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In vitro modulation of LPS/calcimycin induced inflammatory and allergic mediators by pure compounds of *Andrographis paniculata* (King of bitters) extract

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ABSTRACT

The aim of the current study is to probe the anti-inflammatory/anti-allergic potential of seven phytoconstituents (andrographolide, neoandrographolide, isoandrographolide, andrograpanin, 14-deoxy-11,12-didehydroandrographolide, 7-O-methylwogonin and skullcapflavone-I) isolated from *Andrographis paniculata* (King of bitters) on the production of key inflammatory/allergic mediators (NO, PGE_2, IL-1 beta, IL-6, LTB_4, TXB_2 and histamine). The results demonstrated that andrographolide, isoandrographolide, 7-O-methylwogonin and skullcapflavone-I significantly inhibited LPS stimulated NO and PGE_2 release in J774A.1 macrophages. Andrographolide, isoandrographolide and 7-O-methylwogonin showed considerable inhibition of IL-1 beta production in LPS elicited macrophages. LPS induced IL-6 production was significantly inhibited by andrographolide, isoandrographolide and skullcapflavone-I in a concentration dependent manner. The results revealed that andrographolide, isoandrographolide and skullcapflavone-I significantly decreased TXB_2 release in A23187 activated HL-60 promyelocytic cells. Furthermore, the anti-allergic properties of the phytoconstituents was investigated on A23187 induced LTB_4 production (HL-60 cells) and histamine release (RBL-2H3 basophilic cells). The results showed that only skullcapflavone-I and 7-O-methylwogonin showed marked inhibitory effect on LTB_4 production, however, only 7-O-methylwogonin exerted dose-dependent inhibition towards histamine release. Therefore, this study indicates that some of these phytoconstituents exhibit potent anti-inflammatory/anti-allergic effects by modulating different inflammatory/allergic mediators. Hence, these phytoconstituents might provide useful phytomedical treatment against variety of inflammatory and allergic disorders.

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1. Introduction

Andrographis paniculata (King of bitters) presents a strong candidature as a therapeutic anti-inflammatory and anti-allergic pharmacophore. It is known to have wide application against multitude of clinical conditions [1]. Number of active principles has been reported from this plant, which mainly includes diterpene lactones, flavonoids, polyphenols and stigmasterols [2]. The aerial part of the plant is used to extract its core components, the bicyclic diterpene lactones viz. andrographolide, isoandrographolide, neoandrographolide and 14-deoxy-11,12-didehydroandrographolide [3,4]; among these, andrographolide is the prime component which is biologically most active and constitutes about 70% of the plant extract fraction [2]. Andrograpanin is a minor compound and a hydrolysate obtained from neoandrographolide [5]. As reported by Li et al. [6] 14-hydroxy of andrographolide is rearranged to form isoandrographolide, which is chemically more stable than andrographolide. Flavones like 7-O-methylwogonin and skullcapflavone-I have been reported to be isolated from the whole plant of *A. elongata* [7].

Among the andrographolide analogues, 14-deoxy-11,12-didehydroandrographolide is known to possess immunostimulatory, anti-infective and anti-atherosclerotic activity; while, neoandrographolide possesses anti-inflammatory, anti-infective and anti-hepatotoxic properties. Further, among the less abundant compounds from *A. paniculata*, andrograpanin showed both anti-inflammatory and anti-infective; isoandrographolide displayed tumor suppressive properties; among the flavonoids 7-O-methylwogonin is known to exhibit anti-atherosclerotic property [8]. Various pharmacological investigations have revealed that some of these compounds display biological activities that are related to inflammation viz. NO inhibition [9], suppression of chemotactic migration [10], PGE_2 inhibition [11]. In our previous publication we reported the dual inhibitory (COX and LOX) property of *A. paniculata* (AP) extract (KalmCold™) containing seven phytoconstituents [andrographolide (31.3%, w/w), isoandrographolide (0.4%, w/w), neoandrographolide (3.2%, w/w), andrograpanin (0.6%, w/w), 14-deoxy-11,12-didehydroandrographolide (2.8%, w/w), skullcapflavone-I (0.05%, w/w) and 7-O-methylwogonin (0.05%, w/w)]. Wherein, we hypothesized that the anti-inflammatory activity of AP could be correlated to these phytoconstituents since the biological

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activity of the phytoconstituents mainly revolve around modulation of different inflammatory mediators [12].

