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CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

DEPARTAMENTO DE FARMÁCIA

RAFAEL DOS SANTOS ALVES

Antinociceptive effect of *Aristolochia trilobata* stem essential oil and 6-methyl-5-hepten-2-yl acetate, its main compound, in rodents

SÃO CRISTÓVÃO-SE

2016

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Trabalho de conclusão de curso apresentado ao curso de graduação da Universidade Federal de Sergipe, Centro de Ciências Biológicas e da Saúde, Departamento de Farmácia para conclusão do curso de Farmácia. Área de concentração: Ciências da Saúde.

Orientação: Profª. Drª. Adriana Gibara Guimarães

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BANCA EXAMINADORA

Adriana Gibara Guimarães — Profª. Drª. _____
Universidade Federal de Sergipe

Thalita Kelly Rabelo — Prof. Dr. _____
Universidade Federal de Sergipe

Priscila Laíse dos Santos — Msc. _____
Universidade Federal de Sergipe

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Running title: *Aristolochia trilobata* and major compound reduces nociception

Antinociceptive effect of *Aristolochia trilobata* stem essential oil and 6-methyl-5-hepten-2yl acetate, its main compound, in rodents

Jullyana Sousa Siqueira Quintans[a], Rafael dos Santos Alves[a], Darlisson de Alexandre Santos[b], Mairim Russo Serafini[c], Péricles Barreto Alves[d], Emmanoel Vilaça Costa[d], Gokhan Zengin[e], Lucindo José Quintans-Júnior[a], and Adriana Gibara Guimarães[f,*]

[a]Department of Physiology. [c]Department of Pharmacy. [d]Department of Chemistry. [f]Department of Health Education. Federal University of Sergipe, Sergipe, Brazil.

[b]Federal University of Amazonas, Amazonas, Brazil.

[e]Selcuk University, Science Faculty, Department of Biology, Konya/Turkey.

*Corresponding author. Department of Health Education, Federal University of Sergipe-UFS, Av. Marechal Rondom, s/n, São Cristóvão, Sergipe-Brazil. Tel.: +55-79-2105-6645; fax: +55-79-3212-6640. E-mail address: adrianagibara@pq.cnpq.br; adrianagibara@hotmail.com

Abstract

Aristolochia trilobata L. is an aromatic plant, popularly known as ‘mil-homens’, and its essential oil is generally used to treat colic, diarrhea, and dysentery disorders. We evaluated the antinociceptive effect of *A. trilobata* stem essential oil (EO) and of its major compound, the (R)-(-)-6-methyl-5-hepten-2-yl acetate (sulcatyl acetate: SA), using acetic acid (0.65%)-induced writhing response and formalin -induced (20 µl of 2,5%) nociceptive behavior in mice. We also evaluated the EO and SA effect on motor coordination, using the rota-rod apparatus. EO (25, 50 and 100 mg/kg) or SA (25 and 50 mg/kg) reduced nociceptive behavior in the writhing test ($p < 0.001$). EO (100 mg/kg) and SA (25 and 50 mg/kg) decreased the nociception on first phase of formalin test ($p < 0.05$). On second phase, EO (25: $p < 0.01$; 50: $p < 0.05$ and 100 mg/kg: $p < 0.001$) and SA (25 and 50 mg/kg; $p < 0.001$) reduced the nociceptive response induced by formalin. EO and SA were not able to cause changes in the motor coordination of animals. Together, our results suggest that the EO has an analgesic profile and SA seems to be one of the active compounds in this effect.

Keywords: Aristolochiaceae, sulcatyl acetate, pain.

Introduction

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage”(Bogduk and Merskey, 1994). It has the function of protecting the organism from noxious stimuli (Loeser and Treede, 2008). However, sometimes its etiology is heterogeneous and the underlying pathophysiology mechanisms are complex, making treatment difficult and interfering with the quality of life for people with these pain syndromes (Batista et al., 2010). Besides, the wide range of interindividual variability in the perception of pain makes the management of pain a major challenge for medicine (Kissin, 2010).

