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RESEARCH ARTICLE
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SYNERGISTIC EFFECT OF ZINGIBER OFFICINALE (GINGER) AND CURCUMA LONGA L. (CURCUMIN ANALOGS) FOR ANTI-INFLAMMATORY, ANTI-NOCICEPTIVE ACTIVITY AND ANALGESIC POTENTIALS

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ABSTRACT

This research evaluates the combined analgesic, anti-inflammatory, and haemolytic potential of ethanolic extracts from turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Roscoe). The study involves *in vitro* and *in vivo* experiments using albino mice and Wistar rats. Anti-nociceptive activity was determined using hot plate, tail flick and acetic acid-induced writing methods. Carrageenan-induced rat paw edema (0.1 mL of 1 %) model was used for the assessment of anti-inflammatory activity. The haemolytic activity of the plant extracts was performed and was checked against the control (Triton 100X). Different concentrations of the plant extracts were made i.e. *C. longa* (250ul/ml, 500ul/ml and 1000u/mll), *Z. officinale* (250ul/ml, 500ul/ml and 1000ul/ml) and combined concentrations of turmeric and ginger (1:3 and 1:6 ratio respectively). Anti-nociceptive activity was conducted by using 35 albino mice of either sex. Animals were divided into 7 groups (n=5). Group I and Group II were treated with DMSO (10 ml/kg) and Diclofenac sodium (100 mg/kg) respectively. While Group III, Group IV and Group V received Turmeric extract (60mg/kg), Ginger extract (200mg/kg and 400mg/kg), respectively. On the other hand, Group VI and Group VII were given combined extract (ginger 200mg/kg + turmeric 60mg/kg; ginger 400mg/kg + turmeric 60 mg/kg). All the test samples were dissolved in 1% DMSO. 0.2 ml of the drug was administrated orally for anti-inflammatory activity. This study examines heamolytic activity of ethanolic extracts from turmeric and ginger, alone and combined, revealing ethanolic ginger extract at 1000ul with the highest heamolysis percentage (7.54%), offering insights into their cytotoxicity potential. In the hot plate test, all doses significantly delayed latency response compared to the control, with the peak effect observed at 2 hours, except for turmeric 60mg/kg, which extended the response to 2.5 hours. Additionally, in the acetic acid-induced writhing syndrome method, all doses displayed significant analgesic effects compared to the control, with the combined dose of turmeric and ginger (400mg/kg; 60mg/kg) exhibiting the highest inhibition. All doses of ethanolic extracts from turmeric and ginger significantly

reduced inflammation at 1hr, 2hr, 3hr, and 4hr compared to the control group. The combined dose of ginger (200mg/kg) and turmeric (60mg/kg) exhibited the highest inhibition at the 4th hour.

INTRODUCTION

Out of 258,658 different species of the higher plants, about 10% are used for curing minor injuries, burns, major diseases and other medical issues. The efficacy of these medicinal plants depends on the curative energy, synergistic effects and neutralizing combinations they contain. The curative energy of these plants draws its medical qualities based on the relationship between the plant and people (Shinwari, 2010).

Turmeric is a flowering wild plant of the family Zingiberaceae. It contains 5% essential oils and 5% curcumin. It has been investigated that it has the potential to treat Alzheimer's, cancer, osteoarthritis, pancreatitis, Ulcerative colitis, Acute kidney injury chronic anterior uveitis, chronic allograft nephropathy, obesity, type-2 diabetes, hyperlipidemia, hypertension, CKD, ESRD, and many other diseases (Khajehdehi, 2012). Like *Curcuma longa* the other therapeutically important member of the same family Zingiberaceae is *Zingiber officinale* Roscoe. It is used in ancient chinese medicines for its known therapeutic effects. Many different diseases can be cured by its use. These include sprains, fever, and cough. hypertension, stomach disorders like vomiting, constipation and indigestion, pain and many more. It is also effective anti-inflammatory, analgesic, anticancer, anti-microbial, antidiabetic, nephroprotective, hepatoprotective, antioxidant, antifungal, immunomodulatory, larvicidal and anthelmentic agent (Kumar *et al.*, 2011).

In this present research, effort has been made to evaluate the combined analgesic and anti-inflammatory and haemolytic potential of the ethanolic extracts of these two plant extracts. The values obtained for the combined potential of these plant extracts were compared with their individual effects to conclude that whether they pose synergestic or anagonistic effect in both activities when combined in a single formulation (Ali *et al.*, 2008).

