In vitro inhibition of mouse liver microsomal cytochrome P450 (CYP) subfamilies by the diterpenes synagrantol A and G isolated from the latex of *Euphorbia umbellata*

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Key words:
- CYP1A, CYP2A, CYP2B and CYP3A enzyme isoforms
- *Euphorbia umbellata*
- phorbol esters
- synagrantol A
- synagrantol G

Abstract

Phorbol esters are diterpenoids found in the genus *Euphorbia* (Euphorbiaceae). Synagrantol A (*Phorbol-3,4,12,13-tetraacetate-20-phenylacetate*) and G (*20-deoxyphorbol-3,4,12-triacetate-13-phenylacetate*) are phorbol esters from *Euphorbia umbellata* latex that are under investigation for their anti-HIV properties. This study describes their isolation using chromatographic techniques and their inhibitory effects on four cytochrome P450 (CYP) enzyme subfamilies extracted from mouse liver microsomes after induction by β-naphthoflavone (CYP1A, CYP2A), phenobarbital (CYP2B), or dexamethasone (CYP3A). After euthanasia and liver extraction, enzyme activity was assessed in treated and untreated animal's samples via the dealkylation of ethoxyresorufin (CYP1A1), methoxyresorufin (CYP1A2), pentoxyresorufin (CYP2B), and *N*-methyl-erythromycin (CYP3A). Synagrantol A or G (100 μM) and α-naphthoflavone (CYP1A1/2 positive control) were added to microsomal suspensions, followed by substrate addition. Inhibition was measured by the production of resorufin in a spectrofluorometer or formaldehyde in a spectrophotometer (specific for CYP3A). The induction factor (IF) was 2.9 (CYP1A1), 2.43 (CYP1A2), 2.4 (CYP2B), and 1.2 (CYP3A). Synagrantol A and G significantly inhibited CYP1A1 (>90%), followed by CYP1A2 (84% and 64%, respectively). CYP2B inhibition was lower (34% and 49%), and CYP3A inhibition was the least significant (18% and 32%). Since these CYPs metabolize up to 60% of marketed drugs, further research on their metabolic interactions with phorbol esters is essential to ensure the safety of products containing these compounds.

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Palavras-chave:
- inibição das isoformas das enzimas CYP1A, CYP2A, CYP2B e CYP3A
- *Euphorbia umbellata*
- ésteres de forbol
- synagrantol A
- synagrantol G

Resumo

Ésteres de forbol são diterpenoides tetracíclicos comumente encontrados em *Euphorbia* (Euphorbiaceae). Synagrantol A (forbol-3,4,12,13-tetraacetato-20-fenilacetato) e G (20-desoxiforbol-3,4,12-triacetato-13-fenilacetato), os principais diterpenoides presentes no látex de Euphorbia umbellata, estão sendo investigados por suas propriedades anti-HIV. Este estudo descreve seu isolamento usando técnicas cromatográficas e seus efeitos inibitórios em quatro subfamílias de enzimas do citocromo P450 (CYP) extraídas de microssomas de fígado de camundongo após indução por β-naftoflavona (CYP1A, CYP2A), fenobarbital (CYP2B) ou dexametasona (CYP3A). Após a eutanásia e extração do fígado, a atividade enzimática foi avaliada por meio da desalquilação de etoxiresorufina (CYP1A1), metoxiresorufina (CYP1A2), pentoxiresorufina (CYP2B) e N-metil-eritromicina (CYP3A), comparando amostras tratadas e não tratadas. Synagrantol A ou G (100 μM) e α-naftoflavona (controle positivo CYP1A1/2) foram adicionados às suspensões microssomais, seguido pela adição de substrato. A inibição foi medida pela produção de resorufina em um espectrofluorômetro ou formaldeído em um espectrofotômetro (específico para CYP3A). O fator de indução (FI) foi de 3,9 (CYP1A1), 2,43 (CYP1A2), 2,4 (CYP2B) e 1,2 (CYP3A). Synagrantol A e G inibiram significativamente o CYP1A1 (>90%), seguido pelo CYP1A2 (84% e 64%). A inibição do CYP2B foi menor (34% e 49%), enquanto a do CYP3A foi a menos relevantes (18% e 32%). Como esses CYPs metabolizam até 60% dos medicamentos comercializados, pesquisas adicionais sobre interações metabólicas com ésteres de forbol são essenciais para garantir a segurança dos produtos derivados de plantas que os contêm.

