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Chemical and pharmacological investigations of African *Euphorbia* species

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. **Hammadi R**, Kúsz N, Mwangi PW, Kulmány Á, Zupkó I, Orvos P, Tálosi L, Hohmann J, Vasas A. Isolation and pharmacological investigation of compounds from *Euphorbia matabelensis* *Natural Product Communications* **2019**, *14*, 1–5. DOI: 10.1177/1934578X19863509

II. **Hammadi R**, Kúsz N, Dávid CZ, Behány Z, Papp L, Kemény L, Hohmann J, Lakatos L, Vasas A. Ingol and ingenol-type diterpenes from *Euphorbia trigona* Miller with keratinocyte inhibitory activity
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III. **Hammadi R**, Kúsz N, Dávid CZ, Mwangi PW, Berkecz R, Szemerédi N, Spengler G, Hohmann J, Vasas A.
Polyoxypregnane ester derivatives and lignans from *Euphorbia gossypina* var. *coccinea* Pax.
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ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
APCI	atmospheric pressure chemical ionization
COSY	correlated spectroscopy
cryst	crystallization
δ	chemical shift
DMSO	dimethyl sulfoxide
fr	fraction
GIRK	G protein-activated inwardly rectifying K^+ channel
HMBC	heteronuclear multiple-bond correlation spectroscopy
HSQC	heteronuclear single-quantum coherence spectroscopy
HPLC	high-performance liquid chromatography
HRE(S)IMS	high-resolution electron (spray) ionization mass spectroscopy
JMOD	*J*-modulated spin-echo experiment
NMR	nuclear magnetic resonance
MS	mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NOE	nuclear *Overhauser* effect
NOESY	nuclear *Overhauser* enhancement spectroscopy
NP	normal-phase
OCC	open-column chromatography
PLC	preparative thin-layer chromatography
RP	reversed-phase
SAR	structure activity relationship
SD	standard deviation
SI	selectivity index
TLC	thin-layer chromatography
TMS	tetramethylsilane
t_R	retention time
UV	ultraviolet
VLC	vacuum-liquid chromatography

1. INTRODUCTION

Plants synthesize a characteristic mixture of chemical constituents to protect themselves against various biotic and abiotic factors (e.g., diseases, pests, pathogens, herbivores, and environmental stresses). In addition, plants and their constituents have been widely used as sources of medicines since ancient times in various forms of traditional medicinal practices. The medicinal properties of plants are generally associated with their specialized metabolites, especially terpenoids, alkaloids and phenolic compounds.[1] Natural compounds exhibit high chemo-diversity with exceptional molecular scaffolds, and thus offer the possibility of synthetic alterations to increase their bioactivity. Therefore, natural resources are highly preferred in developing new drug molecules with therapeutic efficacy.[2] Over half of currently marketed pharmaceutical products are derived or originated from natural sources.[3,4]

The number of higher plant species (angiosperms and gymnosperms) is estimated at 250 000, of which only about 6% have been screened for biological activity and 15% have been evaluated from phytochemical point of view. The substantial progress of bioactivity driven high-throughput screening of plant extracts in the past decades in combination with the development of new more selective extraction techniques resulted in the identification of increasing number of novel natural products with interesting biological properties.[5] Bioactivity-guided isolation of compounds and identification of their structure virtually opened the box revealing many novel structural scaffolds, which can be served as potential starting points or lead compounds in the search for new, more active and/or more selective inhibitors/activators/modifiers of various biological processes.[6,7] A number of natural products, with diverse chemical structures, have been isolated as anticancer agents. Several potential lead molecules such as camptothecin, vincristine, vinblastine, taxol, podophyllotoxin, combretastatins, galegine, khellin have been isolated from plants and many of them have been modified to yield better analogues for activity, toxicity, or solubility while others were provided as a template for next synthetic generation of drugs.[8,9] Several successful molecules like topotecan, irinotecan, taxotere, etoposide, and teniposide have emerged as drugs upon modification of these natural lead and many more phytoconstituents-based drugs are yet to be discovered.[10]

Different approaches can be used for the identification of biologically active compounds, e.g., 1) random selection followed by chemical screening; 2) random selection followed by one or more biological assays; 3) follow-up of biological activity reports; 4) follow-up of ethnomedicinal (traditional medicine) uses of plants, and 5) intuition. Ethnopharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biological activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines (anthropology, archaeology, history, and linguistics) that contribute to the discovery of natural products with biological activity.[11,12]

The family Euphorbiaceae is one of the largest families of flowering plants with approx. 7500 species belonging to 300 genera worldwide.[13] Important economic plants can be found in this family, such as *Hevea brasiliensis* (pará rubber); *Euphorbia tetragona* and *E. triangularis* (inferior rubber); *Manihot esculenta* (cassava, tapioca); *Croton tiglium* (croton oil); *Ricinus communis* (castor oil); and *Euphorbia resinifera* ('euphorbium'), among others.[14] Members of the largest genus (*Euphorbia*) of Euphorbiaceae are widely distributed throughout mainlands of both tropical and temperate regions and characterized by the production of a milky irritant latex. *Euphorbia* species are widely used in different traditional medicines for the treatment of different ailments, e.g., infections, gonorrhea, migraine, intestinal parasites, rheumatism, snakebites, asthma, obstipation, coughs, sores, and skin diseases.[15]

According to the scientific data published earlier in the literature, plants of the family Euphorbiaceae are sources of compounds with a variety of interesting biological activities. Especially the diterpenes are of considerable interest because of their wide range of potentially valuable biological activities and their broad structural diversity due to their different polycyclic and macrocyclic skeletons and the various aliphatic and aromatic ester groups. Among *Euphorbia* diterpenes, ingenol 3-angelate (ingenol mebutate) has attracted the greatest interest in the past few years as it was approved by the FDA in 2012 and by the EMA in 2013 for the treatment of actinic keratosis, a precancerous skin condition. It has been a considerable time since a natural product without structural modification has been introduced into clinical practice. In addition to ingenol 3-angelate, other promising *Euphorbia* diterpenes are also the subjects of drug development projects. Some phorbol and ingenol derivatives, particularly prostratin, have become of great interest in HIV therapy: they reactivate HIV-1 latency through PKC-dependent NF-κB activation, and avoid the new infection of $CD4^+$ cells.[16]

Phytochemical and pharmacological investigation of *Euphorbia* species in the Department of Pharmacognosy, University of Szeged dates back almost three decades. During this time, many diterpene esters of different skeletal types have been isolated.[17] In continuation of this work, the investigation of three *Euphorbia* species, namely *Euphorbia matabelensis* Pax*, E. trigona* Miller*, and E. gossypina* var. *coccinea* Pax was performed. The present thesis summarizes the results of this preparative work.

2. AIMS OF THE STUDY

The family Euphorbiaceae is a source of biologically active compounds, especially alkaloids, sesqui-, di- and triterpenoids, flavonoids, lignans and other phenolic constituents. Investigation of the specialized metabolites of the *Euphorbia* species dates back to the end of the 20th century in the Department of Pharmacognosy, University of Szeged, and resulted in the isolation and structure determination of dozens of compounds, especially diterpenes from several *Euphorbia* species. In continuation of this work, the objectives of the present research were the isolation and structural characterization of specialized metabolites from further *Euphorbia* species, and the investigation of their pharmacological effects.

In order to achieve the aims, the main tasks of the presented study were:

- A review of the literature on the genus *Euphorbia*, from the aspect of the chemistry and pharmacological properties of the plants.
- Collection of *Euphorbia* plant samples (altogether six species).
- Preliminary pharmacological investigation of the *E. candelabrum*, *E. trigona*, *E. cotinifolia* and *E. ramipressa* against keratinocyte cell line.
- Preparation and fractionation of plant extracts for phytochemical work.
- Isolation and purification of compounds of *Euphorbia matabelensis*, *E. trigona*, and *E. gossypina* var. *coccinea* using a combination of different chromatographic methods.
- Structure determination of isolated compounds by spectroscopic methods (1D and 2D NMR, HR-MS).
- Investigation of the antiproliferative effect of isolated compounds in different test systems.
- Evaluation of the pharmacological properties and structure-activity relationship of the isolated compounds.
- Evaluation of the chemotaxonomical relevance of the isolated compounds.

3. LITERATURE OVERVIEW

3.1. BOTANY OF THE INVESTIGATED *EUPHORBIA* SPECIES

The spurge family (Euphorbiaceae) is one of the foremost complex, largest, and most diverse families of angiosperms. Members of this family occur in arid and semiarid environments as shrubs, weeds, and trees, or as climbing lianas.[10,18] Euphorbiaceae comprises approximately 50 tribes, 334 genera, and approx. 7500 known species. This complex family was established as a vital source of medicines and toxins.[13,18] Plants of this family occur mainly in the tropical and subtropical areas of Africa and America surviving acute climate conditions within several varieties of vegetations and habitats.[18] Among the Euphorbiaceae family, latex is found substantially in members of the subfamilies Euphorbioideae and Crotonoideae but is rarely found within the other subfamilies.[19] *Euphorbia* is the largest genus of the family with approx. 2000 species, subdivided into many subgenera and sections.[20] *Euphorbia* species are widely distributed throughout mainlands (both tropical and temperate regions) and range in morphology from small, annual, or perennial herbaceous plants to trees, woody shrubs, spiny succulents, lianas, and trees and even large desert succulents.[21]

Euphorbia matabelensis Pax (three-forked euphorbia) (Euphorbiaceae) is a monoecious, slightly succulent, deciduous shrub or tree, usually growing up to 3-8 m tall with abundant latex. It belongs to section *Lyciopsis*, a group of tuberous herbs or shrubs, characterized by glandular stipules, terminal or axillary cymes in large umbels, almost sessile capsules, and seeds without a caruncle. Leaves arranged spirally or crowded at branch apex, they are simple and entire. The inflorescence is an axillary cyme consisting of clusters of flowers, each cluster called a 'cyathium', in 3–7-branched umbels; their color is yellowish green. Flowers are unisexual, the fruit is an obtusely 3-lobed capsule. Seeds are globose, 3.5 mm in diameter, smooth, brown, and obscurely speckled. The plant occurs often on rocky outcrops and in open woodland on hillsides in sandy soils, in Somalia, southern Kenya, Tanzania and throughout southern Africa.[22]

Among the succulent, cacti-form *Euphorbia* species, *E. trigona* Miller, known as the African milk tree, is the most widely grown one. It originates from Africa and grows in tropical dry forests and dry deciduous forests. *E. trigona* is a spiny shrub that contains a milky sap in its leaves. The sap (or latex) from the plant is venomous and can cause skin irritations. It has an upright stem that is branched into three or four sides. The stem itself is dark green with V-shaped light green patterns. The about 5 mm long thorns are placed in pairs of two on the stem's ridges. The drop-shaped leaves grow from between the two thorns on each ridge. The flowers are white or light yellow. The flowers appear in spring and summer, but potted versions of the plant may not grow flowers at all.[23] *E. trigona* is widely commercialized as an ornamental, hedge plant and potted plant across tropical and subtropical regions.[24,25] It grows to 6 feet tall, highly tolerant of drought, and some cultivars have red leaves. This

species has the potential to escape from cultivation. In Cuba and India, where this species has become naturalized, it grows to form thickets in disturbed sites and abandoned gardens in dry and semiarid sites. In India, it grows in moist and dry deciduous forests from foothills to 400 m. It mostly spreads vegetatively by cuttings and stem fragments.[26]

E. gossypina var. *coccinea* belongs to the subgenus Tirucalli, section *Tirucalli*.[27] It is a laticiferous, exstipulate, unarmed, much-branched, evergreen, succulent shrub with a sprawling habit; it can grow up to 1-1.5 m tall, sometimes scrambling into surrounding vegetation and enable itself to develop to 4 m high.[28,29] The spreading branches are around 1 cm thick, with dark brown leaf-scares becoming more or less prominent on older branches.[28] Branches are terete, alternately or randomly produced, marked with alternate leaf-scars which become woody with age. Leaves are alternate, caduceus, and sessile, often with an axillary bud. The central cyathium of the umbellate inflorescence, which develops only male flowers, bears 6 to 8 glands and is considerably larger than the lateral bisexual ones with their 4 glands.[27] The latex is rather thin but copious, odorless, and innocuous.[29] It is indigenous to Africa and sometimes is harvested from the wild for local medicinal use.[28]

3.2. CHEMICAL CONSTITUENTS OF *EUPHORBIA* SPECIES

Each plant family, genus, and species produce a characteristic mixture of chemical constituents, and they can sometimes be used as taxonomic characters in classifying plants. The family Euphorbiaceae serves as an outstanding source of specialized metabolites, including alkaloids,[30] triterpenoids,[31,32] flavonoids (both *C*- and *O*-glycosides),[31-33] diterpenoids,[30,34,35] tannins,[31] and peptides (cyclic and linear) as the most important ones.[19,31] These compounds have been isolated from the latex or from different plant parts (e.g. bark, cortex, flowers, leaves, roots and stems). In this section, I focused on those groups of specialized metabolites and their occurrence in Euphorbiaceae species that were isolated from the investigated plants.

3.2.1. Diterpenes from *Euphorbia* species

Diterpenoids are classified as a subgroup of terpenoids which are composed one of the most important groups of specialized metabolites in plants with more than 23 000 known structures.[1,36] They possess a considerable spectrum of important biological activities.[1,37] Diterpenes occurring in plants of the genus *Euphorbia* are the focus of natural product drug discovery because of their wide-range biological activities [e.g., antitumor, cytotoxic, multidrug-resistance-reversing (MDR), antiviral, anti-inflammatory, neuroprotective, antimalarial activity, inhibition of osteoclast formation and various vascular effects].[35,38] Furthermore, their great structural diversity, derived from four isoprene units joined through a head-to-tail cyclization resulting in various macrocyclic and polycyclic skeletons (e.g. cembrane, jatrophane, ingenane, daphnane, tigliane, lathyrane, etc.) and oxygen-containing functionalities, including different aliphatic (e.g. acetyl, *n*-butanoyl, isobutanoyl, methylbutanoyl,

tigloyl, angeloyl, isovaleroyl, etc.) and aromatic (benzoyl and nicotinoyl) acids.[35,37] More than 1700 diterpenoids, incorporating over 25 skeletal types, have been reported from *Euphorbia* plants until 2021; half of them were identified in the last eight years.[34,35,39,40] Diterpenes are considered to be important taxonomic markers of *Euphorbia* species, the morphological diversity (characteristic of this species) is also manifested in the chemical features.[41]

3.2.1.1. Higher diterpenoids

Generally, there are two different methods for diterpenoid cyclization from the precursor geranylgeranyl pyrophosphate (GGPP), resulting in "higher" and "lower" diterpenoids (**Figure 1**). Higher diterpenoids involve the classical "concertina-like" cyclization of the precursor, which finally forms various cyclized hydrocarbon structures (e.g., abietane, kaurane, rosane, labdane, atisane, and bayeran skeletons). Higher diterpenes usually contain a 6/6/6-membered tricyclic ring systems.[35,36] These diterpenes, such as the bicyclic labdane and clerodane, the tricyclic abietane, and the tetracyclic atisane, and kaurane types, are not specific compounds of Euphorbiaceae species; they occur in many other plant families too.[34]

