Assessment of antinociceptive, antipyretic and antimicrobial activity of *Piper cubeba* L. essential oil in animal models

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Abstract: This study was designed to investigate the possible antiniciceptive, antipyretic and antimicrobial activities of the essential oil obtained from the fruits of *Piper Cubeba* (L.). To assess the antinociceptive and antipyretic activities, three doses (150, 300 and 600 mg/kg, i.p.) were tested in acetic acid-induced abdominal writhing, tail flick reaction and hot-plate and Brewer's yeast-induced hyperpyrexia test models in animals. Moreover, the antimicrobial activity was examined using agar diffusion method and broth micro-dilution assay for minimum inhibitory concentrations (MIC). The *Piper Cubeba* essential oil (PCEO) showed a marked antinociception (17, 30 and 54%) and an increase in reaction time in mice in the flick tailed and hot-plate tests. The brewer's yeast induced hyperpyrexia was decreased in a dose dependent manner. PCEO also exhibited a strong antimicrobial potential. These findings confirm the traditional analgesic indications of *P. cubeba* oil and provide persuasive evidence and support its use in Arab traditional medicine

Keywords: *Piper cubeba*, essential oil, antinociceptive, analgesic, antipyretic, antimicrobial.

INTRODUCTION

At the present time, non-steroidal anti-inflammatory drugs (NSAIDs) are often used by a large number of world's population for the treatment of inflammatory diseases and pain relief, despite their use is sometimes associated with severe well-known adverse side effects (Corley *et al.*, 2003, Wallace, 2008). Thus, more effective and safer anti-inflammatory and analgesic drugs with very little side effects are required. Consequently, considerable attention has been given to pharmaceutical and scientific research on drugs of natural origin to find efficient compounds with minimal side effects which could replace some of these chemical therapeutics (Calixto *et al.*, 2000, Calixto *et al*., 2003, Calixto *et al*., 2004, Choi and Hwang, 2003).

The family Piperaceae consists of 14 genera including the genus *Pieper* which is widely distributed in tropical and subtropical regions (Mabberley, 1997). The genus *Piper* is represented with more than 1000 commercially and medicinally interesting species (Mabberley, 1997). Several species of this genus were found to have anti-inflammatory, antinociceptive, cytotoxic, antimicrobial, antiprotozoal and antiproliferative activities (Lima *et al.*, 2012, Iwamoto *et al.*, 2015, Wan *et al*., 2014, Capello *et al.*, 2015, Bagatela *et al.*, 2013, Woguem *et al*., 2013).
Piper cubeba L. is a well-known plant belong to the family Piperaceae and has been used for a long time as a spice and medicinal plant by traditional healers in different places of the world. In Arab traditional medicine, this plant is used for the treatment of several diseases including rheumatism, cough and intestinal disorders (Bos *et al.*, 2007). In addition, the extract as well as essential oil has been shown to demonstrate antiestrogenic, anti-inflammatory, nephroprotective, cytotoxic, tumor inhibitory, antiparasitic and antimicrobial potential (Our previous studies on the essential oil of *P. Cubeba* demonstrated amelioration of CCl_4 liver injuries and oxidative damage (Alsaid *et al*., 2015) as well as anti-inflammatory effect (under publication).

Earlier, an anti-inflammatory and analgesic activity of methanol extract of *P. cubeba* was reported (Choi and Hwang, 2003). Since there is no scientific data available so far on analgesic, antipyretic effect of *Piper cubeba* essential oil (PCEO), the present work was designed to evaluate the analgesic, antipyretic and antimicrobial activities of *Piper cubeba* essential oil (PCEO).

MATERIALS AND METHODS

Plant materials

Piper cubeba fruits were purchased from a local herbal medicine material shop in Riyadh, Saudi Arabia. They were identified by an expert taxonomist (Dr. Mohammed Yusuf) of the Department of Pharmacognosy, College of

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Pharmacy, King Saud University, Riyadh. A voucher specimen (# 123) was deposited in the crude drug museum of the college.

