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Sacred Maya incense, copal (*Protium copal* - Burseraceae), has antianxiety effects in animal models

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ABSTRACT

Ethnopharmacological relevance: The Maya have traditionally used copal, *Protium copal,* as incense during ceremonies since pre-Columbian times. Anecdotally, copal (when burned as incense), is thought to elicit mentally uplifting and calming effects. The main objective of this study was to determine whether the incense elicits anxiolytic-like behavior in animal models using rats. A second objective was to characterize active constituents and discern potential mechanism(s) of action, specifically the involvement of the GABAergic and endocannabinoid (eCB) systems. Despite the extensive Central American use of this resin, there are currently no known scientific behavioral or pharmacological studies done with the incense.

Materials and methods: Quantification of the triterpenes in the copal resin and cold trapped incense was achieved by HPLC MS. Behavioral effects in rats were assessed using the elevated plus maze (EPM), social interaction (SI) test, conditioned emotion response (CER) and Novel object recognition (NOR) paradigms. Rats were exposed to burning copal (200 mg) over 5 min in a smoking chamber apparatus and then immediately tested in each behavioral paradigm. Follow-up SI tests were done using two antagonists flumazenil (1 mg/kg) and AM251 (1 mg/kg) administered systemically. Inhibition of MAGL (monoacylglycerol lipase) was measured by microplate assay with recombinant human enzyme and probe substrate.

Results: Phytochemical analysis revealed that copal resin and incense had high α- and β-amyrins and low lupeol triterpene content. Exposure to *Protium copal* incense significantly reduced anxiety-like behavior in the SI and CER tests. In contrast, no anxiolytic effects were observed in the EPM. The CER effect was time dependent. Both flumazenil and AM251 blocked the anxiolytic activity of copal revealing the involvement of GABAergic and endocannabinoid systems. Copal, as well as the identified triterpenes, potently inhibited monoacylglycerol lipase (MAGL) activity in vitro ($IC_{50} \leq 811$ ng/mL).

Conclusions: This is the first study to show that copal incense from *Protium copal* elicits anxiolytic-like effects in fear and social interaction models as evidenced by a reduced learned fear behavior and an increase in active social interaction. It's high α and β-amyrin content suggests behavioral effects may be mediated, in part, by the known action of these terpenes at the benzodiazepine receptor. Furthermore, *P. copal*'s observed activity through the eCB system via MAGL offers a new potential mechanism underlying the anxiolytic activity.

1. Introduction

Copal resin (Pom in several Maya languages) harvested from the bark of the lowland tropical tree *Protium copal* L. (Burseraceae) is a sacred essence (Itz) through which the Maya Gods manifest themselves on earth (Schele and Freidel, 1993). Documented in the rich Maya archeological record, copal (mainly but not exclusively *P. copal* resin) has been used as an incense throughout the Maya territory of Mesoamerica (Fig. 1), during important religious ceremonies (Case et al., 2003). Today, the incense is still used widely in traditional Maya ceremonies

Abbreviations: EPM, elevated plus maze; CER, conditioned emotional response; SI, social interaction; NOR, novel object recognition; eCB, endocannabinoid; MAGL, monoacyl glyercol lipase; ANOVA, one-way analysis of variance

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Fig. 1. A. Left: Stella (7th century) at Nimli Punit Ceremonial Site showing spiritual leaders placing copal in sacred fire 1B. modern calendar ceremony at Lubantuun ceremonial site with Maya spiritual leaders placing copal in sacred fire.

(Fig. 1) such as celebration of the 260 day ceremonial calendar (the Tzolkin), healing ceremonies to purify and restore patients to health, and to bless agricultural crops. Copal represents one of the most important ritual and ceremonial plants of many species which are widely used not only by indigenous peoples of the Americas (Turi and Murch, 2013), but in religious rituals/ceremonies in certain current cultural practices.

