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Investigation of Some *Piper* Species for Anti-Bacterial and Anti-Inflammatory Property

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Abstract: Methanol extracts *of Piper longum* L., *Piper betle* L., *Piper attanuatum* Buch-Ham. [type-2], *Piper hymenophyllum* Miq., *Piper sarmentosum* Roxb., *Piper argyrophyllum* L., *Piper attanuatum* Buch.-Ham. [type-1] and *Piper chaba* Hunter were checked for their antibacterial efficiency against 15 clinically important bacterial strains. Cefotaxime sodium (100 μg $disc^{-1}$) was used as standard. Crude leaf powder suspensions of these 8 *Piper* species were also evaluated for acute and chronic anti-inflammatory study at a dose of 300 mg kg^{-1}. Diclofenac sodium was used as the standard drug. Carrageenan and dextran models were studied for acute inflammation while cotton pellet-induced granuloma was used for chronic inflammation study. ANOVA followed by Dunnett's t-test were employed for statistical analysis. Piper species showed better anti-inflammatory activity than antibacterial activity. *Piper sarmentosum*, *Piper argyrophyllum*, *Piper longum*, *Piper betle* and *Piper chaba* has biologically important properties and they should be further explored and the active principle should be elucidated in order to bring out the promising antibacterial and anti-inflammatory agent.

Key words: Antibacterial, anti-inflammatory, Piperaceae, *Piper* species, carrageenan, dextran, cotton pellet granuloma

INTRODUCTION

Plants have played a remarkable role in health care since the ancient times. Traditional plant-based medicines still exert a great deal of importance to the people living in developing countries and also lead to discovery of new drug candidates for a variety of diseases that threaten human health. Even with recent advances in modern medicine, traditional medicine practice is gaining more followership. Recently, several experimental studies have contributed scientific evidence for the pharmacological effects of the medicinal plants observed in folk medicine (Nardi *et al*., 2003). Nosocomial bacteraemia associated with resistant organisms and postoperative surgical infections is a serious problem (Nicholas, 2001). Since antibiotic use became widespread 50 years ago, bacteria have relentlessly developed resistance, hence efforts have been made to develop and study new compounds outside conventional antibiotic therapy (Martinez and Baquero, 2002).

Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules (Mantri and Witiak, 1994). Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, colonic adenomas and Alzheimer's disease (Smith and De Witt, 1995; Lipsky, 1999).

Inflammation is a process involving multiple factors acting in a complex network. Inflammation can be induced in animals by many substances. Carrageenan-induced rat paw oedema has been used as inflammation model in order to investigate the anti-inflammatory effect of drug (El-Shenawy *et al*., 2002). Models of chronic inflammation, which is provoked by subcutaneous implantation of foreign bodies, are used to investigate the effects of drugs on a chronic phase of inflammation (Ionac *et al*., 1996). The side effects of the currently available anti-inflammatory drugs pose a major problem in their clinical use. For instance, NSAIDs cause several serious adverse effects like gastric injury and ulceration, renal damage and bronchospasm (Tapiero *et al*., 2002). The use of steroidal drugs as anti-inflammatory agents is also becoming highly controversial due to their multiple side effects (Van den Worm *et al*., 2001). Therefore, a need arises for the development of newer anti-inflammatory agents from natural sources with more powerful activity and with lesser side effects as substitutes for chemical therapeutics. Therefore, plant remedies have become increasingly popular and are often preferred to synthetically derived pharmaceuticals.

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The family Piperaceae comprises approximately 5 genera and 1400 species. The genera *Piper* and *Peperomia* are the most representative of Piperaceae (700 and 600 species, respectively). Some of these species are used in folk medicine to treat many diseases. Analgesic, antibacterial, anthelmintic, aphrodisiac, carminative, expectorant property of *Piper* species has been reported previously by many workers. Phytochemical investigations of Piperaceae species have shown the presence of metabolites from mevalonic acid (monoterpenes and sesquiterpenes), acetic acid/shikimic acid (flavonoids), shikimic acid pathways (lignoids, arylpropanoids and amides); amides (cinnamoyl amides and alkyl amides), aristolactams and other alkaloids also have been isolated (Sengupta and Ray, 1987).

The purpose of the present study is to evaluate antibacterial and anti-inflammatory potentials of eight *Piper* species in order to validate some of its traditional uses.