Natural products emerge as interesting therapeutic resources for the development of new drugs for the management of certain painful states (McCurdy and Scully, 2005). The search for new chemical entities as therapeutic alternatives for pain or inflammatory conditions has constantly progressed (Guimarães et al., 2014, 2013) and presents the medicinal plants as the major source (Brito et al., 2013; Lima et al., 2013; Paula-Freire et al., 2013; Quintans et al., 2013)

Aristolochia trilobata L. (Aristolochiaceae), popularly known as ‘mil-homens’, is found from Central America down to South America, having various applications in folk medicine (Heinrich et al., 2009). *A. trilobata* is used by humans to treat stomachaches, colic, poisoning, diabetes and also for skin affections (Lans, 2007; Sosa et al., 2002). There are few pharmacological data on this plant species. Previous studies have shown that a chloroform extract of *A. trilobata* leaves had antiphlogistic potency close to that of the non-steroidal anti-inflammatory drug indomethacin (Sosa et al., 2002).

Considering the antinociceptive potential of essential oils and their constituents and the absence of publications demonstrating the antinociceptive effect of *A. trilobata*, this study evaluates the antinociceptive property of stem essential oil (EO) of *A. trilobata* and its major

compound, 6-methyl-5-hepten-2yl acetate (sulcatyl acetate, SA), in animal models of nociception.

Material and methods

Plant material

A. trilobata were collected in Estância-SE/Brazil (satellite positioning: S 11° 14' 22.4" and W 037° 25' 00.5"), during October 2011. The voucher specimen was deposited in the Herbarium of the Federal University of Sergipe and identified as *A. trilobata* with voucher ASE 23.161. Dry stem *A. trilobata* was cut into small pieces and crushed in a four-knife mill (Marconi®, model MA680).

Essential oil extracted and isolation of sulcatyl acetate

The essential oil from 200 g of stem was obtained through hydrodistillation (1500 ml of distilled water) for 3 h using a Clevenger-type apparatus. The oil was physically separated from the water, dried over anhydrous sodium sulphate and filtered. Samples of the oil were transferred to amber glass bottles and stored in a freezer until the GC analysis. The distillations were performed in triplicate. Main compounds were isolated through preparative thin layer chromatography (TLC), according to Santos et al. (2014).

Chemicals

Acetic acid, Tween 80, formalin were purchased from Sigma (USA). Morphine, acetylsalicylic acid and diazepam were purchased from União Química (Brazil). Sulcatyl acetate was

solubilized in saline + 0.2% of Tween 80. Morphine, acetylsalicylic acid and diazepam were solubilized in saline.

Animals

Male Swiss mice (28-34 g) were obtained from the Central Animal Care of the Federal University of Sergipe (São Cristóvão, Brazil). Animals were randomly assigned to groups and maintained in plastic boxes at controlled room temperature (22 ± 2°C) with free access to food and water, under a 12 h light/dark cycle. All the experimental procedures were carried out during the light period of the day (08:00 a.m. to 04:00 p.m.) and complied with the guidelines on animal care of the Federal University of Sergipe Ethics Committee for Animal Use in Research (CEPA/UFS # 16/12), which was conducted in accordance with the internationally accepted principles for laboratory animal use and care. The mice submitted to intraperitoneally (i.p.) administration of drugs fasted for 12 h before the experiments and were acclimatized for at least 2 h before the experiments. All efforts were made to minimize the number of animals used and their suffering. The tests were conducted in a blind manner.

Acetic acid-induced writhing

This test was run using the methods described Koster et al. (1959) and Le Bars et al. (2001). Muscular contractions were induced by intraperitoneal injection (i.p.) of a 0.65% solution of acetic acid (0.25 ml/animal) in a group of 8 mice (per group). The number of muscular contractions was counted for 15 min after the injection and the data represent the average of the total number of writhes observed. Mice received treatment with essential oil (EO: 25, 50 and 100 mg/kg, i.p.), sulcatyl acetate (SA: 25 and 50 mg/kg, i.p.) or morphine (MOR: 5 mg/kg, i.p.), which were solubilized in saline + 0.2% of Tween 80 (vehicle). Control group

received only vehicle. All drugs were administered intraperitonealy in different groups of mice 1 h before the acetic acid injection.