MATERIAL AND METHODS

Chemicals and solvents

Ethanol; Glacial acetic acid; Distilled water; Dimethyl Sulfoxide (DMSO); Carrageenan; Diclofenac sodium were purchased from Pacific Pharmaceuticals Ltd. (Lahore, Pakistan). All other reagents were of analytical grades and were freshly prepared at pharmacognosy laboratory, Punjab University College of Pharmacy, Punjab University, Lahore.

Plant materials

Rhizomes of turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Roscoe) were collected from botanical laboratory of Biotechnology Department, Lahore College for Women University, Lahore, Pakistan.

Animals

Animals used in the research were Albino mice (25-30g) and Wistar rats (150-200g) of either sex. All the animals were purchased from the Pharmacology Department, University of Veterinary Sciences, Lahore. These animals were kept under the standard conditions (25°C and 16/8hrs light/dark period) in the animal house of Punjab University, Lahore. Standard food and normal water was used to feed all the animals during experimentation. We followed the guidelines protocol which was approved by Animal Ethical Committee of University College of Pharmacy, Punjab University, Lahore. A reference number was issued by the departmental ethical committee which follows the international guidelines of National Institute of Health.

Preparation of Ethanolic plant extract

180 grams of ginger and 150 grams of turmeric was extracted by help of Soxhlet apparatus using 900ml and 750 ml of ethanol respectively. Extracts were subjects to rotary evaporation and then were dried in oven below 40 ºC for three days.

IN VITRO HAEMOLYTIC ACTIVITY

The haemolytic activity of the plant extracts was performed and was checked against the control (Triton 100X). 3 ml of fresh human blood was poured into a sterile blood serum vial. Afterwards, it was centrifuged for 5 mins. The supernatant was discarded while the blood cells were washed 3 times with chilled PBS buffer whose pH was maintained at 7.4. Then finally 20ml chilled PBS buffer was added in the washed blood cells. Different concentrations of the plant extracts were made i.e. *C. longa* (250ul/ml, 500ul/ml and 1000u/ml), *Z. officinale* (250ul/ml, 500ul/ml and 1000ul/ml) and combined concentrations of turmeric and ginger (1:3 and 1:6 ratio respectively). 20ul of each concentration was taken in separate 2ml eppendorf and 180ul of blood sample was added in each of them. These eppendorfs were incubated at 37 ºC for 35 mins. Triton X was used as positive control while PBS was used as negative control. After incubation, the eppendorfs were subjected to centrifugation for 5 mins. 100 ul of supernatant was carefully taken and was mixed with 900ul of PBS for dilutions. Then 200ul of each dilution (3 replicates) was placed on 96 well plates which already contained positive and negative buffer (Zubair et al., 2017). The absorbance was checked at 576nm. The formula for calculating percentage heamolysis is;

% lysis=abs(sample)/abs(control)*100

IN VIVO BIOLOGICAL ACTIVITIES

Anti-nociceptive activity

Anti-nociceptive activity was conducted by using 35 albino mice of either sex. Animals were divided into 7 groups (n=5). Group I and Group II were treated with DMSO (10 ml/kg) and Diclofenac sodium (100 mg/kg) respectively. While Group III, Group IV and Group V received Turmeric extract (60mg/kg), Ginger extract (200mg/kg and 400mg/kg), respectively. On the other hand, Group VI and Group VII were given combined extract (ginger 200mg/kg + turmeric 60mg/kg; ginger 400mg/kg + turmeric 60 mg/kg). All the test samples were dissolved in 1% DMSO.

Hot plate method

Hot plate was maintained at 55 ^{0}C $\pm$ 1 ^{0}C. After 30 mins of dose administration, mice were placed on the hot plate. The latency time was measured. It is the time period when the mouse was placed on the hot plate till it showed any response (licking, jumping). The cut off time was set 30 sec to avoid any kind of tissue damage. Readings were noted using stop watch. The latency time was measured at 0hr, 1hr, 1.5hr, 2hr, 2.5hr, 3hr, 3.5hr and 4hr after the dose administration.

Acetic acid induced writhing syndrome method

30 mins prior to drug administration each group received 0.2 ml of 0.6% acetic acid. This acetic acid induces writhes (abdominal constriction). Writhes were counted 5 mins after the acetic acid injection for next 10 mins (between 5-15 mins) (Kravchenko *et al.,* 2019).