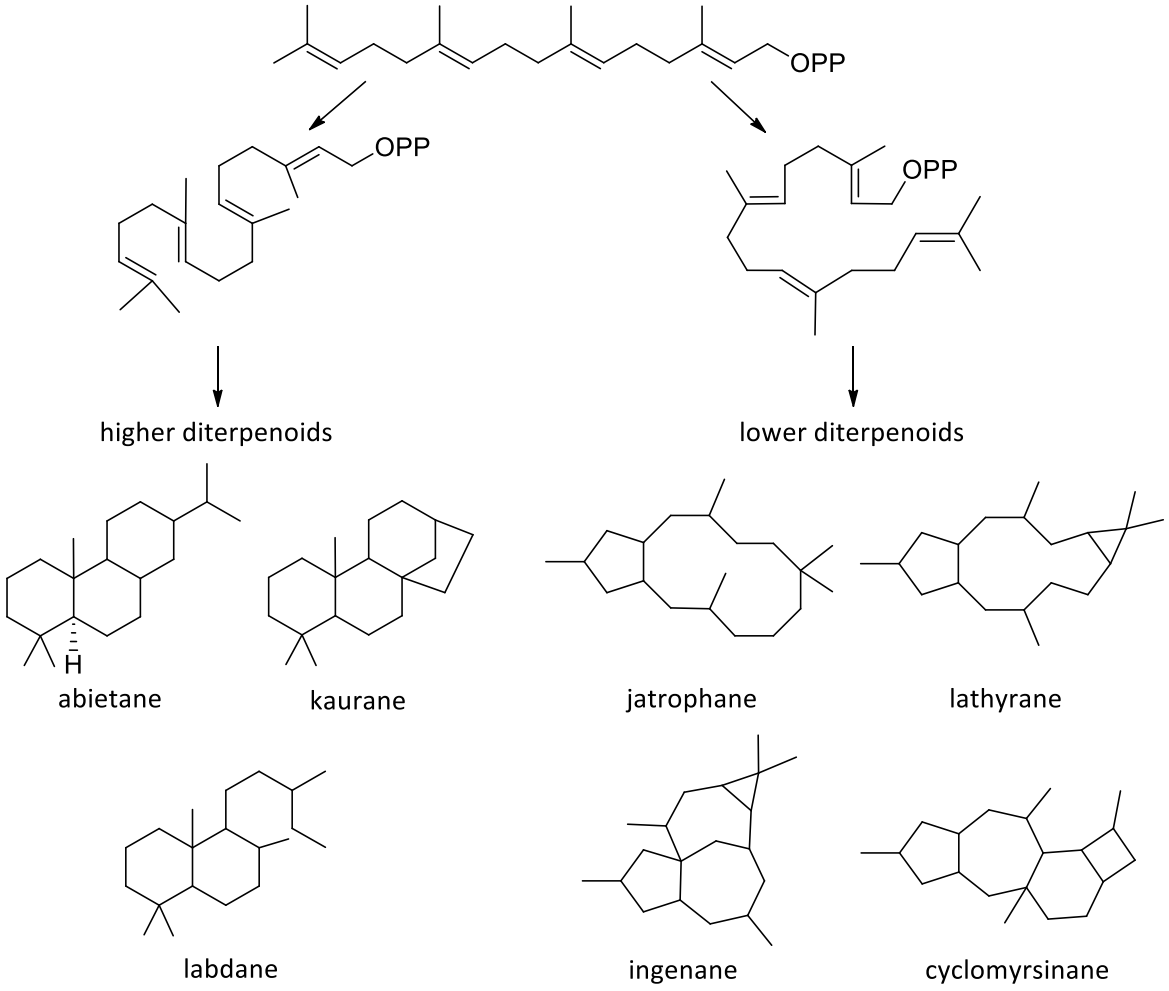


Figure 1. Biosynthesis of "higher" and "lower" diterpenoids

3.2.1.2. Lower diterpenoids

Macrocyclic diterpenoids and their cyclization products can be classified as "lower terpenoids" or "Euphorbiaceae diterpenoids" (**Figure 1**). Lower diterpenoids are characteristic compounds of plant species belonging to the families Euphorbiaceae and Thymelaeaceae, showing their chemotaxonomic relevance.[35] Valuable consideration has been paid to the macrocyclic diterpenoids, having high chemical diversity and therapeutically relevant bioactivities.[36]

Among Euphorbiaceae diterpenoids, jatrophanes are the most abundant diterpenes within *Euphorbia* species, but lathyranes are also very common in this genus. Besides compounds belonging to these two groups, myrsinane, tigliane, ingenane, segetane, paraliane, pepluane, and euphoractine-type compounds are characteristic of Euphorbiaceae and Thymelaeaceae species, and are of chemotaxonomic relevance.[35] As in our work mainly ingenane and ingol-type diterpenoids were isolated, in this section I focused on the literature overview of these compounds.

Ingenane diterpenoids are polycyclic compounds belonging to phorboids that also include daphnanes, rhamnopholanes and tiglianes. Ingenane-type diterpenoids are characterized by a tetracyclic ring system of 5/7/7/3 and a ketone bridge between C-8 and C-10, in addition to *β*-hydroxyl group at C-4. The rings A and B are usually *trans*-fused and have a double bond between C-1 and C-2 in ring A, and between C-6 and C-7 in ring B. In most cases, the carbons C-3, C-5, C-13, C-17, and C-20 are oxygenated or esterified.[35]

The distribution of ingenoids is limited to three closely related genera within the Euphorbiaceace family (*Euphorbia*, *Elaeophorbia* and *Mabea*), but most of them have been reported from *Euphorbia* species.[42] The first ingenol derivative (ingenol 3-palmitate) was reported in 1968 from *Euphorbia lathyris* and from *Croton tiglium*.[43,44] A combination of limited occurrence, chemical instability, very low concentration in plant tissues, and difficulty of detection delayed the discovery of ingenane derivatives.[45] Later, the improvements in analytical techniques and the introduction of modern chromatographic techniques like HPLC-MS have simplified the detection and the isolation of ingenol esters, and approx.. 200 compounds were identified from different *Euphorbia* species (n = 55) (*E. acrurensis*, *E. antiquorum*, *E. biglandulosa*, *E. broteri*, *E. canariensis*, *E. candelabrum*, *E. caducifolia*, *E. coralloides*, *E. cornigera*, *E. cotinifolia*, *E. cyparissias*, *E. deightonii*, *E. desmondii*, *E. drummondii*, *E. drupifera*, *E. ebracteolata*, *E. erythradenia*, *E. erythreaea*, *E. esula*, *E. grandiflora*, *E. helioscopia*, *E. hermentiana*, *E. iberica*, *E. ingens*, *E. kamerunica*, *E. kansui*, *E. kotschyana*, *E. lactea*, *E. lathyris, E. leuconeura, E. memoralis*, *E. millii*, *E. nematocypha*, *E. neriifolia*, *E. nivulia*, *E. palustris*, *E. paralias*, *E. pentagona*, *E. peplus*, *E. petiolata*, *E. portulacoides*, *E. pseudograntii*, *E. quadrialata*, *E. quinquecostata*, *E. resinifera*, *E. robbiae*, *E. royleana*, *E. segetalis*, *E. serrata*, *E. sieboldiana*, *E. sikkimensis*, *E. tirucalli*, *E. trigona*, *E. virgata*, *E. wallichii*).[34,35,46] *Euphorbia esula* (n = 30) and *E. kansui* (n = 23) are the best sources of such compounds.

Chemical diversity within ingenol derivatives is associated with modification of the hydroxylation pattern of the parent polyol and/or the nature of the acyl substitution. The number of esterifying acids in a molecule can be varied between one and four. The most common acyl substituents are acetic, propionic, isobutyric, 2-methylbutyric, angelic, tiglic and benzoic acids, but numerous other aliphatic and cyclic acids were also published in the literature. Some acyl groups are unique to ingenol esters. The most interesting ones are the anthranilate-based tripeptides (**Figure 2**) occurring in milliamines and related compounds isolated from *E. milii*.[47,48]

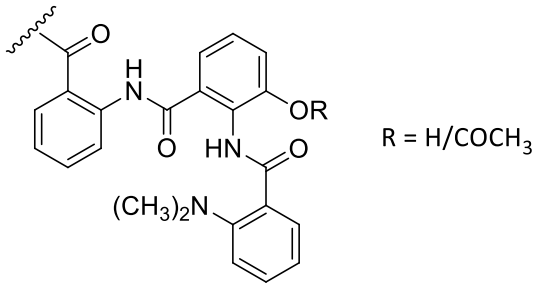


Figure 2. Unique esterifying acids of ingenanes isolated from *E. millii*

Deoxygenation can occur at C-5 and C-20, and hydroxylation at C-13, C-16, and C-19, with the possibility also of combining deoxygenation and hydroxylation, as in 20-deoxy-16-hydroxyingenol derivatives (**Figure 3**).[45]

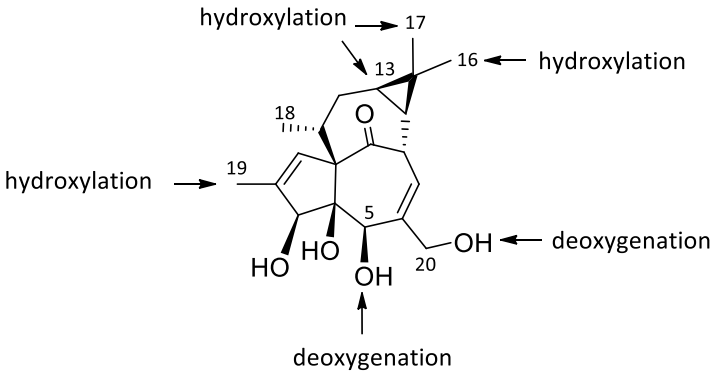


Figure 3. Functionalization diversity within ingenol esters

Ingenol 3-angelate (syn. ingenol mebutate), the best-known ingenol derivative, is used in human therapy (Picato®) for the treatment of actinic keratosis, a precancerous skin disease. Its curiosity, that it is a natural product and without structural modification has been introduced into clinical practice. Ingenol 3-angelate has a dual action by induction of necrosis followed by a PKC-driven immune response and wound healing.[49] Although it is a very effective agent, it occurs in the source plant (*E. peplus*) in very low quantity (ca δ = 1 ppm). The first semisynthesis of ingenol mebutate was developed from its parent polyol (ingenol), available in over 100 mg/kg yield from the seeds of *E. lathyris*. Other possible sources of ingenol are the latices of *E. ingens* and *E. tirucalli*.[50,51] An investigation of the concentration of ingenol in the latex of various *Euphorbia* species was carried out after hydrolysis of

the diterpenoid mixture and GC analysis of the triacetate. It was observed that typical concentration levels ranged between 1% and 3%.[52] The esterification of angelic acid is a long-standing synthetic problem, plagued by isomerization to its more stable *E* isomer (tiglic acid).[53] Nowadays, several ingenol esters are under pharmacological investigations and some of them showed promising activities, e.g., it was reported that treatment of ingenol derivatives such as ingenol triacetate in HIV-infected T cells inhibited HIV replication through downregulation of CD4 receptors and CXCR4 receptors.[54,55]

One of the largest groups of tricyclic diterpenes with a 5/11/3-membered ring system is the lathyrane group. The macrocyclic casbanes are considered the biogenetic precursors of these diterpenes. They can contain an epoxy function between C-4 and C-15 or C-5 and C-6 and double bonds between C-5 and C-6 and/or C-12 and C-13. Those compounds wearing the epoxy functionality between C-4 and C-15 are named ingols. Such compounds were identified from e.g., *E. acrurensis*, *E. antiquorum*, *E. bungei*, *E. hermentiana*, *E. ingens*, *E. kamerunica*, *E. lactea*, *E. marginata*, *E. neriifolia*, *E. nivulia*, *E. officinarum*, *E. poisonii*, *E. portulacoides*, *E. resinifera*, *E. royleana*, *E. saudiarabica* and *E. tirucalli*.[34,35] They are generally esterified at C-3, C-7, C-8, C-12 or C-19, mainly with acetyl, tigloyl, isovaleroyl, angeloyl, benzoyl and nicotinoyl groups. In case of ingols, isolated from *E. poisonii*, five ester groups can be found in the molecules, one of them is the rare phenylacetyl group (**Figure 4**).[56]

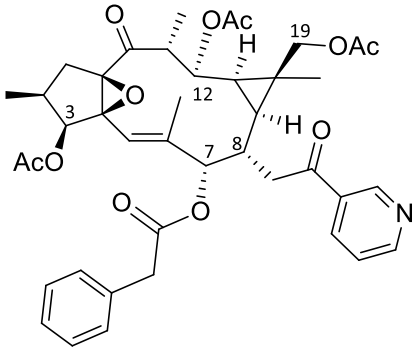


Figure 4. An ingol diterpene isolated from *E. poisonii*

3.2.2. Pregnane glycosides

Pregnanes belong to steroids which are classified as terpenoids. All steroids possess a tetracyclic carbon skeleton and differ in the number and nature of nuclear substituents and sometimes in the degree of unsaturation (**Figure 5**). According to the presence of different groups at different positions, steroids can be divided into many sub-groups, among them pregnanes and cardenolides.[57] In plants, androstanes, estranes, pregnanes, and corticoids have been well-described; these steroids promote plant development: cell divisions, root, and shoot growth, embryo growth, flowering, pollen tube growth, and callus proliferation.[58]

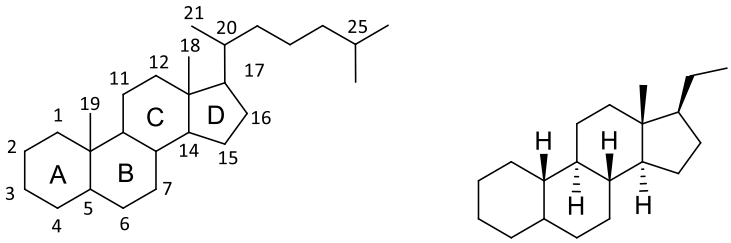


Figure 5. Sterane skeleton and general structure of pregnanes

Steroids in latices including euphol, tirucallol, euphorbol, lanosterol, lanosterol isomers, cycloartenol, 24-methylenecycloartanol, *α*-amyrin, *β*-amyrin, and other pentacyclic steroids have been detected by GC-MS and isolated from many *Euphorbia* species including *E. aphylla*, *E. arbuscula*, *E. balsamifera*, *E. characias*, *E. coerulescens*, *E. cylindrifolia*, *E. drupifera*, *E. globosa*, *E. ingens*, *E. lathyris*, *E. marlothii*, *E. misera*, *E. obtusifolia*, *E. stenoclada*, *E tirucalli* and *E trigona*.[59]

Several ergostane-type steroids have been isolated from *E. chamaesyce*.[60] Three known steroids 5α-stigmastane-3*β*-6*α*-diol and *β*-sitosterol glucopyranoside and their acetylated derivatives, and 5*α*-stigmastane-3*β*-5,6*β*-triol acetylated at C-3 and C-6 were isolated from *E. boetica*.[61] Several geniculatosides [e.g., stigma 16-en-3*α*-*O*-(*β*-D-galactopyranoside), namely geniculatoside F] were isolated from the aerial parts of *E. geniculata*.[62] Additionally, sitosterol and daucosterol present in many *Euphorbia* species, such as *E. quinquecostata*,[63] *E. altotibetic*,[64] *E. segetalis*,[65] *E. aleppica* and *E. latifolia*.[66,67]

Pregnanes are C_{21} steroidal compounds found naturally either as free or glycosidic form having perhydro-1,2-cyclopentanophenanthrene (sterane) ring system with *β*-oriented angular methyl groups at C-10 and C-13 and a two-carbon atom side chain at C-17 (**Figure 5**). Glycosides are polar compounds occurring as a complex mixture.[68,69] The pregnane skeleton is common to progesterone and many synthetic steroids with progestational activity. Moreover, cortisol, aldosterone, and many other steroids with glucocorticoid and mineralocorticoid activities are also pregnane derivatives. Pregnanes occur in two stereoisomeric forms; 5*α*-pregnane (allopregnane – *trans*) and 5*β*-pregnane (epipregnane – *cis*), differing only in the steric positions of the hydrogen and methyl substituents at the C-5 and C-10 carbon atoms of ring A.

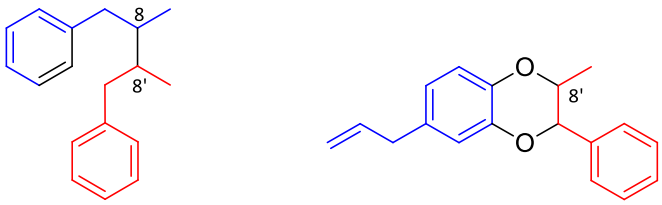
Structurally, pregnane glycosides are based on the pregnane skeleton, the aglycone part and an oligosaccharide chain linearly configured instead of a branched chain, and linked to a hydroxy group through an acetal linkage most often at C-3, C-20 or both.[67,68] In some cases, the sugar moiety is linked to hydroxy functions at C-2, C-4 or C-21.[68] The C and D rings at the aglycones unit are *cis*-fused in most reported glycosides. Additionally, the aglycones might also be substituted with tigloyl, acetyl, isovaleroyl, 2-methylbutyroyl, *o*-hydroxybenzoyl, cinnamoyl, or nicotinoyl moieties.[68]

To date, only two compounds have been isolated from an Euphorbiaceae species, *Croton ruizianus*.[70] Many of the pregnane derivatives can be utilized as a starting material for the partial synthesis of pharmaceutical steroids, such as adrenocortical hormones (corticosteroids) and sex hormones (e.g., estrogens, progestogens, and androgens).

Previous research has not indicated the presence of either pregnanes or its glycosides in *Euphorbia* species.