Introduction

Euphorbia umbellata (Pax) Bruyns (Euphorbiaceae) is a shrubby tree native to East Africa. Its aerial parts and latex have been used in African traditional medicine for human and veterinary purposes (Siani & Abreu 2023). However, most published data on this species refer to it as belonging to the genus *Synadenium* Boiss. and are based on the morphological characteristics described by Edmond Boissier for the genus (Pax 1895). Botanical binomials referring to species and varieties such as *Synadenium grantii* Hook.f. (Hooker 1867), *S. umbellatum* Pax (Pax 1895) or *S. umbellatum* var. *puberulum* N.E. Brown (Pax 1911) lingered until the first decade of this century, when the genus *Synadenium* was transferred to *Euphorbia*, on the basis of phylogenetic studies scoping a broad series of African Euphorbiaceae (Bruyns *et al*. 2006). Initially cultivated as an ornamental in Brazil, *E. umbellata* is known as *janauba* or *leiterinha*, among several other names (Fig. 1). Its cultivation is scattered throughout the country, mainly because of the use of its latex in folk medicine to treat various types of cancer and other diverse health disorders (Ortêncio 2012; De Souza *et al*. 2024). Furthermore, the latex of this species has been popularly reported to be useful in the treatment of HIV infection (Flores 2010).

Recent research has demonstrated that diterpene esters isolated from Euphorbiace species can act as agents for reversing HIV latency (LRAs) in infected cells. This effect suggests that these compounds could be the basis for developing complementary therapies to antiretroviral treatment and that treating asymptomatic patients who still carry the virus in cellular reservoirs is possible (Katlama *et al*. 2013). Some promising results concerning this activity have been obtained from preliminary studies on the latex of *E. umbellata* (Valadão *et al*. 2018). *In vitro* bioguided assays using infected defense cells subsequently led to the identification of three minor diterpenes contained in latex that are highly potent as LRAs (Tostes *et al*. 2021). More recently, this activity was also reported for lyophilized latex and its fraction enriched with diterpenes, as well as the two major diterpene constituents isolated from latex, synagrantol A and synagrantol G (Neves *et al*. 2024). Chemically, these diterpenes belong to the tigliane type, which is characterized by a peculiar tetracyclic conformation in which seven-, six-, five- and three-membered carbon rings are fused. The primary structure is represented by the

phorbol itself, which presents an α,β-unsaturated pentanone and five hydroxyl groups at C-4, C-9, C-12, C-13 and C-20 (Fig. 2). Several phorbol esters were previously isolated or characterized in the latex of *E. umbellata* (Bagavathi *et al*. 1988; Hassan *et al*. 2012). However, the application of phorbol esters as possible therapeutic agents raises several distinct questions. For instance, phorbol esters can inhibit detoxifying liver cytochrome P450 enzymes (CYPs), thus interfering with the ability of CYPs to eliminate exogenous and endogenous material from the body during drug metabolism (Zhao *et al*. 2021). Since CYPs function to catalyze a wide range of critical reactions (Wright *et al*. 2019), verifying their effects on the metabolism of drug candidates is important. In regard to phorbol esters, studies involving synagrantol A and G are exploratory and are not reported in the current literature.

With respect to the diterpene content of *E. umbellata* latex, synagrantol A and G were chosen as tigliane structural models for assaying *in vitro* interactions with four CYP subfamilies. The present study reports their isolation from latex followed by their *in vitro* ability to inhibit the subfamilies CYP1A1/CYP1A2, CYP2B and CYP3A. These enzymes were previously quantified in the microsomal fraction extracted from mouse liver after induction by intraperitoneal injections of β-naphthoflavone, phenobarbital and dexamethasone (Ramos *et al*. 2024). Enzymatic activity was measured by the ability to promote the dealkylation of substrates selected to determine the specificity of each isoform tested.

Material and Methods

Solvents and chemicals

β-Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ethoxyresorufin, methoxyresorufin, β-naphthoflavone, α-naphthoflavone, glycerol, EDTA, zinc sulfate, barium sulfate, and chloroform HPLC-Plus (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (Supelco), potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, silica gel 60 GF 254 glass plates (20 × 20 cm; 500 μm), silica gel 60 RP18 F_{254S} aluminum plates (20 × 20; 200 μm), and LiChroprep® RP-18 (40–63 μm) stationary phase for liquid chromatography were purchased from Merck (Darmstadt, GE). Silica gel 60 RP-18 F_{254S} glass plates (20 × 20, 1000 μm) were obtained from UniplateTM (Miles Scientific, DE, USA).

Plant material

The latex of *E. umbellata* (150 g) was collected from plants cultivated in a private locale in the city of Nova Friburgo, Rio de Janeiro state. Legal access of the genetic heritage component was registered at SISGEN/MMA/Brazil as A37B4B7. The latex was collected by the successive cutting of small pieces from the branches and direct dripping into a suitable container (Fig. 1c). This process requires protective care because the latex may cause irritation to the skin and eyes. The material was kept refrigerated (0–10 °C) until it was processed in the laboratory.