Therefore in this study, we examined the inhibitory activity of seven phytoconstituents of *A. paniculata* viz. andrographolide, neoandrographolide, isoandrographolide, andrograpanin, 14-deoxy-11,12-didehydroandrographolide, 7-O-methylwogonin and skullcapflavone-I on inflammatory and allergic mediators using mammalian cells. The inhibitory activity of these compounds was studied on LPS induced NO, PGE_2, IL-1 beta and IL-6 levels in J774A.1 murine macrophages; A23187 stimulated LTB_4, TXB_2, and histamine levels in human promyelocytic leukemia (HL-60) and rat basophilic leukemia (RBL-2H3) cells respectively. Here we report for the first time, the *in vitro* anti-inflammatory and anti-allergic activity of skullcapflavone-I and 7-O-methylwogonin.

2. Materials and methods

2.1. Source of materials

Lipopolysaccharide (LPS), calcimycin (A23187), acetylsalicylic acid, MTT [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan], ketotifen fumarate, dexamethasone (D-2915), N-[[3-(aminomethyl) phenyl]methyl]-ethanimidamide (1400W) dihydrochloride, sulphanilamide, naphthyl ethylene diamine dihydrochloride (NEDD) and captopril were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Iscove's modified Dulbecco's media (IMDM), Earle's minimum essential media (EMEM) and Dulbecco's modified Eagle's medium (DMEM) were supplied by Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA).

2.2. Preparation and isolation of phytoconstituents

The phytoconstituents, andrographolide, neoandrographolide, isoandrographolide, andrograpanin, 14-deoxy-11,12-didehydroandrographolide, 7-O-methylwogonin and skullcapflavone-I were isolated from *A. paniculata* (Burm.f.) Nees as per the method described in our previous publication [13]. All the pure isolates were dissolved in DMSO and filter sterilized through 0.2 μm positively charged nylon DMSO compatible filter in order to eliminate the endotoxins. The filtered solution was then aliquoted and stored at −20 °C for use in all the *in vitro* assays. The final concentration of DMSO was up to a maximum of (0.2%), which did not show any cytotoxicity or influence the stimulant induced release of any of the inflammatory mediators. All the assays were controlled using respective reference standards.

2.3. Cell culture

J774A.1 murine macrophage cell line (TIB-67™), HL-60 human promyelocytic leukemia cell line (CCL-240™) and RBL-2H3 rat basophilic leukemia cell line (CRL-2256™) were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). J774A.1 cells were cultured in DMEM supplemented with 10% FBS, HL-60 cells were grown in IMDM supplemented with 20% FBS and RBL-2H3 cells were cultured in EMEM conditioned with 15% heat inactivated FBS (HIFBS). All the cells were maintained at 37 °C under humidified atmosphere containing 5% CO_2.

2.4. Preparation of Ringer's buffer

Ringer's buffer was prepared using following reagents: 118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 1.10 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 5.0 mM HEPES, 0.1% BSA and 11.1 mM D-glucose. The pH was maintained to 7.4.

2.5. Cell viability assay

Cell viability assay was performed as per the method described in our previous publication [12]. Based on the results, different non-cytotoxic concentrations were chosen for each study.

2.6. NO scavenging, PGE_2, and IL-6 inhibition assays

Estimation of NO, PGE_2 and IL-6 levels in the supernatant of treated/untreated cells was performed as per the method demonstrated in our prior publication [12]. In brief, J774A.1 cells were pre-incubated with different non-cytotoxic concentrations of phytoconstituents for 1 h and followed by LPS for 24 h. Post-incubation the cell supernatant was collected and analyzed for nitrite (Griess reaction assay), PGE_2 [Homogenous Time Resolved Fluorescence (HTRF) kit from CisBio (France)], IL-6 [ELISA Kit, OptEIA™ from BD biosciences (USA)] production. 1400W dihydrochloride (NO) and dexamethasone (PGE_2 and IL-6) were used as reference standards.

2.7. IL-1 beta inhibition assay

The IL-1 beta estimation was carried out as per the method described in our previous publication [12]. In brief, J774A.1 cells were pre-treated with phytoconstituents for 1 h and then stimulated with LPS (0.1 μg/mL) for 6 h. The treated cells were lysed using cell lysis buffer [0.1% Triton X-100 and protease cocktail inhibitor (1X)] in combination with repeated freeze thaw cycles. Level of IL-1 beta in the treated cell lysates was quantified using ELISA kits (R&D Systems, Minneapolis, MN. USA). Dexamethasone was used as a reference standard.

2.8. LTB_4 and TXB_2 inhibition assay

The differentiated HL-60 cells were pre-incubated with the phytoconstituents for 1 h and stimulated with A23187 (5 μM) for 15 min in Ringer's buffer [12]. The aliquots were removed from the conditioned medium and used for the quantification of LTB_4 (HTRF kit, CisBio, France) and TXB_2 (ELISA kit, Sapphire Bioscience, Australia) levels. The reference standards used for LTB_4 and TXB_2 inhibition assays were captopril and acetylsalicylic acid respectively.

