Metabolism of Safrole by Heraclides thoas brasiliensis (Papilionidae)

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METABOLISM OF SAFROLE BY *HERACLIDES THOAS BRASILIENSIS* (PAPILIONIDAE)

Additional key words: Safrole, *Piper divaricatum*, *Heraclides thoas brasiliensis*, metabolism

As part of the systematic study of chemical interactions between plant-insect (Ramos et al. 2012) we have found the plant *Piper divaricatum* (Piperaceae) as a new source of safrole (Barbosa et al. 2012). Safrole is a natural insecticide useful in the management of insecticide-resistant insects and it also is an important raw material used in the synthesis of piperonyl butoxide, a crucial ingredient in pyrethroid insecticides (Bizzo et al. 2001). Despite the toxicity and insecticidal activity of safrole (Wen et al. 2001), *Heraclides thoas brasiliensis* (Rothschild & Jordan, 1906) caterpillars can be found feeding ravenously on *Piper* species including *P. divaricatum* leaves (Vanin et al. 2008). Thus, the present study aimed to describe mechanisms by which safrole is metabolized by *H. thoas brasiliensis* in order to understand the adaptation allowing the caterpillar to live on a diet rich in safrole.

For this study, fresh young leaves of *P. divaricatum* were collected in the city of Itabuna-BA, Brazil, at 3 a.m., 6 a.m., 9 a.m., noon, 3 p.m., 6 p.m., 9 p.m. and midnight for a previously planned circadian rhythm study. The samples were collected in triplicates. The leaves were stored in hermetically sealed plastic bag and kept under refrigeration at + 5 ºC until an extract of leaf chemicals could be obtained. Dried leaves (40 °C for 24 h) of *P. divaricatum* (8 g) were milled and extracted with dichloromethane (100 mL x 3), which after concentration in vacuum, yielded 0.9 g of crude extract. The botanical material was identified by Dr. Elsie F. Guimarães (Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Brazil) and a voucher specimen (Kato-1063). Heraclides thoas brasiliensis caterpillars were collected from *P. divaricatum* and identified by Dr. Sérgio Antônio Vanin of the Zoology (Department of the Biosciences Institute of the University of São Paulo). A voucher specimen (CSR 001) was deposited at the Zoology Museum of the University of São Paulo.

The caterpillars were reared separately in three cages (5 individuals in each cage at a temperature of 28 ± 2 ºC, relative humidity of 70 ± 10% and under a 15L:9D photoperiod) on an exclusive diet of *P. divaricatum* leaves in the laboratory. Insects were fed with the same plant material as used for the leaf analyses. Dried feces (40 °C for 24 h) were extracted with dichloromethane (100 mL x 3) and the extract obtained was concentrated under reduced pressure and subjected to chromatographic and spectrometric analysis. The GC/MS analyses of the extracts were carried out at a concentration of 2mg/mL dichloromethane. The fecal and leaf extracts were analyzed by GC-MS (60-240 ºC at 3 ºC min. rate) in a Varian 431-GC coupled to a Varian 220-MS instrument using a fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm.) coated with DB-5. MS spectra were obtained using electron impact at 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da. The leaves were also analyzed by HPLC using a Shimadzu chromatograph model SCL-10A with UV–VIS detector (model SPD-M10A) with reversed phase column (Supelco, C18; 5 µm i.d., 4 x 250 mm). Elution was carried out in a gradient mode starting with methanol:water (3:7) for 10 min, rising to (9:1) in 30 min and maintained for to 35 min. The flow rate was 1 mL/min; injection volume 20 µL; UV scan, 200-400 nm, and all chromatograms were obtained at λ max = 254 nm. The content of safrole in plant material was calculated by comparison of HPLC data obtained for samples and standard solutions containing of safrole 0.1 – 2.0 mg/mL (R^2 = 0,985) in dichloromethane. The authentic standards of safrole as well as methyleugenol and eugenol were purchased from Sigma-Aldrich.

The chromatogram (GC-MS) of the dichloromethane extract from leaves of *P. divaricatum* showed the major compound as safrole. In addition, the circadian variation of safrole content in the dried leaves was investigated by HPLC. The results indicated maximum concentration at noon (34.5 ± 0.2 µg/mg) and minimum concentration at 6 a.m. (14.2 µg/mg ± 0.5), on a dry weight basis (Fig. 1).

