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Exploration of anti-inflammatory activity of turmeric and onion combination on phorbol ester induced ear inflammation in mice

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Abstract

The turmeric and onion mixture is used as home remedy for acute inflammation. The anti-inflammatory effect of this mixture was evaluated on acute inflammatory condition induced by 12-O-tetradecanoyl-phorbol-13-Acetate (TPA) in mouse ear. The changes in ear redness, edema, production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IFN-γ), anti-inflammatory cytokine (IL-10), lipid peroxidation assay and nitric oxide assay were evaluated as indicators of acute inflammation. Topically applied turmeric and onion extract mixture (1:1, 250 mg/kg body weight) significantly inhibited the TPA induced acute inflammation, leading to substantial reductions in ear redness, ear weight (edema), pro-inflammatory cytokines and oxidative stress. The data suggest that turmeric and onion extract mixture is topically effective anti-inflammatory agent useful to treat the acute skin inflammatory conditions.

Key words: *Curcuma longa* L., *Allium cepa* L., TPA, anti-inflammatory activity, inflammatory mediators, lipid peroxidation, nitric oxide, cytokines

1. Introduction

The skin is an external organ that covers the entire body surface and protect the organism from outside environment. It is constantly subjected to exogenous stimuli. The skin is capable to activate a defense mechanism aimed at pathogen elimination and tissue repair. The infiltration of neutrophils and the release of several pro-inflammatory mediators, which starts the inflammatory process is a characteristic symbol of initiation of the defense response (Cabrini *et al.,* 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) are used throughout the world for the treatment and management of inflammation, pain and fever (Habib and Waheed, 2013). The currently available therapeutics to treat inflammatory conditions produce many side effects, therefore the search for safer treatment alternatives is the need of the hour.

Plants contain many constituents with local physical impact on body tissues and the topical use of herbal remedies is among the most noticeable in the simplest traditions of healthcare. Recent ethnobotanical surveys conducted in the India (Smita *et al.*, 2012; Ayyanar and Ignacimuthu, 2011; Pandikumar *et al.*, 2011; Sen *et al.*, 2011; Tangjang *et al.*, 2011; Namsa *et al.*, 2009) have recorded interesting uses of very common plant materials applied as topical homemade remedies. Traditional practices include mainly different dosage forms such as plant juices, tinctures and related products (alcoholic or hydro-alcoholic solutions prepared from botanicals). Their use is claimed to have local anti-inflammatory effects on minor wounds and lesions and on the management and relief of inflammations affecting joins, muscles and other subcutaneous tissues (Rafael *et al.,* 2011).

Turmeric (*Curcuma longa* L.) is a tropical plant native to South and Southeast tropical Asia. It is a rhizomatous herb that belongs to the family Zingiberaceae. Turmeric contains essential oils, fatty oils and 2-5% curcuminoids. Turmeric is fairly rich in omega-3 fatty acids. Curcuminoids are polyphenolic compounds with a β-diketone moiety. Whole turmeric or the extracted curcuminoids appear to be active in many disease processes with specific reference to chronic ailments such as cardiovascular, degenerative, infective, carcinogenesis, gastrointestinal effects, wound healing and also proved as hepatoprotective, anticoagulant, antifertility, antifungal, antibacterial, antitumor, antiparasitic, antispasmodic, anti-inflammatory and inhibits cancer growth (Jurenka, 2009; Krishnaswamy, 2008; Araujo and Leon, 2001; Scartezzini and Speroni, 2000; Ammon and Wahl, 1991).