2.9. Histamine inhibition assay

Effect of phytoconstituents on A23187 stimulated histamine release estimation was performed as per the method described in our previous publication [12]. Briefly, RBL-2H3 cells were incubated with various concentrations of phytoconstituents for 1 h in Ringer's buffer, prior to stimulation with A23187 (5 μM). After 30 min incubation, supernatant was collected from the conditioned medium and used for quantification of histamine as directed by the manufacturer of the kit (HTRF kit, CisBio, France). Ketotifen fumarate was used as a reference standard.

2.10. Statistical analysis

Data are represented as mean ± standard deviation (S.D) from three independent experiments. Statistical significance between groups was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego CA). In all data analysis, $P<0.05$–0.01 were considered significant. The half maximal inhibitory concentration (IC_{50}) was calculated using median effect plot [14].

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3. Results

3.1. Cytotoxicity of test substances

Cytotoxic effect of all the phytoconstituents was assessed at different concentrations by MTT assay in J774A.1, HL-60 and RBL-2H3 cells. The results showed that all the phytoconstituents were non-cytotoxic up to the maximum tested concentration under our experimental conditions. Hereafter, the non-cytotoxic concentrations were chosen for further experiments.

3.2. Effect of phytoconstituents on NO production in LPS induced cultured macrophages

Effect of the phytoconstituents on LPS (0.1 μg/mL) stimulated NO release in J774A.1 cells was determined by testing the non-cytotoxic concentrations. Andrographolide (7.1–57 μM) (Fig. 1), isoandrographolide (0.8–57 μM) (Fig. 2), skullcapflavone-I (31.5–63 μM) (Fig. 3) and 7-O-methylwogonin (16.7–67 μM) (Fig. 4) exhibited significant dose-dependent inhibition of NO release at the indicated concentrations. Andrographolide and isoandrographolide displayed 50% inhibition at a concentrations of ~21.3 μM and ~5.8 μM respectively. Maximum inhibition of 87.2% with andrographolide, 95.5% with isoandrographolide, 28.94% with 7–O–methylwogonin and 20.02% with skullcapflavone–I was observed at the highest concentrations. However, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide and andrograpanin failed to exhibit inhibitory effects towards LPS induced NO release at the tested concentrations. The reference standard, 1400W showed an IC_{50} value of ~10.7 μM.

3.3. Effect of phytoconstituents on PGE_2 production in LPS induced cultured macrophages

Inhibition of LPS (0.1 μg/mL) induced PGE_2 production in cultured macrophages was examined by testing the non-cytotoxic concentrations of the phytoconstituents. A significant dose-dependent inhibition was exhibited by andrographolide (28.5–57 μM) (Fig. 1), isoandrographolide (28.5–57 μM) (Fig. 2), skullcapflavone-I (31.5–63 μM) (Fig. 3) and 7-O-methylwogonin (16.7–67 μM) (Fig. 4) at the indicated concentrations. Andrographolide and 7-O-methylwogonin demonstrated 50% inhibition of PGE_2 production at ~23.7 μM and ~19.7 μM respectively. Maximum inhibition of 98.17% for andrographolide, 96.4% for 7-O-methylwogonin, 46.44% for isoandrographolide and 34.87% for skullcapflavone-I was obtained at the highest tested concentrations. However, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide and andrograpanin failed to exhibit inhibitory effects towards LPS induced PGE_2 release at the tested concentrations. The reference standard, dexamethasone exhibited 50% inhibition at a concentration of ~120 nM.

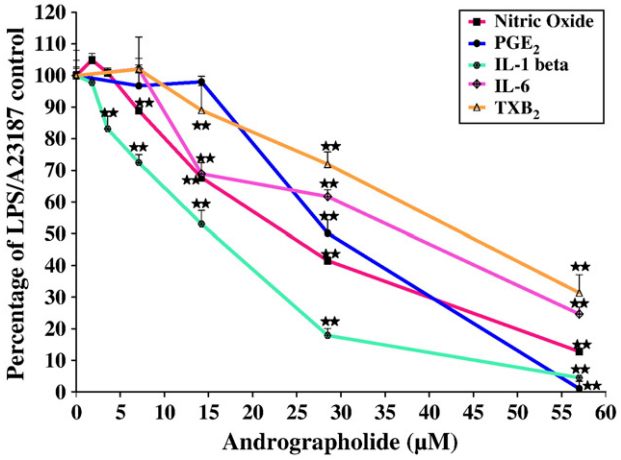

Fig. 1. Effect of andrographolide on LPS induced NO, PGE_2, IL-1 beta, IL-6 and A23187 induced TXB_2 production. Levels of NO, PGE_2, IL-1 beta and IL-6 in the supernatant was determined after stimulating J774A.1 cells with LPS in the presence or absence of andrographolide for 24 h. TXB_2 level was determined in A23187 stimulated differentiated HL-60 cells, after treating the cells with andrographolide for 1 h prior to A23187 stimulation for 15 min. Values are expressed as mean ± S.D. An asterisk indicates a significant (**$P<0.01$) difference from LPS/A23187-induced mediator release.