Formalin test

The observation chamber was a glass box of 30 cm in diameter on an acrylic transparent plate floor. Beneath the floor, a mirror was mounted at a 45° angle to allow clear observation of the paws of the animals. Mice were treated with the vehicle (saline + 0.2% of Tween 80), EO (25, 50 and 100 mg/kg, i.p.), SA (25 and 50 mg/kg, i.p.), or acetylsalicylic acid (ASA 200 mg/kg, i.p.) 1 h before the formalin injection. Each mouse was placed in the chamber more than 5 min before treatment in order to allow acclimatization to the new environment. The formalin test was carried out as described by Hunskaar and Hole (1987). Twenty microliters of a 2.5% formalin solution in a phosphate-buffer were injected into the dorsal surface of the left hind paw using a microsyringe with a 26-gauge needle. Each animal was then returned to the chamber and the amount of time that the animal spent licking the injected paw was considered to be indicative of nociception. Two distinct phases of intensive licking activity were identified: an early acute phase and a late or tonic phase (0-5 and 15-30 min after formalin injection, respectively).

Evaluation of the motor activity

In order to evaluate a possible non-specific muscle-relaxant or sedative effect of the EO or SA, mice were submitted to the rota-rod test, according to what had been described by Quintans-Júnior et al. (2010). Initially, the mice able to remain on the Rota-rod apparatus (AVS®, Brazil) longer than 180 s (9 rpm) were selected 24 h before the test. Mice were treated with EO (25, 50 and 100 mg/kg, i.p.) or SA (25 and 50 mg/kg, i.p.), vehicle or

diazepam (3 mg/kg, i.p.) 1, 2 and 4h before evoluation on Rota-rod apparatus. E each animal remained on the bar for up to 180 s.

Statistical analysis

Data obtained from animal experiments were expressed as mean and standard error of the mean (mean ± S.E.M.). Statistical differences between the treated and the control groups were evaluated by ANOVA followed by Dunnett's test. Differences were considered to be statistically significant when $p < 0.05$. All statistical analyses were performed using Graph Pad Prism 5 (Graph Pad Prism Software Inc., San Diego, CA, USA).

Results and Discussion

In the present study, we evaluated the antinociceptive activity of the *A. trilobata* essential oil (EO) and of the *A. trilobata* majoritary compound 6-methyl-5-hepten-2yl acetate (sulcatyl acetate, SA), both in male mice.

According to Santos et al. (Santos et al., 2014), our sample of the essential oil of *A. trilobata* presented an average yield of 0.22 ± 0.05% (v/w), and is composed mainly by 6-methyl-5-hepten-2-yl acetate (sulcatyl acetate) (21.49 ± 0.43%), germacrene D (15.07 ± 0.23%), bicyclogermacrene (8.84 ± 0.45%), linalool (6.85 ± 0.42%), *(E)*-caryophyllene (5.58 ± 0.12%), *(E)*-β-ocimene (5.56 ± 0.067%) and *p*-cimene (4.68 ± 0.10%).

In the pretreatment with EO or SA, all doses significantly ($p < 0.001$) reduced the number of writhing movements (nociceptive behavior) induced by acetic acid solution (Fig 1). As expected, morphine (5 mg/kg, i.p.), an opioid analgesic drug, produces an inhibition of nociceptive behavior ($p < 0.001$). In this model, pain is elicited by the injection of an irritant such as acetic acid into the peritoneal cavity, which produces episodes of characteristic stretching (writhing) movements, and inhibition of the number of episodes by analgesics is

easily quantifiable. Despite being a non-specific test, acetic acid-induced writhings are a highly sensitive and useful test for analgesic drug development (Le Bars et al., 2001).

INSERT FIGURE 1

After the antinociceptive effects of EO and SA were evaluated in a model of persistent nociception, the formalin test, which has two distinctive phases that can possibly indicate different types of pain (Hunskaar and Hole, 1987). The early and late phases of formalin test have obvious differential properties, and therefore this test is useful not only for assessing the analgesic substances but also for an initial investigation the mechanism of analgesia (Shibata et al., 1989). The early phase, named non-inflammatory pain, is a result of direct stimulation of nociceptors and reflects centrally mediated pain; the late phase, named inflammatory pain, is caused by local inflammation with a release of inflammatory and hyperalgesic mediators (Hunskaar and Hole, 1987). In the present study, we found that EO and SA produced antinociceptive activity both in the early ($p < 0.05$) and late ($p < 0.05$-0.001) phases of formalin test (Fig 2). However, EO was effective in the early phase only in higher dose ($p < 0.05$).

