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Research Article

ANTI-INFLAMMATORY PROPERTIES OF *TAGETES PATULA* L. FLOWER EXTRACTS: AN *IN VITRO* STUDY IN RHEUMATOID ARTHRITIS PATIENTS

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ABSTRACT

Rheumatoid arthritis is a chronic inflammatory disorder characterized by damage of joints. Several cytokines are elevated in active disease state and have been linked with disease severity. Various plant materials have been used to diminished inflammations and proposed to be possible therapeutic element against RA. Reports on usage of *T.patula* L. on treatment of RA and/or *invitro* investigations are very limited. Antioxidant activity and inflammatory cytokines modulation properties of *T. patula* has been reported, we hypothesized that flower extract of *T. patula* may affect cytokines levels in RA patients. In the present study we enrolled five RA patients and three healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with different concentrations of *T. patula* flowers extract (50 ng/ml, 100 ng/ml and 200 ng/ml). TNF-α and IFN-α levels in culture supernatant was measured by ELISA. Stimulation with Maroon colors flower extracts displayed lower concentration of TNF-α and IFN-α compared to orange and yellow colors flower extract. In addition, higher concentration of flower extract (200 ng/ml) irrespective of colors, significantly diminished levels of TNF-α and IFN-α when compared to other flower concentrations (50 ng/ml and 100 ng/ml). These observations collectively suggest that flower extract of *T. patula* is a potent anti-inflammatory agent. The constituents of *T. patula* which is responsible for suppression of inflammatory molecules need to be explored and can be translated in to possible therapeutic.

INTRODUCTION

Rheumatoid arthritis (RA) is chronic inflammatory disease and mainly autoantibodies target various joints and that may be lead to long-term deformities. The prevalence of RA worldwide is about 1% (Chopra and Abdel-Nasser, 2008)and its more frequent in western countries (1-2%) (Alamanos *et al.*, 2006). Data on prevalence of RA in Indian population is limited: reports suggested the occurrence proportion of RA is 0.28-0.7%(Handa *et al.*, 2016). Incidence of RA is more frequent in female compared to male (3:1) and role of hormone is presumed (Linos *et al.*, 1980).

The pathogenesis of RA is still unclear. It is believed that cytokines play an important role in disease susceptibility and severity. Elevated tumour necrosis factor-alpha (TNF-α) (Vasanthi *et al.*, 2007)and interferon-alpha (IFN-α) (Conigliaro *et al.*, 2010)have been reported in RA patients and positively correlated with disease severity. Blocking of TNF-α in culture has shown to reduce production of interleukin-1 (IL-1), IL-6 and IL-8, thus control inflammatory pathways. Anti-TNF therapy has been shown promising results in treatment of RA both in human and experimental model system(Feldmann, 2002; Goronzy and Weyand, 2004; Webb *et al.*, 1996). Application of anti-interferon in RA has been contradictory; however some reports suggest their important role in management of severe rheumatic diseases (van Holten *et al.*, 2002; Vervoordeldonk and Tak, 2002).

Anti-inflammatory properties of plants part have been demonstrated in literatures (Kumar *et al.*, 2013). Wide range of plant from various family viz. Asteraceae, Acanthaceae, Rutaceae, Apocyanaceae etc. have shown anti-inflammatory phenotype. Plant part includes whole plant, leaves, flowers, roots, rhizome, pulp demonstrated to inhibit inflammation (Kumar *et al.*, 2013).Furthermore, suppression of inflammation has shown by *Calendula Officinalis* flower in lipopolysaccharide induced human cells (Preethi *et al.*, 2009), indicating its importance to be tested as a potent anti-inflammatory agent in inflammatory disorders. A recent study showed suppression of arthritis by administration of lutein extracted from *Tageteserecta* in collagen induced mice

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model(Nobuo *et al.*, 2016). Based on above observations, we hypothesized that flower extracts of *T. patula* would anti-inflammatory in nature and significantly reduced inflammatory markers viz. TNF-α and IFN-α in rheumatoid patients and healthy controls.

In the present study we enrolled RA patients and healthy controls and investigated effect of *T. patula* flower extracts on cytokines levels *in vitro*. TNF-α and IFN-α levels in culture supernatant was quantified and compared among different doses of flower extracts and among variant colors of *T. patula*.

MATERIALS AND METHODS

Flower extracts

Three different cultivars were included in the present study viz. maroon, orange and yellow. Shade dried flowers were crushed in mixer grinder and 1 gm of crushed flower was mixed with 20 ml methanol and incubated at 32°C for 24 hours in a rotary shaker. Extracts were filtered, and each filtrate was dried to made concentrated extract. The yield was obtained and re-dissolved in 10 ml of methanol for further analysis.