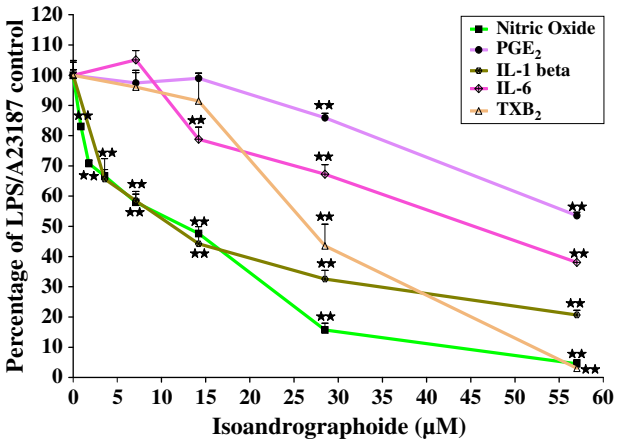

Fig. 2. Effect of isoandrographolide on LPS induced NO, PGE_2, IL-1 beta, IL-6 and A23187 induced TXB_2 production. Levels of NO, PGE_2, IL-1 beta and IL-6 in the supernatant was determined after stimulating J774A.1 cells with LPS in the presence or absence of isoandrographolide for 24 h. TXB_2 level was determined in A23187 stimulated differentiated HL-60 cells, after treating the cells with isoandrographolide for 1 h prior to A23187 stimulation for 15 min. Values are expressed as mean ± S.D. An asterisk indicates a significant (**$P<0.01$) difference from LPS/A23187-induced mediator release.

3.4. Inhibitory effect of phytoconstituents on interleukin-1beta levels in LPS induced cultured macrophages

All the phytoconstituents were examined for their ability to inhibit LPS (0.1 μg/mL) induced IL-1 beta levels in J774A.1 cells by testing the non-cytotoxic concentrations. Andrographolide (3.56–57 μM) (Fig. 1), isoandrographolide (3.56–57 μM) (Fig. 2) and 7-O-methylwogonin

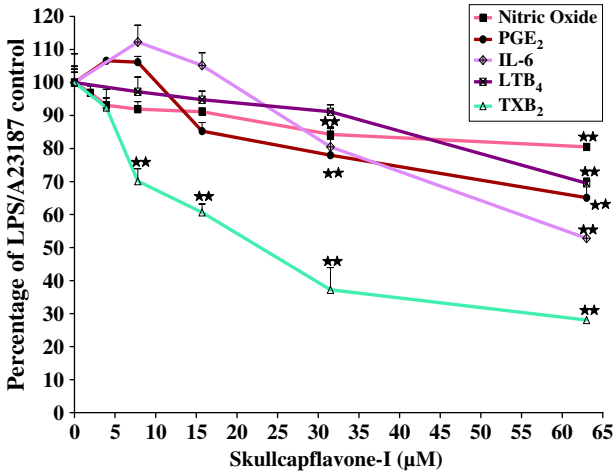

Fig. 3. Effect of skullcapflavone-I on LPS induced NO, PGE_2, IL-6 and A23187 induced LTB_4 and TXB_2 production. Levels of NO, PGE_2, and IL-6 in the supernatant was determined after stimulating J774A.1 cells with LPS in the presence or absence of skullcapflavone-I for 24 h. LTB_4 and TXB_2 levels were determined in A23187 stimulated differentiated HL-60 cells, after treating the cells with skullcapflavone-I for 1 h prior to A23187 stimulation for 15 min. Values are expressed as mean ± S.D. An asterisk indicates a significant (**$P<0.01$) difference from LPS/A23187-induced mediator release.

