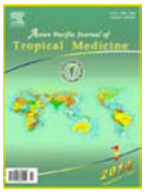
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60275-3

Analgesic and anti-inflammatory activities of *Piper nigrum* L.

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ARTICLE INFO

Article history:
Received 4 Jul 2014
Received in revised form 14 Jul 2014
Accepted 3 Aug 2014
Available online 17 Aug 2014

Keywords:
Piper nigrum Linnacus
Analgesic activity
Anti-inflammatory activity
Acetic acid
Carrageenan

ABSTRACT

Objective: To evaluate and compare the analgesic and anti-inflammatory activity of pure compound, piperine along with hexane and ethanol extracts of *Piper nigrum* L. fruit in mice and rats.

Methods: The analgesic activity was determined by tail immersion method, analgesy-meter, hot plate and acetic acid induced writhing test. While the anti-inflammatory activity was evaluated by carrageenan-induced paw inflammation in rats.

Results: Piperine at a dose of 5 mg/kg and ethanol extract at a dose of 15 mg/kg after 120 min and hexane extract at a dose of 10 mg/kg after 60 min exhibited significant ($P<0.05$) analgesic activity by tail immersion method, in comparison to ethanol extract at a dose of 10 mg/kg using analgesy-meter in rats. However, with hotplate method, piperine produced significant ($P<0.05$) analgesic activity at lower doses (5 and 10 mg/kg) after 120 min. A similar analgesic activity was noted with hexane extract at 15 mg/kg. However, in writhing test, ethanol extract significantly ($P<0.05$) stopped the number of writhes at a dose of 15 mg/kg, while piperine at a dose of 10 mg/kg completely terminated the writhes in mice. In the evaluation of anti-inflammatory effect using plethysmometer, piperine at doses of 10 and 15 mg/kg started producing anti-inflammatory effect after 30 min, which lasted till 60 min, whereas hexane and ethanol extracts also produced a similar activity at a slightly low dose (10 mg/kg) but lasted for 120 min.

Conclusions: It is concluded from the present study that *Piper nigrum* L possesses potent analgesic and anti-inflammatory activities.

1. Introduction

Secondary metabolites are utilized by plants as shielding molecules and accountable for their biological efficacy and are distinctly effective for human beings because of their great influence in healthcare system[1]. Raw materials produced by plants are the absolute onset of curative radical and their chemical structures can be used for the exploration of new compounds[2]. *Piper nigrum* L. (*P. nigrum*) is one of the best known species of Piperaceae family. It is cultivated in Pakistan, the hills of south-western India as well as in South America and Africa. It is robust-woody climbing perennial plant. Piperine is a major alkaloid constituent of *P. nigrum* L. The pungency of the black pepper is because of its alkaloidal constituents present in the fruit. It is commonly used as a spice all over the world and also possesses pharmacological properties and thus used in traditional system of medicine, such as Ayruvedic and Unani medicine for the treatment of various diseases such as fever, pain and inflammation[3].

Pain medication is one of the most common complications managed by the medical practitioners. Acute pain usually comes from a fracture or infection, while chronic pain is usually distinguished by functional loss and triggered by psychosocial problems, economic stressors and exhibits a more complex situation in terms of illnesses and remedy[4]. Currently drugs which are used to regulate the pain are narcotics *e.g.* opioids, non-narcotics *e.g.* salicylates or corticosteroids *e.g.* hydrocortisone. All of these drugs have specific side and toxic effects.

Inflammation represents the four famous signs *i.e.* tumor or redness, heat, pain, and swelling[5]. It is a complex biological feedback of vascular tissues to injurious

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Foundation Project: Supported by the Research & Development Fund of the Dean, Faculty of Pharmacy, University of Karachi-Pakistan (Grant No. 0852469-15/4/13).

stimuli, such as pathogens, damaged cells or irritants. Inflammation is caused by release of chemicals from tissues and migrating cells. Most actively related are the prostaglandins, leukotrienes, histamine, bradykinin, and, further newly, platelet-activating factor and interleukin-1. Anti-inflammatory agents are capable to inhibit the cyclooxygenase COX-1 and COX-2 pathway of arachidonic acid metabolism which produces prostaglandins[6]. Analgesic and anti-inflammatory agents are needed to counter pain and inflammation. Among these, non-steroidal anti-inflammatory drugs (NSAID) are clinically remarkable medicine for the therapy of inflammation. However, the continuous consumption of NSAID may cause gastro intestinal ulcer, bleeding and renal diseases due to non-selective inhibitor of both COX-1 and COX-2[7].

