RESEARCH PAPER

Isolation of Chemical Constituents from Andrographis paniculata and its Anti-inflammatory Activity

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ABSTRACT

Phytochemical investigation of the aerial parts of Andrographis paniculata give diterpenic constituents andrographolide, 14-deoxy-11,12-didehydro andrographolide, 14-deoxy andrographolide, 3,14-dideoxy andrographolide, 14-deoxy-11-oxo andrographolide, 14-deoxy-12-hydroxy andrographolide, neoandrographolide, andrographiside and 14-deoxyandrographiside. The structures of these compounds have been established on the basis of spectral data analysis. Active phytoconstituents andrographolide also extracted from alcoholic extract which play an important role for altering concentration in human body. The present study was planned to investigate the in vivo anti-inflammatory activity of alcoholic extract of A.paniculata and andrographolide following intramuscular administration @ 100, 200, 400 mg/kg bw and andrographolide 10mg and 20mg/kg bw in male albino rats by using carrageenan-induced paw edema model. Forty two rats were divided randomly into 7 groups and each group consists of six male albino rats. All the animals were treated with Lambda carrageenan solution (1%) prepared in 0.9% normal saline subcutaneously into sub plantar region of left hind paw as a local acute edema inducer after 30 min subsequent to intramuscular administration of alcoholic extract of A.paniculata and andrographolide. Rats of carrageenan control groups were kept untreated. Rats of standard control group were treated intramuscularly with indomethacin@ 5 mg/kg body weight as a reference standard drug. Rats of other five group were treatment groups with alcoholic extract @ 100mg, 200mg and 400 mg/kg body weight, and 10mg, 20mg andrographolide respectively. Edema was expressed as the increase in paw volume in ml and measured up to the tibiotarsal articulation. Volume of edematous paw was measured at 0 h (before treatment), 1, 2, 3, 4 and 5 h after treatments. Increase in paw thickness was measured by using digital plethysmometer and percent inhibition was calculated. The anti-inflammatory effect of ellagic was highest at 4 h (58.02 %) at the dose of 150 mg/kg and (45.47 %) at the dose of 100 mg/kg. The anti-inflammatory effect of standard drug indomethacin (63.14 %) was higher than andrographolide at 4 h. alcoholic extract @ 100, 200 and 400 mg/kg post intramuscular administration gave higher anti- inflammatory effect at 4 h in rats. andrographolide showed dose dependent anti-inflammatory activity in male albino rats.

Keywords: Andrographolide, 14-deoxy-11,12-dehydro andrographolide, Nitric oxide (iNOS), Reactive oxygen species (ROS).

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INTRODUCTION

Andrographis paniculata (Acanthaceae), commonly known as Kalmegh is found in the plains of India, Pakistan, Sri Lanka and West Indies. About twenty six different poly herbal formulations of this plant are mentioned in Ayurveda as a popular remedy for the treatment of various liver disorders[1,2]. Aerial parts of the plant are used for jaundice, colic dysentery and dyspepsia[3,4], as a bitter tonic, stomachic, anthelmintic and anti plasmodial[5]. It is widely used in traditional medicine as an antidote against poisons of snakes and insect, and as an antimalarial agents[6-8]. It is beneficial in general debility, asthma, bronchitis, filariasis[9] and in hepatitis during clinical studies[10]. Natural plant products and their secondary metabolites are also of great interest in the drug discovery process. Among the various medicinal plant compounds, polyphenols play an important role for the development of new

therapeutic agents. A.paniculata is a polyphenol compound. A.paniculata and its active constituents has exhibited antioxidant, anticancer, antiallergic and anti-inflammatory activities[11]. Least information is available about investigation on the anti-inflammatory effect of A.paniculata after intramuscular administration in rats. Based on the mentioned above facts, this study was aimed to assess the *in-vivo* anti-inflammatory effect of alcoholic extract of A.paniculata and andrographolide following intramuscular administration in carrageenan-induced paw edema in albino rats.

Experimental Section

Chemical and Reagents:

All the melting points were uncorrected. The UV spectra were determined on a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer. The IR spectra were determined on a Perkin Elmer spectrum RX1 (4000-450 cm-1). The 1H NMR spectra were measured on a Bruker DRX-300 spectrometer using $CDCl_3$ as solvent and TMS as internal standard in δ ppm.