Figure 1 – a-c. *Euphorbia umbellata* – a. cultivated in the Phytomedicines Agroecological Platform, Fiocruz, Rio de Janeiro, RJ, Brazil; b. stems and leaves details; c. latex extraction.

Isolation of synagrantol A and G

The whole latex was initially lyophilized to yield 25% white solid that was manually sieved and homogenized. Part of this material (2.5 g mixed and homogenized with 250 mg of Perfiltra®) was subjected to medium-pressure liquid chromatography (Büchi Sepacore X50 system) on a C_{18}-Reversed-Phase Silica Gel, and acetonitrile was used as the eluent, followed by chloroform. This process yielded 500 mg (20% yield) of the phorbol ester-enriched fraction (DF). DF (200 mg) was submitted to preparative TLC plates (silica gel) developed with 98:2 chloroform-acetone. The higher R_f (0.75) spot visualized under UV light at 254 nm was scratched from the plate and extracted with ethyl acetate to yield, after evaporation, 21 mg (11%) of a colorless solid containing a mixture of synagrantol A and G. The mass of this mixture was accumulated by performing repeated plates. This solid (200 mg) was distributed to five consecutive preparative thin-layer chromatography (TLC) runs on RP-18 silica gel plates and eluted with 7:3 acetonitrile:water. Two spots were separated from each plate (R_f 0.33 and 0.27; UV 254 nm) and extracted with acetonitrile to yield synagrantol A (averaged 99.3 mg, 49%) and synagrantol G (averaged 18.7 mg, 9.4%), respectively. All the processes were monitored by TLC and HPLC analysis, which were accompanied by standards of the substances previously isolated and fully characterized (Neves *et al*. 2024). The chromatographic conditions for TLC were silica gel 60 RP18 aluminum plates; $CHCl_3$-acetone 98:2; and sulfuric anisaldehyde as the staining agent (sample at 5 mg/mL MeOH, 10 μl and reference compounds at 1 mg/mL MeOH, 10 μl). HPLC analysis was performed using a LiChrocart Purospher Star RP-18e column (250 × 4.6 mm; 5 μm) and elution with water (A) and acetonitrile (B) in gradient mode: 20–95% B (40 min), 95% B (10 min), 95–20% B (5 min), and 20% B (10 min). The flow rate was 1 mL/min with an oven at 40 °C, a volume injection of 10 μL and detection at 210 nm. The samples and fractions were solubilized in MeOH at concentrations of 1 or 5 mg/mL. Both compounds were identified by proton nuclear magnetic resonance (^{1}H-NMR) and high-resolution electrospray ionization mass spectrometry in positive mode (HRESIMS$^+$), and the results were compared with data from the literature. The ^{1}H-NMR and HRESIMS data are in accordance with those reported for an authenticated sample by Neves *et al*. (2024).

Synagrantol A (*Phorbol-3,4,12,13-tetraacetate-20-phenylacetate*): white amorphous powder; (+)-HRESIMS *m/z* 675.2667 $[M+Na]^+$ (calcd. for