The chromatogram (GC-MS) of the fecal extract showed one major peak at 23.6 min that was not detected in the chromatogram of the leaves (Fig. 2). The peak was identified as methyleugenol, a product of the biotransformation of safrole by the *H. thoas brasiliensis* caterpillars. The chromatogram of the fecal extract also revealed the presence of eugenol, indicating that the biotransformation of safrole to methyleugenol occurs via eugenol as an intermediate. Three compounds were identified by their mass spectra compared to the authentic standards. Methyleugenol as the main metabolite for cleavage of the methylenedioxy group of safrole has not been observed in studies with mammals. Cleavage of the methylenedioxy group of

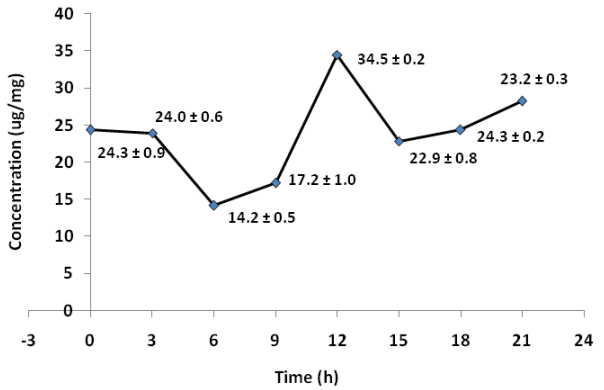

FIG. 1. The circadian variation in safrole content (μg/mg dry weight) in the leaves of *P. divaricatum*. The graphic was plotted using the values means of replicates.

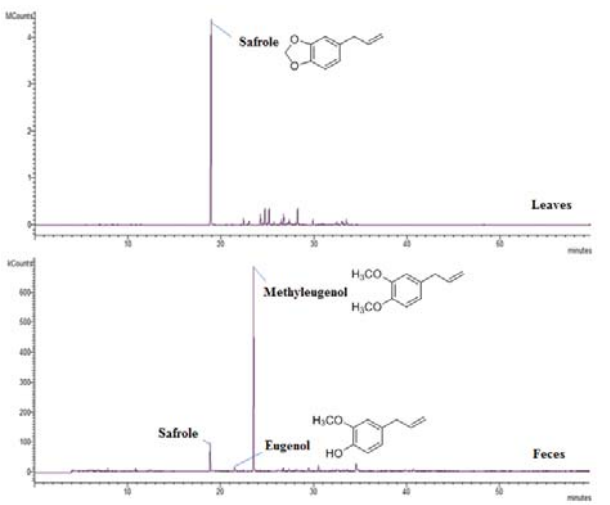

FIG. 2. GC-MS profiles of dichloromethane extracts from P. divaricatum (leaves) and *H. brasiliensis* caterpillar (feces).

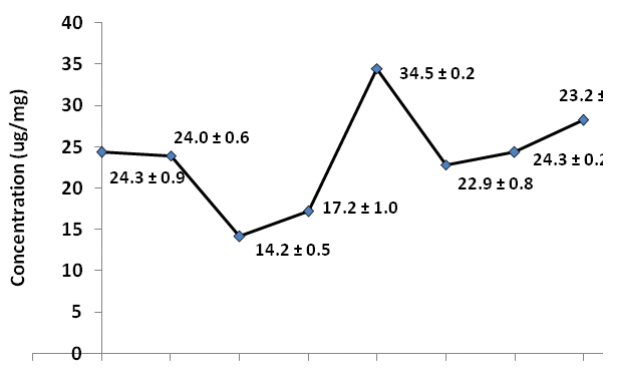

FIG. 3. The main metabolic pathways of safrole in mammals and caterpillar *H. brasiliensis* (Ioannides et al., 1985).

safrole has been observed in rats and humans as the main route of their metabolism, producing allylcatechol as the main compound and eugenol as a minor compound (Fig. 3). In insect in vitro metabolism of myristicin, an analog of safrole, indicated that this is metabolized to 1-(3`,4'-methylenedioxy-5'-methoxyphenyl)-2,3-epoxypropane, an epoxidation of the methylene group (Mao et al. 2008).

In summary, the in vivo metabolism study of safrole by *H. thoas brasiliensis* revealed that this is biotransformed to methyl eugenol (major metabolite) and eugenol (minor metabolite). The cleavage of the methylenedioxy group of safrole has also been identified as a major route of metabolism of piperonyl butoxide in mammals. Piperonyl butoxide is a semisynthetic derived from safrole and is a potent cytochrome P450 inhibitor in animals. This inhibitor is attributed to the methylenedioxy (Gokbulut et al. 2010; Ioannides et al. 1985) Thus, cleavage of the methylenedioxy group of safrole by caterpillars implies a probable mechanism of insect adaptation to a diet rich in safrole. This hypothesis is strengthened because the methylenedioxy group is an enzyme detoxification inhibitor (cytochrome P450).

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