Plant Material

The whole plant except root was collected from rural area, Jaunpur, U.P., India in July 2025 and identified as *Andrographis paniculata* (Acanthaceae) from specimen sample of Botany Department, U.P. College Varanasi, U.P.

Isolation Procedure

The aerial part powder (1 kg) of *A. paniculata* was percolated with 95% ethanol (5 × 1L) with soxhlet apparatus. The combined extracts were concentrated under reduced pressure, affording the residue (70 g). The crude residue (70 g) was defatted with hexane and then fractionated into chloroform (10 g) and Ethanol (20 g). Ethanol fraction (5 g) was subjected to silica gel column chromatography eluted with chloroform-methanol (1.5-21% methanol in chloroform) to yield four fractions (A-D). All fractions were further subjected to repeated column chromatography on silica gel eluted with chloroform-methanol (1-35% methnol in chloroform). Repeated column chromatography of fraction A on silica gel (chloroform-methanol; 1-2%, 2.5-3.5%) afforded compounds **1** (20 mg) and **2** (13 mg). Compounds **3** (18 mg) and **8** (16 mg) were obtained from the fraction B after repeated silica gel chromatography (chloroform-methanol; 5-7% and 8-12% respectively). The fraction C on repeated silica gel chromatography gave compounds **4** (14 mg) and **5** (18 mg) (chloroform-methanol; 14-18% respectively). Compounds **6** (13 mg) compound 7 (6.2mg) obtained from fraction D after repeated silica gel chromatography (chloroform-methanol; 22-35% and 17-20%, respectively). The structure for each compound isolated was determined by IR, 1H NMR and MS studies carried out at Central Drug Research Institute, Lucknow, India.

Biological study:

Experimental animals:

The study was conducted on male albino wistar rats weighing between 300 to 400 grams. The animals were obtained from Animal house Ram Nagar Varanasi, and maintained at the laboratory animal house, Department of applied chemistry, U.P. College, Varanasi. They were kept under constant observation for two weeks prior to commencement of the experiment. The animals were divided into 7 groups and kept in cages. Standard ration and water was provided ad libitum. All necessary managemental procedures were adopted to keep the animals free from stress. The experimental protocol and use of animals for conducting the present study was approved by the Institutional Animal Ethics Committee (IAEC).

***In vivo* anti-inflammatory:**

The carrageenan-induced rat paw edema model was used with minor modification as described[12]. Alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg bw was prepared in 5ml of distilled water. Indomethacin (5 mg/kg) was prepared in DMSO. Experimental animals (n=42) were divided into 7 groups with 6 animals in each group. A mark on the left hind paw was made in each animal and initial volume was measured by immersing in the plethysmometer persplex tube.

Group-I animals were kept as Carrageenan control, Group-II animals were treated with Indomethacin (5 mg/kg, IM), Group-III, IV and V animals were treated with Alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg bw. All the animals were treated with Lambda carrageenan solution (1%) prepared in 0.9% normal saline subcutaneously into sub plantar region of left hind paw. Half an hour before the carrageenan administration, vehicle, test drug and positive control drug were injected via intramuscular route in respective animal groups. Inflammation in the form of edema was measured in paw volume (ml) before carageenan

administration and at 1, 2, 3, 4 and 5 h after carrageenan administration and expressed as percent edema formation in relation to initial paw volume before carrageenan injection for each animal. The paw volume data for test drug and positive control drug were analyzed and expressed as percent inhibition of edema formation in comparison to carrageenan control group.

Results and Discussion

The compounds **1-5** were identified as andrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide, 3,14-dideoxyandrographolide, 14-deoxy-11-oxoandrographolide and compounds **7-9** were identified as neoandrographolide, andrographiside, 14-deoxyandrographiside (Figure 1). Compound **1** was obtained as amorphous powder. The molecular formula, $C_{20}H_{30}O_5$ was established by mass spectrum giving a molecular ion at *m/z* 350(M+) and named as Andrographolide. The IR spectrum showed a broad peak at 3400 cm^{-1} due to hydroxyl group. Peaks at 1727 and 1673 cm^{-1} were due to lactone ring. Peak at 904 cm^{-1} was due to presence of exocyclic methylene group. The ^1H NMR spectrum exhibited a singlet at δ 6.91 for methyl groups present at C18 and C20. Peak at δ 4.21-4.50 (multiplet) and 2.20 (triplet) was obtained for a hydroxyl proton and a proton present at C3. Peak at δ 3.98 gave a doublet for two protons present at C15. Broad singlet was obtained at δ5.20 and 5.66 for hydroxyl group present at C19 and C12, respectively.