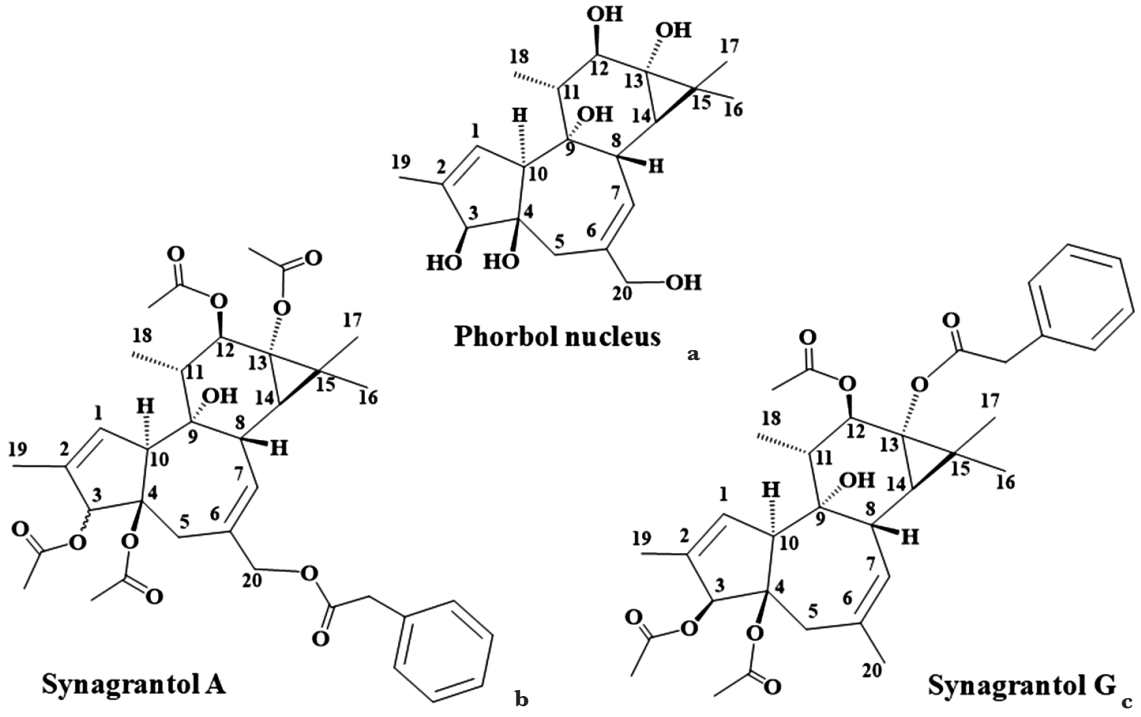

Figure 2 – a-c. Structures – a. phorbol (prototype of tigliane alcohol, $C_{20}H_{28}O_6$); b. synagrantol A (3,4,12,13-tetraacetylphorbol-20-phenylacetate, $C_{36}H_{44}O_{11}$); c. synagrantol G (3,4,12,13-tetraacetylphorbol-20-phenylacetate, $C_{34}H_{42}O_9$)

$C_{36}H_{44}O_{11}Na^+$, 675.2781), 615.2463 [M+Na-$C_2H_4O_2$]$^+$ (calcd. for $C_{34}H_{40}O_9Na^+$, 615.2564), 555.2258 [M+Na-$C_2H_4O_2$-$C_2H_4O_2$] (calcd. for $C_{32}H_{36}O_7Na^+$, 555.2353), 479.1953 [M+Na-$C_2H_4O_2$- $C_8H_8O_2$] (calcd. for $C_{26}H_{32}O_7Na^+$, 479.2040), 419.1752 [M+Na-$C_2H_4O_2$-$C_2H_4O_2$-$C_8H_8O_2$] (calcd. for $C_{24}H_{28}O_5Na^+$, 419.1828); ^{1}H-NMR ($CDCl_3$, 400 MHz, TMS, δ): 5.18 (1H, br*d*, *J* = 11.6 Hz, H-1), 5.38 (1H, *s*, H-3), 2.14-2.20 (2H, *m*, H-5α and β), 5.18 (1H, br*d*, *J* = 11.6 Hz, H-7), 1.41 (1H, *dd*, *J* = 11.9 and 9.3 Hz, H-8), 1.11-1.16 (1H, *m*, H-10), 2.34 (1H, *m*, H-11), 5.12 (1H, br*d*, *J* = 8.4 Hz, H-12), 1.11-1.16 (1H, *m*, H-14), 1.11 (3H, *s*, CH_3-16), 0.99 (3H, *s*, CH_3-17), 0.90 (3H, *d* , *J* = 7.4 Hz, CH_3-18), 1.67 (3H, *s*, CH_3-19), 4.39 (1H, *d*, J = 12.4,H-20)/4.68 (1H, br*d*, *J* = 11.3,H-20) , 2.13/2.14 (3H each, *s*, CH_3CO-3 or 4), 2.03/2.04 (3H each, *s*, CH_3CO-12 or 13), 3.74 (2H, *s*, Ph-CH_2CO-20), 7.29-7.39 (5H, overlapped, PhAc-20).

Synagrantol G (20-*Deoxyphorbol-3,4,12-triacetate-13-phenylacetate*): white amorphous powder; (+)-HRESIMS *m/z* 617.2673 [M+Na]$^+$ (calcd. for $C_{34}H_{42}O_9Na^+$, 617.2721), 557.2466 [M+Na-$C_2H_4O_2$]$^+$, (calcd. for $C_{32}H_{38}O_7Na^+$, 557.2509), 497.2291 [M+Na-$C_2H_4O_2$-$C_2H_4O_2$]$^+$ (calcd. for $C_{30}H_{34}O_5Na^+$, 497.2298), 421.1950 [M+Na-$C_2H_4O_2$-$C_8H_8O_2$]$^+$ (calcd. for $C_{24}H_{30}O_5Na^+$, 421.1985), 361.1742 [M+Na-$C_2H_4O_2$-$C_8H_8O_2$-$C_2H_4O_2$]$^+$ (calcd. for $C_{22}H_{26}O_3Na^+$, 361.1774); ^{1}H-NMR ($CDCl_3$, 400 MHz, TMS, δ): 4.93 (1H, *d*, *J* = 12.1 Hz, H-1), 5.14 (1H, *s*, H-3), 2.19-2.25 (2H, *m*, H-5α and β), 4.93 (1H, *d*, *J* = 12.1 Hz, H-7), 1.32 (1H, *m*, H-8), 1.25 (1H, *m*, H-10), 2.35 (1H, *m*, H-11), 5.10 (1H, *d*, *J* = 7.2 Hz, H-12), 1.15 (1H, *s*, H-14), 1.10 (3H, *s*, CH_3-16), 0.98 (3H, *s*, CH_3-17), 0.88 (3H, *d* , *J* = 7.5 Hz, CH_3-18), 1.56 (3H, *s*, CH_3-19), 1.77 (3H, *s*, CH_3-20), 2.02 (3H, *s*, CH_3CO-3), 2.12 (3H, *s*, CH_3CO-4), 2.04 (3H, *s*, CH_3CO-12), 3.74 (2H, *s*, Ph-CH_2CO-13), 7.26-7.36 (5H, overlapped, PhAc-13).

