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Abstract

Ethnopharmacological relevance Seven studied medicinal plants; *Aconitum laciniatum*, *Ajania nubigena*, *Codonopsis bhutanica*, *Corydalis crispera*, *Corydalis dubia*, *Meconopsis simplicifolia* and *Pleurospermum amabile*, are currently used in the Bhutanese Traditional Medicine (BTM) for the management of different types of disorders including the diseases that bore relevance to various inflammatory conditions.

Aims of the study This study aimed to evaluate the inhibition of TNF- α production in LPS-activated THP-1 monocytic cells by the crude extracts of seven selected Bhutanese medicinal plants. It is expected to; (a) generate a scientific basis for their use in the BTM and (b) form a basis for prioritization of the seven plants for further phytochemical and anti-inflammatory studies.

Materials and methods Seven plants were selected using an ethno-directed bio-rational approach and their crude extracts were prepared using four different solvents (methanol, hexane, dichloromethane and chloroform). The TNF- α inhibitory activity of these extracts was determined by cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs). The results were quantified statistically and the statistical significance were evaluated by GraphPad Prism version 5.01 using Student's t-test with one-tailed distribution. A p-value ≤ 0.05 was considered statistically significant.

Results Of the seven plants studied, the crude extracts of six of them inhibited the production of pro-inflammatory cytokine, TNF- α in LPS-activated THP-1 monocytic cells. Amongst the six plants, *Corydalis crispera* gave the best inhibitory activity followed by *Pleurospermum amabile*, *Ajania nubigena*, *Corydalis dubia*, *Meconopsis simplicifolia* and *Codonopsis bhutanica*. Of the 13 extracts that exhibited statistically significant TNF- α inhibitory activity ($p < 0.05$; $p < 0.01$), five of them showed very strong inhibition when compared to the DMSO control and RPMI media.

Conclusions Six medicinal plants studied here showed promising TNF- α inhibitory activity. These findings rationalize the traditional use of these selected medicinal plants in the BTM as an individual plant or in combination with other ingredients for the treatment of disorders bearing relevance to the inflammatory conditions. The results forms a good preliminary basis for the prioritization of candidate plant species for an in-depth phytochemical study and anti-inflammatory activity screening of the pure compounds contained within those seven plants.

Keywords

activated, tnf, thp, 1, monocytic, cells, crude, extracts, seven, bhutanese, medicinal, plants, production, lps, inhibition, CMMB

Disciplines

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Inhibition of TNF- α production in LPS-activated THP-1 monocytic cells by the crude extracts of seven Bhutanese medicinal plants

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ABSTRACT

Ethnopharmacological relevance

Seven studied medicinal plants; *Aconitum laciniatum*, *Ajania nubigena*, *Codonopsis bhutanica*, *Corydalis crispa*, *Corydalis dubia*, *Meconopsis simplicifolia* and *Pleurospermum amabile*, are currently used in the Bhutanese Traditional Medicine (BTM) for the management of different types of disorders including the diseases that bore relevance to various inflammatory conditions.

Aims of the study

This study aimed to evaluate the inhibition of TNF- α production in LPS-activated THP-1 monocytic cells by the crude extracts of seven selected Bhutanese medicinal plants. It is expected to: a) generate a scientific basis for their use in the BTM and b) form a basis for prioritization of the seven plants for further phytochemical and anti-inflammatory studies.

Materials and methods

Seven plants were selected using an ethno-directed bio-rational approach and their crude extracts were prepared using four different solvents (methanol, hexane, dichloromethane and chloroform). The TNF- α inhibitory activity of these extracts was determined by cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs). The results were quantified statistically and the statistical significance were evaluated by GraphPad Prism version 5.01 using Student's *t*-test with one-tailed distribution. A *p*-value ≤ 0.05 was considered statistically significant.