Anti-inflammatory activity

Carrageenan induced rat paw edema model

0.2 ml of the drug was administrated orally. Paw volume at zero time was measured using Vernier caliper. One hour after drug administration, 0.2 ml of 1% carrageenan was injected intraperitoneally into subplantar region of right hind paw of the rat. After carrageenan administration, the measurement of paw volume was done at hourly intervals for next 4 hrs i.e. 1hr, 2hr, 3hr and 4hr (Naik *et al.,* 2011). Percentage inhibition of paw volume was calculated by using the formula:

% Inhibition = {(A-B)/A}*100

RESULTS

Haemolytic activity

Haemolytic activity was performed to evaluate the cytotoxicity potential of both plant extract and their combination was also subjected to analysis. The results revealed, ethanolic ginger extract (1000ul) has highest percentage haemolysis 7.54. while the order for rest of samples was ginger extract 500ul> ginger extract 250ul>turmeric 100ul>turmeric 500ul> turmeric 250ul> combined concentration 1:6> combined concentration 1:3 as shown in the Table 1.

Anti-nociceptive activity

Results of hot plate clearly showed that all the doses were significantly ($p<0.05$) effective in delaying the latency response as compared to control group. Maximum response was shown at 2 hr for all the doses after which the effect was relapsed except for turmeric 60mg/kg which delayed the response for 2.5 hr as shown in Table 2.

In case of acetic acid induced writhing syndrome method, analgesic effect of all the doses was significant ($p<0.05$) when compared with control. Standard drug was most efficient in inhibiting body contortions. Turmeric was more effective than both doses of ginger but when turmeric was combined with ginger than effect was enhanced as shown in the Table 3. Maximum inhibition was seen in case of combined dose (400mg/kg;60mg/kg).

Table 1: heamolytic activity of ethanolic plants extracts and fractions.

Plant extracts	Concentrations (ul/ml)	% lysis
Turmeric extract	250	6.28±0.10
	500	6.57±0.07
	1000	6.85±0.15
Ginger extract	250	7.08±0.03
	500	7.42±0.02
	1000	7.54±0.03
Combined extract	1:3	2.57±0.05
	1:4	1.71±0.05
PBS	1000	0
Triton X 100	1000	100

Values are reported as mean ± S.D for three separate experiments.

Table 2: Anti-nociceptive effect of Ethanolic extracts of test samples in mice by hot plate method

TREATMENT	DOSE (mg/kg)	0hr	0.5hr	1hr	1.5hr	2hr	2.5hr	3hr	3.5hr	4hr
DMSO	10mg/ml	5.9±0.70	5.9± 0.71	5.6± 0.75	5.6± 0.69	5.9± 0.73	5.8±0.76	5.7±0.76	5.7± 0.74	5.8± 0.74
Turmeric	60	3.5±0.40	5.5*±0.49	6.1*±0.28	7.4*±0.52	8.5*±0.58	9.5±0.63	7.1±0.75	5.5*±0.63	4.3*±0.35
Ginger	200	2.9*±0.27	3.1*± 0.26	3.4*±0.24	3.7*±0.31	3.3*±0.34	2.5*±0.19	2.2*±0.13	2.1*±0.18	2.1*1±0.16
	400	2.4*±0.21	2.8*± 0.23	3.5*±0.33	4.3*±0.32	4.6*±0.48	3.5*±0.49	3.4*±0.39	2.9*±0.32	2.8*±0.31
Ginger + Turmeric	200 + 60	2.7*±0.26	3.4*± 0.49	3.8*±0.49	4.4*±0.49	4.8*±0.66	4.0*±0.59	3.0*±0.31	2.6*±0.27	2.5*±0.27
	400 + 60	3.1*±0.59	4.2*± 0.55	4.8*±0.47	5.3*±0.46	5.3*±0.59	4.4*±0.36	4.0*±0.37	3.5*±0.33	3.4*±0.32
Diclofenac Sodium	100	5.2±0.48	7.8*± 0.84	10.5*± 1.1	12.3*± 1.0	12.5*± 0.8	10.1*± 1.2	10.2*± 1.2	10.6*± 0.8	10.5*±0.6

Values are reported as mean ± S.E.M. for group of five animals. The data was analyzed by one way ANOVA. Results are expressed as mean ± SEM. *P<0.05as compared to control.