MATERIALS AND METHODS

Plant material: The leaves of *Piper longum* L., *Piper betle* L., *Piper attanuatum* Buch-Ham. [type-2], *Piper hymenophyllum* Miq., *Piper sarmentosum* Roxb., *Piper argyrophyllum* L., *Piper attanuatum* Buch.-Ham. [type-1] and *Piper chaba* Hunter were collected during the month of April, 2006 from Tropical Botanical Garden and Research Institute (TBGRI), Kerala. The taxonomic identification was done by Dr. P.J. Mathew, Scientist, TBGRI, Palode, Kerala. The leaves were air dried, pulverized using a mechanical grinder and stored in air-tight containers, at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India.

Extraction: Ten grams of dried leaf powder was taken in 100 mL of methanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 h the extracts were centrifuged at 5000 g for 10 min and supernatant was collected and solvent was evaporated and dry extract was stored at 4°C in airtight bottles.

Microbial strains: The test bacterial strains investigated are shown in Table 1. All the bacterial strains were obtained from National Chemical Laboratory (NCL), Pune, India. The bacteria were grown in the nutrient broth and maintained on nutrient agar slants at 4°C.

Antimicrobial assay: The antibacterial assay was evaluated by the method of agar disc diffusion method (Bauer *et al*., 1966; Parekh and Chanda, 2007). The media Mueller Hinton Agar No. 2 (HiMedia, India) and the test microbial cultures were poured into Petri dishes (Hi-Media). The test strain (200 µL) was inoculated into the media (inoculums size 10^8 cells mL^{-1}) when the temperature reached 40-42°C. 100 µg/20 µL of the test compound was impregnated in to sterile discs (7 mm, HiMedia). The disc was then introduced into medium with the bacteria. The plates were incubated overnight at 37°C for bacteria. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition. The experiment was performed in triplicates and the mean values of the result are expressed as Mean±SEM. Cefotaxime sodium (100 µg $disk^{-1}$) was used as positive control and Dimethylsulphoxide was taken as negative control.

Experimental animals and drug dose: Wistar strain albino rats were obtained from the animal house of Sarabhai Research Centre (SRC), Baroda and the in-house colony was maintained at the animal house of Department of Biosciences, Saurashtra University, Rajkot. The animals were maintained on Pranav Agro Brand rat pellet feed and tap water given *ad libitum* in a normal uncontrolled condition. The rats were kept for one week for

Table 1: Anti-bacterial activity of the 8 *Piper* species studied

Plants used	Extractive yield (%)	Inhibition zone (cm)														
		Sa-1	Sa-2	Se	Mf	Bc	Bs	Ka	Kp	Ec	Cf	Cr	Pa	St	Pm	Pv
P. betle	2.70	-	-	-	-	1±0	-	-	1.00±0.00	-	-	-	-	-	-	-
P. attanuatum [type-2]	2.92	-	-	-	-	-	-	-	1.20±0.00	-	-	-	-	-	-	-
P. hymenophyltum	2.42	-	-	-	-	-	-	-	0.90±0.00	-	-	-	-	-	-	-
P. sarmentosum	2.40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. argyrophyllum	2.60	-	-	-	-	-	-	-	1.00±0.00	-	-	-	-	-	-	-
P. attanuatum [type-1]	2.93	-	-	-	-	-	-	-	1.05±0.02	-	-	-	-	-	-	-
P. longum	2.40	-	-	-	-	-	-	-	0.50±0.28	-	-	-	-	-	-	-
P. chaba	3.02	-	-	-	-	1.35±0.02	-	-	1.05±0.02	-	-	-	-	-	-	-
Cefotaxime sodium	-	2.95±0.02	2.5±0	2.8±0	3.6±0.11	1.25±0.02	4±0	1.5±0	2.85±0.02	2.45±0.02	2.8±0.05	3±0	2.1±0.05	2.5±0	1.6±0	1±0

Values are mean±SEM, n = 3, Disc diameter 0.7 cm, Sa-1 - *Staphylococcus aureus* ATCC 25923, Sa-2 - *Staphylococcus aureus* ATCC 29737, Se - *Staphylococcus epidermidis* ATCC 12228, Mf - *Micrococcus flavus* ATCC 10240, Bc - *Bacillus cereus* ATCC 11778, Bs - *Bacillus subtilis* ATCC 6633, Ka - *Klebsiella aerogenes* NCTC 418, Kp - *Klebsiella pneumoniae* NCIM 2719, Ec - *E. coli* ATCC 25922, Cf - *Citrobacter freundii* ATCC 10787, Cr - *Corynebacterium rubrum* ATCC 14898, Pa - *Pseudomonas aeruginosa* ATCC 27853, St - *Salmonella typhimurium* ATCC 23564, Pm - *Proteus mirabilis* NCIM 2241, Pv - *Proteus vulgaris* NCTC 8313, - Means no activity

acclimatization before the experimental sessions. The studies were approved by the CPCSEA approved local ethical committee.