Results

Of the seven plants studied, the crude extracts of six of them inhibited the production of pro-inflammatory cytokine, TNF- α in LPS-activated THP-1 monocytic cells. Amongst the six plants, *C. crispera* gave the best inhibitory activity followed by *P. amabile*, *A. nubigena*, *C. dubia*, *M. simplicifolia* and *C. bhutanica*. Of the 13 extracts that exhibited statistically significant TNF- α inhibitory activity ($p < 0.05$; $p < 0.01$), five of them showed very strong inhibition when compared to the DMSO control and RPMI media.

Conclusions

Six medicinal plants studied here showed promising TNF- α inhibitory activity. These findings rationalize the traditional use of these selected medicinal plants in the BTM as an individual plant or in combination with other ingredients for the treatment of disorders bearing relevance to the inflammatory conditions. The results form a good preliminary basis for the prioritization of candidate plant species for an in-depth phytochemical study and anti-inflammatory activity screening of the pure compounds contained within those seven plants.

Keywords: Bhutanese traditional medicine; medicinal plants; TNF- α inhibition; anti-inflammatory.

1. Introduction

One of the important mediators that regulates biochemical changes and the symptomatic pathophysiological responses in a body is a pro-inflammatory cytokine known as tumor necrosis factor alpha (TNF- α) which is produced by monocytes, macrophages and other types of cells. (Jang et al., 2004; Dey et al., 2008; Kang et al., 2009). Its over production by these cells causes a wide range of human diseases (Rose et al., 2005; Dey et al., 2008). The inhibition of TNF- α in LPS activated THP-1 monocytic cells is generally used as an *in vitro* model for evaluating the anti-inflammatory effects of various materials including plant extracts. The plant extracts are known to exhibit various anti-inflammatory activities including the inhibition of the pro-inflammatory cytokine TNF- α . For example, the extract of a feverfew plant was found to effectively reduce LPS-mediated TNF- α and monocyte chemoattractant protein-1 (MCP-1) released by THP-1 cells (Chen and Cheng, 2009). Similarly, the ethanol extract of *Cryptolepis buchanani* significantly inhibited TNF- α production in LPS-stimulated monocytic THP-1 cells (Laupattarakasem et al., 2006).

Our study on the inhibition of TNF- α production in LPS-activated THP-1 monocytic cells involved the crude extracts of seven medicinal plants: *Aconitum laciniatum* (Ranunculaceae), *Ajania nubigena* (Compositae), *Codonopsis bhutanica* (Campanulaceae), *Corydalis crispa* (Fumariaceae), *Corydalis dubia* (Fumariaceae), *Meconopsis simplicifolia* (Papaveraceae) and *Pleurospermum amabile* (Umbelliferae) used in the preparation of various polyherbal formulations of the Bhutanese traditional medicine (BTM). These formulations were prepared by mixing them with other ingredients and making them into pills, tablets, capsules and other dosage forms as reported in Wangchuk et al. (2011). These plants contained different types of major classes of phytochemicals (established based on the TLC

profile and phytochemical test protocols) and possess antimalarial, antimicrobial, anti-*Trypanosoma brucei rhodesiense* and cytotoxicity effects (Wangchuk et al., 2011).

However, no anti-inflammatory study have been carried out on these plants although their ethnopharmacological uses (see Table 1) bear relevance to various inflammatory conditions. For example, the common therapeutic indications of these medicinal plants (see Table 1) was for treating *chin-tshad* which is a traditional ethnomedical term used to describe fever associated with liver disorders. It is to be understood in the context of liver inflammation since it is characterized by *tsha-ba* (pain) with a rise in body temperature (Wangchuk et al., 2011). Scientifically, liver inflammation occurs when the hepatic Kupffer cells (macrophages in the liver derived from monocyte) of the liver respond to foreign antigens by regulating the pro-inflammatory cytokine, TNF- α . Its over production can trigger the production of reactive oxygen species (ROS) and for this reason TNF- α has been implicated as a key mediator that plays an important role in inflammation and pathogenesis of many forms of liver diseases, often associated with fever.