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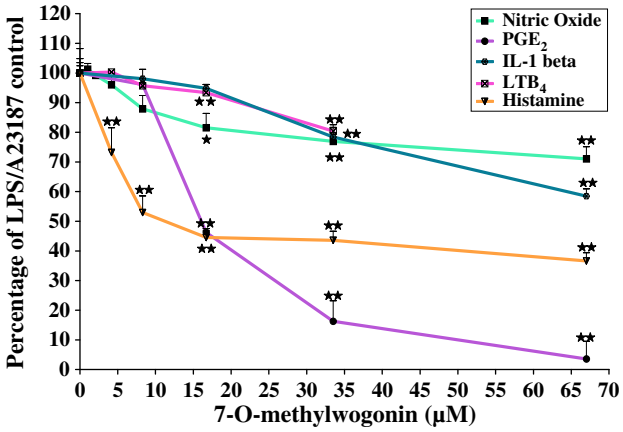

Fig. 4. Effect of 7-O-methylwogonin on LPS induced NO, PGE_2, IL-1 beta, and A23187 induced LTB_4 and histamine release. Levels of NO, PGE_2 and IL-1 beta in the supernatant was determined after stimulating J774A.1 cells with LPS in the presence or absence of 7-O-methylwogonin for 24 h. LTB_4 level was determined in A23187 stimulated differentiated HL-60 cells, after treating the cells with of 7-O-methylwogonin for 1 h prior to A23187 stimulation for 15 min. Histamine release was estimated in the supernatant, after treating RBL-2H3 cells with 7-O-methylwogonin for 1 h prior to A23187 stimulation for 30 min. Values are expressed as mean ± S.D. An asterisk indicates a significant (*$P<0.05$ and **$P<0.01$) difference from LPS/A23187-induced mediator release.

(33.5–67 μM) (Fig. 4) exhibited significant concentration dependent reduction in IL-1 beta levels at the indicated concentrations. Isoandrographolide showed more potent activity (IC_{50} ~9.9 μM), followed by andrographolide (IC_{50} ~12 μM). Isoandrographolide and andrographolide demonstrated maximum inhibition of 79.3% and 95.5% at the highest tested concentration respectively. 7-O-methylwogonin showed a maximum inhibition of 41.3% at the highest concentration. Nevertheless, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide, andrograpanin and skullcapflavone-I failed to demonstrate inhibitory effects towards LPS induced IL-1 beta release at the tested concentrations. The reference standard, dexamethasone showed an IC_{50} value of ~70 nM.

3.5. Inhibitory effect of phytoconstituents on interleukin-6 levels in LPS induced cultured macrophages

Inhibitory effect of LPS (0.1 μg/mL) induced IL-6 levels in J774A.1 cells was determined by testing the non-cytotoxic concentrations of the phytoconstituents. Significant dose-dependent reduction in the levels of IL-6 was shown by andrographolide (14.2–57 μM) (Fig. 1), isoandrographolide (14.2–57 μM) (Fig. 2) and skullcapflavone-I (63 μM) (Fig. 3) at the indicated concentrations. The IC_{50} values indicated that, andrographolide (~33.3 μM) was most potent, followed by isoandrographolide (~43 μM). The maximum inhibition of 75.3% for andrographolide, 62% for isoandrographolide and 47.19% for skullcapflavone-I was obtained at the highest concentrations. But the other phytoconstituents 14-deoxy-11,12-didehydroandrographolide, neoandrographolide, andrograpanin and 7-O-methylwogonin failed to inhibit LPS induced IL-6 release at the tested concentrations. The reference standard, dexamethasone showed an IC_{50} value of ~58 nM.

3.6. Modulatory effect of phytoconstituents on LTB_4 production in A23187 induced HL-60 promyelocytic leukemia cells

Inhibition of A23187 (5 μM) induced LTB_4 production was examined in all the phytoconstituents at various non-cytotoxic concentrations. Skullcapflavone-I and 7-O-methylwogonin displayed moderate inhibition of LTB_4 levels with significance obtained at the highest concentrations of 63 μM and 33.5 μM respectively (Figs. 3, 4). Skullcapflavone-I and 7-O-methylwogonin showed maximum inhibition of 30.5% and 19.6% at the highest tested concentration respectively. However, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide, isoandrographolide, andrographolide and andrograpanin failed to exhibit inhibitory effect on LTB_4 production at the tested concentrations. The reference standard, captopril was found to have an IC_{50} value of ~48 μM.

3.7. Suppression of TXB_2 release by phytoconstituents in A23187 induced HL-60 promyelocytic leukemia cells

HL-60 cells were stimulated by A23187 (5 μM) to augment the production of TXB_2. Significant reduction in TXB_2 levels was displayed by andrographolide (28.5–57 μM) (Fig. 1), isoandrographolide (28.5–57 μM) (Fig. 2) and skullcapflavone-I (7.8–63 μM) (Fig. 3) at the indicated non-cytotoxic concentrations. The IC_{50} values of andrographolide, isoandrographolide and skullcapflavone-I were found to be ~45 μM, ~25.4 μM and ~27.7 μM respectively. Maximum inhibition of 68.6% for andrographolide, 97% for isoandrographolide and 72% for skullcapflavone-I was attained. The other tested phytoconstituents 14-deoxy-11,12-didehydroandrographolide, neoandrographolide, 7-O-methylwogonin and andrograpanin failed to exhibit inhibitory effect on TXB_2 production at the tested concentrations. The reference standard, aspirin displayed an IC_{50} value of ~5.5 μM.

