitogenic response of lymphocytes from normal mice was also significantly higher in presence of the EtOH extract but was less so with **1**. The observation once again suggests the presence of substances other than **1** in the EtOH extract, which are capable of inducing mitogenic response. The data on routes of administration of the two diterpenes shows that stimulation of immune response was higher with respect to MMI ($P<0.05$) by ip route. No significant difference was, however, observed with respect to antibody response to SRBC by the two routes. Since different investigators have employed different parameters for evaluation of immunostimulant activity of natural and synthetic products, it is not possible to compare the immunostimulant activity of *An. paniculata* with any standard immunostimulant. However, with respect to MMI, a parameter employed by us for macrophage activation, the activity of andrographolide is comparable to that of muramyl dipeptide (10).

A large number of plants long been used in the traditional medicine of Europe for rejuvenation therapy and treatment of chronic ailments have now been shown to possess immunostimulant activity (12,13). The activity is associated with substances of varying molecular weights and chemical structures. Among well studied low molecular weight substances (12,13) are: the alkaloids aristolochic acid, isopteropodin, and cepharanthine isolated from *Aristolochia clematitis, Uncaria tomentosa,* and *Stephania cepharantha*, respectively; sesquiterpenlactones euperfolin and eufoliatin from *Eupatorium perfoliatum* and *Eupatorium cannabinum*, respectively; and germacranolides from *Zexmenia brevifolia* and *Coriolus consors*. Most of these substances stimulate macrophages and enhance phagocytosis, and some also possess antitumor activity.

The high molecular weight immunostimulants include krestin, a protein-bound carbohydrate from *Coriolus versicolor,* and polysaccharides from *Echinacea purpurea,*

Echinacea cannabinum, Arnica montana, Calendula officinalis, and *Chamomilla recutita.* These substances act by stimulating macrophage and possibly other immune cell functions. Krestin also shows conspicuous antitumor activity. Recently we have demonstrated strong immunostimulant activity enhancing both humoral and cell mediated immune responses in picroliv (14), an iridoid glycoside fraction from *Picrorhiza kurroa* (15), a perennial herb used in the Indian traditional medicine as tonic and treatment of fever, jaundice, and other liver ailments (16,17). The fraction has also shown nonspecific protection of hamsters against *Leishmania donovani* infection.

An. paniculata has traditionally been also used in India as tonic and for treatment of fever and convalescence following fever, general debility, and liver disorders (12–14). The plant and andrographolides have also been shown to possess antidiarrheal (18) and antimalarial (19) activity. The observations of the study suggest that the aforesaid properties of *An. paniculata* may be ascribed at least partly to andrographolides, which stimulate specific and nonspecific immune responses.

ACKNOWLEDGMENTS

Two of the authors, A.P. and V.S., are grateful to CSIR and Ministry of Environment and Forests for financial support. Technical assistance of Mr. A.K. Srivastava is acknowledged.

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Received 17 August 1992