Within these understandings, the crude extracts of the seven plants, with various therapeutic indications bearing relevance to the symptoms of inflammatory conditions, were subjected to screening for their inhibitory effects on the production of TNF- α cytokine in LPS-activated THP-1 monocytic cells. Through this study, it was expected to generate a scientific basis for their use in the BTM polyherbal formulations and also to find a basis for prioritization of the seven plants for our in-depth phytochemical and their anti-inflammatory studies in the future.

2. Materials and methods

2.1. Plant materials and preparation of crude extracts

Seven medicinal plants of Bhutan; *A. laciniatum* (Ranunculaceae), *A. nubigena* (Compositae), *C. bhutanica* (Campanulaceae), *C. crispa* (Fumariaceae), *C. dubia* (Fumariaceae), *M. simplicifolia* (Papaveraceae) and *P. amabile* (Umbelliferae) were selected based on the selection criteria described by Wangchuk et. al. (2011). The ethnopharmacological uses of these plants (Phuntshok, 1994; Wangchuk et. al., 2011) are described in Table 1. These selected plants were collected from Lingzhi within the altitudinal ranges of 2000 to 6000 metres above sea level in the period of June to December 2009. Herbarium specimens were prepared and preserved at the Pharmaceutical and Research Unit (PRU) in Bhutan after assigning a voucher specimen number (see Table 1) (Wangchuk et al., 2011).

The dried plant material (2 kg) was chopped into small pieces and then repeatedly extracted with methanol over a period of four days to obtain the concentrated viscous methanol extracts of each plant (see Table 1 for their weights). A small portion of the crude methanol extract (10 g each) was then acidified with HCl (5%) and extracted with hexane and dichloromethane (5 x 60 mL of each solvent) to obtain their respective crude extracts (Table 2). The remaining acidified aqueous solution was then basified (pH 9-11) with NH₄OH solution and then extracted with CHCl₃ (5 × 60 mL) to yield the chloroform extracts of each plant (Table 2). Small portions of these different solvent crude extracts of each plant were then evaluated for their inhibitory effects on the TNF- α production in LPS-activated THP-1 monocytic cells.

Table 1

Seven selected Bhutanese medicinal plants with their botanical names, ethnopharmacological uses and associated information.

Botanical name	g.so-ba-rig-pa name	V.No.*	Altitude (meters)	Distribution	Part used	MeOH extract**	Ethnopharmacological uses (Wangchuk et al, 2008; Wangchuk et al., 2011)
<i>Aconitum laciniatum</i> Stapf. (Ranunculaceae)	b.san-dug	93	3500-4570	Himalayas	Tuber, leaf, flower	165.0	Anthelmintic. Allays leprosy (<i>Klus-nad</i>), bone diseases, mumps, gout and chronic infections.
<i>Ajania nubigena</i> DC. (Compositae)	'khen-d.kar	73	3600-4800	Bhutan and India	Aerial	80.1	Allays abscess, swelling of limbs, benign tumor and kidney infection. It is vulnerary, expectorant, styptic and anti-epistaxis.
<i>Codonopsis bhutanica</i> Ludlow (Campanulaceae)	klu-b.dud-rdor-rje-nag-po	71	4200-4800	Endemic to Bhutan	Aerial	170.1	Allays leprosy, evil spirit afflictions (<i>g.don-nad</i>), tingling, nephritis, numbness, gout and blood infections.
<i>Corydalis crispa</i> Prain (Fumariaceae)	sngo-ba-sha-ka	78	4000-4600	Himalayas	Whole		Allays blood, liver and bile disorders and useful for tuberculosis (<i>bad-d.kan-mug-po</i>).
<i>Corydalis dubia</i> Prain (Fumariaceae)	re-skon	14	4500-4900	Himalayas	Whole	65.1	Allays fever (<i>Chin-tshad</i>) arising from the liver, heart, lung, pancreas and kidney infections and also useful for neuralgia and tuberculosis (<i>bad-d.kan-mug-po</i>). Detoxify impure blood.
<i>Meconopsis simplicifolia</i> Walpers (Papaveraceae)	up-pel-sngon-po	2	3500-4600	Bhutan, Nepal and Tibet	Aerial	43.1	Allays fever (<i>Chin-tshad</i>) associated with cough and cold and malaria. Also heals the blood, liver and the lung infections.
<i>Pleurospermum amabile</i> Craib. (Umbelliferae)	Rtsad	29	3950-4700	Himalayas	Aerial	190.0	Anti-dote, febrifuge and useful for dyspepsia.