Onion (*Allium cepa* L., Family: Liliaceae) is found across a wide range of latitudes and altitudes in Europe, Asia, North America and Africa. Onions are rich in two chemical groups that have perceived benefits to human health. These are the flavonoids and the alk(en)yl cysteine sulphoxides. Onion has been reported as one of the major sources of dietary flavonoids in many countries. Quercetin 4'-glucoside and quercetin 3,4'-diglucoside are reported as the main flavonols in onions, accounting for about 80 to 95% of total flavonols (Perez-Gregorio *et al.*, 2014). The downstream products are a complex mixture of compounds which include thiosulphinates, thiosulphonates, mono-, di- and tri-sulphides. Compounds from onion have been reported to have a range of pharmacological activities including anticarcinogenic properties, antiplatelet activity,

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antithrombotic activity, anti-hyperglycaemic, antiasthmatic, cardiovascular and antibiotic effects, *etc*. (Griffiths *et al*., 2002; Block *et al*., 1997). Depression of cutaneous inflammation and edema formation by topical application of onion extracts have recently been reported by Nasri *et al.* (2012).

In the present study, we investigated the anti-inflammatory properties of turmeric and onion extract mixture. For this study, the 95% ethanolic extract of turmeric rhizome and dichloromethane extract of onion bulb, separately as well as 1:1 mixture were evaluated on a classic model of skin inflammation 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear inflammation.

2. Materials and Methods

2.1 Chemical and reagents

Indomethacin, 12-O-tetradecanoylphorbol-13-acetate (TPA), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Malondialdehyde (MDA) were procured from Sigma-Aldrich (USA), biochemical kits for TNF-α, IL-6, IL-1β, IL-10 and IFN-γ from Thermo Scientific (USA) and Phosphate buffer saline tablets were purchased from Himedia, Mumbai. All other chemicals and reagents used in the study were of analytical grade.

2.2 Plant material

Dried rhizomes of *Curcuma longa* L. (turmeric) and fresh bulb of *Allium cepa* L. (onion; red variety) were procured from local market and authenticated by taxonomist, Dr. S.C. Singh, Botany and Pharmacognosy Department of CSIR-CIMAP, Lucknow.

2.3 Preparation of extracts

Dried rhizomes of turmeric were coarsely powdered and extracted with 95% ethanol using Soxhlet apparatus. Fresh bulbs of onion were peeled off, chopped in to fine pieces and macerated in dichloromethane for approximately 72 h with occasional shaking. After filtration both the extracts were concentrated under reduced pressure and temperature using rotary evaporator. Further, dried at 40°C on water bath and yields of extracts were noted for turmeric (6.27%) and onion (8.00%).

2.4 Experimental animals

Swiss albino male mice (outbred strain) of 18-22 g body weight were selected for this study. They were bred in the Animal House Facility of the CSIR-CIMAP, Lucknow and maintained on a standard rodent pellets diet and water *ad libitum*. Permission and approval for animal studies was obtained from CPCSEA (Reg. No. 400/01/AB/CPCSEA), Government of India through the Institutional Animal Ethics Committee.

2.5 Study design

All the mice were acclimatized in groups of five in controlled environmental conditions (23±2°C, 55±10% RH and 12 h day/night cycle), 7 days before commencement of experiment. Prior to inducing inflammation, animals were grouped as given below (six animals in each group; n=6) and all the treatments were given on right ear of each mouse, topically for five days. Successive treatments were given at 30 min interval (Yadav *et al.*, 2009).

Group I (vehicle control group): 20µl of acetone + 20µl of hydro alcohol (50:50; vehicle).

Group II (toxin control group): 20µl of TPA (1µg/20µl acetone)

Group III (positive control group): 20µl of TPA (1µg/20µl acetone) + Indomethacin (5mg/20µl acetone)

Group IV (turmeric treated group): 20µl of TPA (1µg/20µl acetone) + turmeric extract (5mg/20µl of hydro alcohol)

Group V (onion treated group): 20µl of TPA (1µg/20µl acetone) + onion extract (5mg/20µl of hydro alcohol)

Group VI (turmeric + onion treated group): 20µl of TPA (1µg/20µl acetone) + turmeric-onion extract (1:1; 5mg/20µl of hydro alcohol)

2.6 Evaluation of anti-inflammatory activity

2.6.1 Observational study

(i) Redness: Each ear was observed for the difference of redness between each group and graded arbitrary on 1-5 scale.

