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Investigation of the inhibitory effects from the Brazilian medicinal plant *Pothomorphe umbellata* L. (Piperaceae) on the molecular pathways of cyclooxygenase-2 and nuclear factor kappa B

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Presently, medicinal plants, such as *Pothomorphe umbellata* L. (Piperaceae), have been assessed as sources of potential therapeutic medicines. The inhibitory effects of the crude extract, fractions and 4-nerolidylcathecol, a phenolic compound which has been assigned significant activity in several beneficial properties performed by the plant, on the molecular pathways of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) were evaluated. The *in vitro* inhibition of COX-2 was performed by enzyme-linked immunosorbent assay (ELISA). Methylene chloride fraction demonstrated the preeminent inhibition of COX-2. In order to estimate the inhibition of NF-κB, an adaptation of the luciferase plasmid assay was developed. 4-Nerolidylcathecol presented the best inhibition NF-κB activity. The results obtained from the *in vitro* assays were promising, mainly on the molecular pathway of NF-κB, once 4-nerolidylcathecol demonstrated a remarkable inhibition activity.

Key words: Piperaceae, *Pothomorphe umbellata, Piper umbellatum*, 4-nerolidylcathecol, folk medicine, cyclooxygenase-2 (COX-2), nuclear factor-kappaB (NF-kappaB), inflammation.

INTRODUCTION

From thousand years until today, anti-inflammatory therapies have been applied with the use of natural resources. However, only recently, the cellular and molecular mechanisms of the inflammatory process have

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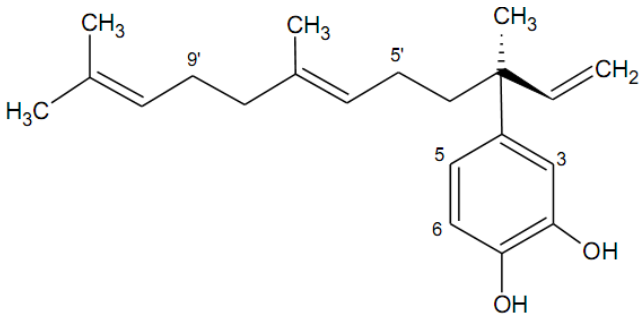

Figure 1. Chemical structure of 4-nerolydilcathecol.

been sufficiently detailed to promote anti-inflammatory strategies with reduced adverse effects (D'Acquisto et al., 2002).

In this context, medicinal plants have been receiving increasing attention. They are an important source of novel structures leading to the expansion of drugs as well as an alternative to the use of synthetic compounds in pharmaceutical technology (Kengne et al., 2016). Plants of the Piperaceae family are purported in the Ayurvedic system of medicine and in folk medicine of Latin America for their numerous curative actions (Parmar et al., 1997; Perazzo et al., 2005).

Innumerous folk preparations developed from Brazilian medicinal plants are generally used for the treatment of inflammation (Stasi et al., 1989). *Pothomorphe umbellata* sin. *Piper umbellatum* L. (Piperaceae), belonging to Brazilian biodiversity, known as "caapeba-do-norte" or "pariparoba", is a green shrubby plant which blooms naturally from the north to the south of Brazil, mainly in the states of Amazonas, Bahia, Espírito Santo, and São Paulo (Angely, 1969).

P. umbellata L. (Piperaceae) had been termed in the first edition of the Brazilian Pharmacopoeia (Silva, 1929). It has been relegated to several therapeutic properties, such as an antioxidant (Lopes et al., 2013), analgesic and anti-inflammatory (Perazzo et al., 2005), antibacterial (Isobe et al., 2002), antifungal (Rodrigues et al., 2012) and antimalarial (Bagatela et al., 2013).

As a part of our research on pharmacological activities of *P. umbellata* L., the inhibitory activities of its extract, fractions and 4-nerolidylcathecol, a phenolic compound which is assigned significant activity in several beneficial properties performed by the plant species, on the molecular pathways of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) have been investigated.

MATERIALS AND METHODS

Plant material

Aerial parts of *P. umbellata* L. were collected in the campus of the University of São Paulo (Ribeirão Preto, SP, Brazil). Professor Pedro de Melillo de Magalhães made the botanical identification of the aerial parts. A sample was deposited in the herbarium of the Botany Department of the University of Campinas – UNICAMP, under register number #UEC 127123.

Preparation of crude extract and fractions

The crude extract and fractions from aerial parts of *P. umbellata* L. were prepared as previously described (Perazzo et al., 2005). Briefly, 500 g of dry material was used to obtain the ethanolic extract, yielding 112 g of dried extract (22.47%). This extract was suspended in methanol:water (9:1) mixture and extracted with methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc), in sequence, to furnish CH_2Cl_2 (7.45 g, 1.49%), EtOAc (12.4 g, 2.48%) and hydromethanol (residual) fractions.

Isolation and identification of 4-nerolidylcatechol

The isolation of 4-nerolidylcatechol was performed by column chromatography and identified by nuclear magnetic resonance (NMR) through ^{13}C NMR and ^{1}H NMR analyses (Kijjoa et al., 1980; Bagatela et al., 2013) (Figure 1).