Carrageenan-induced paw edema in rats: The method of Winter *et al.* (1962) was followed for determining the anti-inflanimatory activity of the extracts in rats. The animals were divided into ten groups, each group consisting of six animals of either sex. Initially the paw volume was measured using a plethysmometer. Thereafter, Crude leaf powder (300 mg kg^{-1}) of 8 *Piper* species, positive control Diclofenac sodium (5 mg kg^{-1}) or vehicle control were orally administered to the respective group of animals. One hour after drug administration, oedema was induced by 0.1 mL of 1% carrageenan in N-saline. The paw volume was measured after 1, 2 and 3 h.

Dextran-induced paw edema in rats: Oedema was induced by subplantar injection of 0.1 mL of 1% freshly prepared suspension of Dextran (60,000-90,000 mw, HiMedia, India) in N-saline into the right hind paw of each rat (Winter and Porter, 1957). All the remaining procedure was same as that employed for carrageenan.

Cotton pellet-induced granuloma in rats: The rats were divided into ten groups, each group consisting of six animals of either sex. After shaving of the fur, the animals were anaesthetized with ether. Two sterile pre-weighed cotton pellets (100±1 mg) were implanted in the dorsal region on both sides of each rat through a single incision (Swingle and Shideman, 1972). The incision was sutured. Crude leaf powder (300 mg kg^{-1}) of the 8 piper species, positive control Diclofenac sodium (5 mg kg^{-1}) or vehicle control (5 mL kg^{-1}) were administered to the respective group of animals for seven days from the day of cotton pellet implantation. On the eighth day, the animals were sacrificed; the cotton pellets were removed surgically and made free from extraneous tissues. The pellets were dried at 60°C to constant weight. The increment in the dry weight of the pellets was regarded as measure of granuloma formation.

Statistical analysis: The observations are expressed as mean±SEM One way analysis of variance (ANOVA) followed by Dunnet's t-test were employed for statistical analysis.

RESULTS

The extractive yield of the 8 *Piper* species is given in Table 1. Maximum extractive yield was in *P. chaba* and minimum was in *P. sarmentosum* and *P. longum*. The results of antibacterial activity are as shown in Table 1. None of the extracts showed any significant activity against the studied bacterial strains used. *P. betle* and *P. chaba* showed moderate activity against *B. cereus*, while 7 *Piper* species screened showed moderate activity against *K. pneumoniae*, except *P. sarmentosum*.

In carrageenan-induced paw edema (Table 2), *P. sarmentosum*, *P. argyrophyllum* and *P. longum* exhibited significant reduction in paw volume by 47.41% ($^{d}p \leq 0.01$), 46.72% ($^{d}p \leq 0.01$) and 39.81% ($^{d}p \leq 0.01$) in the 1 h, while they showed 24.78, 42.56 and 31.61% reduction in paw volume in the 3 h, respectively as compared to the control group. Diclofenac sodium showed 35.90% ($^{d}p \leq 0.05$) and 41.03% decrease in paw volume in the 1 and 3 h as compared to the control group. *P. attanuatum* exhibited poor activity while *P. hymenophyllum* showed no activity. *P. betle*, *P. attanuatum* and *P. chaba* exhibited pro-inflanimatory potency.

In dextran-induced edema (Table 3), *P. hymenophyllum*, *P. sarmentosum*, *P. argyrophyllum* and *P. chaba* exhibited significant decrease in paw volume by 44.59% ($^{d}p \leq 0.05$), 44.56% ($^{d}p \leq 0.05$), 45.69% ($^{d}p \leq 0.05$) and 43.18% ($^{d}p \leq 0.05$) in the 1 h. *P. sarmentosum*, *P. argyrophyllum* and *P. longum* exhibited very significant decrease in paw volume by 63.63% ($^{d}p \leq 0.01$), 63.66% ($^{d}p \leq 0.01$), 67.51% ($^{d}p \leq 0.001$), while *P. chaba* showed significant decrease in paw volume by 39.31% ($^{d}p \leq 0.05$). *P. longum* was inactive in the 1 h, while *P. hymenophyllum* was inactive in the 3 h. Diclofenac sodium exhibited 20.45 and 23.36% inhibition in paw volume after 1 and 3 h, respectively.