(ii) Ear edema: The edema was assessed in terms of difference in weight of right and left ear. On the 5th day after 6 hr of treatment, all the animals of each group were sacrificed by cervical dislocation and both the ears were removed. The 8 mm discs were excised with the help of biopsy punch from both the ears, weighted and difference was calculated. Right ear biopsy discs were kept in ice-cold PBS buffer (pH 7.4) until it was further proceed.

2.6.2 Oxidative stress estimation

The part of previously removed right ears tissues were homogenized in tissue homogenizer (Pro 200; Pro Scientific, USA) using ice cold PBS to get 10% ear homogenate. It was centrifuged at 10,000 rpm at 4ºC for 15 minutes and supernatant was collected and stored at -20ºC for further study.

(i) Lipid peroxidation assay (LPO): Briefly, to 200µl of supernatant, equal volumes of 10% w/v TCA was added and mix well to precipitate the protein content. The mixture was centrifuged at 5,000 rpm for 5 min and aliquot of 200µl supernatant was reacted with equal volume of 200µl of 0.67% of TBA. The above sample was kept in boiling water bath for 10 min. Blank was prepared only with TCA and TBA reagent. The absorbance was measured at 535 nm and 600 nm and estimation of LPO was carried out using standard plot.

(ii) Nitric oxide assay (NO): The 75µl of ear tissue supernatant was taken in a micro titer plate and mixed with equal volume of griess reagent and incubated at 37°C for 10 min. The absorbance was read out at 540 nm. The concentration of nitrite was calculated from a standard curve plotted with serial dilutions of sodium nitrite.

2.6.3 Pro-inflammatory cytokines estimation

The levels of TNF-α, IL-1β, IL-6 and IFN-γ in the supernatants were measured using commercially available ELISA kits (Thermo Scientific, USA) according to the manufacturer's manual.

2.6.4 Anti-inflammatory cytokine estimation

The levels of IL-10 in the supernatants was also measured using commercially available ELISA kits (Thermo Scientific, USA) as per the manufacturer's guidelines (Yadav *et al.*, 2012).

2.7 Statistical analysis

Data is reported as the Mean ± Standard error of mean. Statistical differences between groups were determined by one-way-analysis of variance (ANOVA) following Dunnett's multiple comparison test using GraphPad PRISM® version 5.01 (GraphPad Software, Inc., USA). $p < 0.05$ was considered indicative of significance.

3. Results and Discussion

Inflammatory diseases are accompanied by the chronic release of cytokines and reactive oxygen (ROS) and nitrogen (RNS) species, which may be involved in increased tissue injury. Much evidence has shown that the production of reactive species such as superoxide anion radical, hydrogen peroxide, hydroxyl radical and peroxynitrite occurs at the site of inflammation and contributes to tissue damage (Mittal *et al.*, 2014).

The edema, or swelling, one of the cardinal signs of acute inflammation, is an important parameter to be considered when evaluating compounds with a potential anti-inflammatory activity (Morris, 2003). TPA cause increase in ear redness and weight due to hyperemia, cellular infiltration and accumulation of exuded serum (Ferrandiz *et al.*, 1996). Application of TPA on mouse ear induced an edematogenic response as evidenced by significant ($p<0.001$) increment in ear redness and edema. Topical application of turmeric extract (group IV), onion extract (group V) as well as combination of both extracts (group VI) caused a significant ($p<0.01$) suppression in ear redness while significant ($p<0.01$) reduction in edema was observed only in group VI (Figure 1). The results of this investigation provide evidence that the alcoholic extract of *C. longa*, dichloromethane extract of *A. cepa* and mixture of both extract are topically active in attenuation of inflammation induced by TPA.