Extraction of the essential oil

The dried fruits of *P. cubeba* were pulverized and hydro-distilled for 3h by using Clevenger-type apparatus according to the European Pharmacopoeia. The obtained oil was dried over anhydrous sodium sulfate. Then the oil was filtered and stored at 4°C until use.

Animals

The experiments were conducted using healthy male Swiss mice (25-35g) and adult male Wistar albino rats (150-180g), which were procured from the Experimental Animal Care Centre, College of Pharmacy, King Saud University, Riyadh. The animals were kept at a 22±2°C, humidity (55%) under 12h light-dark conditions with free access to water.

Acute toxicity assay

The acute toxicity was tested in male Albino rats according to the guidelines of OECD. The animals were divided into three group comprising of six animals in each group. The test was performed using single dose of 500, 1000 and 1500 mg/kg. All the rats were observed continuously for 2 h for any toxic symptoms, behavioral change and general motor activities and further up to 24 and 72 h for any mortality. The oil does not cause any significant behavioral changes and no mortality was observed.

Studies on antinociceptive activity

Acetic acid-induced abdominal writhing

The test was carried out as previously described (Amresha *et al*., 2007). The mice were divided into five groups of six animals each. Group I was injected intraperitoneally with 0.2 ml of 1 % acetic acid solution only. Group II to IV received three doses of PCEO (150, 300 and 600 mg/kg p.o.). Group V received indomethacin (10mg/kg b.w., p.o.), as a positive control, after an overnight fast. One hour after the treatment, the mice from groups II to V were injected intraperitoneally with 0.2ml of 3 % acetic acid solution in order to induce the characteristic writhing. After that the number of writhings between 5 and 15 min following acetic acid administration was counted and was recorded.

Tail-flick test method

The procedure used was similar to (Tadele *et al.*, 2009) with modification. The tail flick latency was assessed by the analgesiometer (IITC Life Sciences Series, Woodland hills, USA). Prior to treatment, the thermal reaction time of each animal (flicking or removing the tail) was performed at 0 and 10min interval. The average of the two readings was obtained as the initial reaction time. Only rats that showed nociceptive responses within 4 s were used in the experiments. The mice were divided into four groups of six animals each. After 30 min of treatment with PCEO (150, 300 and 600mg/kg b.w., p.o.) and indomethacin 10mg/kg p.o. (positive control), the tail-flick response was measured by gently placing the tail at a central position of a focused light beam from a 45-W projection bulb. The time taken by the animal to withdraw (flick) its tail from heat induced by the light beam was recorded as the reaction time. The reaction time was measured at intervals of 30, 60 and 120 min. The cut-off time was 10 s to prevent injury to the rat tail.

Hot-plate test method

The test was performed in order to measure the latency of the response as described previously by (Asongalem *et al.*, 2004) with minor modifications. The temperature of the hot-plate was maintained at 56±1°C. The mice were placed in a 24cm diameter glass cylinder on the heated surface and the time between placement and licking of the paws or jumping was recorded as response latency. Animals were divided into four groups of six animals each. Group II to IV were treated with four doses of PCEO (150, 300 and 600mg/kg b.w., p.o.). Group V was treated with indomethacin (10mg/kg b.w., p.o.), as a positive control. Mice were selected 24h before the experiment on the basis of their reactivity to the test. The animals tested at 0, 30, 60, and 120 minutes after administration of PCEO (0.4, 1.2 and 4ml/kg) and indomethacin. The cutoff time was 30.

Determination of antipyretic activity in mice

Hyperpyrexia was induced in mice by s.c. injection of 20 ml/kg b.w. of a 20% aqueous suspension of brewer's yeast in the back below the nape of the neck (Makonnen *et al*., 2003). Animals were divided into four groups of six animals each. The animals were then fasted for the duration of the experiment (approximately 24h), having free access to water. Control temperatures were taken 24 h after the yeast injection to determine the pyretic response to yeast. Temperature was taken 1h prior to drug administration in fevered animals served as a pre-drug control. PCEO (150, 300 and 600 mg/kg b.w., p.o.) was given 24 h after the yeast injection and the temperatures were recorded at 30, 60 and 120 minutes after its administration. Group IV was treated with indomethacin (10 mg/kg b.w., p.o.), as a positive control.