In view of this, exploration of new analgesic and anti-inflammatory drugs to restrict these side effects is still a difficult project and researches are being carried out in order to find alternatives to NSAID and opiates. Plant-based drugs provides a lot of clues for finding new drugs. The aim of the present study is to evaluate the analgesic and anti-inflammatory activities of the major constituents of *P. nigrum* L fruit, piperine as well as hexane and ethanol extracts. Diclofenac sodium and acetylsalicylic acid were used as standard drugs for comparison. In view of the earlier studies and references available, it is evident that not many studies have been reported on analgesic and anti-inflammatory activity of piperine and its hexane and ethanol extracts. Therefore the present study was undertaken to evaluate its analgesic and anti-inflammatory activity on animal modle, so as to support the local herbal drug manufacturing industry and to develop some topical preparations as an economical way to manage pain and inflammation of moderate severity. To ascertain the safety profile of piperine and the hexane and ethanol extract acute toxicity studies have also been performed. Acute toxicity refers to the adverse effects that occur on first exposure to a single dose of a substance, which can be manifested by the abnormal behaviors and other physiological activities like sedation, hyperactivity, hypo activity, pupil shrinkage, paralytic effect on hind limbs, writhes, erection of tail and body hairs, shivering of body, increase or decrease heart beat and furthermore the rate of mortality[8,9].

2. Materials and methods

2.1. Plant materials

The fruit of *P. nigrum* L was purchased from local market of Karachi, Pakistan and identified by Professor Dr. Usman Ghani Khan, The identified sample specimen code (*Piper nigrum* L PN-03-09) is available in Herbarium of the Department of Pharmacognosy University of Karachi, Karachi, Pakistan.

The pure piperine, hexane and ethanol extract of *P. nigrum* L. (Piperaceae) fruit were solubilized in 10% dimethylsulfoxide for intra-peritoneal and oral administration to the test group at different doses according to the body weight.

2.2. Isolation of piperine from P. nigrum L.

Black pepper (100 g) was ground into fine powder and extracted with 95% ethanol (500 mL) in a Soxhlet extractor for 2 h. The solution was filtered and concentrated under reduced pressure at 60 °C. Then 50 mL of 10% alcoholic KOH solution was added to it and after a while, the clear liquid was decanted from the insoluble residue. The alcoholic solution was left overnight. Yellow needle shaped crystals of piperine were obtained. The identification and purity of piperine was done by chemical tests of M.P. TLC, UV, high performance liquid chromatography and Fourier transform infrared spectroscopy[10].

2.3. Animals

Swiss albino mice of both sex weighing 25-30 g and Swiss albino rats of both sex weighing 150-200 g were used in the study. Animals were kept and maintained under laboratory condition of temperature (23±3) °C with 12 h/12 h light and dark cycles and were allowed free access to food and water and marked with their identification.

2.4. Experimental design for biological activities

The experimental animals were divided separately into five major groups, *i.e.* Group A, B, C, D and E. Group A (control) received saline (10 mL/kg), Group B were administered with diclofenac sodium (5 mg/kg), while Group C, D and E were further divided into nine groups (n=5). Three different doses, *i.e.* 5, 10, and 15 mg/kg of body weight of piperine, hexane and ethanol extracts were administered to groups of Gp-1C, Gp-2C, Gp-3C, Gp-1D,Gp-2D,Gp-3D, Gp-1E, Gp-2E and Gp-3E, respectively.

2.5. Chemicals and drugs

Dimethylsulfoxide (10%), normal saline (0.9%), acetic acid (1%), carrageenan (1%), hexane, ethanol, distilled water and

standard drugs (acetyl salicylic acid and diclofenac sodium) were used in the study. All chemicals and drugs used were of analytical grade.

2.6. Equipments

Eddy's hot plate (Life Science No. 8 Model 39), Ugo Basile analgesy meter (No. 7200), Ugo Basile plethysmometer (No. 7140), stop watch and water bath (Bushi Heating Bath B-490) were used in the study.