In vitro inhibition of cyclooxygenase-2

Mouse macrophages were cultured in cell tubes (75 cm^2) in RPMI-1640 medium (Gibco™, Carlsbad, CA, USA) supplemented with 10% of bovine serum (HyClone Laboratories, Logan, UT, USA) and 60 mg/L of amikacin (Sigma-Aldrich, St. Louis, MO, USA). They were maintained in a temperature equal to 37°C in an atmosphere with 95% humidity and 5% carbon dioxide (CO_2). Then, cells were sprinkled in 96-well enzyme-linked immunosorbent assay (ELISA) microplates (Greiner Bio-One, São Paulo, SP, Brazil) and incubated for twenty-four hours at 37°C. Cells had been treated with 250 µM of aspirin for thirty minutes for complete inactivation of the activity of cyclooxyenase-1 (COX-1) enzyme. After washing with the culture medium described previously, cells were incubated with 5 mg/mL of lipopolysaccharide (LPS, Sigma-Aldrich Co., St. Louis, MO, USA) for sixteen hours to induce the production of cyclooxyenase-2 (COX-2) enzyme. The induced cells were washed again with culture medium for the completed removal of LPS and, then, treated with different concentrations (50, 10 and 2 µg/mL solubilized in DMSO) of the samples for two hours. It was added 300 mM of arachidonic acid (Sigma-Aldrich, St. Louis, MO, USA) and the cells were incubated for thirty minutes at 37°C. The supernatant was recovered for quantitation of prostaglandin E_2 (PGE_2) using the PGE_2 enzyme immunoassay reagent (Cayman Chemical Company, Ann Arbor, MI, USA). Preliminarily, the activity of COX-2 was determined by conversion of exogenous arachidonic acid into PGE_2 and expressed as percentage of control (DMSO, 0.5%). And, subsequently, the IC_{50} (inhibition concentration which decreases the investigated response by 50%) was calculated. N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398; Cayman Chemical Company, Ann Arbor, MI, USA), a specific inhibitor of COX-2, was used as control in the assay.

In vitro inhibition of nuclear factor kappa B

Chondrosarcoma cells SW1353 were cultured in a mixture of DMEM/F12 supplemented with 10% FBS (1:1), 100 U/mL of penicillin G sodium, and 100 mg/mL of streptomycin. The *in vitro*

Table 1. Inhibition of COX-2 by *P. umbellata* L. (Piperaceae) species.

Tested drug	Concentration (µg/ml)			IC_{50} (µg/ml)
	50	10	2	
	Inhibition percentage (%)			
Crude extract	20.27 ± 3.42	10.86 ± 3.06	6.95 ± 4.15	NA
CH_2Cl_2 fraction	57.38 ± 2.61	22.79 ± 2.40	10.86 ± 2.09	39.21 ± 2.22
EtOAc fractions	20.95 ± 2.37	13.87 ± 2.21	6.15 ± 3.10	NA
Residual fraction	12.71 ± 2.11	2.29 ± 2.05	0.00	NA
4-Nerolydilcathecol	11.24 ± 2.13	1.18 ± 2.10	0.00	NA
NS-398	100	100	98.73 ± 2.17	0.013 ± 0.02

NA, not active.

inhibition of nuclear factor kappa B (NF-κB) assay is an adaptation of a procedure previously described (Subbaramaiah et al., 2005). Preliminarily, the sensitivity of NF-κB to high dosages (30, 20 and 10 µg/mL solubilized in DMSO) of the drugs had been measured. The data is presented as percentage. And, thus, the IC_{50} was calculated. N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma-Aldrich, St. Louis, MO, USA), a specific inhibitor of NF-κB, was used as control in the assay.

Statistical analysis

Analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests were applied (Sokal and Rohlf, 2012). The results with $P < 0.05$ were considered significant. Data were expressed as mean (M) ± standard deviation (SD).

RESULTS AND DISCUSSION

Phytochemical constituents of *P. umbellata* L.

Phytochemical studies on aerial parts of *P. umbellata* L. (Piperaceae) demonstrated the presence of several chemical constituents, such as β-stigmasterol, β-sitosterol, and campesterol (Perazzo et al., 2005; Pino et al., 2005; Baldoqui et al., 2009; Bagatela et al., 2013). Plants containing these bioactive molecules have previously demonstrated a significant anti-inflammatory activity (Navarro et al., 2001). *P. umbellata* L, among its relevant properties, evidenced a significant *in vivo* anti-inflammatory action (Perazzo et al., 2005).

In vitro inhibition of cyclooxygenase-2

The *in vitro* inhibition activity of *P. umbellata* L. (Piperaceae) on the molecular pathway of COX-2 is exhibited in Table 1. The obtained data are presented as inhibition percentage of COX-2 compared to untreated cultures. Thus, the tested material exhibited remarkable inhibition percentages in the primary assay. However, when the material were tested at lower concentrations in the secondary test, the preeminent result obtained was that of CH_2Cl_2 fraction, the only tested plant component that presented a considerable IC_{50} value and, consecutively, a substantial inhibition activity in the secondary assay.