Antimicrobial susceptibility testing

Test microorganisms

A test microorganisms included *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* wild strain, *Cryptococcus neoformans* wild strain, *Candida albicans* ATCC 60193 and *Aspergillus fumigates* AUMC 8794. All microbial strains were obtained from the Botany and Microbiology Department, King Saud University in Saudi Arabia, Riyadh.

Disk diffusion testing

Disk diffusion testing of the investigated extracts and several standard antimicrobial agents was performed according to Ortez (2005). A Mueller-Hinton agar (Oxoid, Germany) and potato dextrose agar (Oxoid, Germany) were used to cultivate the bacteria and the fungi respectively. Standard inoculum suspensions of microbial strains were prepared by direct colony assay from growing up colonies. The turbidity of the microbial suspension standardized to match that of a 0.5 McFarland standard. The Mueller-Hintone agar and potato dextrose agar plates were placed at room temperature for 1 hour to remove a excess moisture. The microorganism suspensions were well mixed by vortex after that a sterile cotton swab was dip into the suspension then the excess liquid was removed from swab by slightly pressing it on side of the tube. The surface of plates were completely streaked by the swab then incubated for 15 minutes before applying the standard antimicrobial and investigated extracts disks. A mastring-S 50 (MAST, UK) were used in this study that include eight standard antibacterial disks (Ampicillin (25μg), Cephalothin (30μg), Cotrimoxazole (25μg), Gentamicin (10μg), Mecillinam (33μg), Nalidixic acid (30μg), Nitrofurantion (300μg) and Norfloxacin (10μg)) whereas caspofungin (20μg) was used as standard antifungal agent. The investigated extracts disks were prepared by impregnating sterile filter paper discs of 6 mm diameter (Schleicher and Schuell, ref. No. 10321260, lot. DG0274-1) with 10µl (8.1mg) of the PCEO. All disks were applied on surface of media which inoculated by the microbial strains then kept for 2 hours in refrigerator to enable prediffusion of the essential oil into the agar. Negative controls were carried out using sterile filter paper discs loaded with 20µl of H_2O. After the incubation, the antibacterial activity was evaluated by measuring the diameter (mm) of inhibition zones.

Minimum inhibitory concentration (MIC) testing

Broth microdilution MIC testing of PCEO and numerous standard antimicrobial agents was carried out according to Rankin (2005). A polystyrene panels containing 96 wells, Mueller-Hinton broth and Potato dextrose broth were used in this investigation. The pH of media were between 7.2 to 7.4 at 25ºC. Standard inoculum suspensions of microbial strains were the same as those described in disk diffusion testing. Dilution of standardized inoculum was adjusted to 5×10^4 colony forming unit (CFU)/0.1ml well (5×10^5 CFU/ml). Duplicate two-fold serial dilutions of PCEO (100 µl/well) were prepared in the suitable broth containing 5% (v/v) dimethyl sulfoxide (DMSO) to produce a concentration range of 1.62 to 0.00015mg/ml. Two-fold dilutions of standard antimicrobial agents were used as a positive control. Two well were served as a positive and negative growth control (broth plus inoculums and broth only respectivily). The plates were incubated in several conditions depending into the microbial strains. The MIC of PCEO was recorded as the lowest concentration at which no growth was observed in the duplicate wells. A p-iodonitro-tetrazolium violet (INT) solution (0.04%, w/v) (Sigma, USA) was used as indicator to microbial growth. Twenty microliters of INT was then added to each well. The plates were incubated for a further 30 min, and estimated visually for any change in color from yellow to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC. Experiments were performed in duplicate.