3.2.3. Lignans

Lignans are a large group of naturally occurring compounds that are derived from the shikimic acid biosynthetic pathway.[71] Structurally, lignans contain a basic scaffold of two or more phenylpropanoid (C_6–C_3) units.[72] Basically, lignans are phenylpropanoid dimers, linked by the central carbon (C-8, *ß*,*ß*'-linkage) of their propyl side chains ("classical lignans"). In case of neolignans, the main structural units are coupled in any other way but in position 8–8' (**Figure 6**). Nowadays, lignans are classified into eight subgroups: furofurans (e.g., pinoresinol, sesamin), furan (e.g., lariciresinol, olivil), dibenzylbutane (e.g., secoisolariciresinol, dihydrocubebin), dibenzylbutyrolactone (e.g., matairesinol, hinokinin), aryltetralin (e.g., podophyllotoxin, *ß*-peltatin), arylnaphthalene (e.g., taiwanin, justicidin A), dibenzocyclooctadiene (e.g., steganacin), and dibenzylbutyrolactol (e.g., cubebin) based upon the way in which oxygen is incorporated into the skeleton and the cyclization pattern. Lignans of each subgroup vary in oxidation levels of both the aromatic rings and the propyl side chains. In addition, these lignans can be further classified depending on the oxidation state of the terminal C-9 (C-9') of the propyl side chain.[73]



classical lignan neolignan

Figure 6. Basic structure of lignans

An interesting class of lignans is termed "non-conventional lignans"; they are biogenetically related to true lignans or neolignans but bear some features not discerned in conventional lignans, and include coumarinolignans, flavonolignans, and stilbenolignans.[74]

Lignans are widely distributed in the plant kingdom, and they exist in plant roots, rhizomes, stems, leaves, flowers, fruits, seeds, xylem, and resins. Plants, such as the members of the Lauraceae family, especially the genera of *Machilus*, *Ocotea*, and *Nectandra* are rich sources of lignans. Additionally, Annonaceae, Orchidaceae, Berberidaceae, and Schisandraceae family contain a large number of constituents of lignans and neolignans.[75] Up to date, lignans are found in over 70 families

in the plant kingdom, and more than 200 classical lignans and 100 neolignans have been characterized.[76] They are usually present as dimers, but some of them are trimers or tetramers. Most of the lignans in plants are in a free state, while some of them occur in glycosidic form or as other derivatives.[77]

Podophyllotoxin is one of the best-known representative of lignans, used in therapy as an antimitotic agent (**Figure 7**). Currently, its semisynthetic derivatives etoposide, teniposide, and etoposide phosphate, are used in clinics for anticancer chemotherapy.[78]

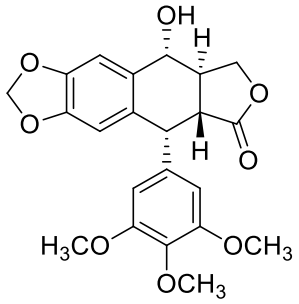


Figure 7. The structure of podophyllotoxin

Lignans have been detected and isolated from the Euphorbiaceae family from various *Croton* species (*C. caudatus* var. *tomentosus*, *C. erythrochilus C. kongensis*, *C. tiglium*),[79-82] and *Euphorbia* species like *E. pekinensis* which is classified as a lignan producing plant;[83] carolignans were isolated from *E. sikkimensis*,[84] while coumarinolignans were detected in *E. lunulata* (syn. *E. esula*),[85] and neolignans in *E. antiquorum*.[86] 21 Lignans were identified from *E. hirta*.[87]

7,7-diarylbutanol *seco*-lignans are an interesting class of lignans from both chemical and pharmacological points of view. Previously cytotoxic, anti-HIV-1 and antioxidant activities have been reported.[88-90]

3.2.4. Chemical constituents of the investigated species

3.2.4.1. Chemical constituents of *Euphorbia matabelensis*

E. matabelensis had been analysed for crude protein (CP), neutral and acid detergent fiber (NDF and ADF), acid detergent lignin (ADL), insoluble proanthocyanidins (IPAs), and protein precipitating polyphenolics (PPPs) content. The result showed that the plant contains the highest level of crude protein among other 17 Zimbabwean species with 210 g kg^{-1} of dry material and the other measured values were as follows; NDF 505 g kg^{-1}, ADF 475 g kg^{-1}, ADL 167 g kg^{-1}, IPAs 151 g kg^{-1}, and PPPs 14.23 g kg^{-1}.[91]

Comprehensive phytochemical investigations of the plant have not been reported previously.

3.2.4.2. Chemical constituents of *Euphorbia trigona*

Previously, triterpenes including taraxeryl acetate, friedelin, friedelan-3*β*- and 3*α*-ols, taraxerol, cycloartenol, 24 methylene-cycloartanol, and amyrins, lupeol, sitosterol, mortenol triterpene along with 24-epimeric cycloart-25-ene-38,24 diols had been reported from the stems of *E. trigona*.[92] Moreover, four diterpenes, among them three ingenol esters and one ingol ester were isolated from the plant.[25]

Extensive phytochemical investigations of this plant have not been reported previously.

3.2.4.3. Chemical constituents of *Euphorbia gossypina* var. *coccinea*

Neither phytochemical nor pharmacological investigations were performed previously with the plant.

3.3. TRADITIONAL USES OF THE INVESTIGATED SPECIES

Euphorbia species are widely used in traditional medicines for the treatment of different ailments, e.g., infections, gonorrhea, migraine, toothache, intestinal parasitic diseases, abdominal pain, rheumatism, paralysis, snakebites, asthma, obstipation, coughs, sores and skin diseases.[15,93,94] Several *Euphorbia* species were documented in Greek and Roman medical literature.[20,95] In a comprehensive ethnopharmacological survey, the three most-referred plants used as traditional medicines were *E. hirta, E. thymifolia*, and *E. lathyris*.[96]

Plants of this genus are characterized by the secretion of a milky irritant latex, the responsible substance of their toxicity which acts as a pesticidal agent against insects and herbivores (self-defence substance).[97,98] In some African and Asian countries, these plants (e.g., *E. helioscopia*. *E. poisonii*) have often been used as sources of ingredients in arrow poisons, not only for their cohesive properties, but also to produce irritation at the site of the arrow wound, thereby promoting absorption of the poison.[99] Some species have been classified based on the sort of their poisons and within the context of this classification, species could be divided into different groups; first group includes the species used as fish poisons e.g., *E. tirucalli*, *E. scheffleri*, and *E. inaequilatera*; the second group refers to the species combined human poisons e.g., *E. ledienii*, *E. heterophylla*, *E. cooperi*, *E. candelabrum*, *E. poissonii*, *E. unispina* and *E. venenifica*; the third group includes species used as poisons of domestic animals e.g., *E. caput-medusae, E. silenifolia*, *E. ingens*, in addition to irritating species: *E. tirucalli*, *E. poissonii*, *E. unispina* and *E. venenifica*.[31] Latex of *E. tirucalli* and *E. royleana* has been confirmed to cause conjunctivitis in contact with eyes.[100] Additionally, some members are claimed to cause typical body ailments such as *E. tirucalli*, *E. leuconeura* and others are known to be co-carcinogenic and can therefore excessively induce cell division resulting in tumor growth.[101]

Besides medicinal and toxicological uses, several species are grown as ornamental plants (*E. milli*, *E. tirucalli*, *E. obesa*, and *E. pulcherrima*), and a lot of them are of considerable economic

importance, like cassava (*Manihot esculenta*), the castor oil plant *Ricinus communis*, the rubber tree *Hevea brasiliensis*, and the biodiesel source *Jatropha curcas*.[13]

E. matabelensis is harvested from the overland for local medicinal use and sometimes to form a gum.[102] It is a traditional medicine and widely used in Africa, in Malawi; a root decoction, combined with the leaves of *Dichrostachys cinerea*, is drunk to treat depression, hypertension, and swollen lymph glands,[102] while in Zimbabwe, where it is vernacularly known as Murimbo, the powdered root is rubbed into scarifications on the breasts as a galactagogue for foster mothers.[103] Moreover, some drops of decoction of the chopped roots or latices are infused into glass of milk and taken orally as a purgative in case of poisoning and to induce abortion. The roots are chopped and boiled with water until softness, then the liquid is taken orally to act as a purgative.[104,105] The latex of *E. matabelensis* is also taken into the drinkable soup of chickens to treat diarrhoea (Newcastle disease) and respiratory problems.[102,105] It is also considered as an important browse feed resource during the hot season in Zimbabwe, particularly in marginal ecological regions.[104]

E. trigona is a succulent plant indigenous to Africa used for decorative aspects. It is called the African milk tree because of the high latex content.[106] *E. trigona* from African region, Angola, and Malawi are ordinarily planted as a ceremonial and ritual plant and hedges near villages, especially in Gabon. It is possibly of hybrid origin, as it is only known in cultivation and is not known to flower.[102] The latex is used as an additive to *Periploca nigrescens*, in arrow poison preparation, it is used as a fish poison or as a criminal poison.[102] In Congo, a few drops of latex in palm wine are taken in severe cases of constipation or in case of an epileptic attack.[102]

In Kenya, a decoction of the pounded stems of *E. gossypina* is drunk to treat swollen legs and general body aches, while in Tanzania, the latex is applied as eye drops to treat conjunctivitis, as eardrops to treat oral infections, and to treat cut-opened warts. The diluted latex of small twigs is taken to treat laryngitis. In Somalia, the latex is applied to treat mange in livestock.[28,102]

3.4. PHARMACOLOGY OF THE GENUS *EUPHORBIA* AND THE INVESTIGATED SPECIES

Generally, the entire plants, stems, leaves, latex, roots, and seeds of *Euphorbia* species were investigated in chemical and pharmacological studies and proved that they accumulate a wide range of biologically active specialized metabolites.[34] The isolated diterpenes from different *Euphorbia* species are mainly characterized by their antimicrobial, antiviral, and antiproliferative activities.[1] Moreover, *Euphorbia* diterpenes have the ability to reverse multidrug resistance (MDR), the phenomenon occurs when a cell line, initially sensitively responds to cytotoxic agents, thereafter gradually develops cross-resistance to a wide range of structurally unrelated drugs after exposure to only one drug due to the presence of an increased level of P-glycoprotein (P-gp) multidrug transporter

that efflux the drug out of the cell.[107] In this section, I focused on the pharmacological activities of the investigated species and those groups of specialized metabolites that were isolated from them.

The extract of *E. matabelensis* from Zimbabwe where it is used as a medicinal plant for treating diarrhoea and for lactation was tested for mutagenicity, using *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA102. The results indicated that the *E. matabelensis* is nonmutagenic.[94]

E. trigona contains lectins a group of proteins (or glycoproteins) of non-immune origin that bind specifically and reversibly to carbohydrates, leading to cell agglutination and precipitation.[108] Due to this capability, lectins are implicated in diverse cellular mechanisms. Three lectins (ETR1, ETR2, and ETR3) have been purified from the latex of *E. trigona* by anion exchange chromatography, all of the three are suggested to be the sort of II RIP family and bind specifically to galactose-derivative sugars and inhibit protein synthesis in a cell-free system and showed remarkable cytotoxic activity against A549, HeLa, H116, HL-60 cell lines.[108] The agglutinating ability helps in differentiation between *E. trigona* (protein content 13.00 mg/mL) and *E. hermentiana* (protein content 25.8 mg/mL), the agglutinating ability of *E. trigona* is twice as that of *E. hermentiana*.[109]

The antibacterial activity of *E. trigona* latex-based extracts (petroleum ether, chloroform, and acetone) in various solvents has been assessed against Gram-positive and Gram-negative pathogens, e.g., *Proteus mirabilis* and *Pseudomonas aeruginosa*. It was found that the latex of the plant may contain some compound(s) that act against the urinary pathogens by blocking and/or inactivating their quorum-sensing (QS) molecules or interfering with their signaling system in some manner. The anti-QS compounds present in *E. trigona* can retract and weaken the virulence of the pathogens without challenging their growth, thereby preventing the emergence of resistant strains and facilitating the elimination of pathogens by the host's immune system, consequently, considerable immunostimulant effect possessed by the plant extracts.[110-112] The main constituent of the bioactive fraction was 9,12-octadecadienoyl chloride (*Z*,*Z*).[111] Furthermore, the extracts of *E. trigona* could serve as free radical scavengers, acting possibly as primary antioxidants because of the presence of flavonoids, phenolics, quinones and saponins.[113]

Ingenol-3-acylates carrying a wide variety of 3-acyl groups have been isolated and identified as the irritant principles of the *Euphorbia* plants.[46,114] The skin-irritant properties of most ingenoids make it reasonable that they play an ecological role as feeding deterrents, complementing the physical protection of spines in the succulent species of the genus *Euphorbia*.[45] This strategy is successful on cattle, but goats and sheep can feed on spurges, while camels, dromedaries and rhinos can also consume the thorny succulent species with impunity.[115] The physiological basis for the insensitivity to oral irritation by phorboids observed in these animals is unknown, but excretion in milk represents a major detoxification route in lactating animals, making the milk and the dairy products derived therefrom unsuitable for human consumption.[115] Ingenoids often co-occur with different macrocyclic

diterpenoid esters.[35,116] These, by inhibiting transport proteins like P-gp that are overexpressed in lactating mammary tissues could interfere with the transfer of diterpenoids from plasma to milk, potentially exacerbating the toxicity of spurges.

Interestingly, ingenoids are the only class of phorboids to have so far been subjected to clinical development. Clinical research of ingenol-3-angelate is expected to provide a better comprehension of the biological profile of this class of compounds, clarifying the differences, if any, with the analogous parameters of phorbol esters, while the increased availability of the parent polyol will expand the resultant knowledge on their chemical reactivity and the medicinal chemistry.[45]

From medicinal point of view, *E. peplus* can be considered as one of the most important *Euphorbia* species. In Australian folk medicine, the latex of this plant has been used topically for the treatment of skin cancers. The constituent responsible for this effect was identified as ingenol angelate (syn. ingenol mebutate), and the compound in its original form (Picato®) was approved by the FDA (Food and Drug Administration) and thereafter the EMA (European Medicinal Agency) for the treatment of actinic keratosis (AK, a precancerous skin condition) in 2012 and in 2013. Ingenol angelate was isolated for the first time from the source plant *E. peplus* at the Department of Pharmacognosy, University of Szeged,[117] and later it was also identified from other *Euphorbia* species (*E. lathyris*, *E. nivulia, E. esula*, *E. antiquorum*, *E. serpens*, and *E. fischeriana*).[118,119]

Ingenol mebutate offered a short treatment schedule (2–3 days), providing effective and sustained clearance of AK lesions with a predictable onset and short duration of local skin responses.[120] Ingenol mebutate acts on keratinocytes in two different ways: (i) induces the cell death of aberrant keratinocytes, and (ii) induces a lesion-directed immune response that is mediated by, at least partially, the enzyme family protein kinase C (PKC).[120] The ingenol mebutate-induced cell death is mediated through the PKC*δ*/MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) pathway. PKC*δ* is a key mediator in cell differentiation and inhibition of proliferation in various tissues. Ingenol mebutate directly binds to PKC*δ* and leads to its phosphorylation, which activates of MEK/ERK signaling, resulting in decreased viability and cell proliferation.[121] Topical application of ingenol mebutate causes rapid cell death via necrosis. Subsequently, the concentration of TNF-*α* and IL-8 promptly increases that leads to the recruitment and infiltration of neutrophils into the inflamed region followed by the activation of apoptotic cell death.[122]

Unfortunately, Picato is no longer authorized in the EU market, as the European Medicinal Agency (EMA) has concluded that the medicine may increase the risk of skin cancer and its risks outweigh its benefits.[123] Therefore, searching for other active analogous is a rational purpose for research aimed at phytochemical investigation of various *Euphorbia* species.