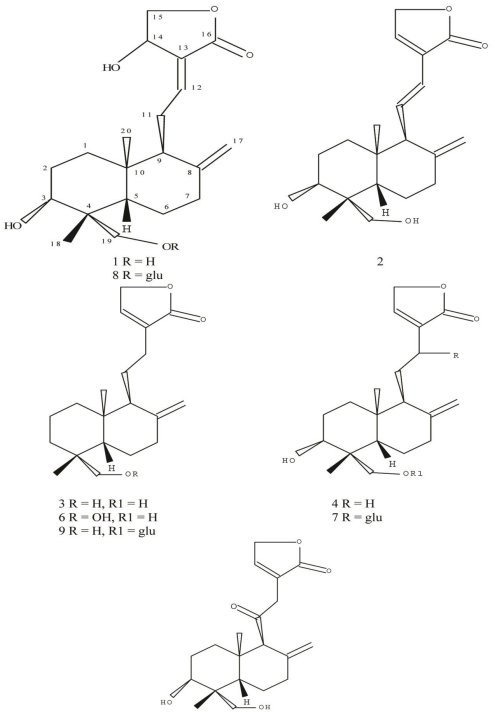

Figure 1 — Diterpenes and diterpenoidal glycosides isolated from *Andrographis paniculata*

Eight isolated compound were process for physical examination melting point elemental analysis which data are presented in table 1.

Table 1. Physical Data of Isolated compound

Compound Code	Compound Name	Physical Stutus	M.P. ^0C observed	M.P. ^0C Reported
AP-1	Andrographolide	Colourless cubes	218-22	219-223[14]
AP-2	14-Deoxy-11,12-didehydroandrographolide	White crystal	204-205	203-205[14]
AP-3	14-Deoxyandrographolide	Fine needles	170	173-174[14]
AP-4	3,14-Dideoxyandrographolide	Colourless needles	106-107	106-108[14]
AP-5	14-Deoxy-11-oxoandrographolide	White crystalline solid	101	100-102[14]
AP-6	14-Deoxy-12-hydroxyandrographolide	Amorphous power	106-108	105-106[14]

AP-7	Neoandrographolide	Long colourless needles	160-62	159-161[14]
AP-8	Andrographiside	Amorphous power	193	191-193[14]

These compound were characterized on the basis of IR and ^{1}H NMR spectral study presented in table 2.