2.7. Acute toxicity

Acute systemic toxicity test of compounds and extracts were carried out as per earlier reported methods[11,12], while LD_{50} was calculated by using below mentioned formula[13]:

$LD_{50}=\sqrt{}$ Highest nonlethal dose×least lethal dose

The therapeutic index (also known as therapeutic ratio) is a comparison of the amount of a therapeutic agent that causes the therapeutic effect to the amount that causes death in animal studies or toxicity in human studies[14]. Therapeutic index was calculated using the following formula:

$$\text{Therapeutic index}=\frac{LD_{50}}{ED_{50}}$$

Where ED_{50} is 50% effective dose.

Animals were divided into three major groups. Group-1 (GP-1) treated with saline water served as control group. Group-2 (GP-2) was the drug treated group relating to mice and was further divided in four sub groups, *i.e.* GP-2A, GP-2B, GP-2C, and GP-2D. Group-3 (GP-3) was the drug treated group relating to rats and further divided into four sub groups, *i.e.* GP-3A, GP-3B, GP-3C, and GP-3D. Each group contain six animals (n=6). The animals were maintained under laboratory condition of temperature (23±3) °C with 12 h/12 h light and dark cycles and were allowed free access to food and water and marked with proper identification.

Four different doses of piperine, hexane and ethanol extracts, *i.e.* 75, 50, 25 and 15 mg/kg body weight were administered intraperitoneally to the mice of each tested group. While four different doses of piperine and hexane and ethanol extracts, *i.e.* 15, 50, 100 and 200 mg/kg body weight were administered orally to the rats of each tested group. The mice and rats in both test and control groups were allowed for free access to water and feed. The animals were observed continuously just after drug administration at 30 min, 60 min and 120 min interval of time for changes in general behaviors and physiological activities. Furthermore the occurrence of mortality was noted up to 72 h.

2.8. Analgesic activity

Analgesic activity was investigated using following methods: (1) tail immersion method based on thermal radiant heat as a source of pain[15]; (2) analgesy-meter based on physical pressure as a source of pain[16]; (3) hot plate method based on jumping from hot plate at 55°C[17]; (4) acetic acid induced writhing based on chemical radiant as a source of pain[18].

2.8.1. Tail immersion method

After administration of designed drugs as above mentioned, the base line latency was measured before and after drug treatment in a regular interval of 30 min, 60 min and 120 min by immersing the tail tips (1-2 cm) of the mice in water bath thermostatically maintained at temperature of (45±1) °C. The actual flick response of mice was measured by stop watch and results were compared with control and standard group. The maximum cutoff time for immersion was 180 seconds to avoid the injury of the tissues of tails.

2.8.2. Analgesy-meter

Analgesic activity was determined by placing the left hind paw of the rat on a plinth under a cone-shaped pusher of the Ugo Basile analgesy-meter. It generates a linearly increasing mechanical force or pressure on hind paw. As the applied pressure increases, it gets to a point where the animal struggles to free its paw. The strength at which each rat withdrew its paw was recorded and considered as indicative of pain. The reaction strength of each rat was determined before and after drug treatment in a regular interval of 30 min, 60 min and 120 min after treatment with tested drugs and standard drugs. Stimulus was terminated and force threshold was read in grams. The groups administered with tested extracts and pure compound were compared to control and standard drug groups.

2.8.3. Hot plate

Animals in all groups were individually exposed to the hot plate. The time taken in seconds for fore paw licking or jumping was taken as reaction time and was measured in a regular time interval and the reaction strength of each rat was determined before and after drug treatment in a regular interval of 30 min, 60 min and 120 min. A cutoff period of 15 seconds was set up to avoid damage to the paws. The groups administered with tested extracts and pure compound were compared to control and standard drug groups.

2.8.4. Acetic acid induced writhing

Tested extracts and pure compound were administered intraperitoneally using different doses as defined in study design, 30 min prior to administration of 0.1 mL acetic acid (1%). Animals were observed individually and the number of writhes was counted for 20 min commencing 5 min after injection of acetic acid. The significant reduction in number of writhes of treated groups was compared to that of the control and standard groups.