Patients and controls

Five rheumatoid arthritis patients those reported to S.C.B. Medical college Cuttack were included in the present study. In addition, three healthy controls were enrolled in this report for comparison. About 5 ml of venous blood was collected in EDTA vial. The study protocol was approved by Institutional Human Ethical Committee of Central University of Jharkhand and written informed consent was obtained from each participant.

PBMCs culture and stimulation

Blood was layered on equal amount of HISTOPAQUE-1077 (Sigma) carefully and centrifuged at 400 g for 30 min in room temperature as instructed by the manufacturer. Middle white ring which is mostly contain peripheral blood mononuclear cells (PBMCs) were removed and washed thrice (10 min, 250 g) in culture medium. Number of cells were counted and 5 X 10^6 cells were incubated per well in 8 well culture plate. Dulbecco's Modified Eagle Medium (DMEM) was used as culture media and 1% antibiotics (Penicillin Streptomycin solution from Sigma) and 10% autologous plasma was added. Culture plates were incubated in a 5% CO_2 incubator at 37°C for 4 hours. Non-adherent cells were removed by rinsing of medium. Adherent cells were stimulated with different concentration of flower extracts (50 ng/ml, 100 ng/ml and 200 ng/ml) and to one well only medium was added which act as unstimulated. After 48 hours of incubation, supernatants were collected and preserved at -80°C till further use.

Quantification of TNF-α and IFN-α

Levels of TNF-α and IFN-α in culture supernatant was quantified by enzyme linked immunosorbent assay (ELISA) by kit according to manufacturer's instructions (eBioscicences).

Statistical Analysis

All statistical analysis was performed by Graphpad prism 7.04 version. Mean TNF-α and IFN-α in different concentration of flower extract or unstimulated culture supernatant was compared by one-way analysis of variance (ANOVA) followed by Tukey's post-test. A P value <0.05 was considered as significant.

RESULTS

Baseline data of patients and controls

In the present study we enrolled five RA patients including 3 female and 2 males. Mean age of patients was38.38 years. Duration of disease was also noted in each patients and mean disease duration as 4.82 years. Only three healthy controls were included in the present investigation with 40.28 years of mean age (Table 1).

Table 1 Baseline characteristics of patients and controls

Variables	RA	HC
Male/female	2/3	1/2
Mean age ± SD	38.38 ± 7.35	40.28 ± 4.68
Duration of disease (mean±SD)	4.82 ± 2.12	--

Note: RA: rheumatoid arthritis, HC: healthy control, SD: standard deviation.

Anti-inflammatory properties of flower extracts

PBMCs were extracted from blood samples of rheumatoid arthritis patients and healthy controls and cultured in CO_2 incubator. Different concentrations of flower extracts were stimulated for 48 hours and cytokines (TNF-α and IFN-α) were quantified by ELISA. As shown in Figure 1, a significant difference of TNF-α levels in culture supernatant was observed in healthy controls and those without stimulation. In addition, irrespective of flower colors, higher concentrations of flower extracts lowered TNF-α levels compared to lower concentrations of flower extracts (Figure 1 a, b, c). Interestingly, in a given flower extract concentrations maroon flower showed highest level of TNF-α suppression compared to orange and yellow flower. Collectively, these observations suggest that flower extracts of *T.patula* is anti-inflammatory in nature and differential anti-inflammatory capacity depend on color of the flower.

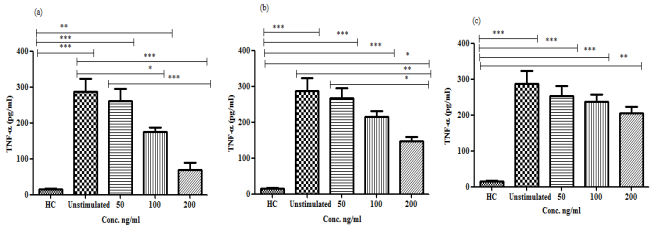

Figure 1 TNF-α levels in culture supernatant of controls and RA patients. PBMCs were isolated from whole blood samples of RA patients (n=5) and healthy controls (n=3) and cultured for 48hrs in CO2 incubator. PBMCs were stimulated with different concentrations (50 ng/ml, 100 ng/ml and 200 ng/ml) of flower extracts (a: maroon, b: orange and c: yellow). A group of cells were kept unstimulated. Levels of TNF-α was scored by ELISA. Mean TNF-α levels were compared by one-way ANOVA followed by Tukey's post-test. A P value <0.05 was considered as significant.