Table 2. Spectral study of isolated compound

Compound Code	**IR** (KBr) cm^{-1}	**^{1}H NMR** (300Mz) $CDCl_3$ δ
AP-1	3400, 3299, 1727, 1673, 908	6.91 (1H, t, C12), 4.90 (1H, d C14), 4.21- 4.50 (2H, m, C15), 1.81-1.96 (5H, m, C2, C7, C9), 0.72 (2 × CH3, s, C18, C20)
AP-2	3328, 2930, 2849, 1727, 1669 and 901	0.71 (3H each, s, C18, C20), 2.51(1H, s (br), C3-OH), 2.39 (1H, t, C3), 4.51(2H, d, C15), 4.87 (1H, t, C14), 5.16 (1H, s (br), C19-OH), 5.68 (1H, d, C12)
AP-3	3399, 3280, 2930, 2850, 1728, 1632, 1458, 1369, 908	0.66 and 1.26 (each 3H, s, 2 × CH3,C18,C20), 1.59 (12H, S, 6 × CH2), 3.32 (1H, d, H19), 3.56 (1H, t, 3β-H), 3.78 (H, s (br), C3-OH), 4.17(1H, ABd, C19), 4.58 (2H, s, C17), 4.73 (2H, m, C15), 5.10 (H, s (br), C19-OH) 6.97 (1H, m, C14)
AP-4	3261, 2935, 2861, 1720, 1640, 1556, 1306, 890	0.88 (3H each, s, C18, C20), 3.14 (2H, d, C12), 3.88 (2 H, s, C17), 3.99 (1H,t, C14), 1.42 (10H, s, 5 × –CH2), 5.12 (1H, s, C19 - OH)
AP-5	3340, 2929, 2849, 1726, 1713, 1679, 1458, 1365 and 901	0.69 (3H each, s, C18, C20), 2.40 (1H, t, C3), 3.41 (1H, s, (br), C3-OH), 4.33 (2H, d, C15), 4.42 (1H, t, C14), 4.98(1H, s (br), C19-OH), 5.59 (2H, s, C12)
AP-6	3420, 2932, 2828, 1730, 1656 and 904	0.69 (3H each, s, C18, C20), 2.20 (1H, t, C3), 3.01 (1H, s, C3-OH), 3.39 (1H, t, C14), 3.98 (2H, d, C15), 5.20 (1H, s, C19 - OH), 5.66 (1H, s, C12-OH)
AP-7	3449, 2931, 2852, 1748, 1600, 1447, 1357, 910	0.65 and 1.07 (each 6H, s, 2 × CH3, C18,C20), 1.19-2.37 (14H, m,7 × CH2), 3.44 - 4.80 (11H, m, sugar protons, C19) 7.18 (1H, ABd, *J* = 4.0Hz, H14)
AP-8	3399, 3307, 2929, 2849,	0.88 and 1.07 (each 6H, s, 2 ×

	1727, 1674, 1458, 1365, 907	CH3,C18, C20), 1.47-2.09 (10H, m, 5 × CH2), 3.20 - 4.62(11H, m, sugar protons, C1'-6')

Anti inflammatory Study

In the present experiment of *in vivo* anti-inflammatory activity of Alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg bw following intramuscular administration was performed. No any clinical signs of toxicity were observed in experimental rats. Carrageenan-induced paw edema model is widely used to assess the anti-inflammatory activity of several natural and synthetic compounds[13]. The carageenan-induced rat paw edema is used as a distinct acute inflammatory model. Carrageenan dilates postcapillary venules that result in exudation of inflammatory fluid and cells. This process involves the release of several proinflammatory mediators. These events represent the early exudative inflammatory phase and its inhibition terminates the inflammatory process[14]. Another feature of the inflammatory process is infiltration of polymorphonuclear cells into the tissue[15]. The details of information in rat paw presented in table 3 and Fig.1.

Table 3: Carrageenan induced paw edma volume (ml) of treated group with carrageenan, indomethacin, alcoholic extract of A.paniculata (100mg, 200mg, 400mg/kg bw and andrographolide 10mg, 20mg/kg bw) in albino rats.

Group		Edma (vol ml.)				
		1hr.	2hr.	3hr.	4hr.	5hr.
1	Carrageenan	1.23	1.55	1.87	2.10	2.14
2	Indomethacin	0.51	0.89	1.24	0.73	0.84
3	Extract 100mg	0.66	1.25	1.42	1.04	1.12
4	Extract 200mg	0.50	1.09	1.19	0.79	1.02
5	Extract 400mg	0.39	0.96	1.11	0.71	0.93
6	Andrographolide 10mg	0.69	1.28	1.41	1.01	1.09
7	Andrographolide 20mg	0.47	1.04	1.23	0.73	0.98

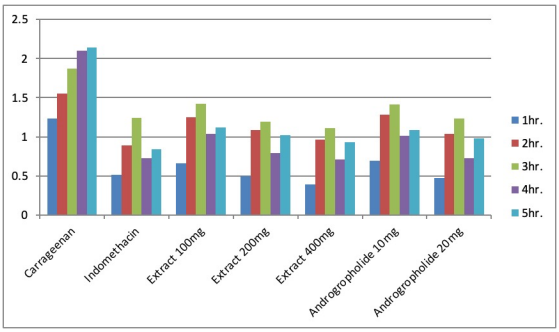

Fig.2: Carrageenan induced paw edma volume (ml) of treated group with carrageenan, indomethacin, alcoholic extract of A.paniculata (100mg, 200mg, 400mg/kg bw and andrographolide 10mg, 20mg/kg bw) in albino rats.