STATISTICAL ANALYSIS

The results are given as mean ± the standard error of mean (SEM). Statistical significance was determined by one-way ANOVA using a multiple comparison procedure (Dunnett's multiple test). A p-value of less than 0.05 was considered significant. All statistical analyses were performed employing GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

RESULTS

Antinociceptive activity

Effect of PCEO on acetic acid-induced writhing

The results of the antinociceptive activity of PCEO assessed using acetic acid-induced writhing test in Swiss albino mice are presented in table 1. It was shown that PCEO at the doses of 300 and 600mg/kg exhibited significant (P>0.01) reduction of the number of writhes at the rate of 30.5 and 54.0% respectively. Moreover, indomethacin showed potent inhibition of writhing (70.3%). These results clearly indicate that PCEO possess analgesic activity.

Effect of PCEO on tail flick reaction time

Treatment with PCEO showed dose dependent increase in the reaction time when compared to control group. The results of administered PCEO on tail flick latencies in mice are summarized in table 2. PCEO (600mg/kg), at 1 and 2h after its administration, significantly (P<0.05) increased the tail flick latency, when compared with the control group. In addition, the reference drug indomethacin (10mg/kg) significantly (P<0.001) increased the tail flick latency at 1 and 2h, as compared to the control group. The antinociceptive activity of PCEO at 600mg/kg was the highest and comparable with that of the reference drug.

Effect of PCEO on hot plate reaction time

A significant (P<0.001) raise in the reaction time on hot plate was observed at 30, 60 and 120 min. The analgesic activity of PCEO is shown in table 3. In this testing model, the high dose of PCEO (600mg/kg) prolonged significantly the reaction time of animal with relatively extended duration of stimulation. At that dose level and 30 and 60 min reaction time, the antinociceptive activity

(14.66 and 16.83 s) was the highest and comparable with that of the reference drug indomethacin (15.50 and 15.50 s).

Antipyretic activity

Effect of PCEO on yeast-induced hyperthermia in mice

The results of the antipyretic effect of PCEO are presented in table 4. Treatment with PCEO reduced yeast-induced fevere in mice (table 4). PCEO showed significant dose-dependent decrease in rectal temperature at all administered doses 150, 300 and 600mg/kg.

Antimicrobial activity of PCEO

The results of the antimicrobial activity are shown in table 5. It was shown that the essential oil had varying degrees of growth inhibition against the bacterial as well as fungal strains (inhibition zones: 10 and 30mm). The fungal strains *Candida albicans* and *Aspergillus fumigates* as well as the methicillin-resistant *Staphylococcus aureus* were the most sensitive strains to PCEO with inhibition zones between 24 and 36mm and MIC-values ranging 6-25µg/ml. The bacterial and fungal strains showed more susceptibility to PCEO than some standard antimicrobial agents (table 5).

DISCUSSION

Piper cubeba represents a valued spice and medicinal plant which is widely used in the traditional medicine. Previous biological works on its alcoholic extract reported several pharmacological effects e.g. antiestrogenic, anti-inflammatory, nephroprotective, cytotoxic, tumor inhibitory, antiparasitic and antimicrobial potential (Yam *et al*., 2008, Bos *et al.,* 2007, Ahmad *et al.*, 2012, Graidist *et al.,* 2015, Viviane *et al.*, 2013, Khan and Siddiqui, 2007). We previously investigated the chemical composition and anti-inflammatory effect of PCEO (under publication). Those results showed that PCEO could decrease lung inflammation in rats treated with carrageen an and demonstrated a significant attenuation of the levels of TNF-α, IL-1β and myeloperoxidase (MPO) in the lungs of carrageen an-injected rats leading to anti-inflammatory effect.

In the present study, the analgesic and antipyretic effects of the essential oil of *P. cubeba* using *in vivo* models nociception in rodents assays were assessed. In addition, the antimicrobial activity was determined using the agar diffusion assay and Broth micro-dilution assay for minimum inhibitory concentrations. Reviewing the available current literature, it is important to point out that this work represents the first report on the, antinociceptive and antipyretic activities of *P. cubeba* essential oil (PCEO).

The present study clearly demonstrates that *P. cubeba* essential oil (PCEO) possesses a potent analgesic activity tested by employing three different testing methods. The aim of using these methods was to identify the peripheral and central effects of PCEO.