Induction of CYP expression in mice, liver microsomal fraction and protein quantification

The study protocol was approved by the institutional Ethics Committee for the Use of Animals (P-41/19-1). After one week of acclimatization, a group of five female Swiss mice received the inducer of a specific CYP. The production of CYP1A and CYP1A2 was induced by intraperitoneal administration of β-naphthoflavone suspended in corn oil, with a single daily dose of 80 mg/kg, for 4 consecutive days. The same procedure was followed with phenobarbital (4 days) and dexamethasone (100 mg/kg, 3 days) to induce the production of CYP2B and CYP3A, respectively (Santos *et al*. 2021). The animals were euthanized using anesthetics on the fifth (β-naphthoflavone) or fourth (phenobarbital) day of treatment, except for mice induced with dexamethasone, which were euthanized 12 h after the last dose. The livers were removed, individually cooled in an ice bath, weighed, wrapped in aluminum foil, and stored in liquid nitrogen.

The hepatic microsomal fraction was obtained according to the methods of De-Oliveira *et al*. (1997). The livers were transferred from the liquid nitrogen to an ice bath to avoid abrupt thawing, and the subsequent steps were carried out at ≤ 4 °C. The livers were subsequently washed with 250 mM sucrose solution, dried with filter paper and then subjected to a glass homogenizer (50 mL capacity, 1,200 rpm) and a Teflon pestle in 100 mM Tris buffer solution with 150 mM KCl (pH 7.4). The volume of the buffer solution corresponded to four times the weight of the organ. The liver homogenate was ultracentrifuged (Beckman® XL-90 with a Ti 70.1 rotor) at 4 °C and 9,000x g for 30 min. The pellet, containing the nucleus, mitochondria and cell debris, was discarded. The supernatant was separated, filtered through gauze and ultracentrifuged (Novatécnica® NT136, Piracicaba, BRA) at 100,000x g for 1 h at 4 °C. This procedure was repeated, and the pellet containing the enzymes bound to the smooth endoplasmic reticulum (microsomes) was homogenized in 100 mM dibasic potassium phosphate buffer with 20% glycerol and 1 mM EDTA (pH 7.4) at a speed of approximately 250 rpm. The microsomes thus obtained were distributed in cryogenic tubes and frozen in liquid nitrogen until use. The total protein concentration in the microsomal fraction of treated and untreated control animals was determined by the Bradford method. An analytical curve was constructed using 0.28, 0.56, 0.84 and 1.4 mg/mL bovine serum albumin (5 μL) in a 96-well plate containing 250 μL/well of Bradford reagent. The absorbance was read in a spectrophotometer with a microplate reader (EZ Read 2000 Biochrom®) at 595 nm 30 min after the addition of the reagent to the diluted proteins. The CYP induction factor (IF) for each subfamily was calculated as the increase in enzyme activity produced by the microsomal fraction of induced animals compared with that of untreated animals.

EROD, MROD, PROD and ERMd activity assays

To evaluate the inhibitory effects of the tested substances on the catalytic activity of CYP1A, CYP1A2, CYP2B and CYP3A, assays of 7-ethoxyresorufin-*O*-deethylation (EROD), 7-methoxyresorufin-*O*-demethylation (MROD), pentoxyresorufin-*O*-dependentylation (PROD) and erythromycin-demethylation (ERMd) of the substrates ethoxyresorufine, methoxyresorufine, pentoxyresorufin and erythromycin, respectively,