The result of anti-inflammatory effect is presented as change in paw edema volume (ml) in Table 3 and Fig. 1. The paw edema volume (ml) in the carrageenan group was significantly ($p<0.01$) higher as compared to other treatment groups upto 5h of observation period. As compared to carrageenan group, the paw edema volume of vehicle treated group was non-significantly differed upto 5h of observation period. The paw edema volume in the test drug groups (Alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg bw) were significantly ($p<0.01$) differed as compared to carrageenan group. The paw edema volume in positive control (Indomethacin) group was non-significantly differed with alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg groups at 3h. In this experiment the peak inflammation was observed at 3h in standard drug Indomethacin, Alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg treated groups and then it was subsided the edema volume of these three groups at 4h and to increase again at 5h. Edema formation in the rat paw is a triphasic event with involvement of several inflammatory mediators. The initial phase (during the first 2h after carrageenan injection) is attributed to the release of chemical mediators such as histamine and serotonin. The intermediate phase (2-2.5 h) of edema is due to the release of kinin, protease, and lysosome. The last phase (2.5-6h) just begins after the intermediate phase and is subsequent to the emancipation of bradykinin and prostaglandins

such as PGE2 in tissue[16]. Increase in paw volume as an index of inflammation reaches a peak of 4h following carrageenan injection and is modulated by some inhibitory molecules of the inflammatory cascade[17]. It may be due to the inhibition of chemical mediators such as histamine and serotonin at initial phase and they may contribute in its anti-inflammatory effects. Some studies have shown that inhibition of H1 and H2 receptors and histamine release, serotonin and muscarinic receptors can suppress the inflammation[15]. In support to our findings, similar observations were reported for the anti-inflammatory activity of *Punica granatum* (pomegranate) ethanolic whole fruit extracts and synthetic ellagic acid at 0.1 ml/10 g body weight in carrageenan induced paw edema in mice revealed 49.4% and 77.3% inhibition of inflammation, respectively[17]. Gupta *et al.*[19] reported that *in vitro* antiinflammatory activity of ethanol and aqueous extracts of *Terminalia bellirica* plant and ellagic acid by inhibition of heat-induced albumin denaturation[20]. Results showed that the both the extracts and active compound exhibited concentration dependent anti-inflammatory activity. Maximum inhibition of albumin denaturation by EA (86.62±0.65%) was observed at the concentration of 16.7 mg/ml while AQ and EtOH extract produced 67.57% and 77.67% inhibition, respectively at the concentration of 50mg /ml.[21].

Based on these reports, it can be inferred that the inhibitory effect of the extract and andrographolide on carrageenan induced inflammation in rat may be due to inhibition of the mediators responsible for inflammation[22]. The present study revealed that intramuscular administration of alcoholic extract 100mg, 200mg, 400mg and andrographolide 10mg, 20mg/kg showed dose-dependent *in vivo* anti-inflammatory activity against carrageenan-induced rat paw edema[23-25]. The highest anti-inflammatory activity of all three doses of extract was observed at 4h post intramuscular administration in wistar rat. Thus, it is one of the best bioactive compounds have shown *in vivo* anti-inflammatory activity[26]. However, Preliminary pharmacokinetic study of andrographolide following intramuscular administration in rat is required because this study paves the way for further attention and research to identify the active metabolite products of active constituents responsible for anti-inflammatory activity[27-28]. Additional analysis of cytokines and histo-pathological examination of rat paw tissue would be performed because it might be helpful to find out the actual molecular mechanism underlying inhibition of the first, intermediate and second phase edema is of interest and helpful in developing the new anti-inflammatory herbal compound[29].

CONCLUSION

All eight isolated compounds are active phytoconstituents of A.paniculata. The showed that Alcoholic extract and andrographolide is one of the best bioactive compounds that have shown *in vivo* anti- inflammatory activity. However, Preliminary pharmacok- inetic study of androgropholide following intramuscular administration in rats is required because this study paves the way for further attention and research to identify the active metabolite products of alcoholic extract and andrographolide responsible for anti-inflammatory activity. Additional analysis of cytokines and histo-pathological examination of rat paw tissue would be performed because it might be helpful to find out the actual molecular mechanism underlying inhibition of the first, intermediate and second phase edema is of interest and helpful in developing the new anti-inflammatory herbal compound.

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