The percentage inhibition of abdominal constrictions was calculated using the following formula.

$$\text{Inhibition (\%)} = \frac{\text{Mean No. of writhes (Control)} - \text{Mean No. of writhes (Test)}}{\text{Mean No. of writhes (Control)}} \times 100$$

2.9. Anti-inflammatory activity

The screening of anti-inflammatory activity was investigated on crude extracts and pure compound by adopting carrageenan method[19].

Edema was induced by sub plantar injection of carrageenan (0.1 mL of 1% solution in 0.9% saline solution) into the left hind paw an hour after oral administration of standard drug, tested drugs and control. Paw volume was measured after 30 min, 60 min and 120 min of carrageenan treatment by means of volume displacement method using the Ugo Basile plethysmometer. The difference between initial and after treatment paw volumes indicated the degree of inflammation. Edema was expressed as a percent increase in paw volume due to carrageenan administration referred to the non-injected paw. The average increase in paw volume of each group was calculated and compared with the control and the standard groups.

2.10. Statistical analysis

SPSS version 20.0 statistic software was used for statistical analysis. Values are expressed as mean±SD. Student's *t*-test was carried out to compare the results of control and test drug groups. Data were considered to be significant if $P<0.05$ and $P<0.01$ most significant.

3. Results

3.1. Acute toxicity

The pure compound piperine, hexane and ethanol extracts of *P. nigrum* L. were evaluated for acute toxicity in mice and rats by intraperitoneal and oral administration of extracts at 15, 25, 50 and 75 mg/kg and 15, 50, 100 and 200 mg/kg, respectively. Results are presented in Tables 1 and 2.

Table 1

Toxicity assessment of *P. nigrum* L. in mice.

Extracts and Compound	Doses (mg/kg)	Quantal incidence of symptoms	Quantal incidence of mortality	% of mortality
Piperine	15	0/6	0/6	0
	25	6/6	6/6	100
	50	6/6	6/6	100
	75	6/6	6/6	100
P. nigrum	15	0/6	0/6	0
L. (hexane	25	6/6	6/6	100
extract)	50	6/6	6/6	100
	75	6/6	6/6	100
P. nigrum	15	0/6	0/6	0
L. (ethanol	25	6/6	6/6	100
extract)	50	6/6	6/6	100
	75	6/6	6/6	100
Control	–	0/6	0/6	0

During the experimental procedure it was observed that mice felt irritation just after intra peritoneal administration of 25, 50 and 75 mg/kg of piperine and observed drowsy after 30 min, while paralytic effect on hind limbs were noted after more than three hours with no intake of water and feed. After 24 h all six animals treaded with 75 mg/kg of dose were found dead, while animals that were treated with doses of 25 and 50 mg/kg were found dead after 48 h.

Table 2

Toxicity assessment of *P. nigrum* L. in rats.

Extracts and Compound	Doses (mg/kg)	Quantal incidence of symptoms	Quantal incidence of mortality	% of mortality
Piperine	15	6/6	0/6	0
	50	6/6	0/6	0
	100	6/6	0/6	0
	200	6/6	0/6	0
P. nigrum	15	6/6	0/6	0
L. (hexane	50	6/6	0/6	0
extract)	100	6/6	0/6	0
	200	6/6	0/6	0
P. nigrum	15	6/6	0/6	0
L. (ethanol	50	6/6	0/6	0
extract)	100	6/6	0/6	0
	200	6/6	0/6	0
Control	–	6/6	0/6	0

On the other hand, during acute toxicity of hexane extract mice felt shivering and irritation just after intra peritoneal administration of doses of 25, 50 and 75 mg/kg after 30 min. Breathing problem, shivering and paralytic effect of hind limbs was observed in one animal, while one animal at a dose of 50 mg/kg was found dead. In the remaining animals blood traces in urine was seen after one hour with flat body and closed eyes. At a dose of 75 mg/kg, two animals were found dead after two hours, while the remaining survived with fits and paralytic effect of hind limbs without intake of water and feed. After three hours, the remaining four survived animals were detected dead at a dose of 75 mg/kg,

while all survived animals were found without loco motory movement. After 24 h all survived animals at a dose of 50 mg/kg were noticed dead and animals at a dose of 25 mg/kg were discovered completely paralyzed without intake of water and feed. All these animals were dead after 48 h.