Some structure-activity relationship observations were determined for ingenane diterpenes. According to the existing hypothesis, esters of unsaturated acids (e.g., 2,4,6-decatrienoic, angelic) are

biologically less active than the saturated monoesters (e.g., hexadecanoic, dodecanoic acids); similarly, triesters are less active than diesters of saturated fatty acids.[124] Moreover, ingenol-3-acylates carrying a wide variety of 3-acyl groups have been isolated and identified as the irritant principles of *Euphorbia* species. Depending on the particular acyl groups present, many of the irritant ingenol-3-acylates show tumor-promoting activity comparable to that of phorbol-12,13-diacylates.[114] It is interesting that 8-*epi*-ingenol is a biologically active diterpene with tumor-promoting activity, while its isomer isoingenol, is considered biologically inactive and used as a negative control in biological assays.[125]

Tigilanol tiglate (TT, also known as EBC-46) is a novel diterpene ester under clinical evaluation as a simple-to-administer, intratumoral treatment for a range of cancers (head and neck) in humans and companion animals, including MCTs (mast cell tumors) in dogs.[126,127] It is a potent cellular signaling molecule with activation of protein kinase C responsible, in part, for its efficacy. Intratumoral injection of TT elicits a rapid but highly localized inflammatory response, disruption of tumor vasculature, and induction of tumor cell death by oncosis.[126] These processes lead to hemorrhagic necrosis and destruction of the tumor mass within 2 to 7 days followed by resolution of the resulting wound with good cosmetic and functional outcomes between 28 and 84 days after treatment.[128,129]

Besides antitumor activity, *Euphorbia* diterpenes exhibited various pharmacological activities, such as antibacterial effect of diterpenes from *E. guyoniana* and *E. pubescens*,[38,130] antiviral activity of diterpenes from *E. hyberna*,[131] *E. cotinifolia* and *E. tirucalli*,[132] antifungal activity of diterpenes from *E. hirta* and *E. tirucalli*,[133] nematicidal activity of diterpenes from *E. tirucalli*, *E. helioscopia*, *E. splendens*, *E. pulcherrima*, and *E. kansui*,[13,134] and molluscicidal effect of diterpenes from *E. conspicua* and *E. paralias*.[135,136] Ingenol derivatives such as ingenol triacetate proved to inhibit HIV replication in HIV-infected T cells through downregulation of CD4 and CXCR4 receptors.[137,138] Resiniferatoxin (RTX) obtained from the latex of *E. resinifera* acts on TRPV1 (transient receptor potential V1) receptors with a potency that is several orders of magnitude greater than that obtained for capsaicin.[139] Interestingly, the ameliorating effects of intracisternal and lumbar intrathecal RTX on pain have been shown in dogs with osteoarthritis and osteosarcoma.[140]

In a retrospective study, the effect of *E. prostrata* on patients (n = 120) with hemorrhoids was observed over a follow-up period of 12 weeks. Patients consumed one tablet of *E. prostrata* every morning for two weeks. The tablet contains 100 mg plant extract (containing 3.15–8.25 mg total flavonoid calculated as apigenin-7-glucoside and 12.6–44.0 mg total phenolics calculated as gallic acid). It was observed that *E. prostrata* can be used as an effective and well-tolerated pharmaceutical agent in the treatment of early grades of hemorrhoids.[141]

Several lignans, isolated from Euphorbiaceae species were investigated in different test systems. Two lignans (niranthin and 7-hydroxy-hinokinin) from *E. hirta*, showed antiproliferative activity against the Hep G2 cells with IC_{50} values 7.2 ± 0.17 and 8.5 ± 0.36 µM.[87] Phyllamycin B and retrojusticidin B,

obtained from *Phyllanthus myrtifolius*, exhibited inhibitory effects on HIV-1 RT with EC_{50} values 3.5 and 5.5 μM, respectively.[142] Interestingly, their antiviral activities led to the synthesis and bioassay of 18 derivatives, including nitrogen containing azalignans, among them 1-aryl-pyrronaphthalenes and 3-*N*-alkylaminomethyl-1-arylnaphthalenes. Some of them also showed good anti-HIV activity.[143] (±)-*Erythro*-7'-methylcarolignan E, isolated from *E. sikkimensis* showed anti-HIV activity (EC_{50} = 6.3 (–) and 5.3 (+) μM, respectively).[84]

4. MATERIALS AND METHODS

4.1. PLANT MATERIAL

The aerial parts of *E. candelabrum*, *E. cotinifolia*, *E. ramipressa*, and *E. trigona* were collected in the Botanical Garden of the University of Szeged (Hungary), and in the Botanical Garden of the Eötvös Loránd University, Budapest (Hungary), and were identified by Anikó Németh (director of the Botanical Garden of the University of Szeged) and László Papp (Botanical Garden of the Eötvös Loránd University, Budapest). Voucher specimens (No. 891-1–5, respectively) have been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

The stems and roots (2.5 kg, fresh weight) of *E. matabelensis* Pax were collected in Kenya (Matuu subcounty, Machakos county, GPS coordinates 01°04.579’ S, 037°35.065’ E), Africa, in June 2016, and identified by Patrick Chalo Mutiso taxonomist (Department of Biological Sciences, Faculty of Science and Technology, University of Nairobi). A voucher specimen (No. UON 2016/501) has been deposited at the Herbarium of the School of Biological Sciences, University of Nairobi, Kenya.

The aerial parts (5.7 kg, fresh weight) of *E. trigona* Miller were collected in June 2018, in the Botanical Garden of Eötvös Loránd University, Budapest (Hungary), and was identified by László Papp (Botanical Garden, Eötvös Loránd University, Budapest, Hungary). A voucher specimen (No. 891) has been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Aerial parts of *E. gossypina* var. *coccinea* Pax were collected in Kenya (GPS coordinates 1◦24024.3177700 S, 36◦42053.8612500 E), Africa, in July 2018, and identified by Patrick Chalo Mutiso taxonomist (Department of Biological Sciences, Faculty of Science and Technology, University of Nairobi). A voucher specimen (No UON 2018/249) has been deposited at the Herbarium of the School of Biological Sciences, University of Nairobi, Kenya.

4.2. EXTRACTION

4.2.1. Preparation of extracts for pharmacological screening

The fresh plant materials of *E. candelabrum* Trémaux ex Kotschy (**ECA**), *E. cotinifolia* (L.) Millsp (**ECO**), *E. ramipressa* Croisat (**ER**), and *E. trigona* Miller. (**ET**) (100 g, each) were extracted with methanol (3 × 500 mL, each) in an ultrasonic bath (3 × 15 min) at room temperature. After filtration, the extracts were concentrated to dryness *in vacuo* [14.2 g (**ECA**), 8.0 g (**ECO**), 4.2 g (**ER**), and 7.17 g (**ET**), respectively], and then dissolved in MeOH–H_2O 1:1 (150 mL, each). Thereafter, solvent-solvent partitions were performed with *n*-hexane, $CHCl_3$ and EtOAc (3 × 150 mL, each). The *n*-hexane, $CHCl_3$ and EtOAc extracts were evaporated to dryness (*n*-hexane extracts 1.53 g (**ECA**), 2.30 g (**ECO**), 0.44 g (**ER**), 1.04 g (**ET**); $CHCl_3$ extracts 0.12 g (**ECA**), 0.11 g (**ECO**), 0.12 g (**ER**), 0.93 g (**ET**); EtOAc extracts 0.63 g (**ECA**), 0.80 g (**ECO**), 0.2 g (**ER**), 0.61 g (**ET**), respectively] and used for pharmacological investigation.

4.2.2. Extraction of the plant materials for preparative phytochemical work

The fresh plant materials of *E. matabelensis* [1.6 kg, stem (EMS); 0.9 kg, root (EMR)] and *E. trigona* (5.7 kg, aerial parts) were crushed with a blender and percolated with MeOH (35 L and 30 L) at room temperature. The dried ground aerial parts of *E. gossypina* var. *coccinea* (1 kg) has been percolated with MeOH (30 L) at room temperature. The methanol extracts were then concentrated *in vacuo* [51 g (EMS), and 28 g (EMR)], dissolved in 50% aqueous methanol and solvent-solvent partitions were performed with $CHCl_3$ and EtOAc (EMS and EMR), with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH (*E. trigona*) and *n*-hexane, $CHCl_3$ and EtOAc (*E. gossypina* var. *coccinea*).

4.2.3. Purification and isolation of compounds

Open-column chromatography (OCC)

OCC was carried out on polyamide for column chromatography (MP Biomedicals). Mixtures of MeOH–H_2O (1:4, 2:3, 3:2 and 4:1) were used as mobile phases for both plant extracts.

Vacuum-liquid chromatography (VLC)

VLC was performed on silica gel [NP-VLC: silica gel 60 GF254, 15 µm, Merck 1.11678; RP-VLC: LiChroprep RP-18, 40–63 µmm, Merck 113900]. Elutions were performed with a stepwise gradient.

NP-VLC 1: $CHCl_3$–MeOH (98:2, 95:5, 9:1, 8:2, 7:3, and 1:1; 100 mL of each).

NP-VLC 2: cyclohexane–EtOAc–MeOH (1:1:1; 25 mL of each).

NP-VLC 3: cyclohexane–EtOAc–MeOH (99:1:0, 95:5:0, 9:1:0, 8:2:0, 7:3:0, 6:4:0, 60:30:3, 60:30:5, 6:3:1, and 1:1:1; 500 mL of each); volume of collected fractions 10 mL; sorbent 100 g

NP-VLC 4: cyclohexane–EtOAc–MeOH (95:5:0, 9:1:0, 8:2:0, 7:3:0, 6:4:0, and 6:3:0.5; 150 mL of each); volume of collected fractions 10 mL; sorbent 10 g

NP-VLC 5: cyclohexane–EtOAc–MeOH (8:2:0, 7:3:0, 6:4:0, 6:3:1; 1.5 L of each), and then $CHCl_3$–MeOH (9:1, 4:1, 7:3, 3:2, 1:1, 0:1; 2 L of each); volume of collected fractions 20 mL; sorbent 120 g

NP-VLC 6: toluene–acetone [9:1, 4:1, 7:3, 3:2, 1:1; 200 mL of each); volume of collected fractions 5 mL; sorbent 10 g

NP-VLC 7: cyclohexane–EtOAc–MeOH (9:1:0 ,8:2:0, 7:3:0, 6:3:0.3, 6:3:0.5, 6:3:1; 150 mL of each); volume of collected fractions 5 mL; sorbent 2 g

RP-VLC 1: MeOH–H_2O [1:4, 3:7, 2:3, 1:1, 3:2, 7:3, and 4:1; 250 mL of each); volume of collected fractions 10 mL; sorbent 7 g

RP-VLC 2: MeOH–H_2O [1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1; 400 mL of each); volume of collected fractions 20 mL; sorbent 60 g

Preparative layer chromatography (PLC)

Preparative thin-layer chromatography (PLC) was performed on silica gel 60 F_{254} (Merck 105715). Separation was monitored in UV light at 254 nm and 366 nm. The OCC, VLC, and PLC fractions obtained were monitored by TLC on silica gel 60 F_{254} (Merck 105554) and on reversed-phase silica gel 60 F_{254} (Merck 105559). Compounds were eluted from the scraped adsorbent with CH_2Cl_2–MeOH (4:1). Mobile phases:

NP-PLC 1: toluene–acetone (4:1)

NP-PLC 2: toluene–acetone (7:3)

NP-PLC 3: $CHCl_3$–MeOH (4:1)

NP-PLC 4: $CHCl_3$–MeOH (95:5)

NP-PLC 5: $CHCl_3$–MeOH (85:15)

NP-PLC 6: toluene–acetone (1:1)

RP-PLC 1: MeOH–H_2O (4:1)

RP-PLC 2: MeOH–H_2O (7:3)

Visualization methods: UV light at 254 nm and 366 nm, and at daylight after spraying with vanillin-sulfuric acid reagent and then heating at 110 °C for 5 min.

High-performance liquid chromatography (HPLC)

HPLC was carried out on normal (LiChrospher Si 100 (250 × 4 mm, 5 µm) and reversed-phase [LiChrospher RP-18 (5 µm, 250 × 4 mm), and Kinetex Phenyl-Hexyl 100A° 150 × 4.6 mm] columns, using a Waters instrument (Controller 600, a Pump 600, a Photodiode Array Detector 2998, and an injector Rheodyne 7725i).

NP-HPLC 1: cyclohexane–EtOAc gradient [80% solvent A (cyclohexane) for 1 min, changed linearly to 20% (1–10 min), held 2 min (10–11 min), then followed by a return to the initial conditions within 1 min (11–12 min) and kept 2 min (12–14 min) for the equilibrium; flow rate 1.5 mL/min]

NP-HPLC 2: cyclohexane–EtOAc gradient [80% solvent A (cyclohexane) for 1 min, changed linearly to 60% (1–10 min), then changed linearly to 80% (10–10.5 min) and held at 80% for 1 min (10.5–

11.5 min), then followed by changing to 100% mobile phase A within 30 s (11.5–12 min) and kept 3 min (12–15 min) for the equilibrium; flow rate 1.5 mL/min]

RP-HPLC 1: MeOH–H_2O gradient [60% solvent A (MeOH) for 1 min, changed linearly to 80% (1–10 min), then changed linearly to 60% (10–10.5 min) and held at 60% for 1 min (10.5–11.5 min), and maintained for 3 min (12–15 min) for the equilibrium. The flow rate was 1 mL/min]

RP-HPLC 2: MeOH–H_2O gradient [10% A (MeOH) for 1 min, changed linearly to 50% A (1–10 min), held at 50% for 1 min (10–11 min), then followed by a return to the initial conditions within 1 min (11–12 min) and kept 2 min (12–14 min) for the equilibrium; flow rate 1 mL/min

RP-HPLC 3: MeOH–H_2O gradient [20% A (MeOH) for 1 min, changed linearly to 80% A (1–10 min), held at 50% for 30 sec (10–10.5 min), then followed by a return to the initial conditions within 1 min (10.5–11.5 min) and kept 2 min (11.5–13.5 min) for the equilibrium; flow rate 1 mL/min

RP-HPLC 4: MeOH–H_2O (8:2) isocratic, 15 min, flow rate 1mL/min

RP-HPLC 5: MeOH–H_2O gradient [20% A (MeOH) for 1 min, changed linearly to 50% A (1–10 min), held at 50% for 30 sec (10–10.30 min), then followed by a return to the initial conditions within 1 min (10.30–11.30) and kept 2 min (11.30–13.30 min) for the equilibrium; flow rate 1 mL/min

RP-HPLC 6: MeOH–H_2O (7:3) isocratic, 17 min, flow rate 1mL/min

4.2.4. Structure elucidation of the isolated compounds

Optical rotations were determined in $CHCl_3$ at room temperature with a Perkin-Elmer 341 polarimeter.

NMR spectra were recorded in CD_3OD, $CDCl_3$, DMSO-d_6 or C_5D_5N on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C). The signals of the deuterated solvents were taken as a reference. The chemical shift values (δ) were given in ppm and coupling constants (J) were in Hz. Two-dimensional (2D) experiments were performed with standard Bruker software. In the ^1H-^1H COSY, HSQC and HMBC experiments, gradient-enhanced versions were applied.

High-resolution MS (HRMS) data were recorded on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer equipped with an ESI ion source in positive ionization mode. The data were acquired and processed with MassLynx software. APCI-MS measurements were performed on an API 2000 Triple Quad mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface, using positive and negative polarity.

4.3. PHARMACOLOGICAL TESTS

Pharmacological investigations were performed in cooperation with the Department of Pharmacodynamics and Biopharmacy, University of Szeged, and the Department of Medical Microbiology, Albert Szent-Györgyi Medical School, University of Szeged (antiproliferative assays); the Department of Pharmacology and Pharmacotherapy, Albert Szent-Györgyi Medical School, University

of Szeged, (GIRK channel-inhibitory assay); and the Department of Dermatology and Allergology, Albert Szent-Györgyi Medical School, University of Szeged (keratinocyte inhibitory assay).

4.3.1. Antiproliferative assays

MTT assay

Antiproliferative effects of the compounds isolated from *E. matabelensis* (**1**–**3**) and *E. gossypina* var. *coccinea* (**13**–**23**) were measured *in vitro* in human carcinoma cell lines [cervical (HeLa and C33a), breast (MCF7 and MDA-MB-231), in case of *E. matabelensis*, and HeLa, in case of *E. gossypina* var. *coccinea*], by means of an MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay.[144,145] Cisplatin and doxorubicin were used as positive controls. The reduced MTT was assayed at 545 nm, using a microplate reader, and the IC_{50} values were calculated utilizing GraphPad Prism 4.0.

Keratinocyte inhibitory assay

In the keratinocyte inhibitory assay, immortalized human keratinocyte cell line (HPV-Ker) was used. The HPV-Ker cells were seeded in 96-well E-plates and keratinocytes were allowed to attach to the bottom of the wells and grow for 24 h. Cells were then treated with the extracts, prepared from *E*. *candelabrum*, *E*. *cotinifolia*, *E*. *ramipressa*, and two collections of *E*. *trigona*, and the compounds, isolated from *E. trigona* at concentrations of 5 and 0.5 μg/mL for another 72 h. Real-time measurements of impedance were monitored every 15 min, then data at 24 and 48 h were used for calculations. Ingenol mebutate as a positive control was applied at 5 μg/mL for 24 h. The half-maximal inhibitory concentration (IC_{50}) values for the compounds were calculated and GraphPad Prism 9.0 was used to plot the results. All experiments were performed in duplicate, in three independent repeats.

4.3.2. GIRK channel-inhibitory assay

Experiments were performed on HEK293 (human embryonic kidney) cells stably expressing the G protein-activated inwardly rectifying K^+ channels, GIRK1/4 (Kir3.1/3.4). GIRK ion current was measured using planar patch-clamp technology in the whole-cell configuration with a 4-channel semi-high-throughput automated patch clamp system (Nanion Technologies GmbH), according to the protocol published in ref.[146] Propafenone (1 μM) was used as a positive control. For each compound isolated from *E. matabelensis*, a stock solution of test compound (10 mM) was prepared with DMSO and added to the cells in increasing concentrations (1 and 10 μM). Before experiments, stock solutions were further diluted with high K^+ external solution to give appropriate concentrations for the measurements. The final DMSO concentrations in the tested samples were ≤ 0.1%. Data acquisition and online analysis were performed with an EPC-10 Quadro patch-clamp amplifier using PatchMaster 2.65 software.