Note: *Voucher specimen number, **Weight of methanol extracts (in g) obtained from 2 kg of dried powdered materials.

2.2. Bioassay method

Based on the ethnopharmacological uses of the plants, the crude extracts of the seven Bhutanese medicinal plants were used to determine the inhibition of TNF- α production in LPS-activated THP-1 monocytic cells by *in vitro* bioassay method as detailed in Wangchuk et al. (2012a). A total of 28 plant extracts (20 mg each), obtained using four different solvents (methanol, hexane, dichloromethane and chloroform) from the seven plants (Table 1) were used to determine inhibition of TNF- α production in LPS-activated THP-1 human monocytic cells. The hexane extract of *C. dubia* was not tested due to its poor solubility. All water extracts contained salts formed from the acid-base extraction process (see extraction methods above) and were therefore not tested.

The cell viability was studied for all the test samples and was determined from the ratio of viable cells over total cells. The percentage of cell viability was calculated by the formula: % cell viability = $100 \times (1 - (\text{dead cells} \div \text{total cells}))$ and all the samples gave the % cell viability of $\geq 90\%$. TNF- α production in THP-1 cell culture supernatants were measured using the cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R & D Systems, USA). Absorbance was measured at 450 nm using a BioTek[®] Synergy[™] HT (Multi-Detection Microplate Reader, USA). A commercial anti-inflammatory drug, dexamethasone (1 $\mu\text{g/mL}$), (Atlantic LabsComp. Ltd., Thailand) was used as a positive control. The experiments were performed three times in triplicate (3x3). The percentage of TNF- α inhibition was calculated by the formula: % TNF- α inhibition = $100 \times ((\text{observed secreted TNF-}\alpha \text{ of experiment (pg/mL)} \div \text{baseline secreted TNF-}\alpha \text{ (DMSO) (pg/mL)} - 1))$. The statistical significance or differences

were evaluated by GraphPad Prism version 5.01 using Student's *t*-test with one-tailed distribution. A *p*-value ≤ 0.05 was considered statistically significant.

3. Results and discussion

Usually, prior to the TNF- α bioassay, the concentration of test sample needs to be optimized through cell viability study and this optimization was carried out on one representative test sample, the MeOH extract of *C. dubia* in varying concentrations (0-200 $\mu\text{g/mL}$). At 50 $\mu\text{g/mL}$, there was no significant cell reduction/death and had the highest activity and thus, this dose/concentration was selected as the optimum concentration for determining the inhibitory activity of the other 27 crude extracts on TNF- α production in LPS-activated THP-1 cells. This further established that their inhibition of TNF- α production was not associated with the cytotoxic effects to THP-1 cells. In addition, the THP-1 cells in RPMI and THP-1 cells incubated with the crude plant extracts alone (without LPS) did not activate TNF- α production (data not shown) which established that their inhibition of TNF- α production was not associated with the cytotoxic effects to THP-1 cells. Usually, the secretion values of TNF- α of resting cells are very low at 0-<0.5 pg/mL . In the previous study where the cytotoxicity was determined on the normal Vero cells from kidney of African green monkey, *Cecopithecus aethiops* and the human oral carcinoma KB cells, only the chloroform extract of *C. crispa* was reported to exhibit moderate cytotoxicity against KB cells and rest of the crude extracts were found to be non-cytotoxic (Wangchuk et al., 2011).