Table 2: Anti-inflammatory activity of some *Piper* species in carrageenan-induced rat paw edema

	1 h		2 h		3 h	
Treatments	Paw volume	% change	Paw volume	% change	Paw volume	% change
Control	32.36±3.60	-	24.77±4.12	-	33.04±5.19	-
Diclofenac sodium (5 mg kg^{-1})	20.74±3.67	35.90↓*	25.77±5.62	4.05↑	19.48±2.94	41.03↓
P. betle (300 mg kg^{-1})	19.26±2.62	40.48↓**	36.10±7.58	45.73↑	36.88±6.25	11.63↑
P. attanuatum [type-2] (300 mg kg^{-1})	27.21±2.51	15.93↓	25.45±6.02	2.76↑	40.53±4.68	22.66↑
P. hymenophyltum (300 mg kg^{-1})	22.60±2.00	30.16↓*	29.97±7.26	20.97↑	32.88±13.53	0.48↓
P. sarmentosum (300 mg kg^{-1})	17.02±1.67	47.41↓**	24.77±4.91	0.00	24.85±4.87	24.78↓
P. argyrophyllum (300 mg kg^{-1})	17.24±3.26	46.72↓**	18.00±2.91	27.33↓	18.98±4.86	42.56↓
P. attanuatum [type-1] (300 mg kg^{-1})	20.40±3.21	36.97↓*	15.98±2.73	35.49↓	29.58±3.28	10.48↓
P. longum (300 mg kg^{-1})	19.48±2.97	39.81↓**	21.01±3.13	15.18↓	22.60±2.50	31.61↓
P. chaba (300 mg kg^{-1})	35.98±4.62	11.19↑	46.12±6.40	86.20↑	41.76±3.77	26.40↑

Values are Mean±SEM, n = 6, F = (0.05*) for 1 and 2 h, ^{d}p = (0.05*, 0.01**)

Table 3: Anti-inflammatory activity of some *Piper* species in dextran-induced rat paw edema

	1 h		2 h		3 h	
Treatments	Paw volume	% change	Paw volume	% change	Paw volume	% change
Control	42.72±7.76	-	39.00±4.94	-	39.93±5.94	-
Diclofenac sodium (5 mg kg^{-1})	33.98±3.21	20.45↓	26.80±6.78	31.28↓	30.60±4.69	23.36↓
P. betle (300 mg kg^{-1})	28.24±6.17	33.89↓	21.37±5.48	45.20↓	31.10±3.96	22.11↓
P. attanuatum [type-2] (300 mg kg^{-1})	38.22±2.29	10.53↓	33.79±4.16	13.35↓	32.17±2.80	19.43↓
P. hymenophyltum (300 mg kg^{-1})	23.67±6.48	44.59↓*	18.85±3.25	51.66↓*	35.51±5.96	11.06↓
P. sarmentosum (300 mg kg^{-1})	23.68±6.02	44.56↓*	21.08±5.33	45.94↓	14.52±3.56	63.63↓**
P. argyrophyllum (300 mg kg^{-1})	23.20±3.76	45.69↓*	24.71±4.71	36.64↓	14.51±1.47	63.66↓**
P. attanuatum [type-1] (300 mg kg^{-1})	44.54±9.83	4.26↑	38.38±12.02	1.58↓	37.80±6.04	5.33↓
P. longum (300 mg kg^{-1})	48.91±4.02	14.48↓	28.71±6.89	26.38↓	12.97±4.98	67.51↓***
P. chaba (300 mg kg^{-1})	24.27±8.62	43.18*	30.50±6.76	21.79↓	24.23±7.00	39.31↓*

Values are Mean±SEM, n = 6; F = (0.05[#]) for 1 and 3 h, [d]p = (0.05*, 0.01**, 0.001***)

Table 4: Anti-inflammatory activity of some *Piper* species in cotton pellet-induced granuloma in rats

Treatments	Pellet weight (mg)	% change
Control	106.55±8.50	-
Diclofenac sodium (5 mg kg^{-1})	101.35±3.70	4.87↓
P. betle (300 mg kg^{-1})	81.52±1.37	23.48↓*
P. attanuatum [type-2] (300 mg kg^{-1})	101.36±4.00	4.86↓
P. hymenophyltum (300 mg kg^{-1})	99.95±6.30	6.18↓
P. sarmentosum (300 mg kg^{-1})	109.86±8.69	3.11↑
P. argyrophyllum (300 mg kg^{-1})	94.20±7.04	11.59↓
P. attanuatum [type-1] (300 mg kg^{-1})	118.08±4.62	10.83↑
P. longum (300 mg kg^{-1})	105.70±8.59	0.78↓
P. chaba (300 mg kg^{-1})	96.50±6.12	9.42↓