5. RESULTS

5.1. SCREENING OF *EUPHORBIA* SPECIES FOR KERATINOCYTE INHIBITORY ACTIVITY

As part of our screening program for biologically active compounds in plants, four *Euphorbia* species (*E. candelabrum*, *E. cotinifolia*, *E. ramipressa*, and *E. trigona*) were investigated for their keratinocyte inhibitory effect. The extracts were prepared with methanol, the crude extracts were concentrated *in vacuo* and solvent-solvent partitions were performed with *n*-hexane (1), $CHCl_3$ (2), and EtOAc (3). The extracts were evaluated at concentrations of 5 and 0.5 µg/mL (**Figure 8**). Ingenol mebutate administered at 5 µg/mL for 24 h exerted the strongest cytotoxic effect, and after a 48-h treatment, lower cytotoxicity was measured. The treatment of keratinocytes with ingenol mebutate at a 0.5 µg/mL concentration resulted in a weaker, but still significant, cytotoxic effect after 24 h and, interestingly, after 48 h, the inhibitory activity was comparable to the treatment when 5 µg/mL was used for 48 h. The extracts with a 5 µg/mL concentration were applied for 24 h, and ETP1 had similar, but lower, cytotoxic activity than that of ingenol mebutate. Interestingly, the 48-h treatment of cells with 5 µg/mL extracts ECA1, ECO2, ER1, ER2, and ETP1 showed a very similar cytotoxic property as ingenol mebutate. Cytotoxic activity was the lowest when the extracts were used at a 0.5 µg/mL concentration for 24 h, but in the case of ER2 and ETP1, cytotoxic activity was significant compared to the control and quite similar to that of ingenol mebutate. After administration of the extracts at a 0.5 µg/mL concentration for 48 h, only ETP1 displayed cytotoxicity comparable to that of ingenol mebutate. Based on these results, the *n*-hexane extract of *E. trigona* (ETP1) could be considered the most promising one for further investigation.

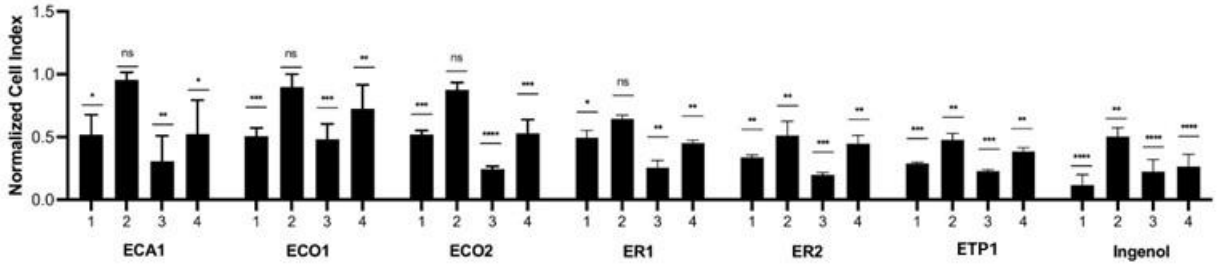


Figure 8. Inhibitory activity of different *Euphorbia* extract against keratinocytes. ECA1: *E. candelabrum n*-hexane extract, ECO1: *E. cotinifolia n*-hexane extract, ECO2: *E. cotinifolia* $CHCl_3$ extract, ER1: *E. ramipressa n*-hexane extract, ER2: *E. ramipressa* $CHCl_3$ extract, ETP1: *E. trigona n*-hexane extract; 1: 5 µg/mL, 24 h, 2: 0.5 µg/mL, 24 h, 3: 5 µg/mL, 48 h, 4: 0.5 µg/mL, 48 h; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

5.2. ISOLATION OF COMPOUNDS

5.2.1. Isolation of compounds from *E. matabelensis*

The methanol extracts were concentrated *in vacuo* [51 g (EMS), 28 g (EMR)], dissolved in 50% aqueous methanol and solvent-solvent partitions were performed with $CHCl_3$ and EtOAc. The $CHCl_3$-soluble

fractions [21 g (EMS) and 17 g (EMR)] were separated on a polyamide column (OCC) with gradient system of MeOH–H_2O (2:3, 3:2, 4:1, and 1:0; each eluent was collected as a fraction), to get four-four fractions (RI–IV and SI–IV) from roots and stems (**Figure 9**). The fractions were monitored by normal phase thin-layer chromatography (NP-TLC), and it could be observed that fractions of stems and roots were differed from each other; therefore, their further purification was performed separately. Fraction RI (1.2 g) was separated by vacuum-liquid chromatography (VLC) on silica gel with a gradient system of $CHCl_3$–MeOH (98:2, 95:5, 9:1, 8:2, 7:3, and 1:1; 100 mL of each) (NP-VLC 1). Fraction RI/4 (47 mg) was purified by preparative TLC on reversed-phase silica gel with MeOH–H_2O (4:1) (RP-PLC 1) to yield compound **1** (16.7 mg). Fraction SII (1.59 g) was separated by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH (9:1:0, 8:2:0, 7:3:0, 6:4:0, 60:30:3, 60:30:5, 6:3:1, and 1:1:1; 25 mL of each) (NP-VLC 2). Fraction SII/5 (11.6 mg) was subjected to NP-HPLC purification with gradient system of cyclohexane–EtOAc (flow rate 1.5 mL/min) (NP-HPLC 1) to yield compound **2** (t_R = 6.3 min, 0.8 mg). From SII/6, compound **3** (t_R = 7.5 min, 1.4 mg) was isolated by the same NP-HPLC method as used for the purification of compound **2**.

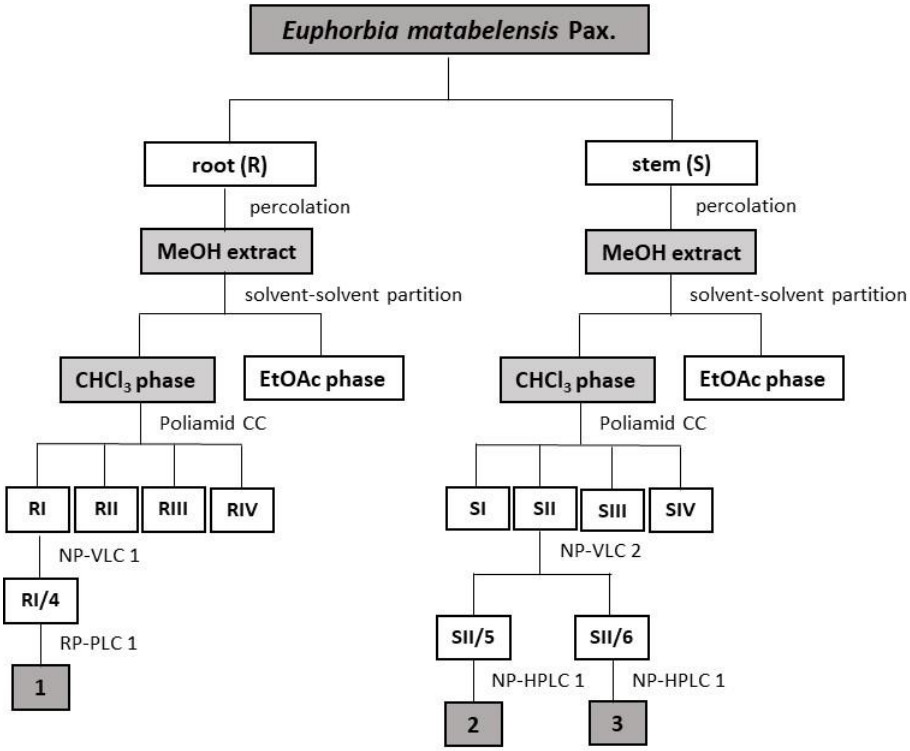


Figure 9. Isolation of compounds from *E. matabelensis*

5.2.2. Isolation of compounds from *E. trigona*

The methanol extract was concentrated *in vacuo*, dissolved in 50% aqueous methanol, and solvent-solvent partition was performed with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The fractions were monitored by NP-TLC. Interestingly, based on the TLC determination, instead of the $CHCl_3$ phase, diterpenes were accumulated in the *n*-hexane fraction. *n*-Hexane fraction (26.5 g) was therefore

further purified at first by polyamide column chromatography (OCC) with a MeOH–H_2O gradient system (3:2, 4:1, and 1:0; each eluent was collected as a fraction) to obtain three main fractions (I–III) (**Figure 10**). Fraction I (10.0 g) was then separated by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH (99:1:0, 95:5:0, 9:1:0, 8:2:0, 7:3:0, 6:4:0, 60:30:3, 60:30:5, 6:3:1, and 1:1:1; 500 mL of each) (NP-VLC 3). TLC determination and combination of the fractions afforded 20 main fractions (I/1–20). Fractions I/7 (164.8 mg), I/8 (326.7 mg), I/9 (160.9 mg), and I/12 (96.8 mg) were purified by VLC on NP silica gel with a cyclohexane–EtOAc–MeOH gradient solvent system (95:5:0, 9:1:0, 8:2:0, 7:3:0, 6:4:0, and 6:3:0.5; 150 mL of each) (NP-VLC 4) to yield combined fractions I/7/1–4, I/8/1–4, I/9/1–4, and I/12/1–6, respectively. Fractions I/7/2 (89 mg), I/8/3 (189 mg), I/8/4 (161 mg), and I/9/4 (82 mg) were further purified by preparative TLC on NP silica gel with toluene–acetone (8:2) (NP-PLC 1) as the mobile phase, and then by NP-HPLC with a gradient system of cyclohexane–EtOAc [80% solvent A (cyclohexane) for 1 min, changed linearly to 60% (1–10 min), then changed linearly to 80% (10–10.5 min) and held at 80% for 1 min (10.5–11.5 min), then followed by changing to 100% mobile phase A within 30 s (11.5–12 min) and kept 3 min (12–15 min) for the equilibrium; flow rate was 1.5 mL/min (NP-HPLC 2) to yield compounds **7**+**8** (13.3 mg) from I/7/2; **5** (45.7 mg) from I/8/3; **6** (1.4 mg) from I/8/4; and **10** (6.8 mg) and **12** (4.8 mg) from I/9/4. Compound **4** (12.1 mg) was isolated from I/9/4 by preparative TLC using toluene–acetone (8:2) (NP-PLC 1) as a mobile phase.

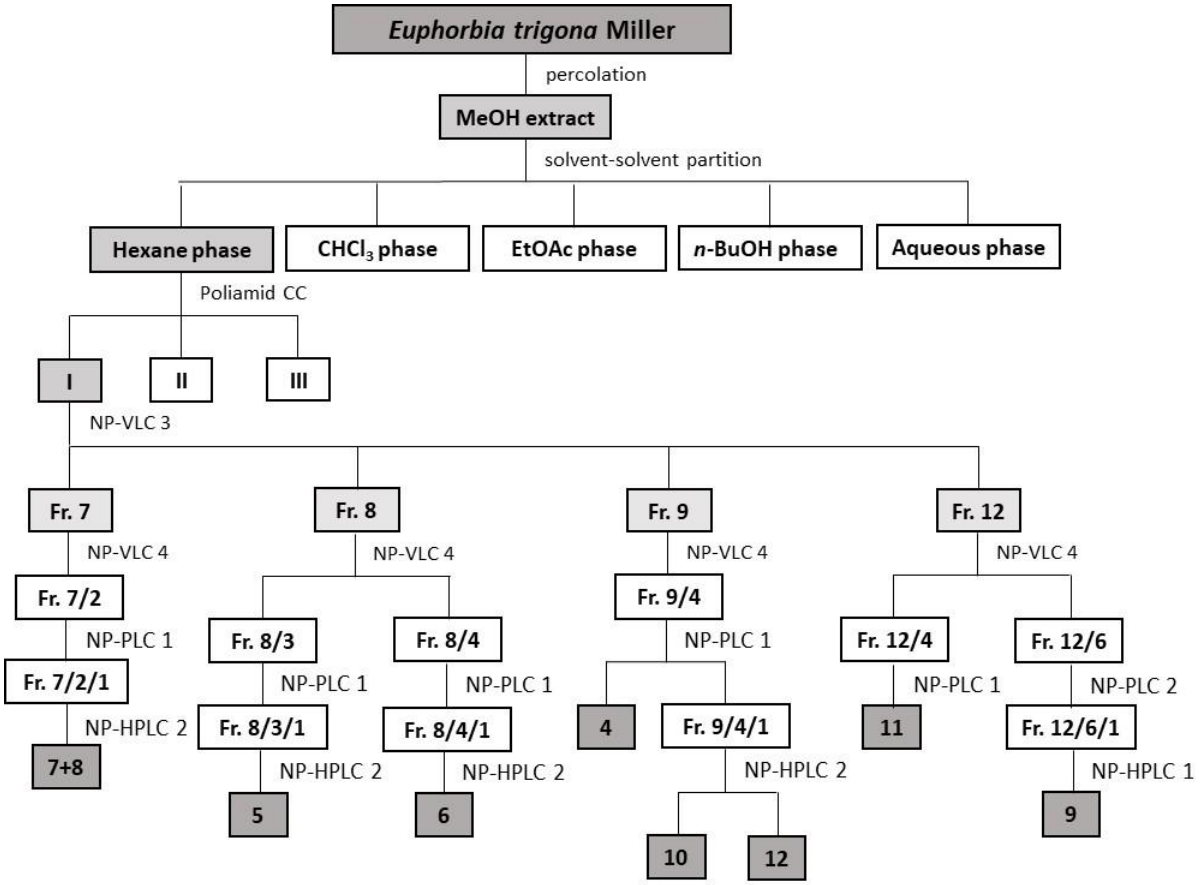


Figure 10. Isolation of compounds from *E. trigona*

Purification of I/12/4 (9.6 mg) by preparative TLC (toluene–acetone 8:2) (NP-PLC 1) resulted in the isolation of compound **11** (1.9 mg). Fraction I/12/6 (12 mg) was separated by preparative TLC with toluene–acetone (7:3) (NP-PLC 2), and then by RP-HPLC with a gradient system of MeOH–H_2O [60% solvent A (MeOH) for 1 min, changed linearly to 80% (1–10 min), then changed linearly to 60% (10–10.5 min) and held at 60% for 1 min (10.5–11.5 min), and maintained for 3 min (12–15 min) for the equilibrium]; flow rate was 1 mL/min] (RP-HPLC 1) to obtain compound **9** (6.5 mg).

5.2.3. Isolation of compounds from *E. gossypina* var. *coccinea*

After concentration of the methanolic extract (45 g), it was dissolved in 50% aqueous methanol, and solvent–solvent partitions were performed with *n*-hexane, $CHCl_3$, and EtOAc. The $CHCl_3$ fraction (28.6 g) was purified by polyamide column chromatography (OCC) using MeOH–H_2O (4:1) to remove chlorophyll. Thereafter, the yielded fraction (22.8 g) was purified by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH (8:2:0, 7:3:0, 6:4:0, 6:3:1; 1.5 L of each), and then $CHCl_3$–MeOH (9:1, 4:1, 7:3, 3:2, 1:1, 0:1; 2 L of each) (NP-VLC 5) (**Figure 11**). The TLC monitoring and combination of the fractions afforded 20 main fractions (Fr. 1–20). Fraction 9 (1.4 g) was further chromatographed by RP-VLC using MeOH–H_2O gradient solvent system (1:4, 3:7, 2:3, 1:1, 3:2, 7:3, and 4:1; 250 mL of each) (RP-VLC 1), and 9 fractions (Fr. 9/1–9) were obtained. Fraction 9/3 (87.9 mg) was subjected to preparative TLC using $CHCl_3$–MeOH (8:2) (NP-PLC 3) as eluent, and was, subsequently, purified by RP-HPLC. Gradient elution was applied, starting at 10% A (methanol) and 90% B (H_2O) for 1 min, then changed linearly to 50% A (1–10 min), held at 50% for 1 min (10–11 min), then followed by a return to the initial conditions within 1 min (11–12 min), and kept 2 min (12–14 min) for the equilibrium (flow rate 1 mL/min) (RP-HPLC 2) to yield compound **22** (t_R = 3.1 min, 2.6 mg). Fraction 10 (1.6 g) was separated by NP-VLC using a toluene–acetone gradient system (9:1, 4:1, 7:3, 3:2, and 1:1; 200 mL of each) (NP-VLC 6) to obtain 9 subfractions (Fr. 10/1–9). Fraction 10/4 (371 mg) was chromatographed by NP-VLC using cyclohexane–EtOAc–MeOH gradient system (9:1:0 ,8:2:0, 7:3:0, 6:3:0.3, 6:3:0.5, 6:3:1; 150 mL of each) to yield 6 fractions (Fr. 10/4/1–6). Compound **24** (5.8 mg) was isolated from Fr. 10/4/3 (87 mg) by preparative TLC using $CHCl_3$–MeOH (95:5) (NP-PLC 4) as mobile phase. Fraction 10/5 (188 mg) was purified by RP-TLC using MeOH–H_2O (8:2) (RP-PLC 1) as eluent, and further purified by RP-HPLC by using gradient elution, started at 10% A (methanol) and 90% B (H_2O) for 1 min, then linearly increased to 50% A (in 10 min) (method RP-HPLC 2) to yield compound **21** (t_R = 7.3 min, 12.1 mg). Fraction 10/6 (123 mg) was separated by RP-TLC using MeOH–H_2O (8:2) (RP-PLC 1) as a solvent system and compound **13** (11.2 mg) was obtained. Fraction 11 (4.9 g) was chromatographed by NP-VLC using the gradient system of toluene–acetone (from 9:1 to 1:1) (NP-VLC 6) to yield 11 subfractions (Fr. 11/1–11). Fraction 11/7 (56.2 mg) was first separated by VLC on reverse phase silica gel using MeOH–H_2O gradient elution (RP-VLC 1) and then by RP-HPLC (RP-HPLC 4) to obtain compound **15** (10.2 mg).