The results of the inhibition of TNF- α secretion and the percentage of TNF- α inhibition of the 27 crude extracts including that of reported *C. crispa* (for easy comparison) (Wangchuk et al., 2012a) by THP-1 monocytic cells is listed in Table 2. In the presence of each of the

plant extracts, TNF- α production was mostly inhibited and in a few cases, stimulated. Of the seven plants studied, the crude extracts of six of them inhibited the production of TNF- α cytokine with varying degrees and effects. Amongst the six plants, *C. crispera* gave the best inhibitory activity followed by *P. amabile*, *A. nubigena*, *C. dubia*, *M. simplicifolia* and *C. bhutanica* (Table 2). The hexane and dichloromethane extracts of *C. crispera* (reported with pure compounds in Wangchuk et al., 2012a) displayed the most potent TNF- α inhibitory activity (see Table 2) by inhibiting/suppressing the TNF- α production to the level of 142 pg/mL (77% inhibition) and 71 pg/mL (89% inhibition), respectively.

Analysis of statistical significance or variances using Student's *t*-test with one-tailed distribution showed that these two crude extracts had the significance *p*-value of $p < 0.01$ indicative of the content of good anti-TNF- α compounds. The in-depth phytochemical analysis of these crude extracts resulted in the isolation of nine alkaloids as protopine, 13-oxoprotopine, 13-oxocryptopine, stylophine, coreximine, rheagenine, ochrobirine, sibiricine and bicuculline (Wangchuk et al., 2012a). Unlike its parent crude extracts, none of the tested pure alkaloids gave significant anti-TNF- α activities (Wangchuk et al., 2012a) which suggested that there must be some other minor or non-alkaloid components in the crude extracts responsible for the significant anti-TNF- α activities.

Similarly, the hexane and dichloromethane extracts of *P. amabile* showed significant TNF- α inhibitory activity with the production values of 206 pg/mL (67% inhibition) and 300 pg/mL (52% inhibition), respectively. The methanol and chloroform extracts of *C. dubia* displayed good TNF- α inhibitory activity with the suppression of TNF- α production to the level of 236 pg/mL (62% inhibition) and 258 pg/mL (58% inhibition), respectively.

Table 2

Inhibition of TNF- α production in LPS-activated THP-1 monocytic cells by different solvent extracts of seven Bhutanese medicinal plants.

Plant name	Controls and crude extracts	Extract yields*	Level of TNF- α production**	% inhibition/ stimulation
Controls	RPMI ^a		502 \pm 43	Media
	DMSO ^b		620 \pm 54	Solvent
	Dexamethasone ^c		258 \pm 65	-58 [‡]
<i>A. laciniatum</i>	Methanol	NA	582 \pm 20	-6
	Hexane	79	763 \pm 74	23
	Dichloromethane	460	820 \pm 35	32
	Chloroform	134	806 \pm 81	30
<i>A. nubigena</i>	Methanol	NA	399 \pm 28	-36 [†]
	Hexane	218	614 \pm 34	-1
	Dichloromethane	420	220 \pm 1	-65 [‡]
	Chloroform	186	381 \pm 65	-39 [†]
<i>C. bhutanica</i>	Methanol	NA	726 \pm 66	17
	Hexane	535	570 \pm 14	-8
	Dichloromethane	318	503 \pm 42	-19
	Chloroform	72	422 \pm 22	-32 [†]
<i>C. crispa</i> ^{RR}	Methanol	NA	778 \pm 3	25
	Hexane	340	142 \pm 17	-77 [‡]
	Dichloromethane	475	71 \pm 17	-89 [‡]
	Chloroform	150	284 \pm 102	-54 [†]
<i>C. dubia</i>	Methanol	NA	236 \pm 45	-62 [‡]
	Hexane	NT	NT	NT
	Dichloromethane	678	669 \pm 19	8
	Chloroform	76	258 \pm 42	-58 [‡]
<i>M. simplicifolia</i>	Methanol	NA	568 \pm 31	-8
	Hexane	213	513 \pm 32	-17
	Dichloromethane	238	399 \pm 14	-36 [†]
	Chloroform	90	610 \pm 63	-2
<i>P. amabile</i>	Methanol	NA	672 \pm 95	8
	Hexane	495	206 \pm 10	-67 [‡]
	Dichloromethane	790	300 \pm 35	-52 [†]
	Chloroform	69	421 \pm 65	-32 [†]