3.8. Inhibition of A23187 induced histamine release in RBL-2H3 rat basophilic leukemic cells

RBL-2H3 cells were stimulated with A23187 (5 μM) to enhance the release of histamine. The phytoconstituents were tested for their ability to inhibit A23187 induced histamine release at various non-cytotoxic concentrations. The results demonstrated that only 7-O-methylwogonin (4.18–67 μM) potently inhibited histamine release at the indicated concentrations (Fig. 4). 50% inhibition was observed at a concentration of ~17.8 μM, with a maximum inhibition of 63.3%. The reference standard, ketotifen fumarate demonstrated an IC_{50} value of ~36.4 μM.

4. Discussion

The pharmacological aspects of *A. paniculata* have been well-documented [15]. In the present study, we have examined the inhibitory potential of seven phytoconstituents isolated from *A. paniculata* on different inflammatory and allergic mediators. Results showed that andrographolide, isoandrographolide, 7-O-methylwogonin and skullcapflavone-I actively inhibited NO and PGE_2 production in LPS stimulated J774A.1 cells. Inhibitory activity of andrographolide on LPS induced NO and PGE_2 production are possibly by attenuating the expression of inducible nitric-oxide synthase and cyclooxygenase-2 proteins respectively [16]. On comparing the IC_{50} values of the phytoconstituents, we observed that isoandrographolide was most potent and interestingly it was found equipotent to 1400W dihydrochloride in inhibiting LPS induced NO production. Further, among the seven phytoconstituents, 7-O-methylwogonin was most potent in inhibiting PGE_2 production, but it showed lesser potency than dexamethasone. However, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide and andrograpanin failed to exhibit inhibitory effects towards NO and PGE_2 release at the tested concentrations. Although a study by Liu et al., 2008 has reported significant inhibitory effect of andrograpanin on NO release at higher concentrations (30–90 μM) in bone marrow derived macrophages [17]. Another study reported that neoandrographolide inhibited LPS stimulated NO and PGE_2 release in bone marrow derived murine macrophages [11]. Nevertheless, several factors such as higher concentrations, longer incubation time with phytoconstituents, methodology used and different cell types utilized may be involved in the contradictory results.

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NF-kappa B is known to play a critical role in the regulation of genes mediating various inflammatory and immune responses, including NOS-2, COX-2, and TNF-alpha [18,19]. In this study we observed that LPS stimulated IL-1 beta production was inhibited by andrographolide, isoandrographolide and 7-O-methylwogonin while LPS stimulated IL-6 production was inhibited by andrographolide, isoandrographolide and skullcapflavone-I. This inhibitory effect on pro-inflammatory mediators might have resulted through modulation of NF-kappa B-dependent gene transcription. Further, the IC_{50} values showed that isoandrographolide and andrographolide were equipotent in inhibiting IL-1 beta and IL-6 production; however showed lesser potency than dexamethasone. Some studies have shown that andrographolide, its derivatives and andrograpanin showed potent inhibitory effect towards different pro-inflammatory cytokines viz. IL-1 beta, IL-6, TNF-alpha and GM-CSF [6,17,20,21]. Tsai et al. reported that, andrographolide significantly attenuated phosphorylation of ERK1/2 and its upstream activators, MAP kinase-ERK kinase (MEK1/2) in C5a-induced RAW264.7 macrophages and also strongly blocked Akt phosphorylation which is a down-stream target protein for phosphatidylinositol-3-kinase (PI3K) [10]. These reports also suggested that the suppression of cytokine release may, in part, be attributed to the down-regulation of intracellular signaling pathways of COX-2 which may be proposed as an alternative mechanism. On the other hand, neoandrographolide, andrograpanin and 14-deoxy-11,12-didehydroandrographolide did not exhibit any inhibitory effect on LPS induced IL-1 beta and IL-6 release.