Tail-flick test as well as hot plat model have been suggested appropriate to evaluate central antinociceptive activity because of some advantages, specially the sensitivity to antinociceptives and limited tissue damage (Kou *et al*., 2005). Prostaglandins and bradykinins were claimed to play a substantial role in analgesia (Vinegar *et al.,* 1969). Acetic acid-induced writhing model was employed to assess the peripheral analgesic activity. Acetic acid is thought to act indirectly causing the release of noxious substances such as bradykinins, serotonin, histamine and prostaglandins, which induce the writhing response (Bartolini *et al*., 1987). It is suggested that the acetic acid-induced abdominal constriction is dependent on the production and release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukine-1β (IL-1 β) and IL-8, from resident peritoneal macrophages and mast cells (Ribeiro *et al*., 2000). Moreover, it is assumed that the acetic acid releases the endogenous mediators indirectly by which it stimulates nociceptive nerve endings, sensitive to both opioids and non-steroidal anti-inflammatory drugs (NSAIDs) (Do Monte *et al*., 2004). The obtained data showed that the PCEO administration exhibited a profound analgesia in all models used. The observed activity indicates that PCEO possesses both writhing inhibition (peripheral) and lengthened the thermal reaction time (central effects). In addition to that, PCEO also exhibited antipyretic activity in mice. These properties of the PCEO resemble those of the non-steroidal anti-inflammatory activities. NSAIDs are generally cause to block the biosynthesis of prostaglandins (Ferreira and Vane 1974). The obtained results indicate towards that the PCEO exerted analgesic and antipyretic activity probably by interfering with endogenous generation of prostaglandins (Di Rosa, 1974, Di Rosa *et al*., 1971). The results of this study are in agreement with previous studies on other *Piper* species e.g. *P. solmsianum, P. aleyreanum, P. tuberculatum* and *P. laetispicum* (Da Silva *et al*., 2010, Lima *et al.*, 2012, Rodrigues *et al.*, 2009, Xie *et al*., 2011).

The probable mechanism of the analgesic and antipyretic activities of PCEO needs more clarification. There are reports indicating that some monoterpenes from EOs are strong inhibitors of certain inflammatory mediators such as prostaglandins and other arachidonic acid metabolites (Juergens *et al.*, 1998). Furthermore, PCEO exhibited a high antimicrobial potential against Gram positive and negative bacteria as well as against fungal strains. These results are in agreement with the antimicrobial activity of the essential oils of certain *Piper* species e.g. *P. abbreviatum, P. erecticaule, P. lanatum, P. divaricatum* and *P. flaviflorum* (Da Silva *et al*., 2014, Wan Salleh *et al*., 2014, Li *et al.*, 2014).The major components found in PCEO e.g. sabinene, 4-terpineol, γ-terpinene and α-thujene could be responsible for the observed activities.

Table 1: Effect of PCEO on acetic acid induced writhing in mice

Treatments (*n=6*)	Dose (i.p.)	Number of writhing	Inhibition in %
Control (only 3% Acetic acid)	0.1ml/kg	38.83±3.93	-
PCEO + 3% Acetic acid	150mg/kg	31.16±2.16	17.16
PCEO+ 3% Acetic acid	300mg/kg	27.00±2.12**	30.47
PCEO+ 3% Acetic acid	600mg/kg	17.83±1.57***	54.07
Indomethacin + 3% Acetic acid	10mg/kg	11.50±1.38***	70.38

All values represent mean ± SEM. ***p*<0.01, ****p*<0.001; ANOVA, followed by Dunnett's multiple comparison test.

Table 2: Effect of PCEO on tail flick test in rats

Treatments (*n=6*)	Dose (i.p.)	Pre Drug	Reaction time in seconds		
			Post drug after		
			30m	60m	120m
PCEO	150 mg/kg	4.00±0.36	5.16±0.30*	5.83±0.30**	4.50±0.42
PCEO	300 mg/kg	4.50±0.42	5.33±0.66	6.16±0.47*	6.33±0.49*
PCEO	600 mg/kg	4.1±0.47	6.83±0.54**	8.66±0.42***	7.00±0.57**
Indomethacin	10 mg/kg	3.6±0.33	7.83±0.47***	8.66±0.42***	7.83±0.47***

Table 3: Effect of PCEO on hot plate reaction time test in mice.