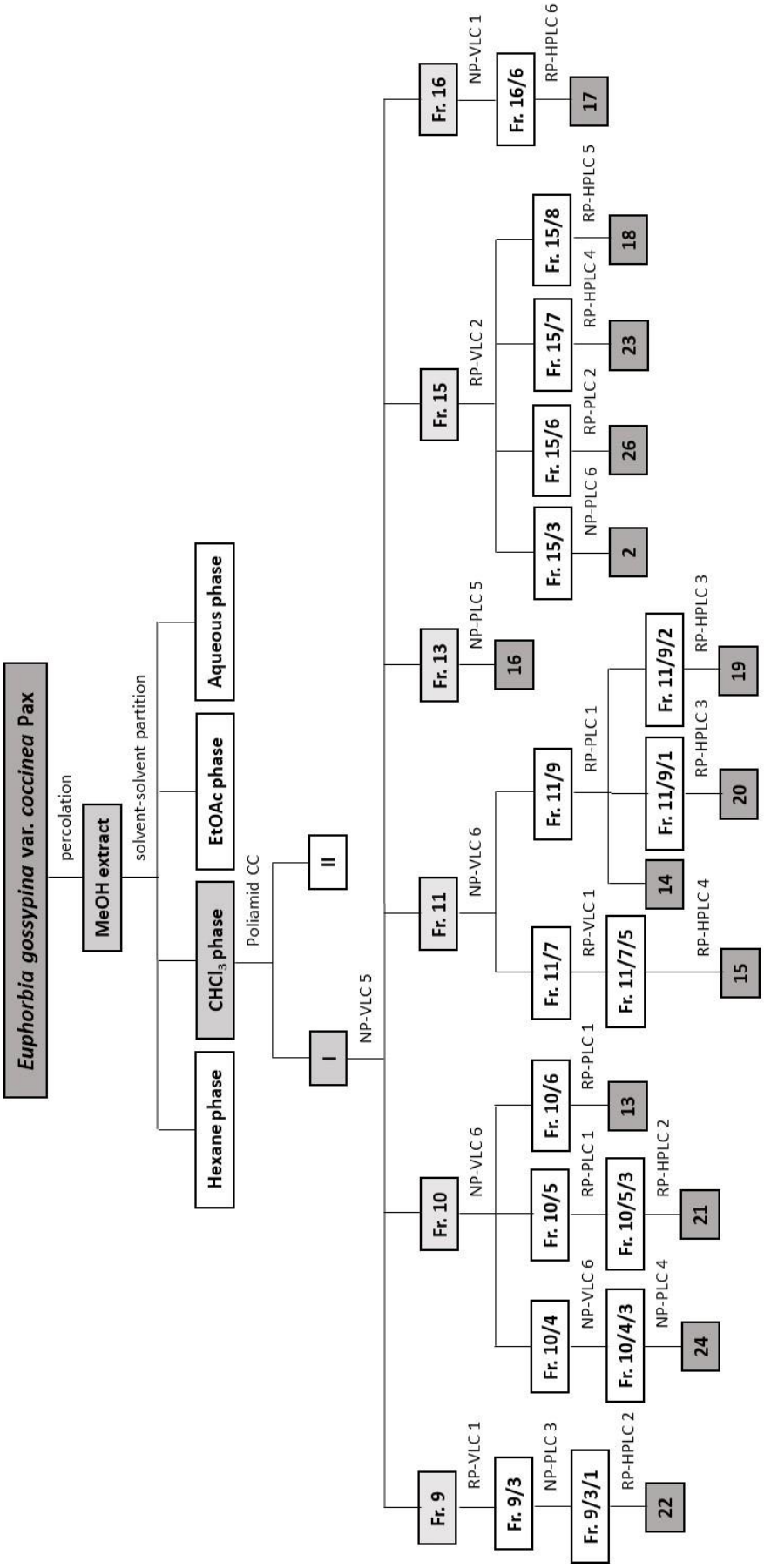


Figure 11. Isolation of compounds from *E. gossypina* var. *coccinea*

Fraction 11/9 (193.5 mg) was purified by RP-TLC using MeOH–H_2O (8:2) (RP-PLC 1) as an eluent to yield compound **14** (19.7 mg), and two subfractions [Fr. 11/9/1 (8.1 mg) and 2 (6.9 mg)]. Both subfractions were purified by RP-HPLC by gradient elution, starting at 20% A (MeOH) and 80% B (H_2O) for 1 min, then changed linearly to 80% A (1–10 min), held at 50% for 30 sec (10–10.5 min), then followed by a return to the initial conditions within 1 min (10.5–11.5 min) and kept 2 min (11.5–13.5 min) for the equilibrium (flow rate 1 mL/min) (RP-HPLC 3) to yield compounds **19** (t_R = 6.1 min, 5.1 mg) and **20** (t_R = 5.2 min, 6.4 mg) . Fraction 13 (165 mg) was purified by NP-TLC using $CHCl_3$–MeOH (85:15) (NP-PLC 5) as mobile phase to isolate compound **16** (12.7 mg). Fraction 15 (5.9 g) was separated by RP-VLC using a gradient system of MeOH–H_2O (1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 7:3, 4:1, and 9:1; 400 mL of each; volume of collected fractions 20 mL) (RP-VLC 2) to yield 7 subfractions (Fr. 15/1–7).

Fraction 15/3 (50 mg) was subjected to an NP-TLC using toluene–acetone (1:1) (NP-PLC 6) as an eluent to obtain compound **2** (5 mg). Fraction 15/6 (75 mg) was subjected to RP-TLC using MeOH–H_2O (7:3) (RP-PLC 2) to yield compound **25** (8.9 mg). Fraction 15/7 (329 mg) was purified by RP-HPLC using MeOH–H_2O (8:2) isocratic solvent system (17 min, flow rate 1mL/min) (RP-HPLC 4) to obtain compound **23** (t_R = 11 min, 47.1 mg). Fraction 15/8 (75 mg) was purified by RP-HPLC starting at 20% A (MeOH) and 80% B (H_2O) for 1 min, changed linearly to 50% A (1–10 min), held at 50% for 30 sec (10–10.5 min), then followed by a return to the initial conditions within 1 min (10.5–11.5 min) and kept 2 min (11.5–13.5 min) for the equilibrium; flow rate was 1 mL/min (RP-HPLC 5) to yield compound **18** (t_R = 5.5 min, 13.9 mg). Fraction 16 (1.6 g) was subjected to an NP-VLC using $CHCl_3$–MeOH gradient system (98:2, 95:5, 9:1, 8:2, 7:3, and 1:1; 500 mL of each) (NP-VLC 1) to yield 9 subfractions (Fr. 16/1–9). Fraction 16/6 (106 mg) was further purified by RP-HPLC using MeOH–H_2O (7:3) isocratic solvent system (17 min, flow rate 1mL/min) (RP-HPLC 6) to obtain compound **17** (t_R = 7.2 min, 24.3 mg).

5.3. CHARACTERIZATION AND STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

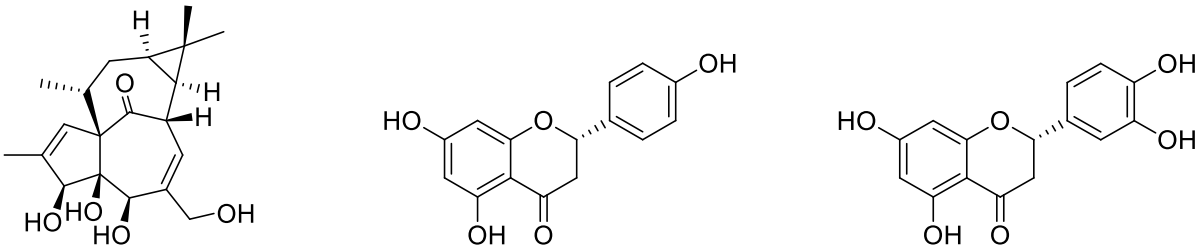
The structure elucidation of the compounds was performed by means of MS and NMR measurements. HRESIMS measurements revealed the molecular masses and molecular compositions of the novel compounds. Information from 1D (^{1}H NMR and JMOD) and 2D (^{1}H-^{1}H COSY, NOESY, HSQC and HMBC) NMR experiments proved to be the most valuable for the structure determination.

5.3.1. Compounds from *E. matabelensis*

With the combination of different chromatographic methods (e.g., CC, VLC, PLC and HPLC), three compounds (**1**–**3**) were isolated from the methanol extract of *E. matabelensis* roots and stems. The structure determination of the compounds was performed by 1D and 2D NMR, and MS investigations and with comparison of the spectral data with those reported in the literature. The identified compounds were ingenol (**1**), 4′,5,7-trihydroxyflavanone (**2**, naringenin) and 3',4',5,7-

tetrahydroxyflavanone (**3**, eriodictyol) (**Figure 12**).[147-149] All compounds were isolated for the first time from the plant.

Ingenol (**1**) was previously detected in the alcoholic extract of *E. resinifera* at concentration of 5–17 mg/L, while only traces were determined in the extracts of *E. amygdaloides* and *E. pilulifera*.[150] However, ingenol esters has been detected in several *Euphorbia* species (e.g. *E. candelabrum*, *E. coralloides*, *E. deightonii*, *E. desmondii*, *E.drupifera*, *E. erythreaea*, *E. grandiflora*, *E. kamerunica*, *E. kotschyana*, *E. lactea*, *E. lathyris*, *E. memoralis*, *E. millii*, *E. nerifolia*, *E. nivulia*, *E. pentagona*, *E. pseudograntii*, *E. robbiae*, *E. royleana*, and *E. sikkimensis*).[151]

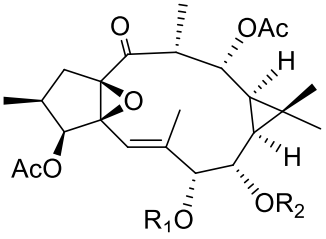


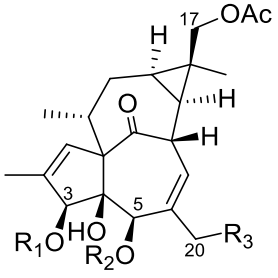
1 **2** **3**

Figure 12. Structures of the compounds (**1**–**3**) isolated from *E. matabelensis*

5.3.2. Compounds from *E. trigona*

Chromatographic separation of the methanolic extract, prepared from the fresh aerial parts of *E. trigona* yielded nine compounds (**4**–**12**). The structures of the compounds were determined by comparison of their spectroscopic data with those of reported literature value, and they proved to be diterpene esters of ingol and ingenane types, namely ingol 3,12-diacetate 7-tigliate (**4**),[152] 8-*O*-methyl-ingol 3,12-diacetate 7-tigliate (**5**),[152] 8-*O*-methyl-ingol 3,12-diacetate 7-benzoate (**6**),[152] ingol 3,7,12-triacetate 8-benzoate (**7**),[152] and ingol 3,7,12-triacetate 8-tigliate (**8**),[153] 17-acetoxyingenol 3-angelate 20-acetate (**9**),[154] 17-acetoxyingenol 3-angelate 5,20-diacetate (**10**),[154] 17-acetoxy-20-deoxyingenol 3-angelate (**11**),[154] and 17-acetoxy-20-deoxyingenol 5-angelate (**12**) (**Figure 13**).[154]





Compound	R_1	R_2
4	Tig	H
5	Tig	Me
6	Bz	Me
7	Ac	Bz
8	Ac	Tig

Compound	R_1	R_2	R_3
9	Ang	H	OAc
10	Ang	Ac	OAc
11	Ang	H	H
12	H	Ang	H

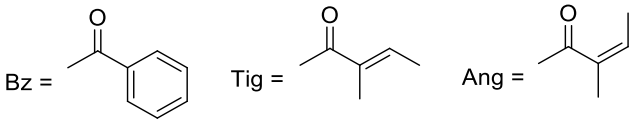
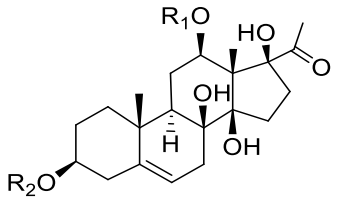


Figure 13. Structures of the compounds (**4**–**12**) isolated from *E. trigona*

The identified diterpenoid esters **4**–**12** were described earlier from only a few plant species. Compound **4** was previously isolated from *E. hermentiana*,[152] compound **5** from *E. acrurensis*,[155] *E. hermentiana*,[152] and *E. kamerunica*,[156] compound **6** from *E. antiquorum*,[153] *E. hermentiana*,[152] and *E. kamerunica*,[156] compound **7** from *E. antiquorum*,[153] *E. nivulia*,[157,158] *E. hermentiana*,[152] and *E. kamerunica*,[159] and compound **8** from *E. antiquorum*,[153] and *E. kamerunica*.[159] The ingenane-type compound **9** was previously reported from *E. canariensis* and *E. hermentiana*,[154,160] compound **10** from *E. hermentiana*,[154] *E. kamerunica*,[156] *E. royleana*,[161] and *E. trigona*,[25] **11** from *E. acrurensis*,[155] *E. hermentiana*,[154] and *E. trigona*,[25] and **12** from *E. hermentiana*.[154]

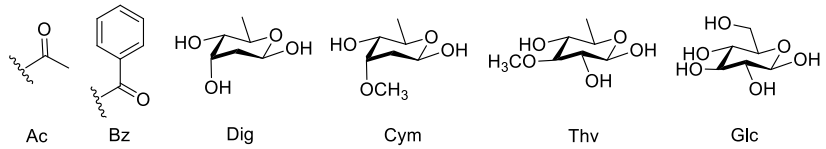
5.3.3. Compounds from *E. gossypina* var. *coccinea*

By using various chromatographic methods, fourteen compounds (**2**, **13**–**25**) were isolated from the methanolic extract of the fresh aerial parts of *E. gossypina* var. *coccinea* (**Figure 14**).



Compound	R_1	R_2 sugar chain			
		Sugar 1	Sugar 2	Sugar 3	Sugar 4
3	Bz	Cym	Cym	Thv	
14	Bz	Dig	Dig	Thv	
15	Bz	Cym	Dig	Thv	
16	Bz	Cym	Cym	Thv	Glc
17	Bz	Dig	Dig	Thv	Glc
18	Bz	Cym	Dig	Thv	Glc
19	Ac	Dig	Dig	Thv	-
20	H	Cym	Dig	Thv	

Sugar 3 (**13**-**15**, **19**, **20**) and sugar 4 (**16**-**18**) are in terminal position.



Ac = acetyl, Bz = benzoyl, Dig = digitoxose, Cym = cymarose, Thv = thevetose, Glc = glucose

Figure 14. Newly isolated pregnane glycosides (**13**–**20**) from *E. gossypina* var. *coccinea*

Compound **13** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{49}H_{72}O_{17}$ by the HRESIMS ion at *m/z* 955.4660 $[M + Na]^+$ (calcd for $C_{49}H_{72}O_{17}Na$, 955.4662). The ^1H NMR spectrum of **13** showed the resonances of three anomeric protons at δ_H 4.85 (dd, *J* = 2.0 and 9.0 Hz), 4.76 (dd, *J* = 1.8 and 9.5 Hz), and 4.30 (d, *J* = 7.8 Hz), three methoxy groups at δ_H 3.42, 3.44, and 3.65 (each 3H, s), and three secondary methyl groups at δ_H 1.22 (d, *J* = 6.3 Hz), 1.27 (d, *J* = 6.2 Hz), and 1.31 (d, *J* = 6.2 Hz), suggesting the presence of a trisaccharide unit in **13** composed of deoxymethyl sugars (**Table A1**). Moreover, the ^1H NMR spectrum contained signals ascribable to three methyl groups displayed at δ_H 1.12 (3H, s), 1.54 (3H, s), and 2.06 (3H, s), and to an olefinic proton at δ_H 5.38 (1H, br s). The proton signals at δ_H 7.93 (2H, dd), 7.43 (2H, t), and 7.55 (1H, t) showed the presence of a benzoyl group in the molecule. The JMOD spectrum indicated that compound **13** contained two carbonyls, seven non-protonated carbons (of which three were oxygenated), twenty-two methines (of which eleven were oxygenated), nine methylenes, and nine methyl carbons (of which three were methoxy). The ^1H and ^{13}C JMOD NMR data dictated that **13** was a pregnane glycoside (**Table A1**). Of these, 21 carbons were assigned to a pregnane skeleton, 7 to a benzoyl function, and 19 to a trisaccharide moiety. The HMBC correlations between H-19 (δ_H 1.12) and C-1 (δ_C 38.9), C-5 (δ_C 140.8), C-9 (δ_C 43.8), and C-10 (δ_C 37.3) suggested the position of a double bond at C-5/C-6 (**Figure 15**).