At the cellular level, the poly unsaturated fatty acids (PUFAs) liberated from the membrane phospholipids act as substrates for the enzymes COX and LOX and their resulting metabolites, viz., eicosonoids (prostaglandins and leukotrienes) which are involved in various inflammatory and allergic disorders. Hence, modulation of AA metabolism by inhibiting either of these enzymes can be considered effective in controlling several inflammatory diseases [22]. In our study, andrographolide, isoandrographolide and skullcapflavone-I elicited potent inhibitory effect towards A23187 stimulated TXB_2 release in differentiated HL-60 cells. Simultaneous inhibition of PGE_2 and TXB_2 production indicates that the inhibitory effect might have occurred at the COX level. However, despite strongly inhibiting the PGE_2 release, 7-O-methylwogonin, did not show any inhibitory effect towards TXB_2 production indicating that it probably inhibited PGE_2 production at the PG synthases level and not COX level and hence it has no effect on TX synthase. On comparing the IC_{50} values isoandrographolide and skullcapflavone-I displayed equipotent inhibitory effect on TXB_2 production than andrographolide, although, both the compounds exhibited lesser potency than aspirin. Nevertheless, neoandrographolide, andrograpanin and 14-deoxy-11,12-didehydroandrographolide failed to inhibit TXB_2 release at the tested concentrations.

The anti-allergic property of the phytoconstituents was examined by their ability to inhibit A23187 induced LTB_4 and histamine release in HL-60 and RBL-2H3 cells respectively. Skullcapflavone-I showed moderate inhibition of LTB_4 release at the highest concentration, while 7-O-methylwogonin inhibited both LTB_4 and histamine release. However, 7-O-methylwogonin was more potent in inhibiting histamine release (with an IC_{50} value of ~17.8 μM) and showed nearly three fold higher potency than ketotifen fumarate. But both the flavones showed lesser potency than captopril in inhibiting LTB_4 levels. In addition, skullcapflavone-I showed inhibitory propensity towards PGE_2, TXB_2 and LTB_4, so it can be anticipated that the modulating effect probably occurred at the phospholipaseA2 (PLA2) enzymatic level (the enzyme that catalyzes the formation of AA pathway metabolites, COX and LOX). Further, we observed that andrographolide, neoandrographolide, isoandrographolide, andrograpanin and 14-deoxy-11,12-didehydroandrographolide failed to demonstrate inhibitory property on release of any of the allergic mediators at the tested concentrations. In this study, andrographolide was tested up to a maximum concentration of 57 μM and it did not show any inhibitory effect towards LTB_4 and histamine release. This result is consistent with the findings of Amroyan et al. which stated that andrographolide does not influence biosynthesis of any lipoxygenase pathway products such as LTB_4, 6E-LTB_4 etc. [23]. However, an *in vitro* and *in vivo* study has reported that andrographolide at higher concentrations of 85.5–855 μM showed significant inhibition in histamine release in mesenteric mast cells [24].

In our recent study [12], we demonstrated the dual inhibitory (COX and LOX) property of *A. paniculata* extract (KalmCold™). Now, in view of the present results we contemplate that the PGE_2 inhibitory effect of AP extract correlates to the potent activity of andrographolide, isoandrographolide, 7-O-methylwogonin and skullcapflavone-I. Similarly, the TXB_2 alleviating propensity of the extract can be attributed to andrographolide, isoandrographolide and skullcapflavone-I. Likewise, the suppressive tendency of the extract towards LTB_4 production was influenced by 7-O-methylwogonin and skullcapflavone-I. Surprisingly, the AP extract failed to show any modulatory effect towards histamine release [12], but 7-O-methylwogonin as an individual showed significant inhibitory effect on histamine levels. The lack of inhibitory effect towards A23187 induced histamine release might have resulted due to the presence of very low levels of 7-O-methylwogonin (0.05%, w/w) in the AP extract. Based on the outcome of our experiments AP extract exhibited poly-pharmacological action which may be due to the presence of these phytoconstituents individually or synergistically. Hence, usage of plant extracts containing mixture of phytoconstituents scores over individual active phytoconstituents in treating various diseases [25].

In conclusion, this study illustrates for the first time that other than andrographolide; isoandrographolide, skullcapflavone-I and 7-O-methylwogonin also exhibit potent modulating response towards different inflammatory and allergic mediators. However, the immunomodulatory activity of these phytoconstituents may mediate through multiple mechanisms, which require further elucidation. Besides, this study also suggests that studying individual active principles present in extract allows distinguishing the exact bioactive component responsible for the pharmacological property of the crude extract. It also offers significant opportunities in finding novel low molecular weight lead compounds. Hence, these phytoconstituents can be used as promising leads in development or synthesis of the new anti-inflammatory/allergic drugs.