As interferon alpha also has been demonstrated as an important molecule and linked with pathogenesis of RA, further we tested ability of *Tagetes* flowers to suppress type I interferon. As shown in Figure 2, flower extracts of *T.patula* diminished IFN-α levels in culture supernatant in dose dependent manner: 200 ng/ml of flower extracts showed lowest levels of IFN-α in cultured supernatant and gradational increased of IFN-α

observed in lowering concentration of flower extracts. Importantly, maroon flower extracts showed higher anti-inflammatory potential compared to orange and yellow flower.

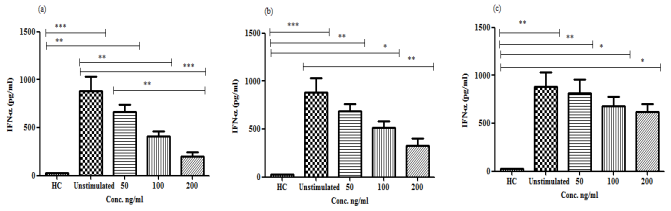

Figure 2 IFN-α levels in culture supernatant of controls and RA patients. PBMCs were isolated from whole blood samples of RA patients (n=5) and healthy controls (n=3) and cultured for 48hrs in CO2 incubator. PBMCs were stimulated with different concentrations (50 ng/ml, 100 ng/ml and 200 ng/ml) of flower extracts (a: maroon, b: orange and c: yellow). A group of cells were kept un-stimulated. Levels of IFN-α was scored by ELISA. Mean IFN-α levels were compared by one-way ANOVA followed by Tukey's post-test. A P value <0.05 was considered as significant.

DISCUSSION

Possible anti-inflammatory role of *T. patula* flowers extracts were explored in the present investigation and we observed highest anti-inflammatory activity of maroon color flower compared to orange and yellow variants. In addition, dose dependent suppression of TNF-α and IFN-α was also observed suggesting *T. patula* flower as a potent source of anti-inflammatory molecules and should be explored in various inflammatory disorders.

Molecules which are responsible for anti-inflammatory properties of *T. patula* flowers are not known. Polyphenols, carotenoids and ascorbic acid are major constituents of flower involved in antioxidant and anti-inflammatory properties. Several reports suggested role of flavonoid as an anti-inflammatory and immunomodulatory agent(Guardia *et al.*, 2001; Knekt *et al.*, 2002; Lee and Kim, 2010; Lin *et al.*, 2003). A recent report highlighted importance of dietary flavonoids against joint inflammation and alleviate arthritis symptoms in both human RA and animal models of arthritis (Hughes *et al.*, 2017). Furthermore, anthocyanin a subset of flavonoids diminished expression and activity of pro-inflammatory cytokines by affecting NF-κBsignalling pathway(Pergola *et al.*, 2006; Wang *et al.*, 1999). Another independent study deciphered role of lycopene on controlling inflammation: in an oxysterol-induced production of pro-inflammatory cytokine model, lycopene significantly reduces inflammatory molecules secretion through inhibition of NF-κB activation(Palozza *et al.*, 2011). Interestingly, differential anti-inflammatory activity was observed among various flower colors of *T. patula*. Maroon color flower had highest anti-inflammatory properties then orange cultivars and yellow flower demonstrated the least suppression of TNF-α and IFN-α levels. These observations indicate importance of flower color determining molecules on anti-inflammatory activity. Carotenoids are synthesized and stored in chromoplasts as secondary metabolites. The conjugated double bonds present in carotenoids absorb visible range of light and determine colors of the flowers. Earlier anti-inflammatory activity of carotenoids has already been demonstrated. Possibly maroon colors flowers contains higher concentration of carotenoids compared to other two colors (Yellow and Orange) and this could be reason for higher anti-inflammatory properties of maroon flower over yellow and orange flowers.

CONCLUSION

In conclusion, *T. patula* flower extracts significantly reduces inflammatory molecules concentrations in culture supernatant of RA patients and healthy controls. Maroon flower extract is more anti-inflammatory than orange and yellow color flowers extracts. However, molecules responsible for such anti-inflammatory characteristics of *T. patula* flower extract is not known. Future studies are essential to screen potent anti-inflammatory molecules of *T. patula* flower extracts and that may open new avenue towards therapeutic application of inflammatory disorders.

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