INSERT FIGURE 2

Several studies have shown the antinociceptive and anti-inflammatory effects of terpenes or essential oils, as reviewed by Almeida et al. (2001), Da Silveira e Sá et al. (2013), De Sousa (2011) and Guimarães et al. (2013). Studies performed with linalool and *p*-cymene, major compounds found in EO of *A. trilobata*, demonstrated the antinociceptive

activity of these monoterpenes in different experimental models of nociception (Batista et al., 2010; Menezes et al., 2014; Quintans et al., 2013; Quintans-Júnior et al., 2013; Santana et al., 2011). Furthermore, the major compound, which has its pharmacological activity described for the first time in this study, can act synergistically with these compounds thus contributing to the pharmacologic response observed.This therapeutic property has gained prominence among research groups of academic community and industry, which increased interest in developing new options for pain management. Furthermore, the high market acceptance of drugs containing terpenes and essential oils for pain control, such as Acheflan® and Salompas® motivate further research in this area and has led to an increase in patent filings in several countries (Guimarães et al., 2014).

Previous studies suggested that the CNS depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination and might invalidate the behavior tests results (Quintans-Júnior et al., 2010). We observed that EO or SA-treated mice did not show any significant motor performance alterations with all doses tested (data not shown). Thus, we refute the hypothesis of a possible relaxing or motor deficit effects of EO and SA at the therapeutic doses.

Together, our results suggest that EO and sulcatyl acetate, its main compound, modulates neurogenic and inflammatory pain in the tests used (Fig 3). Thus, it was observed that the sulcatyl acetate is responsible, at least in part, for the analgesic profile of this essential oil. The antinociceptive actions demonstrated in *A. trilobata* in this study support the ethnomedicinal use of this plant. More studies are needed to determine the ED50 and the dose conversion ratio (DCR) of compounds with standard drugs.

INSERT FIGURE 3

Declaration of interest

The authors report no conflicts of interest.

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FIGURE LEGENDS

Figure 1: Effects of EO or SA on the acetic acid-induced writhing test in mice. Vehicle (control), EO (25, 50 or 100 mg/kg), SA (25 or 50 mg/kg) or morphine (MOR, 5 mg/kg) were administered i.p. 1 hr before acetic acid injection. Each column represents mean ± S.E.M. (n = 8, per group). $^{***}p < 0.001$ versus control (ANOVA followed by Dunnett's test).

Figure 2: Effects of EO or SA on the formalin-induced nociception in mice. Vehicle (control), EO (25, 50 and 100 mg/kg), SA (25 or 50 mg/kg) or aspirin (200 mg/kg) were administered i.p. 1 h before formalin injection. (A) Represents the first phase and (B) represents second phase of formalin-induced nociception in mice. Each column represents mean ± S.E.M. (n = 8, per group). $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ versus control (ANOVA followed by Dunnett's test).

Figure 3: Summary of the data obtained.