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References

[1] Coon JT, Ernst E. *Andrographis paniculata* in the treatment of upper respiratory tract infections: a systemic review of safety and efficacy. Planta Med 2004;70:293–8.
[2] Siripong P, Konckathip B, Preechanukool K, Picha P, Tunsuwan K, Taylor WC. Cytotoxic diterpenoid constituents from *Andrographis paniculata* Nees leaves. J Sci Soc Thailand 1992;18:187–94.
[3] Pholphana N, Rangkadilok N, Thongnest S, Ruchirawat S, Ruchirawat M, Satayavivad J. Determination and variation of three active diterpenoids in *Andrographis paniculata* (Burm.f.) Nees. Phytochem Anal 2004;15:365–71.
[4] Pramanick S, Banerjee S, Achari B, Das B, Sen AK, Mukhopadhyay S, et al. Andrographolide and isoandrographolide, minor diterpenoids from *Andrographis paniculata*: structure and X-ray crystallographic analysis. J Nat Prod 2006;69:403–5.
[5] Ji LL, Wang Z, Dong F, Zhang WB, Wang ZT. Andrograpanin, a compound isolated from anti-inflammatory traditional Chinese medicine *Andrographis paniculata*, enhances chemokine SDF-1alpha-induced leukocytes chemotaxis. J Cell Biochem 2005;95:970–8.
[6] Li J, Huang W, Zhang H, Wang X, Zhou H. Synthesis of andrographolide derivatives and their TNF-alpha and IL-6 expression inhibitory activities. Bioorg Med Chem Lett 2007;17:6891–4.
[7] Jayakrishna G, Harikishore P, Rao CV, Gunasekar D, Blond A, Bodo B. Two new 2-oxygenated flavones from *Andrographis elongata*. Chem Pharm Bull 2001;49:1555–7.

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[8] Chao WW, Lin BF. Review isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). Chin Med 2010;5:17.
[9] Chiou WF, Chen CF, Lin JJ. Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW264.7 cells by andrographolide. Br J Pharmacol 2000;129:1553–60.
[10] Tsai HR, Yang LM, Tsai WJ, Chiou WF. Andrographolide acts through inhibition of ERK1/2 and Akt phosphorylation to suppress chemotactic migration. Eur J Pharmacol 2004;498:45–52.
[11] Liu J, Wang ZT, Ge BX. Inhibitory effects of neoandrographolide on nitric oxide and prostaglandin E_2 production in LPS-stimulated murine macrophage. Mol Cell Biochem 2007;298:49–57.
[12] Chandrasekaran CV, Anumita G, Amit A. Effect of an extract of *Andrographis paniculata* leaves on inflammatory and allergic mediators *in vitro*. J Ethnopharmacol 2010;129:203–7.
[13] Chandrasekaran CV, Thiyagarajan P, Sundarajan K, Goudar KS, Deepak M, Murali B, et al. Evaluation of the genotoxic potential and acute oral toxicity of standardized extract of *Andrographis paniculata* (KalmCold™). Food Chem Toxicol 2009;47:1892–902.
[14] Wolfe KL, Liu RH. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. J Agric Food Chem 2007;55:8896–907.
[15] Jarukamjorn K, Nemoto N. Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent Andrographolide. J Health Sci 2008;54:370–81.
[16] Wang T, Liu B, Zhang W, Wilson B, Hong JS. Andrographolide reduces inflammation mediated dopaminergic neurodegeneration in mesencephalic neuron-glia cultures by inhibiting microglial activation. J Pharmacol Exp Ther 2004;308:975–83.
[17] Liu J, Wang ZT, Ge BX. Andrograpanin isolated from *Andrographis paniculata*, exhibits anti-inflammatory property in lipopolysaccharide-induced macrophage cells through down-regulating the p38 MAPKs signaling pathways. Int Immunopharmacol 2008;8:951–8.
[18] Kim Y, Fischer SM. Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. J Biol Chem 1998;273:27686–94.
[19] Ghosh S, Karin M. Missing pieces in the NF-kappa B puzzle. Cell 2002;109(Suppl):S81–96.
[20] Sheeja K, Guruvayoorappan C, Kuttan G. Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. Int Immunopharmacol 2007;7:211–21.
[21] Abu-Ghefreh AA, Canatan H, Ezeamuzie I. *In vitro* and *in vivo* anti-inflammatory effects of andrographolide. Int Immunopharmacol 2009;9:313–8.
[22] Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective nonsteroidal anti-inflammatory drugs. Ann Rheum Dis 2003;62:501–9.
[23] Amroyan E, Gabrielian E, Panossian A, Wikman G, Wagner H. Inhibitory effect of andrographolide from *Andrographis paniculata* on PAF-induced platelet aggregation. Phytomedicine 1999;6:27–31.
[24] Madav S, Tripathi HC, Tandan SK, Dinesh K, Lal J. Antiallergic activity of andrographolide. Ind J Pharm Sci 1998;60:176–8.
[25] Williamson EM. Synergy and other interactions in phytomedicines. Phytomedicine 2001;8:401–9.