were performed using the respective microsomal fraction, as described by Ramos *et al*. (2024), with some adaptations. A NADPH regenerator system was provided with 0.25 mM β-NADP, 2.5 mM $MgCl_2$, 5 mM glucose 6-phosphate, and 0.5 U/mL glucose 6-phosphate dehydrogenase (De Oliveira *et al*. 1999). The reactions (100 mM K_2HPO_4 buffer, pH 7.8) were carried out in a 2 mL quartz cuvette held at 37 °C in a water bath connected to a Shimadzu RF-5301PC spectrofluorometer (Kyoto, Japan) (550 nm excitation, 582 nm emission, band slit width of 5 nm). The following assays were performed: (i) 5 μM of ethoxyresorufin (EROD for CYP1A) or methoxyresorufin (MROD for CYP1A2) and 0.05 mg/mL of the β-naphthoflavone-treated mouse liver microsomal fraction; (ii) 5 μM of pentoxyresorufin (PROD for CYP2B) and 0.06 mg/mL of the phenobarbital-treated mouse liver microsomal fraction; and (iii) 100 μM of erythomycine (ERMd for CYP3A) and 0.5 mg/mL of the dexamethasone-treated mouse liver microsomal fraction. For the first three reactions, the accumulation of released resorufin was measured in real time and recorded 60 s after initiation by the addition of the NADPH cofactor system. An aliquot (10 μL) of buffer (negative control), synagrantol A, synagrantol G or α-naphthoflavone (100 μM) was added before the addition of the cofactor system (Ramos *et al*. 2024). The data were processed by RF-530XPC® software. For these three cases, a resorufin analytical curve was constructed with volumes of 0, 10, 20, 50 and 100 μL from a 1 μM resorufin stock solution in 100 mM K_2HPO_4 buffer (pH 7.8). For ERMd, the analytical curve employed a serial dilution of formaldehyde (100, 50, 25, 12.5, 6.25, 3, 125, 1.5625 and 0.78125 μM) in 50 mM Tris/150 mM KCl buffer (pH 7.4). After serial dilutions, 100 μL of 25% zinc sulfate solution was added, and the solution was vortexed and then centrifuged at 13,000 rpm for 10 min. Then, 100 μL of saturated barium sulfate solution was added, and the procedure was repeated. The supernatant from each dilution (700 μL) was transferred to Eppendorf® tubes, and 300 μL of freshly prepared Nash reagent, including the blank (water and buffer), was added. The mixture was incubated at 50 °C for 30 min in a water bath. Afterward, 300 μL of each dilution was transferred in triplicate to a 96-well microplate. The reading was performed at 412 nm in the spectrophotometer with an EZ Read 2000 Biochrom® microplate reader (Berg-Candolfi *et al*. 1996). All the determinations were performed in independent triplicate reactions.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) and were analyzed by *t* test or ANOVA followed by the Bonferroni comparison test, when applicable. Differences were considered significant when $p < 0.05$. GraphPad Prism 9® was used to construct the graphs and perform the statistical analysis.

Results and Discussion

Synagrantol A and G were isolated from the lyophilized raw latex by first being subjected to medium-pressure liquid chromatography to separate the diterpene-rich fraction using reversed-phase silica gel and acetonitrile as the eluent. From this fraction, only two sequences of preparative plates, first on silica gel and then on reversed-phase silica, were enough to successfully separate the two target diterpenes. The isolated compounds were > 90% pure according to area normalization by HPLC (data not shown) and were suitable for the tests performed. This procedure proved to be faster and less expensive than that reported by Neves *et al*. (2024), which was based on semipreparative purification by HPLC.

The expression of CYP1A1 and CYP1A2 in mouse liver microsomal fractions was evaluated by EROD and MROD assays. Compared with the control (100% for untreated animals), the increases in enzyme production were 290% (IF 2.90, $p < 0.01$ by *t* test) and 243% (IF 2.43, $p < 0.001$), respectively (Fig. 3a-b). Similarly, compared with that of the

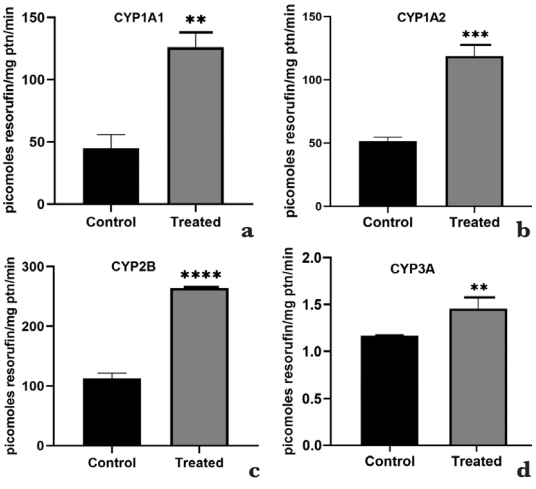

Figure 3 – a-d. Induction of CYP expression (mean ± SD, n = 3) in rat liver microsomal extract after administration of β-naphthoflavone – a. CYP1A1 assessed by resorufin release (EROD activity); b. β-naphthoflavone: CYP1A2 assessed by resorufin release (MROD activity); c. phenobarbital: CYP2B assessed by resorufin release (PMROD activity); d. dexamethasone: CYP3A assessed by formaldehyde release (ERMd activity). Control: microsomal extract from non-treated animals. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ by t-test. See experimental for details.

control, the phenobarbital-induced expression of CYP2B increased to 240% (IF 2.4, $p < 0.0001$) when assessed by the PROD assay (Fig. 3c). In turn, treatment with dexamethasone increased CYP3A expression to 120% (IF 1.2, $p < 0.001$), as assessed by ERMd activity (Fig. 3d).