Values are Mean±SEM, n = 6; F = (0.05[#]), [d]p = (0.05*)

In cotton pellet-induced granuloma (Table 4), only *P. betle* produced 23.48% ([d]p = 0.05) inhibition in granuloma formation, while *P. argyrophyllum* produced 11.59% of inhibition of granuloma weight. Diclofenac sodium produced poor inhibition (4.87%) in granuloma weight as compared to the control group. *P. sarmentosum* and *P. attanuatum* exhibited pro-inflammatory property. All the remaining 4 *Piper* species exhibited poor or no activity in reducing the granuloma.

DISCUSSION

Antibiotic resistance has become a global concern (Westh *et al*., 2004). The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents that lead to repeated use of antibiotics and insufficient control of the disease (NCID, 2002). Traditional healing systems around the world that utilize herbal remedies are an important resource for the discovery of new antibiotics (Okpekon *et al*., 2004). From the results, it can be said that *P. betle* and *P. chaba* can be explored further as they are able to resist bacterial growth up to a certain extent.

Inflammation or phlogosis is pathophysiological response of living tissue to injuries that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be induced, maintain or aggravate many diseases (Sosa *et al*., 2002). It is also known that anti-inflammatory effects can be checked by a variety of chemical agents and that there is little correlation between their pharmacological activity and chemical structure (Sertie *et al*., 1990). The present study establishes the anti-inflammatory activities of *Piper* species in a number of experimental rat models, representing different phases of inflammation.

Carrageenan-induced rat paw edema is a test which has significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Mossa *et al*., 1995). Oedema formation due to carrageenan in the rat paw is the biphasic event (Vinegar *et al*., 1969). The initial phase is attributed to the release of histamine and serotonin (Maity *et al*., 1998) whereas the kinins and prostaglandins mediate the more prolonged delayed onset responses (second phase) (Vane and Botting, 1987). It has been shown that COX-2 reaches maximal expression 1 h from carrageenan local injection (Nantel *et al*., 1999). From the results obtained, it can be inferred that the inhibitory effects of the drugs *P. sarmentosum*, *P. argyrophyllum* and *P. longum* on carrageenan-induced inflammation in rats could be due to suppressed COX-2 expression after 1 h of carrageenan injection.

The dextran-induced edema is a well known experimental model in which the edema is a consequence of liberation of histamine and serotonin from the mast cell (Van Wauve and Goosens, 1989). The result tends to suggest that the anti-inflammatory activity of *P. sarmentosum*, *P. argyrophyllum*, *P. longum* and *P. chaba* is possibly backed by its anti-histamine or anti-serotonin activity.

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of

development of proliferative cells which can either spread or form granuloma. Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation (Gupta *et al*., 2003). The crude leaf powder suspension of *P. betle* exhibited significant anti-inflammatory activity in the cotton pellet-induced granuloma in rats; this reflected its efficacy to inhibit the proliferative phase of the inflammation process (Recio *et al*., 1995).

The present study demonstrates the efficacy of *Piper betle* and *Piper chaba* as antibacterial agents and *Piper sarmentosum*, *Piper argyrophyllum*, *Piper longum* and *Piper betle* as anti-inflammatory agents and also scientifically justifies the medicinal uses of *Piper* species in folk medicine. Further detailed investigation of the active plant is underway to determine the exact phytoconstituents, which are responsible for the antibacterial and anti-inflammatory activities.

CONCLUSIONS

The antibacterial screening of the 8 *Piper* species revealed that the species studied did not possess antibacterial property except *Piper betle* and *Piper chaba* while, in acute anti-inflammatory studies, *Piper sarmentosum*, *Piper argyrophyllum* and *Piper longum* exhibited potential anti-inflammatory activity. *Piper betle* proved to be a better alternate for chronic inflammation. Our results conclude that *Piper sarmentosum*, *Piper argyrophyllum*, *Piper longum*, *Piper betle* and *Piper chaba* has biologically important properties and they should be further explored and the active principle should be elucidated in order to bring out the promising antibacterial and anti-inflammatory agent.

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