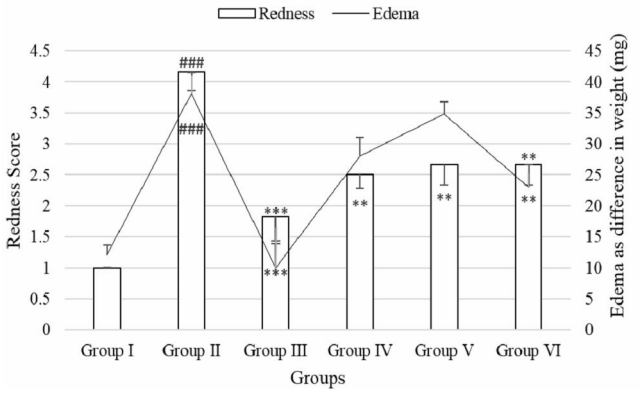

Figure 1: Redness score and edema

Data are expressed as Mean ± SE, n=6. ###p < 0.001 compared to group I, **p < 0.01, ***p < 0.001 group II.

Free radicals are important mediators that provoke inflammatory processes and consequently, their neutralization by antioxidant and radial scavengers can attenuate inflammation (Backhouse *et al.*, 2008; Geronikaki and Gavalas, 2006). Lipid peroxidation is a free radical mediated process and acts as a potential marker of susceptibility of early and irreversible tissue damage. Lipid peroxidation destroys biological membrane leading to change in fluidity and permeability (Torres *et al.*, 2004). TPA produces a generalized change in the physical properties of the lipid phase of the membranes, which results in increased membrane fluidity, changed cell surface morphology, and cellular adhesion. Thus, the lipid peroxidation is an important aspect of the inflammatory response (Weinstein *et al.*, 1979). The oxidative stress in ear homogenate was found to be significantly reduced in test treated groups corresponding to toxin control group (Figure 2). The LPO levels were found to be 14.19±1.26 ($p<0.001$), 22.70±3.57 and 18.55±2.05 μM/ml ($p<0.01$) for groups IV, V and VI, respectively compared to group II (29.38±2.01 μM/ml). These results suggested that combination of extracts is effective in reducing the reactive oxygen species.

NO is another important mediator in the inflammatory process and is produced at inflamed site by iNOS. High levels of NO have been reported in a variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis (MacMicking *et al.*, 1997; Szabo, 1995; Ohshima and Bartsch, 1994). Topical application of extracts of both plants individually as well as in combination reduced the NO concentration to extremely significant ($p<0.001$) level compared to toxin control group (Figure 2). The production of NO was estimated from the accumulation of nitrite in ear homogenates, which is a stable product of the NO metabolism. The levels of NO were found to be 8.95±0.23, 9.33±0.46, 8.59±0.16 μM/ml in group IV, V and VI, respectively compared to group II (12.50±0.56 μM/ml). The results are clearly indicating that the extracts in combination were effective in reducing the reactive nitrogen species, hence decreasing the oxidative load to affected tissues.

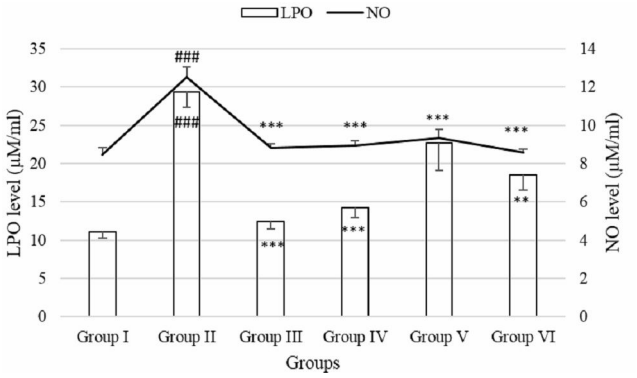

Figure 2: Level of lipid peroxidation (LPO) and nitric oxide (NO)

Data is expressed as Mean ± SE, n=6. ###p < 0.001 compared to group I, **p < 0.01, ***p < 0.001 group II.