The two phorboid compounds were evaluated by comparison with the inhibitory potential of α-naphthoflavone, a classic inhibitor of CYP1A that was used as a positive control in homologous cases of CYP1A. On this basis, CYP1A1 activity was strongly inhibited ($p < 0.0001$ by ANOVA followed by the Bonferroni comparison test) by synagrantol A (85%) and synagrantol G (73%) (100 μM) in the EROD assay (Fig. 4a). Similarly, synagrantol A and synagrantol G (100 μM) inhibited CYP1A2 activity by 64% ($p < 0.0001$) and 14% ($p < 0.05$), respectively, in the MROD assay, whereas α-naphthoflavone inhibited activity by 92% (Fig. 4b).

The percent inhibition of CYP2B and CYP3A by the tested phorboid compounds at 100 μM in the PROD and EMRd assays was evaluated by comparison with the phenobarbital- and dexamethasone-induced microsomal activity as a blank (control). On this basis, CYP2B activity was moderately inhibited ($p < 0.0001$) by synagrantol A (49%) and synagrantol G (52%) (Fig. 5a), whereas weak inhibition ($p < 0.0001$) of CYP3A activity (19% and 30%, respectively) was detected (Fig. 5b).

Many CYPs catalyze a broad range of biotransformation reactions and are critically important for drug response (Wright *et al*. 2019). The CYP families and subfamilies tested in the murine model adopted in this study were CYP1A1, CYP1A2, CYP2B and CYP3A, which have already been established to modulate the metabolism of several classes of drugs. The catalytic activities of the CYP1 members overlap regarding the hydroxylation or other oxidative transformations of aromatic substances, which mainly include aromatic hydrocarbons. Among these, planar structures are preferred by CYP1A1 (including flavonoids and lignans) (Santos *et al*. 2021; Ramos *et al*. 2024), while CYP1A2 acts preferentially on aromatic amines and heterocyclic compounds (Zanger & Schwab 2013). Common variants of CYP1A1 are associated with the oxidation of 17β-estradiol and estrone (Dutour & Poirier 2017).

CYP1A2 expression in the liver is relatively high, where it plays a significant role in the metabolism of many important drugs (Zhou *et al*. 2009) These drugs include acetaminophen, phenacetin, and lidocaine (analgesic); olanzapine and clozapine (antipsychotic); duloxetine (antidepressant); nabumetone (anti-inflammatory); propranolol, guanabenz, and triamterene (cardiovascular); tizanidine (muscle relaxant); zolpidem (hypnotic); and riluzole (multiple sclerosis) (Zanger & Schwab 2013). CYP1A2 activates the actions of the antiandrogen flutamide, in addition to some endogenous substrates such as arachidonic acid, prostaglandins, estrogens, melatonin and retinoic acid (Nebert & Dalton 2006). CYP1A1 (and CYP1B1) mediate the metabolic pathways of dolutegravir, an HIV-1 integrase inhibitor (Zhu *et al*. 2018).

Several CYP2B isoforms have been described in different mammalian species. In humans, CYP2B is expressed mainly in the liver, where it represents approximately 4% of the total hepatic microsomal count; it is involved in the metabolism of approximately 25% of marketed drugs. These include the anticancer drugs cyclophosphamide and

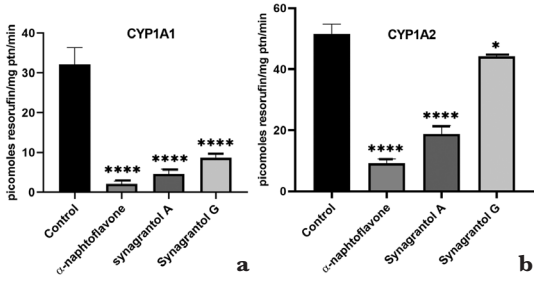

Figure 4 – a-b. Percentual of Inhibition (mean ± SD, n = 3) of: a. EROD (CYP1A) and b. MROD (CYP1A2) by synagrantol A, synagrantol G and α-naphtoflavone (100 μM), positive control) in picomoles/mg protein/min, as assessed by the released dealkylated resorufin. Control: mice liver microsomal extract after a consecutive 4-day *i.p.* treatment of mice with β-naphthoflavone (80 mg/kg). *$p <$ 0.05 and ****$p < 0.0001$ to control by ANOVA followed by Bonferroni comparison test. See experimental for details.

