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Evaluation and Comparative *in vitro* and *in vivo* Analysis of Anti-inflammatory Potential of *Ocimum sanctum* Extracts from Tropical and Alpine Regions of Nepal

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Abstract

The purpose of this study was to evaluate and compare the anti-inflammatory efficacy of *Ocimum sanctum* (OS) (Tulsi) extracts obtained from two different regions; Butwal (tropical) and Gorkha (alpine) of Nepal. The anti-inflammatory activities of the extracts were determined *in vitro* using heat-induced albumin denaturation assay and *in vivo* by using formalin-induced rat paw edema model. In both of these experiments, commercial anti-inflammatory drug, indomethacin, was utilized to justify the results. The extracts from both the regions were effective in inhibition of heat-induced albumin denaturation (HIAD) and attenuation of formalin-induced rat paw edema development. Notably, the extract from alpine Gorkha region was more potent in reduction of HIAD and edema development in rats injected with formalin in their paws, suggesting that OS from alpine regions could have greater contents of constituents mediating the anti-inflammatory actions of OS. The present data may provide the scientific basis for the traditional use of OS and suggests that OS from alpine regions could be studied for design and development of anti-inflammatory therapeutic agents. Further research is required to address the mechanisms and difference in contents of OS between the two regions of Nepal to elicit anti-inflammatory actions.

Keywords

Ocimum Sanctum, Tulsi, Anti-Inflammation, Alpine, Tropical, Nepal

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1. Introduction

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury (Okin and Medzhitov, 2012). Several pathological processes such as trauma, acute transplant rejection, cardiovascular disorders, and cancer has been shown to stimulate systemic or local inflammation (Okin and Medzhitov, 2012; Shen *et al.*, 2013; Yang *et al.*, 2014). Variety of inflammatory mediators, including cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, and reactive oxygen species (ROS) are produced during this process of inflammation (Yang *et al.*, 2014; Mohammad *et al.*, 2014).

Currently, non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are normally used for the treatment of

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inflammation (Suke *et al.*, 2013). However, several serious adverse side effects such as renal, gastrointestinal and cardiovascular problems have been known to arise when such drugs are used for therapy (Ali *et al.*, 2012). Therefore, it is necessary to develop a novel anti-inflammatory agent that could overcome the disadvantages of NSAIDs and glucocorticoids. Furthermore, identification of such agent from natural origin could confer safety and efficacy for the treatment of inflammation.

Since long time, traditional natural plants have been used throughout the world for treatment of inflammation. Moreover, it has been reported that pharmacological efficacy of such plants differs according the altitude, climate and temperature where they are grown. A study analyzed antioxidant properties in the same plant species at different altitudes and thus varying environmental conditions: as altitude increases, for instance, temperature at day decreases and light intensity increases. The study found that the total amount of antioxidant increases as altitude increases, suggesting that the altitude influence antioxidant contents in the plants (Wildi and Lutz, 1996).

Ocimum sanctum (OS) Linn. (Lamiaceae) widely known as Holi basil/Tulsi is an herbaceous sacred plant found across India and Nepal. Earlier studies with OS indicated that the plant has hypoglycemic, hypolipidemic, adaptogenic, antidepressant, antieplileptic, hepatoprotective, anticancer, analgesic, and radioprotective properties (Cohen *et al.*, 2014). Although the plant has been in traditional use for thousands of years, its anti-inflammatory potential and if so, how the growth of the plant in distinct regions that differ by altitude and climatic conditions affects the anti-inflammatory efficacy of it has still not been studied. This study aimed to identify and compare the anti-inflammatory efficacy of aqueous leaves extract of OS from two different regions of Nepal: Butwal (tropical) region) and Gorkha (alpine region).

2. Materials and Methods

2.1. Plant Materials

Fresh leaves of OS were collected from the different regions of Gorkha (alpine region) and Butwal (tropical region), Nepal in September, 2014 (Table 1). The material was identified and authenticated by Mr. Puran Prasad Kurmi (Birpur-5, Kapilvastu, Nepal). The voucher specimen of collected plants was preserved in the Pharmacognosy Laboratory of the Crimson College of Technology, Pokhara University, Nepal.

2.2. Drugs

Indomethacin was purchased from the S.R. Laboratories Pvt. Ltd., Satungal, Kathmandu, Nepal.

Table 1. Details of the plant collection site, part of plants utilized and its scientific and local names.