Given the chemical complexity of medicinal plants, the use of crude extracts, and their fractions can offer significant advantages compared to isolated compounds. Although the reductionist path consists in a logical approach to drug development, crude extracts and fractions must be investigated in order to maintain the benefits of synergy (Orlando et al., 2010), as evidenced below.

In vitro inhibition of nuclear factor kappa B

The *in vitro* inhibition activity of *P. umbellata* L. (Piperaceae) on the molecular pathway of NF-κB is exhibited in Table 2.

This data presents the inhibition percentage of NF-κB compared to untreated cultures. Moreover, the tested drugs exhibited notable inhibition percentages in the primary assay. However, when the drugs were tested at lower concentrations in the secondary test, the preeminent result obtained was that of 4-nerolydilcathecol, the only tested plant component that presented an interesting IC_{50} value and, consecutively, a newsworthy inhibition activity in the secondary assay.

Notably, this assay demonstrated that complex plant extracts, fractions or isolated molecules can be investigated with the proposed experimental systems (Kim et al., 2004). Inflammation has been associated with several pathological disorders, especially several forms of cancers. NF-kB is one of the elements responsible for the link between inflammation and cancer. Several studies aim the search for new compounds that act on the molecular mechanisms involved in the process of inflammation and carcinogenesis. NF-kB is a transcription facto r present in the cytoplasm in an inactive form. When

Table 2. Inhibition of NF-κB by *P. umbellata* L. (Piperaceae) species.

Tested drug	Concentration (µg/ml)			IC_{50} (µg/ml)
	30	20	10	
	Inhibition percentage (%)			
Crude extract	0.00	0.00	0.00	NA
CH_2Cl_2 fraction	7.21 ± 2.11	4.13 ± 1.90	0.00	NA
EtOAc fractions	17.19 ± 2.29	0.00	0.00	NA
Residual fraction	8.62 ± 2.17	0.00	0.00	NA
4-Nerolydilcathecol	72.02 ± 2.68	47.93 ± 2.33	21.47 ± 3.34	22.73 ± 2.23
TPCK	100	72.38 ± 2.58	36.62 ± 2.42	15.07 ± 3.19

NA, not active.

not stimulated, NF-kB is bound to an inhibitory protein: IkB. This complex prevents the translocation of NF-kB to the nucleus, but when it is activated, either by free radicals, inflammatory stimuli, carcinogens, endotoxins or radiation, moves from the cytoplasm to the nucleus where it induces the expression of genes associated with inflammation, pro-angiogenic genes, pro-metastatic and anti-apoptotic genes, and others (Karin et al., 2002; Basak and Hoffmann, 2008; Wong and Tergaonkar, 2009).

Several isolated substances of aromatic plants demonstrated remarkable pharmacological actions through inhibition of NF-kB activity, as 4-nerolydilcathecol. Previous studies have shown that limonene, perillyl alcohol and menthol inhibited the activation of NF-κB (Berchtold et al., 2005; Salminen et al., 2008). α-Pinene, which is also a monoterpene, revealed a robust activity by inhibiting translocation of NF-κB to the cell nucleus, increasing the expression of the inhibitory protein IκB (Zhou et al., 2004). Genipin is one of the substances present in *Gardenia jasminoides* Ellis. This monoterpene exhibited anti-inflammatory activity by inhibiting the production of nitric oxide and blocked IκB degradation, which resulted in inhibition of NF-κB activation (Galvez et al., 2005; Koo et al., 2004; Salminen et al., 2008).

The strategy to use isolated compounds as precursors for synthesis was used for 4-nerolydilcathecol. This compound has been changed to (E)-4-(3,7-dimethylocta-2,6-dienylamino)phenol and showed *in* vivo antinociceptive and anti-inflammatory effects, related to the inhibition of COX-2 and phospholipase A2 (PLA2), demonstrated by *in vitro* assays (Lino et al., 2013). Besides 4-nerolydilcathecol, several natural products such as resveratrol, gingerol, capsaicin and ginsenosides, act towards NF-κB (Kim et al., 2006; Kim et al., 2004; Swales et al., 2006; Lee et al., 2004).

Thus, in these *in vitro* assays, it was possible to study the beneficial potential of *P. umbellata* L. (*Piperaceae*). In a reference survey, no other reports about the inhibition effects of this Brazilian medicinal plant on the molecular pathways of COX-2 and NF-κB. This is the first *in vitro* demonstration of the probable anti-inflammatory mechanism of this promising medicinal plant. However, further research is needed in *in vivo* and in clinical studies to confirm these findings.

Conclusion

The results obtained from the *in vitro* assays were promising, mainly on the molecular pathway of NF-κB, once 4-nerolidylcathecol demonstrated a remarkable inhibition activity. In consequence, this molecule should be considered a notable basis for the development of new beneficial medicines for alternative treatments.

Conflict of Interests

The authors have not declared any conflict of interests.

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