Treatment (n=6)	Dose (i.p.)	Pre Drug	Reaction time in seconds		
			Post drug after		
			30m	60m	120m
PCEO	150 mg/kg	8.33±0.71	10.33±0.49*	9.83±0.87	11.33±0.95*
PCEO	300 mg/kg	8.16±0.70	11.16±0.87*	13.33±0.66***	12.66±0.49***
PCEO	600 mg/kg	8.33±0.49	14.66±0.98***	16.83±0.65***	14.00±0.73***
Indomethacin	10 mg/kg	7.50±0.42	15.50±0.84***	15.50±0.84***	24.16±1.24***

Table 4: Effect of PCEO on yeast-induced hyperthermia in mice

Treatment (n=6)	Dose (i.p)	Pre Drug	Rectal temperature (^{o}C)		
			Post drug after		
			30m	60m	120m
PCEO	150mg/kg	39.13±0.14	38.80±0.09	37.75±0.13***	38.11±0.15* [b]
PCEO	300mg/kg	39.13±0.29	38.08±0.29*	37.88±0.13***	37.25±0.22***
PCEO	600mg/kg	39.06±0.1	37.01±0.06***	36.75±0.17***	37.01±0.13***
Indomethacin	10mg/kg	37.95±0.19	36.43±0.13***	35.63±0.17***	36.13±0.15***

All values represent mean ± SEM. *p<0.05, ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test. Post drug compared with pre drug.

Table 5: The inhibition zones (I.Z) and minimal inhibitory concentration (MIC) of *Piper Cubeba* essential oil (PCEO) against some clinical microorganisms comparing by several standard antimicrobial agents

Organisms tested	PCEO		I.Z of standard antimicrobial Agent								
	I.Z. (mm)	MIC mg/ml	AP	KF	TS	GM	MEC	NA	NI	NOR	CA
S. aureus ATCC 25923	17	0.101	30	28	27	24	-	-	30	30	N.T
E. faecalis ATCC 29212	14	0.202	20	-	-	15	-	-	20	12	N.T
Methicillin-resistant *S. aureus* (MRSA) ATCC 43300	18	0.050	-	-	-	22	-	-	34	32	N.T
E. coli ATCC 25922	14	0.101	16	-	-	20	13	16	18	10	N.T
P. aeruginosa ATCC 27853	10	0.202	12	-	-	24	-	-	13	-	N.T
S. enteritidis wild strain	14	0.101	19	-	-	15	14		18	11	N.T
C. neoformans wild strain	16	0.012	N.T	N.T	N.T	N.T	N.T	N.T	N.T	N.T	12
C. albicans ATCC 60193	20	0.006	N.T	N.T	N.T	N.T	N.T	N.T	N.T	N.T	22
A. fumigatus AUMC 8794	18	0.012	N.T	N.T	N.T	N.T	N.T	N.T	N.T	N.T	23

AP: Ampicillin (25 µg), KF: Cephalothin (30 µg), TS: Cotrimoxazole (25 µg), GM: Gentamicin (10 µg), MEC: Mecillinam (33 µg), NA: Nalidixic acid (30 µg), NI: Nitrofurantion (300 µg), NOR: Norfloxacin (10 µg) and CA: Caspofungin (20 µg). -: no activity; N.T.: not tested; Inhibition zones including the diameter of the paper disc (6 mm)

CONCLUSIONS

In conclusion, the study showed that the essential oil of *P. cubeba* possesses peripheral and central antinociceptive activity along with antipyretic activity. Moreover, it showed a strong antibacterial and antifungal activities. These findings support the use of *P. cubeba* in painful and inflammatory conditions and confirm its use in Arab traditional medicine. Nevertheless, further studies are needed to confirm its clear mechanism of action and to isolate the possible active constituents.

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