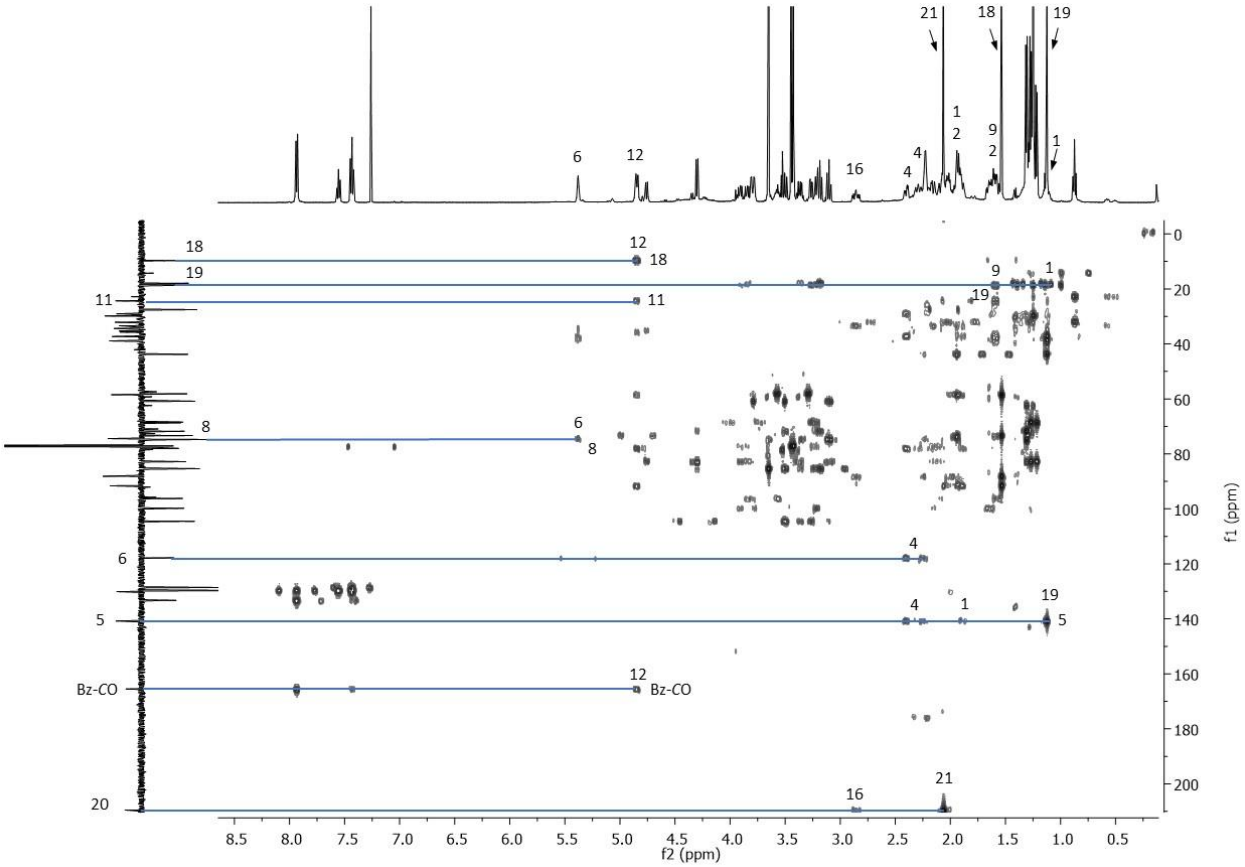


Figure 15. HMBC spectrum of compound **13** and key HMBC correlations (–).

The HMBC correlations between H-18 (δ_H 1.54) and C-12 (δ_C 73.3), C-14 (δ_C 88.1), and C-17 (δ_C 91.6), between H-21 (δ_H 2.06) and C-17 (δ_C 91.6), and H-6 (δ_H 5.38) and C-8 (δ_C 74.4) demonstrated the positions of hydroxy groups at C-8, C-14, and C-17 (**Figures 15** and **16**). The aglycone moiety of **13** was, therefore, determined to be 12*β*-benzoyloxy-3*β*,8*β*,14*β*,17*β*-tetrahydroxypregn-5-ene (cynanforidine), a C/D-*cis*-polyoxypregnane ester.[162] The relative configuration of the molecule was determined by the analysis of NOESY correlations and literature data of similar structures reported previously.

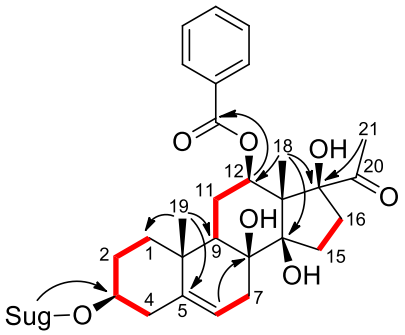


Figure 16. The ^{1}H-^{1}H COSY (—) and key HMBC (H→C) correlations of **13**.

The aglycone of **13** was supposed to have the same configuration as those of pregnanes isolated from *Gymnema sylvestre*.[163] The multiplicity of H-12 [δ_H 4.83 (dd, *J* = 4.2, 11.9 Hz)] implied that the configuration of H-12 was axial (*α*-configuration, **Figure 16**). The NOESY correlations between H-3 (δ_H 3.57), H$_\alpha$-1 (δ_H 1.89), and H$_\alpha$-4 (δ_H 2.40), and between H-12 (δ_H 4.83) and H-9 (δ_H 1.59) determined the configurations of the oxygenated groups at C-3 and C-12 to be *β*. NOESY correlations detected between H$_\beta$-1 (δ_H 1.13) and H$_\beta$-4 (δ_H 2.29) showed the *α*-position of these protons. The C-12 benzoyl group was confirmed by HMBC correlation from H-12 (δ_H 4.83) to the benzoyl carbonyl C-1' (δ_C 165.5). The large coupling constants between the H-1 and H-2 of monosaccharide moieties, and the HMBC correlations between Thv H-1 (δ_H 4.30) and Cym II C-4 (δ_C 82.8), Cym II H-1 (δ_H 4.76) and Cym I C-4 (δ_C 82.7), and between Cym I H-1 (δ_H 4.85) and aglycone C-3 (δ_C 78.0) indicated the sugar linkages as *β*-D-thevetopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside and at C-3 of aglycone. Based on the above evidence, the structure of **13** was elucidated as cynanforidine 3-*O*-*β*-D-thevetopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside, a new compound named euphogossypin A.

The ^{1}H and JMOD spectra of compound **14** exhibited the characteristic signals of a pregnane aglycone, one benzoyl unit, and three sugar moieties (**Table A1**). In addition, the NMR data of **14** were similar to those of euphogossypin A (**13**), except for the difference in sugar units at C-3. A careful analysis of NMR data led to the conclusion that two cymarose units were replaced by two digitoxose monosaccharides. The HMBC correlations between Thv H-1 (δ_H 4.82) and Dig II C-4 (δ_C 84.1), Dig II H-

1 (δ_H 5.41) and Dig I C-4 (δ_C 83.9), and between Dig I H-1 (δ_H 5.48) and aglycone C-3 (δ_C 78.1) confirmed the sugar linkages to be 3-*O*-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside. Consequently, compound **14** was identified as a new compound, cynanforidine 3-*O*-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside, and named euphogossypin B.

Compound **15** differed from **13** and **14** in monosaccharide units in the C-3 sugar chain determined by the ^{1}H and JMOD NMR (**Table A1**), and HRESIMS data. The sugar moieties were found to be D-cymarose, D-digitoxose, and D-thevetose. The sugar linkages (*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranoside) to each other and to the pregnane skeleton were confirmed by the HMBC correlations from Thv H-1 (δ_H 4.35) to Dig C-4 (δ_C 83.8), from Dig H-1 (δ_H 4.89) to Cym C-4 (δ_C 83.9), and from Cym H-4 (δ_H 3.24) to C-3 (δ_C 79.3). Similarly to those of **13** and **14**, the aglycone was found to be 12*β*-benzoyloxy-3*β*,8*β*,14*β*,17*β*-tetrahydroxypregn-5-ene (cynanforidine), therefore, **15** was determined as cynanforidine 3-*O*-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranoside and named euphogossypin C.

HRESIMS and NMR data of compounds **16**–**18** suggested that they were tetrasaccharide derivatives due to the presence of four anomeric carbon and proton signals, one more than observed for compounds **13**–**15**. The additional sugar unit was identified as D-glucopyranose (**Table A2**). The polyoxypregnane ester aglycone was the same as in compounds **13**–**15**. The large coupling constant between H-1 and H-2 of the glucose unit, and the HMBC correlations between Glc H-1 (δ_H 4.43) and Thv C-4 (δ_C 82.8) in **16**–**18**, indicated the sugar linkages as *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside in **16**, *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside in **17**, and *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranoside in **18**. Based on the above evidence, the structure of **16** was deduced to be cynanforidine *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-*β*-D-cymaropyranoside, and was named euphogossypin D, **17** was determined as cynanforidine *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside and named euphogossypin E, and **18** was characterized as cynanforidine *β*-D-glucopyranosyl-(1→4)-*β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranoside and named euphogossypin F.

The NMR data of compound **19** were very similar to that of **14**, with the only difference of the replacement of the benzoyl moiety into an acetyl substituent at C-12. Therefore, the aglycone of **19** was identified as metaplexigenin (**Table A3**).[164] The connecting sugar chain was determined as *β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside based on the 1D and 2D NMR spectral data. Compound **19** was, thus, characterized as metaplexigenin *β*-D-

thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-digitoxopyranoside and named euphogossypin G.

Compound **20** wore the same C-3 trisaccharide sugar chain, *β*-D-thevetopyranosyl-(1→4)-*β*-D-digitoxopyranosyl-(1→4)-*β*-D-cymaropyranoside, as **15** (**Table A3**). By the NMR data, it was determined to be a deacetylmetaplexigenin derivative and named euphogossypin H.

Gossypilignan A (**21**) was obtained as a pale yellow, amorphous solid. Its molecular formula was determined as $C_{22}H_{30}O_7$ from its HRESIMS ion observed at *m/z* 429.1889 $[M + Na]^+$ (calcd for $C_{22}H_{30}O_7Na$, 429.1884). The ^1H and ^{13}C JMOD NMR data indicated the presence of four methoxy (δ_H 2 × 3.82, s, and 2 × 3.83, s; δ_C 4 × 56.8), two methyl (δ_H 0.68, d, *J* = 6.9 Hz, and 0.76, d, *J* = 7.0 Hz; δ_C 10.0 and 12.1), one methylene (δ_H 3.35 and 3.45; δ_C 67.3), and three methine groups (δ_H 1.77, m, 2.62, m, 3.52, d; δ_C 37.0, 37.2, and 57.9) (**Table A4**). Additionally, the ^1H NMR spectrum of **21** showed aromatic protons at δ_H 6.64, s and 6.66, s (2H each), implying that the aromatic rings were tetrasubstituted. The ^{13}C NMR spectrum of **21** also supported the presence of six oxygenated aromatic carbons at δ_C 149.1, 149.2, and 134.7 (each 2C). According to the HMBC correlations from H-2, H-6, H-2′, and H-6′ to C-7, both aromatic rings were attached to C-7. The ^1H-^1H COSY correlations of H-7/H-8/H-8′/H-7′, H-8/H-9, and H-8′/H-9′, as well as the HMBC correlations from H-7 to C-8, C-8′, and C-9, indicated the presence of a 2,3-dimethylbutane moiety (**Figure 17**). By comparison, the skeleton of **21** was found to be the same as that of kadangustin J.[88] According to the chemical shift of δ_C 67.3 (C-7′), a hydroxy group should be placed at C-7′. Four methoxy groups were located at C-3, C-5, C-3′, and C-5, respectively, while C-4 and C-4′ were substituted with hydroxy groups. Thus, the structure of compound **21** was determined to be 4,4-di-(4-hydroxy-3,5-dimethoxyphenyl)-2,3-dimethylbutanol and named gossypilignan A. As compound **21** was a 7,7-diaryl-8,8′-dimethylbutan-1-ol lignan, it had two chiral centers. Based on the investigation of Davidson et al., *syn*- and *anti*-isomers of such compounds can be distinguished based on the significant differences between their ^1H NMR data;[165] therefore, in the case of **21**, both methyl groups were *β*-oriented, proving that this compound was a *syn*-isomer.

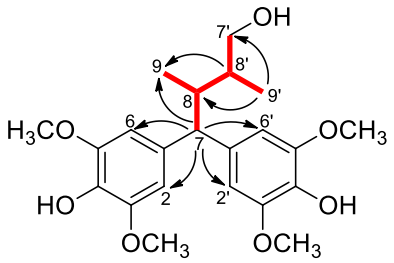
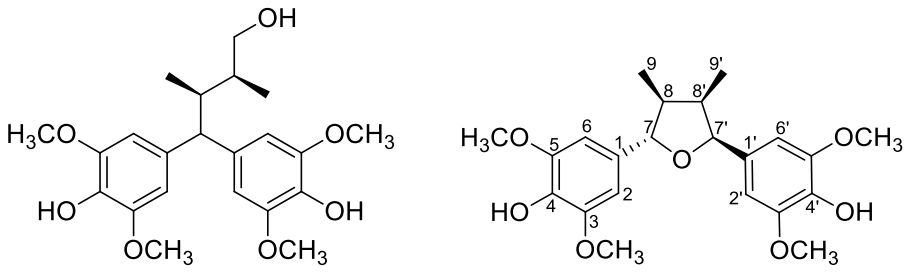


Figure 17. Selected ^1H-^1H COSY (—) and HMBC (→) correlations of **21**

The ^1H NMR spectrum of **22** showed four aromatic hydrogens as two similar systems, one at δ_H 6.69 (2H, br s), and another at 6.63 (2H, br s), indicating the presence of two 1,3,4,5-tetrasubstituted

benzene rings (**Table A4**). The chemical shifts observed for aromatic hydrogens along with the presence of four singlets corresponding to methoxy hydrogens at δ_H 2 × 3.84 and 2 × 3.86 (s, 3H each) indicated the presence of 3,5-dimethoxy-4-hydroxyphenyl groups in this compound. The ^{13}C NMR data corroborated the structural determination of these aromatic rings. Moreover, ^{1}H NMR spectral data suggested a nonsymmetric tetrahydrofuran lignan, through signals corresponding to two methyl groups at δ_H 1.00 (d, *J* = 6.4 Hz) and 0.63 (d, *J* = 7.0 Hz) in addition to two oxybenzyl methines at δ_H 4.64 (d, *J* = 9.3) and 5.47 (d, *J* = 4.4 Hz). The attachment of aromatic rings to the tetrahydrofuran ring was determined by an HMBC experiment. In the HMBC spectrum, interactions were observed between H-2′ and C-7′ and C-4′, between H-6′/C-4′ and C-2′, and between H-2 and C-7. These data indicated the presence of the two 3,5-dimethoxy-4-hydroxyphenyl structural parts at C-7 and C-7′, respectively. Based on previous literature data,[166,167] the coupling constant of 9.3 Hz for the doublet at δ_H 4.64 (H-7) indicated that this hydrogen was in a *trans* configuration with the adjacent H-8, while the coupling constant of 4.4 Hz of H-7′ demonstrated its *cis* relationship with H-8′. NOE correlations confirmed the relative stereochemistry at the tetrahydrofurane ring as *trans* (C-7/C-8), *cis* (C-8/C-8′), and *cis* (C-8′/C-7′). Moreover, NOE effects proved the *cis* configuration of the aromatic group at C-7′ and methyl groups at C-9 and C-9′. These data permitted the establishment of the structure of the new tetrahydrofuran lignan **22** as *rel*-(7*S*,8*R*,7′*S*,8′*S*)-3,5,3′,5′-tetramethoxy-4,4′-dihydroxy-7,7′-epoxylignan and named gossypilignan B (**Figure 18**).