Cytokines are regulatory proteins that are not constitutively produced under normal physiological conditions. However, inflammatory stimuli induce gene expression of cytokines, initiating the inflammatory response (Thomson and Lotze, 2003). The pro-inflammatory cytokines levels were significantly ($p<0.05$) increased in toxin control group (group II) compared to normal control group (group I) but TNF-α and IL-6 levels reduced non-significantly in all treatments groups. Though, the IL-1β level was significantly ($p<0.001$) reduced in all treatment groups. Further, the IFN-γ level

was found to be 353.3±67.29 ($p<0.05$), 487.52±68.53 and 472.36±24.9 pg/ml in group IV, V and VI, respectively compared to group II (531.10±35.29 pg/ml). The acquired data suggested that all the treatments were able to reduce the proinflammatory cytokines levels (Figure 3).

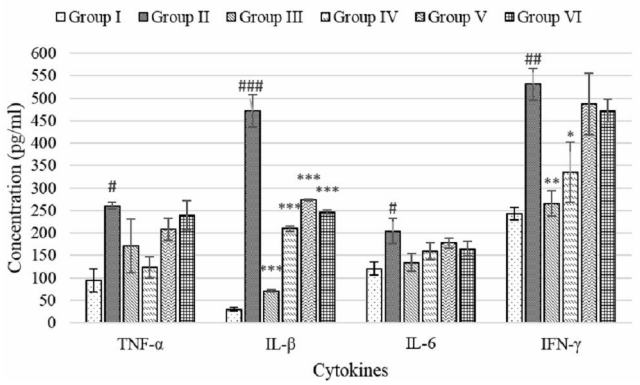

Figure 3: Levels of pro-inflammatory cytokines

Data is expressed as Mean ± SE, n=6. $^{###}p < 0.001$ compared to group I, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ group II.

As IL-10 is anti-inflammatory cytokine, the inverse correlation was observed between proinflammatory cytokines and IL-10. The estimated levels of IL-10 in group IV, V and VI were found to be 1754.31±70.09 ($p<0.05$), 1282.43±45.58 and 1356.00±69.50 pg/ml, respectively compared to group II (1010.34±87.56 pg/ml). The data obtained proposes that all the treatments were effective (Figure 4).

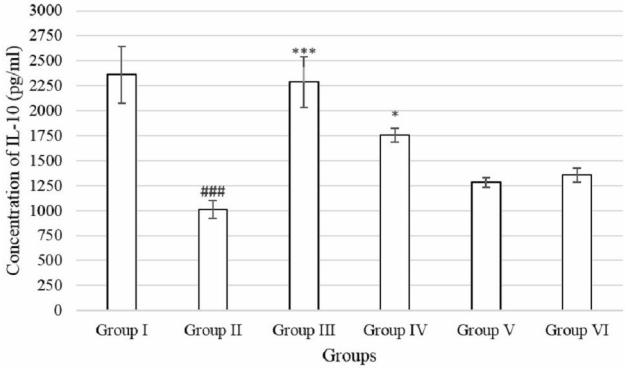

Figure 4: Level of anti-inflammatory cytokine

Data is expressed as Mean ± SE, n=6. $^{###}p < 0.001$ compared to group I, $*p < 0.05$, $***p < 0.001$ group II.

4. Conclusion

Turmeric and onion both are traditional herbs and also used as anti-inflammatory agent in combination as homemade remedy. The present work evidences that alcoholic extract of turmeric and dichloromethane extract of onion and mixture of these two extract possesses topical anti-inflammatory action and supports the claim. However, further studies are needed to evaluate the mechanism of action and responsible chemical constituents of these two plants.

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Conflict of interest

We declare that we have no conflict of interest.

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