FIGURES

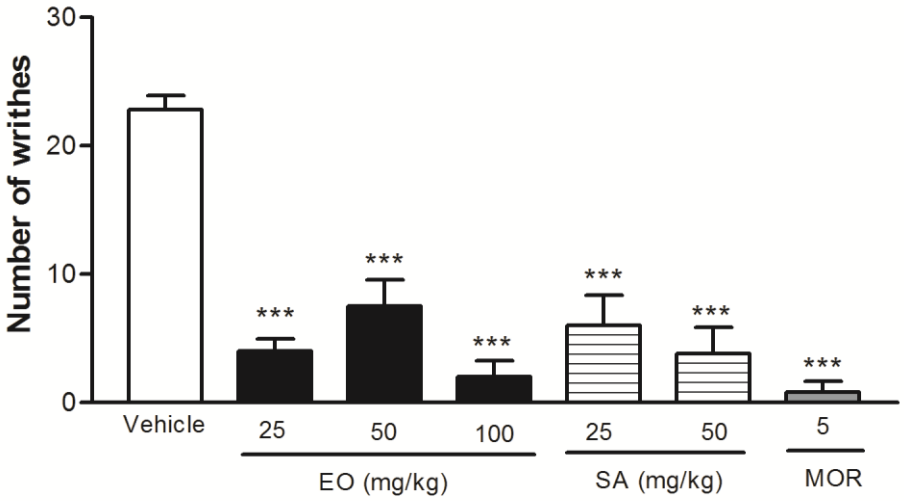
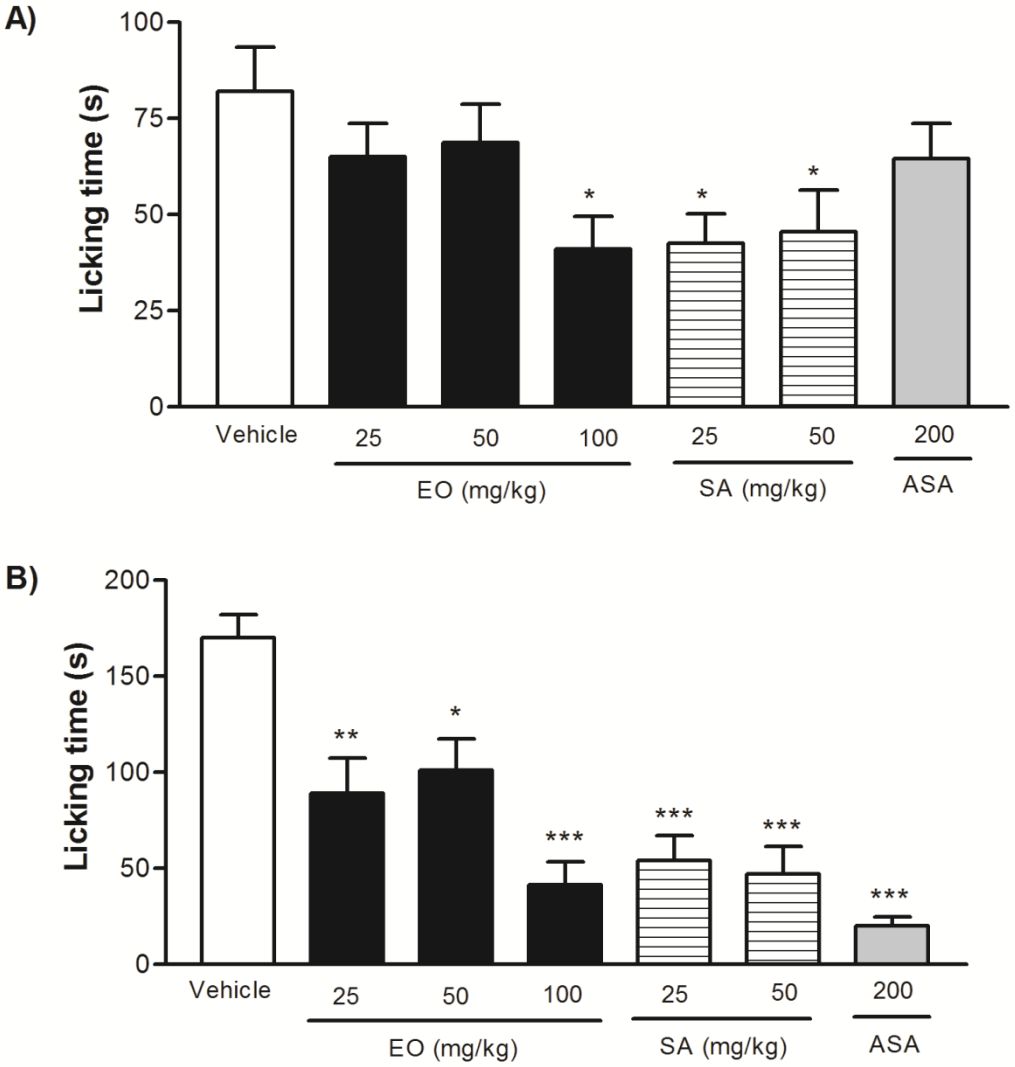


Figure 1


A)
Licking time (s)
100
75
50
25
0
Vehicle
25
50
100
25
50
200
EO (mg/kg)
SA (mg/kg)
ASA
B)
Licking time (s)
200
150
100
50
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Vehicle
25
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100
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50
200
EO (mg/kg)
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ASA