Table 3: Analgesic potential of ethanolic extracts of test samples evaluated by acetic acid induced writhing syndrome method

TREATMENT	DOSE (mg/kg)	No. of wriths	%Inhibition
Acetic acid	10 ml/kg of 0.6%	33.2 ± 1.52	0
Diclofenac Na	100	5.4 *± 0.50	83
Turmeric	60	5.8* ± 0.37	82
Ginger Extract	200	8.6* ± 0.67	74
	400	6.8* ± 1.11	79
Ginger+Turmeric	200+60	4.8* ± 0.37	85
	400+60	3.8* ± 0.37	89

Values are reported as mean ± S.E.M. for group of five animals. The data was analyzed by one way ANOVA. Results are expressed as mean ± SEM. *P<0.05as compared to control

Anti-inflammatory activity

All the doses of ethanolic extracts of turmeric and ginger were effective in reducing inflammation significantly at 1hr, 2hr, 3hr and 4hr when compared with the control group. The maximum inhibition was seen in case of combined dose of ginger 200mg/kg and turmeric 60mg/kg at 4th hour as shown in Table 4.

Table 4: Anti-inflammatory potential of ethanolic extracts of test sample evaluated by carrageenan induced rat paw edema model

TREATMENT	DOSE (mg/kg)	0hr	1hr	2hr	3hr	4hr
DMSO	10mg/ml	3.47±0.21	5.87±0.04	5.97±0.05	6.14±0.06	6.35±0.06
Turmeric	60	3.33±0.08	5.63±0.08	5.40±0.08	5.18±0.12	4.81±0.13
Ginger	200	3.58±0.04	5.78±0.02	5.59±0.01	5.36±0.07	5.19±0.07
	400	3.44±0.12	5.70±0.11	5.48±0.14	5.30±0.15	5.15±0.014
Ginger + Turmeric	200 + 60	3.37±0.05	4.82±0.18	4.25±0.22	4.11±0.11	3.82±0.12
	400 + 60	3.08±0.12	5.65±0.37	5.18±0.27	4.73±0.22	4.37±0.22
Diclofenac Sodium	100	3.50±0.10	5.55±0.03	5.35±0.02	4.73±0.22	4.37±0.22

Values are reported as mean ± S.E.M. for group of five animals. The data was analyzed by one way ANOVA. Results are expressed as mean ± SEM. *P<0.05as compared to control

DISCUSSION

Inflammation is complex natural biological process which occurs in response to infection, pathogens, irritants, allergic reactions or damaged cells. Inflammation can be characterized by several signs like edema, redness, pain, heat and most important loss of function. If left untreated it can lead to serious

problems including rheumatoid arthritis, vasomotor rhinorrhea and atherosclerosis (Singh *et al.*, 2008).

Carrageenan induced rat paw edema model is one of the most efficient model to display anti-inflammatory potential. The results of experiment displayed the fact that all the doses of turmeric and ginger were significantly effective ($P<0.05$) in reducing inflammation when compared to control group. The maximum inhibition was seen in case of combined dose of ginger 200mg/kg + turmeric 60mg/kg i.e. 39.5% at 4^{th} hr.

Turmeric extract probably inhibits inflammation by several mechanisms. They inhibit cytokines by regulating the transcriptional factors such as activating protein-1 thus blocking the expression of cytokine gene. It also inhibits cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase (iNOS) enzymes playing a vital role as inflammatory mediators (Menon and Sudheer, 2007). The anti-inflammatory property of ginger rhizomes might be due to the fact that they are dual inhibitors. They don't only inhibit the COX enzymes (involved in prostaglandin synthesis) but also inhibit LOX enzymes (involved in leukotriene synthesis). Leukotriene is derivatives of Arachidonic Acid and LOX are involved in the metabolism of Arachidonic Acid. Specifically gingerol and shagoals that are the main constituents of *Zingiber officinale* Roscoe that probably fall in the categories of dual inhibitors (Grzanna *et al.*, 2005).

On the other hand Analgesia is a state in which a patient is unable to feel pain and in other word this is a medication that is used as a relief to pain. Turmeric had neutralized pain response may be by initiating cortisol production by adrenal gland thereby inhibiting the bTREK-1 potassium channels. Moreover, it also causes the inhibition of cyclooxygenase-2 gene expression (Sahebkar and Henrotin, 2016). The results of both methods; hot plate and acetic acid induced writhing syndrome method suggested that ginger had both central and peripheral mediated analgesic potential. The central response is due to inhibition of central pain receptors as shown in hot plate method while the peripheral response is in accordance with the inhibition of COX or LOX (and other inflammatory mediators) as depicted in writhing syndrome method.

In case of haemolytic activity all the doses of both *curcuma longa* and *Z. Officinale* showed null effect or void effect. Not a single dose has shown the toxic levels of 50%. Ginger 1000ul showed the maximum effect of 7.54%. phytochemical analysis of ginger had shown high contents of flavonoids and saponins. The high composition of these compounds could account for its haemolytic activity (Duarte *et al*., 2016).

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