S.N	Plant	Local	Parts	Collection site	Collection date
1.	Ocimum sanctum	Tulsi	Leaf	Butwal	2014/09
2.	Ocimum sanctum	Tulsi	Leaf	Gorkha	2014/09

2.3. Experimental Animals

Albino rats of both sexes, aged 2-4 weeks, average weight 25-35 gm were used for the study. They were obtained from department of plant resources, Kathmandu, Nepal. Animals were maintained in standard environmental condition (23-25^{o}C, 55-65% relative humidity and 12 hour light / 12 hour dark cycle) for 2 week for acclimatization before commencement of experiment, and were fed with standard rodent diet and water ad libitum. All animal experiments were approved by the Animal Care and Use Committee at Crimson College of Technology, Pokhara University, Nepal.

2.4. Drying of Fresh Leaves

Upon collection, plant materials were air dried in shade at room temperature in a well-ventilated room. The drying was carried out for 15 days with proper checking at regular interval.

2.5. Comminution of Dried Plants

Dried leaves were grinded to a fine powder using a portable grinding machine and powder was passed through the sieve of mesh size 40. The sieved powder was sealed in air tight plastic container and stored at room temperature in a dark place until used.

2.6. Extraction

The dried leaves of OS (100 g) from Butwal and Gorkha were macerated separately for two days with 1000 ml of distilled water, with frequently shaking and stirring; about 5 to 10 drops of chloroform was added each day during maceration process. The both extracts were filtered through a piece of clean, white cotton material followed by final filtration through Whatman filter paper.

2.7. Preparation of Dry Extracts

The solvent from both filtrates was evaporated to dryness using rotary vacuum evaporator keeping water bath temperature below 45ºC. The gummy mass of chocolate color obtained from both filtrates were dried completely using vacuum pump and percentage yields of each extracts

were calculated. The completely dried extracts were kept at freezer until required for analysis.

2.8. Anti-Inflammatory Activity Test

Extracts were screened for *in-vitro* and *in-vivo* anti-inflammatory activity by albumin denaturation and formalin induced rat paw edema method respectively.

2.9. Albumin Denaturation Method

Anti-inflammatory activity was measured by calculation of inhibition of albumin denaturation. The sample of two concentrations (300 µg/ml and 600 µg/ml) of both extracts was prepared and 1% aqueous solution of bovine albumin fraction was added. The samples were incubated at 37ºC for 20 min followed by heating to 51ºC for 20 min. The samples were cooled down to bring to room temperature and absorbances were measured at 660 nm using spectrophometer UV 1800, Shimadzu. Indomethacin (100 µg/ml) was prepared using same methods as samples and used as a standard. The percentage inhibition of protein denaturation was calculated by using the following formula:

Percentage inhibition = (Abs standard –Abs Sample) X 100/ Abs standard.

2.10. Formalin-Induced Edema in Rat Paw

Experimental rats were randomly selected and allocated into four different groups of six healthy rats each. Different groups were separately treated orally; control group with distilled water; standard group with indomethacin 10mg/kg and two groups test rats with extracts of OS 200 mg/kg and 400 mg/kg, respectively. The inflammation was produced by subcutaneous injection of 0.1 ml of 2 % formaldehyde in the right hind paw of the first and third day. The different group animals were treated once a day with the extracts and indomethacin. The changes in paw size were measured by using Vernier Caliper after 12 h, 36 h, 48 h and 72 h. The percentage inhibition of edema was calculated as follows:

$$\text{Percentage inhibition} = (Vc - Vt) \times 100 / Vc$$

Where,

Vc = Volume of paw edema in control animals.

Vt = Volume of paw edema in treated animals.

2.11. Statistical Analysis

All values are presented as means ± SEM. Statistical significance was determined using the Student's t-test. *p values lower than 0.05 were considered statistically significant.

3. Results

3.1. Phytochemical Analysis

The yield of the aqueous extract of OS from Butwal and Gorkha region of Nepal was found to be 10.80% and 11.16%, respectively, as shown in Table 2.

Table 2. Yield of the extracts from tropical (Butwal) and alpine (Gorkha) regions of Nepal.

S. N.	Plants Extract(s)	Distilled Water
1.	Ocimum sanctum(Leaves) Butwal extract	10.80% (Yield)
2.	Ocimum sanctum(Leaves) Gorkha extract	11.16% (Yield)