For the acute toxicity of ethanol extract, animals felt shivering and irritation just after intra peritoneal administration of doses of 25, 50 and 75 mg/kg. After 30 min, animals felt breathing problem and erection of hairs were observed, while two animals were found dead at a dose of 50 mg/kg. After one hour blood traces in urine was seen and after three hours paralytic effect of hind limbs and flat body were observed without intake of water and feed. Further one animal was found dead at 50 mg/kg after three hours, while all remaining animals were found dead after 24 h. No adverse reaction after intra peritoneal administration of 15 mg/kg piperine, hexane and ethanol extracts were seen even after for 72 h.

The acute toxicity of piperine, hexane and ethanol extracts were determined on rats after oral administration at doses of 15, 50, 100 and 200 mg/kg. No adverse effect was observed after 72 h.

The calculated LD_{50} and values in mice by intra peritoneal treatment of piperine, *P. nigrum* hexane extract and *P. nigrum* ethanol extract are the same as 19.36 mg/kg.

The calculated therapeutic index values in mice by intra peritoneal treatment of piperine, *P. nigrum* hexane extract and *P. nigrum* ethanol extract are the same as 1.66.

3.2. Analgesic activity

3.2.1. Tail immersion method

Results of the analgesic activity of pure compound piperine, hexane and ethanol extracts measured by tail immersion method are given in Table 3. At a dose of 5 mg/kg and 10 mg/kg the piperine showed good analgesic effect as compared to control and standard drug. Piperine exhibited maximum activity after 120 min at a dose of 5 mg/kg as compared to control and standard drug while at a dose of 15 mg/kg the compound did not showed any further enhancement in analgesic activity. The hexane extract showed maximum analgesic activity at a dose of 10 mg/kg as compared to control and standard drug and the analgesic effect increased with passage of time and achieved peak level after 60 min. The ethanol extract showed good analgesic

Table 3

Analgesic activity of *P. nigrum* L. using tail immersion method in mice.

Drugs	Dose (mg/kg)	Reaction time (seconds)				
		0 min	30 min	60 min	120 min	Mean
Standard drug (diclofenac sodium)	5	3.620±0.170	6.180±0.070	7.140±0.060	7.700±0.130	6.160±0.107
Control (saline water)	N/R	1.622±0.001	1.708±0.008	1.772±0.001	1.798±0.004	1.725±0.003
Piperine	5	2.612±0.023	7.168±0.248	3.990±0.011**	11.658±0.129	6.357±0.102
	10	3.566±0.003	6.556±0.008**	9.152±0.024*	3.000±0.095	5.568±0.032*
	15	0.232±0.294	3.600±0.004**	0.286±0.129	4.030±0.379	2.037±0.201
Piper nigrum L. (hexane extract)	5	0.854±0.079	2.060±0.083	2.054±0.083	2.060±0.083	1.757±0.082
	10	1.322±0.103	5.284±0.111	8.284±0.207	4.400±0.113	4.822±0.133
	15	0.990±0.113	2.074±0.175	2.440±0.123	2.996±0.115	2.127±0.131
Piper nigrum L. (ethanol extract)	5	1.040±0.117	3.376±0.144	4.596±0.172	5.064±0.070	3.519±0.125
	10	3.080±0.018	3.780±0.030*	5.166±0.003**	3.206±0.011**	3.808±0.015*
	15	1.170±0.013	2.020±0.007**	3.940±0.045*	9.602±0.041*	4.183±0.026*

n=5. *: P<0.05 significant, **: P<0.01 most significant as compared to control. Reaction time is the time taken by mice to withdraw the tail, recorded by stop watch in seconds.

Table 4

Analgesic activity of *P. nigrum* L. tested by analgesy-meter method in rats.