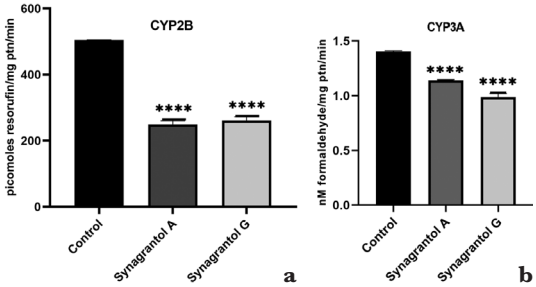

Figure 5 – Inhibition (mean ± SD, n = 3) of: a. PROD (CYP2B), and b. ERM (CYP3A) reactions by synagrantol A and synagrantol G (100 μM) as assessed by the released resorufin (picomoles/mg protein/min) and formaldehyde (nanomoles/mg protein/min), respectively. Control: enzymatic activity of liver microsomal extract after a consecutive 4-day *i.p.* treatment of mice with phenobarbital or dexamethasone. See experimental for details. ****$p <$ 0.0001 to control by ANOVA followed by Bonferroni comparison test.

tamoxifen; the anesthetics ketamine and propofol; and procarcinogenic environmental contaminants, such as aflatoxin B1 and dibenzanthracene (Martignoni *et al*. 2006). Some isoforms, also expressed in extrahepatic tissues, are strongly induced by barbiturates, contributing significantly to the metabolism of drugs such as artemisinin, efavirenz, halothane, pilocarpine, and valproic acid (Tornio & Backman 2018). Isoforms such as CYP2A6, CYP2B6 and CYP2C19 are important in the metabolism of antiretrovirals such as efavirenz and nevirapine.

Four isoforms of CYP3A are expressed in human tissues. In addition to the liver, CYP3A is present in the stomach, lungs, kidneys, and small intestine. Although its members oxidize several endogenous substrates (steroids, retinoic acid, and bile acids), CYP3A is the most important family of enzymes that metabolize xenobiotics in humans, representing 30% of the cytochrome P450 in the liver and being involved in the biotransformation of approximately 50% of marketed drugs (Martignoni *et al*. 2006; Zanger & Schwab 2013). Drugs metabolized by CYP3A include benzodiazepines (midazolam and triazolam), lidocaine, quinidine, carbamazepine, erythromycin, nifedipine, dapsone, and tacrolimus. The isoform CYP3A4/5 is also important in the metabolism of efavirenz and etravirine.

Few reports on the interactions between phorbol esters and CYPs exist in the literature. Most examples are related to their influence on gene expression and function (Okino *et al*. 1992), usually involving the plant ubiquitous phorbol 12-myristate 13-acetate (PMA) or a semisynthetic derivative such as phorbol 12,13-dibutyrate. For instance, the elevated activity of 7-ethoxyresorufin *O*-deethylase (EROD) induced by dibenz[a,c]anthracene in mouse skin was suppressed (up to 80%) in a dose-dependent manner by prior application of PMA; this result was correlated with the content of CYP1A1 mRNA (Reiners *et al*. 1992). Similar results were obtained for the same induction and CYP family in 1c1c7 (murine hepatoma) cells cultured *in vitro* (Reiners *et al*. 1993).

Since the most striking feature of phorbol esters is the ability to activate protein kinase C (PKC) translocation and modulate its regulation of numerous cellular processes, a relationship between PKC activation by phorbol esters and the suppression of the enzymatic activity of different CYPs has been proposed. In fact, in the case of 1c1c7 cells, some PKC inhibitors were able to block the mediation of CYP1A1 by PMA (Reiners *et al*. 1993). Although the outcomes of these experiments might not be directly related to the pharmacodynamics of phorboid compounds, they contribute to the understanding of their interactions with CYP450 enzymes.

Nevertheless, quite a few trials have focused on phorbol esters as therapeutic candidates. Exceptions are those conducted with PMA, as suggested for the treatment of refractory malignancies (Schaar *et al*. 2006) or hematologic disorders (Sharifi *et al*. 2014), among other applications. However, studies on the anticancer and anti-HIV potential of phorbol esters and related substances isolated from natural sources have intensified in recent years (Tostes *et al*. 2021; Wang *et al*. 2015). For instance, esters derived from the ingenane structure, which is chemically very close to tiglianes (phorboids), constitute the chemical basis of products to treat precancerous lesions, as exemplified by ingenol mebutate (Picato®) (Shafombabi *et al*. 2025).

The results of the present study demonstrated that the inhibition of CYPs produced by synagrantol A and G, mainly on CYP1A1/A2 and CYP2B6, could interfere with the metabolism of some antiretroviral drugs. This issue is important to investigate when developing anti-HIV phytomedicines from the latex of *E. umbellata*. More in-depth and detailed studies on CYP inhibition should be performed to clarify this issue. As a further step, concentration–response curves for the inhibition of these enzymes by the phorboid compounds must be constructed.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author A.C.S.

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