^aculture media control; ^bsolvent control (1% DMSO); ^cpositive control drug at 1 μ g/ml concentration; *weight of crude solvent fraction/extracts (in mg) obtained from 10 g of crude MeOH extracts where NA for not applicable has been indicated; **secretion value of TNF- α

of resting cells = 0-<0.5 pg/mL and the level of TNF- α production were determined for the optimized dose/concentration of 50 μ g/mL of each solvent extracts of the plants; †statistical significance (in relative to solvent control) was $p<0.05$; ‡statistical significance with $p<0.01$; – : inhibited; +: activated; NT: Not tested. ^{RR}reported results from Wangchuk et al. (2012a).

In-depth phytochemical analysis identified seven isoquinoline alkaloids as scoulerine, cheilanthifoline, protopine, capnoidine, bicuculline, corydecumbine and hydrastine and they await testing for anti-TNF- α activities (Wangchuk et al., 2012b). The dichloromethane extract of *A. nubigena* showed the TNF- α inhibitory value of 220 pg/mL (65% inhibition). *A. laciniatum* did not inhibit the production of TNF- α cytokine but instead stimulated the production of TNF- α cytokine.

Segregating the activities by the level of statistical significance, of the total of 27 crude extracts obtained from seven plants, 13 of them inhibited the TNF- α production in LPS-activated THP-1 cells with the statistical significance of $p<0.05$ and $p<0.01$ (in comparison to DMSO solvent control and the RPMI culture media control) (see Table 2 for inhibition values). Of these 13 extracts, six of them inhibited the production of pro-inflammatory cytokine TNF- α with 58-89% percent inhibition which gave the statistical significance of $p<0.01$. Another seven extracts (out of 13) suppressed the production of pro-inflammatory cytokine TNF- α with 32-52% percent inhibition which gave the statistical significance of $p<0.05$. The positive control drug, dexamethasone (1 μ g/mL), significantly inhibited the secretion/production of TNF- α at the values of 258 pg/mL (58% of inhibition) with the statistical significance of $p<0.01$.

We observed that, amongst the four different solvent extracts, the chloroform extract consistently showed the best TNF- α inhibitory activities (Table 2) closely followed by the

dichloromethane and hexane. This indicated that chloroform was the best solvent for extracting the bioactive secondary metabolites from seven medicinal plants. Since the chloroform extracts contained basic components (see the extraction procedures), it may be also concluded that basic compounds are responsible for better biological activities.

4. Conclusion and future directions

Of the seven plants studied, the extracts of six of them exhibited TNF- α inhibitory activities with the highest being that of *C. crispata* (Wangchuk et al., 2012a) followed by *P. amabile*, *A. nubigena*, *C. dubia*, *M. simplicifolia* and *C. bhutanica*. Thirteen extracts, obtained from the selected seven plants, significantly ($p < 0.05$ and $p < 0.01$) inhibited the production of TNF- α cytokine in LPS-activated THP-1 cells when compared to DMSO solvent control and the RPMI culture media control which indicated that these crude extracts possess compounds with promising anti-inflammatory properties. Thus, our study forms a good preliminary basis for the prioritization of candidate plant species for further in-depth phytochemical and anti-inflammatory investigations which will be carried out. When in-depth investigation of an individual plant will be studied, it would be worthwhile to assess the anti-inflammatory potency of these medicinal plants on other inflammatory parameters as well as determine their IC₅₀ values both on *in vitro* and *in vivo* models.

This study also generated, for the first time, the preliminary scientific basis for the traditional use of these seven medicinal plants in the BTM for the treatment of various disorders related to inflammation. None of the crude extracts studied here were found to be cytotoxic to the human monocytic cell line, THP-1 and therefore further research with these potential crude extracts could: a) elucidate new mechanism of action of the plants, b) generate

new anti-inflammatory compounds, and c) ultimately open up new vista in the management of inflammatory disorders especially in Bhutan.

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