When used in ceremonies, copal creates a sacred atmosphere for participants. For those who have participated in Maya ceremonies, the effect of copal incense on mood – at least anecdotally – is often rapid and intense. Clearly, this mood is created, in large part, by the reverence the Maya have for their traditional gatherings and the customary and widespread use of the incense at the start of these ceremonies. In this report we examined whether *P. copal* may also have direct pharmacological effect on neurochemical processes affecting mood, through the rapid absorption of inhaled volatiles.

Protium copal resin is generally characterized by its distinct odor and relatively high melting point. Copal resins have a hard, adhesive, gum-like texture and are typically quite malleable, but can harden into amber through polymerization, oxidation, and the loss of essential oils (Rao et al., 2013). Phytochemical analyses reveal that *P. copal* resins are mainly composed of mixture of terpenoids. The essential oil of *P. copal* is dominated by alpha-pinene, sabinene and limonene (Hernández-Vázquez et al., 2010; Lucero-Gómez et al., 2014). Analysis of the related South American *Protium hepataphyllum* and *P icicariba* by Gas Chromatography-Mass Spectrometry revealed that α-amyrin, and β-amyrin are the major pentacyclic triterpenes in the resin. Aragão et al. (2006) reported anxiolytic and antidepressant effects of bark extracts of Brazilian *P. heptaphyllum* when administered orally or intraperitoneally in some animal models. Examination of the mechanisms responsible for these effects suggested that α- and β-amyrins interact with benzodiazepine-type $GABA_A$ receptors to produce sedative and/or anxiolytic effects but also increase noradrenergic activity to evoke antidepressant action (Aragão et al., 2006). Other researchers (Chicca and Gertsch, 2012), have reported that the isolated triterpene, β-amyrin, is also an inhibitor of monoacylglycerol lipase (MAGL). By blocking endocannabinoid (2-arachidonoylglycerol) degradation, MAGL inhibitors enhance endocannabinoid signaling and elicit antidepressant and anxiolytic effects (Wang et al., 2016). Essential oils and resins from *Protium* species also demonstrate anti-inflammatory activity in both animal- and cell-based experimental models (Siani et al., 1999; Melo et al., 2011)

The behavioral effects of inhaled *Protium copal* incense have not been examined. The purpose of this study was to assess potential anxiolytic and cognitive effects of *P. copal* incense using multiple validated behavioral paradigms. We hypothesized that rats exposed to copal incense will exhibit lower levels of anxiety-like behaviors in the elevated plus-maze (EPM) test, social interaction (SI) test, and conditioned emotional response (CER) paradigms, and could elicit cognitive exploratory behavior (NOR). While *Protium* components interact with benzodiazepine-type $GABA_A$ receptors, the chemistry and anxiolytic-like activity of *P. copal* and, more pertinently, copal incense was unknown until studied here.

2. Materials and methods

2.1. Plant material

A sample of *Protium copal* resin was collected in the Toledo district Southern Belize under permit from the Belize forest service, then stored in a dry area at room temperature away from direct sunlight. A voucher specimen was collected and deposited in the University of Ottawa Herbarium (OTT No19199).

2.2. Phytochemical analysis

A sample of *P. copal* resin was ground through 1 mm mesh. A portion (0.5 g) of ground material was extracted in triplicate with 250 mL ethyl acetate using a soxlet apparatus for 2 h. The extract was dried by rotatory evaporation at 40 °C, and re-dissolved in 20 mL of methanol (Lot# SHBB8243V) by sonication for 2 min. The pellet was re-extracted in 20 mL methanol by sonication for 2 min. Total volume was adjusted to 50 mL in a volumetric flask. A 1.0 mL sample of extract was filtered through 0.22 µm PTFE filter analysis by HPLC-MS-MS (using a Sciex3200 QTRAP). The extract was stored at −20 °C.

To analyze the chemistry of *P. copal* vapor, we vaporized 200 mg of resin as described for animal trials (below) and connected two cold traps in series from the vaporization chamber to a Fisherbrand™ Air Cadet™ Vacuum/Pressure Station (Fisher Scientific, Ottawa). Resin vapor was drawn through fritted glass gas dispersion tubes into