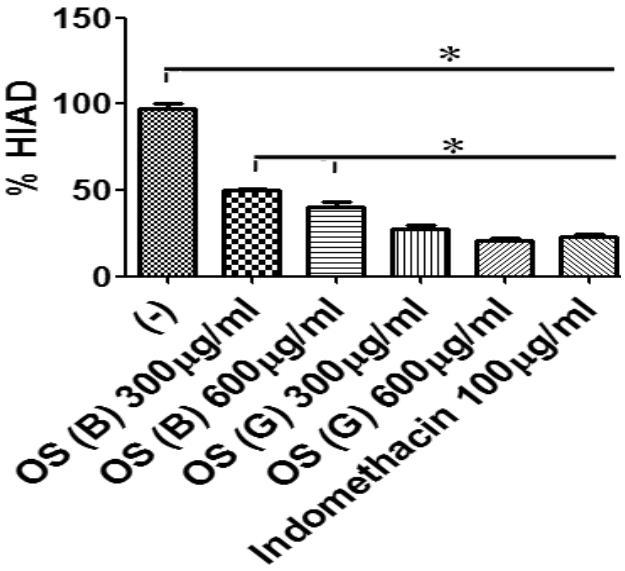

Fig. 1. OS extracts could inhibit HIAD, and that from alpine region has more potent when compared to tropical region of Nepal. The reaction mixture consisting of indicated does of two OS extracts were mixed with1% aqueous solution of bovine albumin fraction. Then the mixture was incubated at 37ºC for 20 min and then heated to 51ºC for 20 min. After this, mixture was cooled to room temperature and the turbidity was measured at 660nm against a blank. Indomethacin was used as standard drug and the percentage inhibition of protein denaturation was calculated as described in Material and Methods section. Data are presented as means ± SEM (*, $p<0.05$). OS (B); Butwal extract, OS (G); Gorkha extract, HIAD; Heat-induced albumin denaturation.

3.2. OS Inhibits Albumin Denaturation

Denaturation of proteins contributes to inflammation. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory agents) is able to thermally induce protein denaturation (Jagtap *et al.*, 2011). In order to examine the potential anti-inflammatory activities of OS, ability of the extracts to inhibit protein denaturation was investigated. Results indicated that both of the OS extracts from Butwal and Gorkha regions of Nepal were effective in inhibition of heat induced albumin denaturation (HIAD) at different concentrations as shown in Fig. 1. Moreover, OS extract from Gorkha was more potent to suppress HIAD when

compared to OS extract from Butwal, suggesting that OS extract from alpine region of Nepal is more effective than that from tropical region. Indomethacin, a standard anti-inflammatory agent was used as positive control in the experiments which showed about 75.59% inhibition of HIAD at the concentration of 100 µg/ml.

3.3. OS Extract Attenuated Formalin-Induced Rat Paw Edema

This experiment is a simple model of sub-chronic inflammation and is utilized to investigate anti-inflammatory potential of drugs (Shojaii *et al.*, 2015). Our data showed that OS extracts could significantly reduce edema development when compared to non-treated group. Moreover, reductions in edema development by OS extract from Gorkha region was more potent than that of Butwal region (Fig. 2), suggesting that OS from alpine region (Gorkha) bears more potency to attenuate inflammatory reactions than that from tropical region (Butwal) of Nepal.

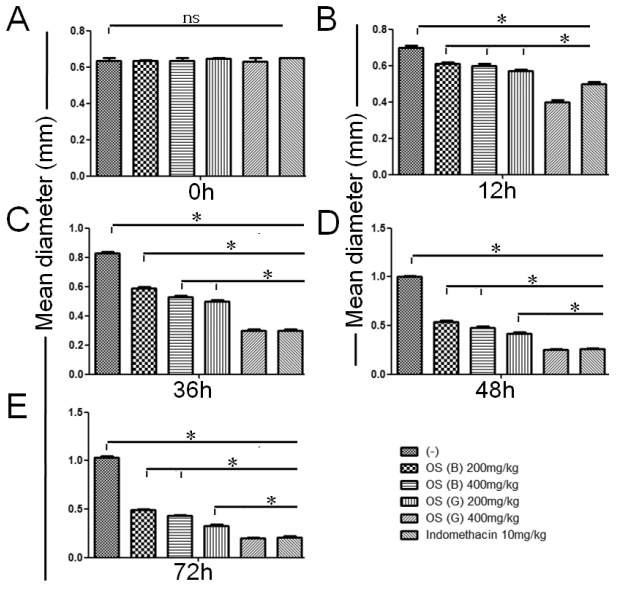

Fig. 2. OS extracts attenuate formalin-induced rat paw edema, and extract from alpine region is more effective than that from tropical region of Nepal. Effective reduction of formalin-induced paw edema in rats by OS extracts. Grapts depicts diameter of oedema measured at 0h (A), 12h (B), 36h (C), 48h (D) and 72h (E) in rats treated with or without indicated doses of OS extracts, or indomethacin. Data are presented as means ± SEM (*, $p<0.05$). OS (B); Butwal extract, OS (G); Gorkha extract.