Extracts and Compounds	Dose (mg/kg)	Reaction time (seconds)[a]				
		0 min	30 min	60 min	120 min	Mean
Standard drug (acetyl salicylic acid)	10	2.480±0.001	8.572±0.002	8.660±0.002	9.294±0.001	7.250±0.001
Control (saline water)	N/R	2.240±0.005	3.800±0.003	5.080±0.006	3.400±0.005	3.630±0.004
Piperine	5	4.680±0.023	5.440±0.033*	8.280±0.001**	4.600±0.050*	5.750±0.026*
	10	2.800±0.002	4.500±0.008**	8.700±0.011**	6.400±0.001**	5.600±0.005**
	15	5.200±0.002	6.700±0.001**	9.400±0.004**	8.100±0.021*	7.350±0.007**
P. nigrum L. (hexane extract)	5	2.600±0.008	4.900±0.003**	13.000±0.006**	3.200±0.013**	5.925±0.007**
	10	1.500±0.003	2.600±0.035*	12.000±0.003**	4.800±0.005**	5.225±0.011
	15	4.200±0.013	6.200±0.001**	8.900±0.045*	6.200±0.001**	6.375±0.015**
P. nigrum L. (ethanol extract)	5	1.800±0.006	13.500±0.002**	14.300±0.007*	2.400±0.008**	8.000±0.005**
	10	2.300±0.002	4.400±0.020*	20.900±0.023*	10.400±0.074	9.500±0.029*
	15	1.600±0.005	8.400±0.068	9.200±0.021*	2.600±0.020*	5.450±0.028*

n=5, *: P<0.05 significant, **: P<0.01 most significant as compared to control. [a]: Sensitivity of animals to pain by reaction time recorded in second.

Table 5
Analgesic activity of *P. nigrum* L. tested by hot plate method in rats.

Extracts and Compounds	Dose (mg/kg)	Reaction time (seconds)				
		0 min	30 min	60 min	120 min	Mean
Standard drug (acetyl salicylic acid)	20	4.330±0.030	7.890±0.050	7.250±0.040	7.020±0.015	6.622±0.033
Control (saline water)	N/R	4.330±0.030	6.412±0.013	5.130±0.011	4.840±0.009	5.178±0.016
Piperine	5	3.500±0.001	3.390±0.003**	4.450±0.001**	9.990±0.005**	5.332±0.002**
	10	3.474±0.001	12.870±0.003**	9.040±0.017*	10.248±0.016*	8.908±0.009**
	15	2.878±0.001	8.756±0.006**	6.536±0.002**	4.404±0.003**	5.643±0.003**
P. nigrum L. (hexane extract)	5	1.938±0.006	1.508±0.004**	2.382±0.025*	2.660±0.053	2.122±0.022*
	10	1.676±0.002	2.018±0.016*	1.452±0.094	1.7080±0.022*	1.713±0.033*
	15	1.954±0.006	1.370±0.145	1.974±0.051*	2.738±0.035*	2.009±0.059
P. nigrum L. (ethanol extract)	5	1.102±0.020	2.022±0.390	2.486±0.023*	2.356±0.030*	1.991±0.115
	10	1.926±0.008	1.328±0.132	1.708±0.002**	0.796±0.019*	1.439±0.040*
	15	1.244±0.007	1.646±0.009**	1.768±0.057	0.722±0.037*	1.345±0.027*

n=5, *: P<0.05 significant, **: P<0.01 most significant as compared to control. Reaction time is a licking time in seconds of animals for fore paw.

activity at the dose of 5, 10 and 15 mg/kg as compared to control and less analgesic activity, as compared to standard at all dose level. Maximum analgesic effect was achieved at a dose of 15 mg/kg after 120 min as compared to control and standard drug. Statistical calculation showed that *P. nigrum* L., possesses non-significant analgesic activity by thermal stimuli.

3.2.2. Analgesy-meter method

Results of analgesic activity of pure compound piperine, hexane and ethanol extracts measured by analgesy meter method are given in Table 4. The piperine exhibited good analgesic effect up to 60 min at a dose of 5 mg/kg, 10 mg/kg and 15 mg/kg as compared to control while at a dose of 10 mg/kg and 15 mg/kg it showed a comparable analgesic effect with that of standard. The maximum analgesic effect was achieved at a dose of 15 mg/kg after 60 min. The hexane extract showed good analgesic effect at doses of 5 mg/kg, 10 mg/kg and 15 mg/kg as compared to control and standard drug. However maximum activity was noted at a dose of 5 mg/kg after 60 min. The ethanol extract indicated maximum analgesic effect at all doses (5, 10 and 15 mg/kg) as compared to control and standard drug. However, ethanol extract exhibited maximum analgesic activity at a dose of 10 mg/kg after 60 min.