Figure 2

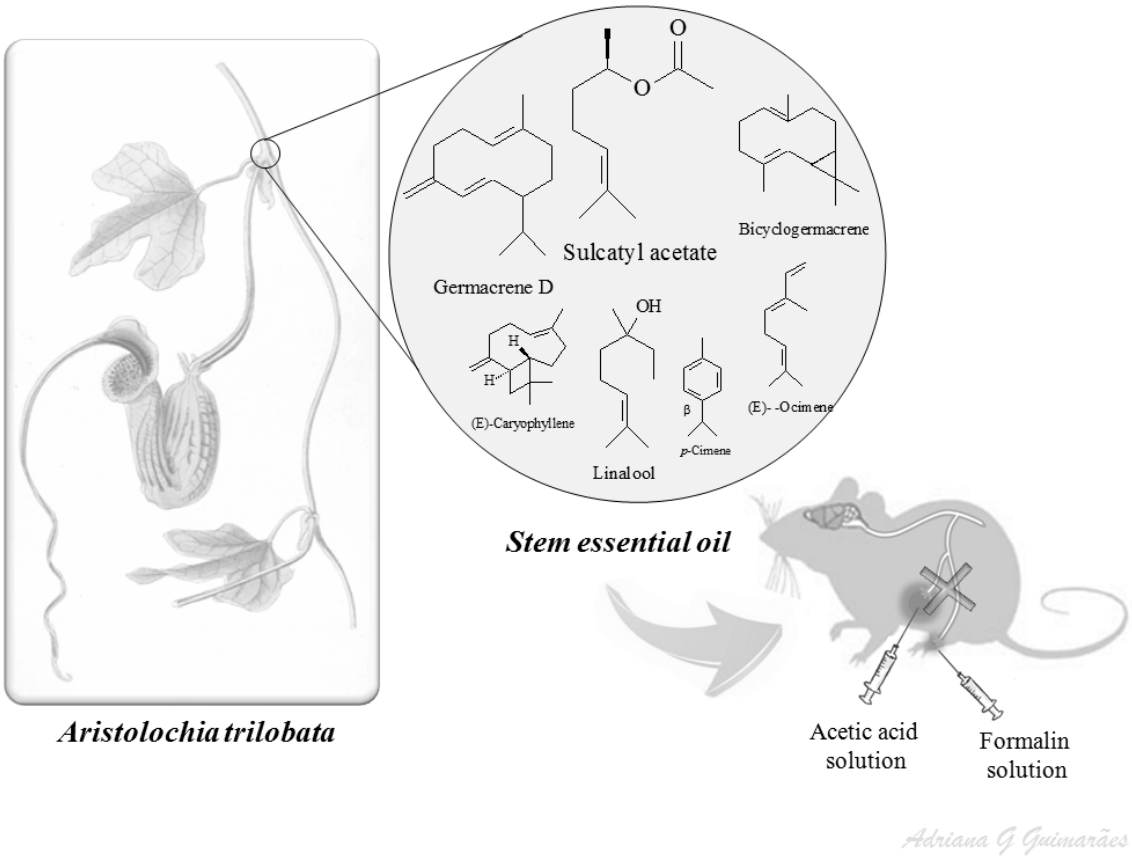
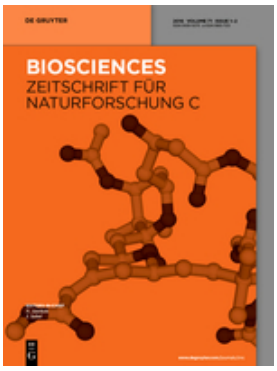

Sulcatyl acetate
Bicyclogermacrene
Germacrene D
(E)-Caryophyllene
Linalool
p-Cimene
(E)- -Ocimene
Stem essential oil
Aristolochia trilobata
Acetic acid solution
Formalin solution
Adriana G Guimarães

Figure 3

DE GRUYTER



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2. Wells D, Bjorksten A. Monoamine oxidase inhibitors revisited. Can J Anaesth 1989;36:64–74.
3. Parsons MR, Convery MA, Wilmot CM, Yadavt KD, Blakeley V, Corner AS, et al. Crystal structure of a quinoenzyme: copper amine oxidase of *Escherichia coli* at 2 A resolution. Structure 1995;3:1171–84.

Examples for book references:

1. Sambrook J, Russell DW. Molecular cloning: a laboratory manual, 3rd ed. New York: Cold Spring Harbour Laboratory Press, 2001.
2. Gower JC, Lubbe S, le Roux NJ. Understanding biplots. Chichester, UK: Wiley, 2011.
3. Moss JN, Dajani E. Antihyperlipidemic agents. In: Turner RA, Hebborn P, editors. Screening methods in toxicology. New York: Academic Press, Vol. 2, 1971:121.

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