4. Discussion

In recent years, use of herbal medicines has been steeply increased for their use as antioxidants and good health supplements (Choudhury *et al.*, 2014). In traditional medicine, OS leaves are found to have wide health benefits. Although NSAIDs and glucocorticoids are prescribed for anti-inflammation therapy, they are known to have adverse side effects. Thus, many researchers have focused on medicinal plants derived natural products such as flavonoids, polyphenols, alkaloids, coumarins, and terpenes because of their broad pharmacological implications including anti-inflammatory properties with comparatively lesser side effects (Wang *et al.*, 2012). However, it is crucial to identify the plant that exhibits maximal pharmacological efficacy, as several factors such as altitude, climate, and temperature are known to affect the pharmacological efficiency of plant extracts, and consequently the constituents of it (Wildi and Lutz, 1996). Moreover, as plants still represent a huge source of structurally novel phytocompounds that might serve as leads for the development of novel compounds for the prevention and treatment of several disorders (Chukwurah *et al.*, 2014), the collection and processing of plants for the identification of novel agents from them should be made from the adequate region so that peak pharmacological activities could be obtained.

The present study investigated an in-vitro and in-vivo anti-inflammatory activity of aqueous extracts of leaves of OS from different regions, tropical and alpine region of Nepal. Experiments were carried out using in-vitro heat induced protein denaturation method and formalin- induced edema in rat paw after feeding the test extract and reference drug (indomethacin) to determine and compare anti-inflammatory activities.

Inflammation is characterized by increase of protein denaturation. Thus, in this study we applied protein denaturation assay for in-vitro assessment of anti-inflammatory properties of OS extracts. Denaturation of tissue proteins is one of the well-documented causes of inflammatory diseases. Production of auto antigens in certain inflammatory diseases could be due to protein denaturation in vivo (Katla *et al.*, 2013; Chandra et al., 2012). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development.

The reduction in absorbance of test samples when compared to control indicates protein stabilization or inhibition of heat-induced albumin denaturation by OS extracts and positive control drug (Jagtap *et al.*, 2011), indomethacin. In line with this study, our data showed that OS extract treatment significantly inhibited heat-induced albumin denaturation when compared to non-treated group. Moreover, there was significant inhibition of heat-induced protein denaturation by OS extracts from alpine Gorkha region when compared to that of tropical Butwal region. Similar to our data, previous reports have utilized this assay to investigate in-vitro anti-inflammatory effects of potential plant products and have shown that inhibition of protein-denaturation accounts for their anti-inflammatory properties (Katla *et al.*, 2013; Jagtap *et al.*, 2011).

Next we applied in vivo formalin-induced rat paw edema model to assess the efficacy of OS extracts in suppression of edema development. This model has been utilized by many researchers world-wide to produce experimental inflammation in rodents (Shojaii *et al.*, 2015; Saini *et al.*, 2012). In line with previous findings, our data demonstrated that injection of formalin could produce an edema (Shojaii *et al.*, 2015). The aim of our study was thus to evaluate the role of OS extracts in the development of edema induced by formalin in the rat paw. Rats were fed OS extracts from each region in two different concentrations, and positive control drug indomethacin was also included in the experiments. Results indicated that treatment of rats with OS extracts at indicated doses significantly suppressed edema development when compared to non-treated groups. Moreover, the anti-inflammatory action of OS extracts from alpine Gorkha region was more potent when compared to that of tropical Butwal region. In line with our findings, previous study has indicated that root extracts of Aristolochia ringens (Vahl.) Aristolochiaceae could inhibit rat paw edema progression in rats (Ruth *et al.*, 2014). Notably, the extent of anti-inflammatory activity shown by extracts from alpine Gorkha region was similar to that of the standard drug indomethacin which justifies its activity as more potent than that of topical Butwal region.

The difference in order of anti-inflammatory activity of OS extracts from alpine Gorkha and tropical Butwal region of Nepal could be due to variability in constituents present in the extracts that are responsible for these activities. Several molecules such as sterols, tannins, and flavonoids are known to be responsible for anti-inflammatory actions of plant extracts (Shah *et al.*, 2011). The presence of these components in OS extracts may be responsible for the observed activities. Furthermore, it is reported that eugenol, a major compound present in OS extract, is shown to inhibit expression of inflammatory molecules, thereby suppressing the inflammatory process (Choudhury *et al.*, 2014), suggesting that it might be present in higher concentration in alpine Gorkha region OS extract when compared to that of Butwal region to exhibit marked anti-inflammatory effects in our experiments.

Taken together, our study demonstrated that OS possesses marked anti-inflammatory effect in vitro as well as in vivo. Moreover, OS extract from alpine Gorkha region was more potent in inhibition of heat-induced denaturation of albumin and development of edema in formalin-induced rat paw, when compared to that of tropical Butwal region of Nepal, suggesting that OS from alpine regions could be studied for design and development of new anti-inflammatory therapeutic agent. Further definitive studies are necessary to ascertain the mechanisms and constituents behind its anti-inflammatory actions.

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