3.2.3. Hot plate method

Results of analgesic activity of pure compound piperine, hexane and ethanol extracts measured by hot plate method are given in Table 5. The piperine exhibited good analgesic effect at a dose of 5 and 10 mg/kg as compared to control and standard. Maximum analgesic effect was noted at a dose of 10 mg/kg after 120 min. The hexane and ethanol extracts showed poor analgesic effect at all doses (5, 10 and 15 mg/kg) as compared to control and standard drug.

3.2.4. Writhing method

The results of analgesic activity of pure compound piperine, hexane and ethanol extracts analyzed by writhing method are depicted in Table 6. Piperine exhibited excellent analgesic effect at all doses of 5, 10 and 15 mg/kg as compared to control and standard. The compound showed maximum protection at a dose of 10 mg/kg (100%). The hexane and ethanol extracts showed maximum analgesic effect at all doses of 5, 10 and 15 mg/kg as compared to control and standard. However hexane extract showed significant protection at a dose of 5 and 10 mg/kg (99.71%) and the ethanol extract showed maximum protection at a dose of 15 mg/kg (100%).

Table 6
Analgesic activity of *P. nigrum* L. tested by writhing method in mice.

Extracts and compounds	Dose (mg/kg)	No. of writhes	% Protection
Standard drug (acetyl salicylic acid)	5	23.000±2.480	54.90
Control (acetic acid)	0.1	70.600±0.000	–
Piperine	5	0.200±0.374	99.71
	10	0.000±0.000***	100.00
	15	7.000±0.205	90.08
P. nigrum L. (hexane extract)	5	0.200±0.374	99.71
	10	0.200±0.374	99.71
	15	0.800±0.242	98.86
P. nigrum L. (ethanol extract)	5	0.400±0.178	99.43
	10	1.200±0.109	98.30
	15	0.000±0.000***	100.00

No. of writhes are expressed as mean±SEM. n=5, *: P<0.05 significant, **: P<0.01 most significant as compared to control.

3.3. Anti-inflammatory activity

3.3.1. Carrageenan induced paw edema method

Carrageenan induced paw edema method was used to detect the anti-inflammatory effect of piperine, hexane and ethanol extracts and results are described in Table 7. Piperine exhibited inhibition of edema at all doses of 5, 10 and 15 mg/kg as compared to control, but less activity as compared to standard. The compound showed maximum activity at a dose of 15 mg/kg after 120 min but still less than the standard. The hexane extract showed anti-inflammatory activity at a dose of 5 and 10 mg/kg compared to control,

Table 7
Anti-inflammatory activity of *P. nigrum* L in rats. mL.

Extracts and compounds	Dose (mg/kg)	Reaction time				
		0 min	30 min	60 min	120 min	Mean
Standard drug (diclofenac sodium)	10	0.970±0.030	1.220±0.060	1.330±0.060	1.290±0.060	1.202±0.052
Control (saline water)	N/R	0.128±0.045	0.040±0.005	0.056±0.001	0.060±0.016	0.071±0.016
Piperine	5	0.044±0.022	0.254±0.006**	0.454±0.004**	0.258±0.003**	0.252±0.008**
	10	0.072±0.007	0.282±0.002**	0.362±0.001**	0.234±0.004**	0.237±0.002**
	15	0.362±0.001	0.484±0.001**	0.548±0.001**	0.588±0.001**	0.495±0.001**
P. nigrum L. (hexane extract)	5	0.234±0.002	0.338±0.001**	0.350±0.001**	0.416±0.004**	0.334±0.002**
	10	0.362±0.007	0.248±0.004**	0.470±0.005**	0.442±0.003**	0.380±0.004**
	15	0.422±0.001	0.228±0.001**	0.052±0.024*	0.050±0.170	0.188±0.049*
P. nigrum L. (ethanol extract)	5	0.110±0.001	0.118±0.001**	0.136±0.001**	0.232±0.007**	0.149±0.002**
	10	0.104±0.002	0.380±0.001**	0.484±0.001**	0.432±0.001**	0.350±0.001**
	15	0.104±0.009	0.294±0.013**	0.284±0.006**	0.284±0.006**	0.241±0.008**

n=5, *: P<0.05 significant, **: P<0.01 most significant as compared to control.

but less than the standard. The hexane extract exhibited maximum anti-inflammatory effect at a dose of 10 mg/kg after 60 min. The ethanol extract showed good anti-inflammatory activity at dose of 10 mg/kg as compared to control but less activity at all doses as compared to standard drug. The ethanol extract exhibited maximum activity at a dose of 10 mg/kg after 60 min.

4. Discussion

In the present study analgesic activity of pure compound piperine, hexane and ethanol extracts of *P. nigrum* L. was analyzed by four different methods (tail immersion, analgesy-meter, hot plate and writhing method). While anti-inflammatory activity was determined by carrageenan induced paw edema. Both activities were determined at a dose of 5, 10 and 15 mg/kg. Acetyl salicylic acid and diclofenac sodium were used as standard reference drugs. Tail immersion method which was used for evaluating centrally acting analgesic effects of drugs showed no increase in latency. Analgesic effect against thermal noxious stimuli may be elicited through opioid receptors or through modulation of several neurotransmitters involved in relevant phenomena[20]. The overall analgesic effect of piperine, hexane and ethanol extracts by thermal parameter was increased with passage of time and maximum effect was achieved after 60 and 120 min. Piperine, hexane and ethanol extracts of *P. nigrum* L. exhibited non significant analgesic effect than diclofenac sodium.

The pressure method is most sensitive to centrally acting analgesics[21]. Pain induced by the analgesy-meter provides a model for the study of non-inflammatory pain. The analgesic effect of piperine, hexane and ethanol extracts of *P. nigrum* L. was investigated by using analgesy-meter method. The oral administration of piperine, hexane and ethanol extracts test involved the increased nociceptive threshold in rat and it had increased up to 60 min and then reduced with passage of time. Significant reduction in the animal sensitivity to pain induced by pressure showed central protecting effect of piperine, hexane and ethanol extracts were comparable to acetyl salicylic acid.

The analgesic effect of piperine, hexane and ethanol extracts of *P. nigrum* L. was investigated using eddy's hot plate method. This test involved marked central analgesic effect as evidenced by significant increase in reaction time. The hexane and ethanol extracts of *P. nigrum* L. possess no analgesic at all doses while pure compound piperine showed maximum activity at a dose of 10 mg /kg after 120 min by increase in the reaction time (increase threshold potential of pain) may be due to the inhibition of prostaglandins synthesis.

Writhing test is a chemical method used to induce pain of peripheral origin by injection of irritant principles like phenylquinone or acetic acid in mice. Analgesic activity of the test compound is inferred from decrease in the frequency of writhes. The manifestations of abdominal writhes in mice were first described by Sigmund *et al.*[22], as an arching of back, extension of hind limbs and contraction of abdominal musculature. The writhing response is considered as a reflexive test. Signals transmitted to central nervous system in response to pain due to irritation, cause release of mediators such as prostaglandins which contributes to the increased sensitivity to nociceptors. Piperine, hexane and ethanol extracts of *P. nigrum* L. decreased the number of writhes significantly at all doses compared to reference drug diclofenac sodium (NSAID) and control. Decrease in writhes are generally considered as an important parameter of analgesic activity in acetic acid induced writhing test.

Carageenan induced paw edema method is most widely used method for testing of non steroidal anti inflammatory agents. Anti-inflammatory agents initially inhibit the cycloxygenase enzyme which is involved in prostaglandin synthesis. We observed that the inhibition of edema volume of piperine at all doses had increased with passage of time, however anti inflammatory effect of hexane and ethanol extracts of *P. nigrum* L. was increased till 120 min. These results indicate that piperine possesses inhibition of prostaglandin release mediated anti-inflammatory properties.

In conclusion, from the present study, *P. nigrum* L. possess potent analgesic and anti-inflammatory activity. In the present study, piperine revealed significant analgesic, anti-inflammatory activity. Acute toxicity assessment results indicated zero percentage of mortality of *P. nigrum* L. at a dose of 15 mg/kg, however at high doses (75, 50 and 25 mg/kg) 100% mortality were observed in mice after intraperitoneal administration. The oral administration of piperine, hexane and ethanol extracts showed zero percent quantal incidence of mortality in rats, indicating excellent safety profile.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by the research grant of The Dean, Faculty of Pharmacy, University of Karachi, Pakistan.

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