21 **22**

Figure 18. Newly isolated lignans (**21** and **22**) from *E. gossypina* var. *coccinea*

The known compounds were identified as 12-*O*-benzoyldeacylmetaplexigenin (**23**), 9*α*-hydroxypinoresinol (**24**),[168] naringenin (**2**),[148] and quercitrin (**25**) (**Figure A1**).[169] The oxypregnane **23** is the aglycone of pregnane glycosides **13**–**18**. It was isolated for the first time from roots of *Cynanchum caudatum* M. II,[170] and later from the roots of *Araujia sericifera*[171] and the aerial parts of *Asclepias curassavica*.[172] 9*α*-hydroxypinoresinol (**24**) was isolated for the first time from the branches of *Allamanda neriifolia* (*Apocynaceae*),[168] thereafter it was reported from several plants, e.g., *A. reinwardtii* (a traditional Indonesian herbal medicine),[173] from the aerial parts of *Galactites elegans*,[174]

and from the roots and rhizomes of *L. kanaitizensis*.[175] Enzymatic deglycosylation of petaslignolide A from *Petasites japonicus* leaves yielded **24**.[176]

All compounds were isolated for the first time from the plant. Such polyoxypregnane ester derivatives were isolated previously, mainly from Asclepiadaceae species (e.g., *Cynanchum wilfordii* and *Leptadenia hastata*).[162] *Euphorbia* species are frequently characterized by the abundant presence of various terpenoids, this is only the second report of pregnanes from a plant belonging to the Euphorbiaceae family. Previously, two pregnane glycosides were identified from the aerial parts of *Croton ruizianus*. Their aglycon was 3*β*,14*β*,15*β*,16*α*-tetrahydroxypregnan-20-one, while the connecting sugar parts were either 3-*O*-*β*-D-Glu-(1→4)-*β*-D-Ole-(1→4)-*β*-D-Ole-(1→4)-*β*-D-Dig-(1→4)-*β*-D-Ole or 3-*O*-*β*-D-Ole-(1→4)-*β*-D-Ole-(1→4)-*β*-D-Dig-(1→4)-*β*-D-Ole (Ole – oleandrose; Dig – digitoxose).[70]

6. DISCUSSION

6.1. INVESTIGATION OF *EUPHORBIA MATABELENSIS*, *E. TRIGONA* AND *E. GOSSYPINA* VAR. *COCCINEA*

Phytochemical investigations of *E. matabelensis*, *E. trigona*, *E. gossypina* var. *coccinea* led to the isolation of 25 compounds including 10 new natural products. The structures were established utilizing spectral analyses as two flavanons (naringenin and eriodictyol), a flavonoid glycoside (quercetine 3-*O*-*α*-L-rhamnopyrnoside), ten diterpenes, among them four esters of ingenol diterpenes, five esters of ingol diterpenes and a free ingenol, three lignans, and eight pregnane-glycosides and a pregnane aglycon. Pharmacological investigations revealed that some of the isolated diterpenes possess remarkable activity (keratinocyte inhibitory activity).

6.1.1. Isolation of compounds

Screening of *Euphorbia* species (*E. matabelensis*, *E. trigona*, and *E. gossypina* var. *coccinea*) led to the conclusion that the lipophilic extracts contain a number of specialized metabolites, among them diterpenes and pregnane glycosides, which can be enriched by polyamide OCC. The purification of the compounds generally requires the involvement of multistep separation methods because the plants produce complex mixtures of esters of the same terpenoid nucleus (they may display very similar chromatographic characters), and the compounds occur merely in low quantities in the plants.

The dried plant material was extracted with methanol at room temperature by percolation. Methanol, an amphipolar solvent, is suitable for the extraction of both lipophilic and polar compounds.

In the initial step of separation, solvent-solvent partition with *n*-hexane and chloroform was applied in order to remove the polar constituents. The purification was continued with a classical column chromatographic technique. In accordance with the screening investigations, polyamide proved to be suitable as a stationary phase for the preparative work, with the use of methanol–water

solvent systems. Depending on the plant, the 40–80% methanol fractions were rich in terpenoids. Although diterpenes are accumulated generally in the chloroform phase, in case of *E. trigona* the *n*-hexane phase contained the diterpene esters.

In the following steps, even more selective methods (VLC, PLC and HPLC) were applied. After polyamide OCC, adsorption chromatography on silica gel was used in all experiments. VLC separations of the diterpene fractions afforded crude fractionations of the main components. For final purification, NP- and RP-HPLC were applied since these were the most effective and most selective separation methods.

The preparative work was completed with analytical thin layer chromatography on silica gel with various solvent systems. The aims of the TLC analysis were to model the separation methods, to combine fractions, and to check the purity of the isolated compounds. The detection was carried out in UV light at 254 nm, followed by spraying with cc. H_2SO_4.

As a result of the isolation procedure, 25 compounds, occurring in low concentration, were obtained from the multicomponent samples. In some cases, compounds with very similar structures could be separated: Compounds **9** and **10** differ from each other in only one substituent, compounds **11** and **12** differ in the position of the angeloyl group, while the pregnane glycosides **13**–**15** and **16**–**18** differ in the connecting sugar moieties. After extensive chromatographic purification, 3 compounds were isolated from *E. matabelensis* (**1**–**3**), 9 from *E. trigona* (**4**–**12**), and 14 from *E. gossypina* var. *coccinea* (**2**, **13**–**25**).

6.1.2. Structure elucidation

The isolated compounds are amorphous solids. Most of them are optically active. The structures of the isolated compounds were elucidated by means of spectroscopic methods. From MS measurements, the molecular compositions were determined. The most useful data concerning the chemical structures were furnished by the 1D and 2D NMR spectroscopy. From the ^{1}H and ^{13}C NMR, HSQC, ^{1}H-^{1}H COSY, and HMBC experiments, the constitutions of the compounds were determined, and then, the relative configurations were elucidated with the aid of the NOESY spectra. As a result of the NMR studies, complete ^{1}H and ^{13}C assignments were made for the characterization of the compounds.

Structurally, 10 of the compounds are diterpenes, among them, 5 are ingol-, and 5 are ingenol-type, 9 are pregnanes, 3 are lignans and 2 are flavonoids. *Euphorbia* diterpenes occur mainly as esters, while pregnanes in glycosidic form in nature. Ingenol (**1**) is a diterpene alcohol, substituted with four hydroxy groups. Ingol derivatives (**4**–**8**) are substituted with acetyl, benzoyl, and tigloyl groups, while in case of ingenol esters (**9**–**12**) acetyl and angeloyl groups connect to the diterpene skeleton.

Compounds **7**, **8** and **10** are the most highly esterified diterpenoids, with 4 ester groups. Besides ester groups hydroxy, keto and epoxy functions and, in the cases of **5** and **6** methoxy groups are

present in the molecules. Acetyl group(s) (n = 1-3) occur in all isolated diterpene esters. Interestingly, tigloyl group is typical in ingols (**4**, **5**, and **8**), while angeloyl group is presented in all isolated ingenol-type diterpenes (**9**–**12**).

The pregnane glycoside series isolated from *E. gossypina* var. *coccinea* is homogeneous. The compounds are substituted with thevetose, cymarose, digitoxose, and glucose-containing linear sugar chains and benzoyl (**13**–**18**) or acetyl (**19**) groups. The only isolated compound which is not esterified is compound **20**. Sugar chains are three- or four-membered and join to the aglycone through 3-OH group. Cymarose or digitoxose connect to the aglycone (sugar 1), while thevetose (in case of 3-membered chain, sugar 3) or glucose (in case of 4-membered chain, sugar 4) can be found at the end of the chain. Compounds **13** and **16**, **14** and **17**, and **15** and **18** are pairs differing only in the chain-end sugar glucose.

Among the two new isolated lignans (**21** and **22**), compound **21** belongs to the rare 7,7-diarylbutanol *seco*-lignans, while compound **22** is a tetrahydrofuran lignan derivative. Both compounds have the same substitution on aryl rings, containing four methoxy and two hydroxy groups in the molecules. 7,7-diarylbutanol *seco*-lignans were isolated previously only from a few plant species, e.g., *Schisandra propinqua* (Schisandra lignan),[89] *S. lancifolia* (schilancifolignan D),[177] *S. wilsoniana* (marphenol G),[178] *S. henryi* (henricine B),[179] and from *Kadsura angustifolia* (kadangustins J and K).[88]

6.1.3. Chemotaxonomical significance

Diterpenes are considered to be important taxonomic markers of the Euphorbiaceae family, because of their limited occurrence and structural diversity.

On the basis of the diterpene composition, *E. trigona* displays a close relationship with *E. hermentiana*, *E. antiquorum*, *E. royleana, E. canariensis*, *E. candelabrum* and *E. kamerunica.* According to the classification of Pax & Hoffmann, *E. antiquorum* and *E. royleana* belong to the Section Euphorbium Bentham, Subsection Diacanthium Boiss., Series Trigonae Berger (V), while *E. canariensis*, *E. candelabrum* and *E. kamerunica* belong to the Series Polygonae Berger (VI) of the same Section and Subsection.[180] Therefore, botanically they are very close to each other, thus it is not surprising that they contain structurally similar diterpenoids as it was proven by Evans and Kinghorn.[42] Moreover, all species are succulent and originated from Africa. These plants contain similar diterpenes, differing only in the esterification.

The chemical constituents of *E. gossypina* var. *coccinea* have not been investigated previously. This was the second time, that pregnane glycosides were isolated from Euphorbiaceae species.

6.2. BIOACTIVITY OF THE ISOLATED COMPOUNDS

6.2.1. Bioactivity of compounds from *Euphorbia matabelensis*

All compounds (**1**–**3**) isolated from *E. matabelensis* were investigated for their antiproliferative and GIRK channel inhibitory activities. The antiproliferative effects were tested against 4 human tumor cell lines (HeLa and C33a [cervix adenocarcinomas], MCF-7 and MDA-MB-231 [breast carcinomas]) using MTT assay with cisplatin as a positive control [IC_{50} values 12.43 µM (HeLa), 3.69 µM (C33a), 5.78 µM (MCF-7), and 19.13 µM (MDA-MB-231)]. Ingenol (**1**), naringenin (**2**) and eriodictyol (**3**) exhibited only lower than 20% growth inhibition activity against the utilized cell lines at 30 µM concentration.

In our study, none of the isolated compounds showed GIRK channel blocking activity.

6.2.2. Bioactivity of compounds from *Euphorbia trigona*

After the discovery of ingenol mebutate as an effective drug for the treatment of actinic keratosis (AK), several semisynthetic derivatives were produced or synthesized with full regiocontrol from ingenol.[120,181] Since our compounds, especially the ingenol-derivatives (**9**–**12**), are structurally very similar to ingenol mebutate (**Figure 19**), we were interested in whether they have any effect on the viability of keratinocytes. The HPV-Ker cell line was treated with the isolated ingol- and ingenol-type diterpenoids in the concentration range of 5×10^{-9}–5×10^{-4} M, and the viability was measured with the xCELLigence System RTCA HT instrument for 72 h, data at 24 and 48 h were used for calculations (**Table 1**).

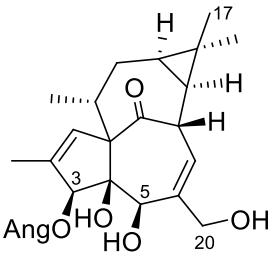


Figure 19. The structure of ingenol mebutate.

Table 1. IC_{50} values (µM ± SD) of the ingol- and ingenane-type diterpenoids (**4**–**12**).

Compound	IC_{50} value (µM)	
	24h	48h
4	14.19 ± 2.85	1.72 ± 0.14
5	17.29 ± 1.65	14.48 ± 3.78
6	inactive	
7+8	4.50 ± 0.93	0.66 ± 0.05
9	0.39 ± 0.09	0.32 ± 0.05
10	0.32 ± 0.02	0.87 ± 0.07
11	4.32 ± 0.92	-
12	14.83 ± 3.83	7.93 ± 1.71
Ingenol mebutate	0.84 ± 0.01	0.96 ± 0.03

Ingenol mebutate, which was used as a positive control, showed IC_{50} values of 0.84 and 0.96 µM at 24 and 48 hours of treatment (**Figure 19**), respectively. The ingenol-type compounds 17-acetoxyingenol 3-angelate 20-acetate (**9**) and 17-acetoxyingenol 3 angelate 5,20-diacetate (**10**) possessed the same order of magnitude as ingenol mebutate (**Figures 20** and **A2**). Moreover, the IC_{50} values of **9** (0.39 µM and 0.32 µM) and **10** (0.32 µM and 0.87 µM) were slightly lower on the HPV-Ker cell line than that of ingenol mebutate. For 17-acetoxy-20-deoxyingenol 5-angelate (**12**), about one and two order of magnitude higher IC_{50} values were recorded after 24 and 48 hours of treatment (14.83 and 7.93 µM) than for ingenol mebutate (**Figure A2**).

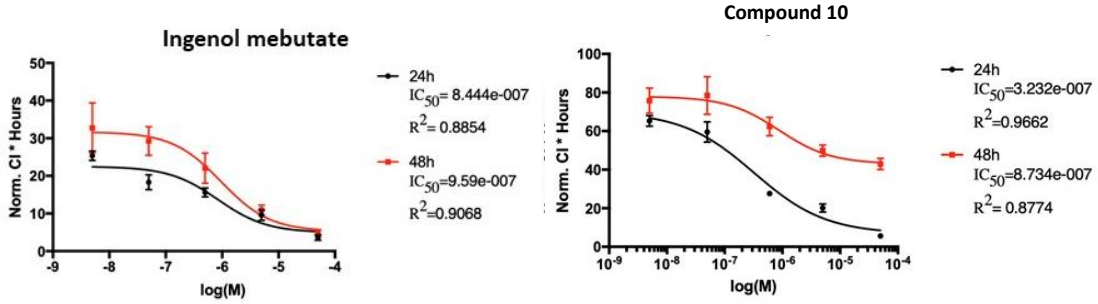


Figure 20. RTCA (real-time cell analysis) measurement of CI (cell index) values of HPV-Ker cells treated with ingenol mebutate and compound **10**. Normalized CI * hours values were plotted as a function of concentration of the indicated diterpenoid (logM).

In case of the ingol-type diterpenoids **4**, **5** and **7**+**8** the measured IC_{50} values were one or two orders of magnitude higher than that of ingenol mebutate, except for **7**+**8** at 48 h (0.66 µM) (**Table 1**).

Based on the pharmacological results in our study, structure–activity relationship (SAR) investigations could also be performed. The ingenol derivatives **9**–**12** are structurally close to ingenol mebutate. The main difference between ingenol mebutate and the isolated compounds is the presence of an acetoxy group at C-17 in these compounds instead of a 17-methyl group in ingenol mebutate. In the less active 17-acetoxy-20-deoxyingenol 5-angelate (**12**), the angeloyl group at C-3, and hydroxy group at C-5, are replaced compared to ingenol mebutate and **9**–**11**; therefore, it was concluded that the presence of the angeloyl group at C-3 seems to be essential for the cytotoxic activity. Since compound **11** differs from ingenol mebutate in only the presence of an acetoxy group at C-17, and its activity was lower, the acetoxy group alone, presumably, is not able to increase the activity. In the case of the most active compounds **9** and **10**, one (at C-20 in **9**) or two (at C-5 and C-20 in **10**) additional acetyl groups are attached to the diterpenoid core; therefore, acetylation of the molecule results in increased cytotoxic activity. It is in accordance with the previously determined SAR statement that the carbonyl moieties of the ester groups are essential for the desired biological effects and the activation of PKC, which likely happens through interaction with Gly23 NH in the C1 domain.[125] Moreover, besides the activation of PKC*δ*, ingenol mebutate was found to reduce the expression of

PKC*α*, which is the PKC isoform responsible for the promotion of cell survival.[182] Thus, further studies are required to evaluate the beneficial effect of our compounds that might result in more effective isoform-specific regulation.

6.2.3. Bioactivity of compounds from *Euphorbia gossypina* var. *coccinea*

As pregnane glycosides and lignans are known to have antiproliferative activities, all isolated compounds were tested for their antiproliferative activity against HeLa cell line using the MTT assay. Doxorubicin and cisplatin were used as positive controls (IC_{50}s 0.02 ± 0.003 μM and 2.07 ± 0.07 μM, respectively). Among the tested compounds, only the pregnane glycoside euphogossypin A (**13**) showed weak antiproliferative activity (IC_{50} 52.4 ± 0.23 μM), while the others proved to be inactive.

From the above results, it could be concluded that ingenane-type diterpenes should be considered therapeutically relevant natural products. In case of other compounds (e.g., pregnane glycosides and lignans) other pharmacological investigations (e.g., antihyperglycaemic or phytoestrogen assays) can be more promising.

7. SUMMARY

The aim of this work was the isolation and structure determination of specialized metabolites from African *Euphorbia* species, *E. matabelensis*, *E. trigona,* and *E. gossypina* var. *coccinea*. First, the contents of the plant materials were investigated by using a CC, TLC-based screening method. The isolation was carried out by a multistep separation procedure, including OCC, VLC, PLC and NP- and RP-HPLC. In some cases, compounds with very similar structures were separated: pregnane glycosides (**13**–**15** and **16**–**18**) are differing only in the connecting deoxy sugars (cymarose or digitoxose) or glucose moieties, while the ingenane diterpenes **11** and **12** differ in the position of the angeloyl group. The structures of the isolated compounds were elucidated by means of spectroscopic methods (HR-MS and NMR). As a result of 1D (^{1}H and JMOD), and 2D NMR studies (COSY, HMBC, HSQC, HMQC), complete ^{1}H and ^{13}C assignments were made for the characterization of the compounds.

As a result of our work, altogether 25 compounds were isolated from the three investigated *Euphorbia* species; three compounds (**1**–**3**), one diterpene (ingenol, **1**) and two flavonoids [naringenin (**2**), and eriodictyol (**3**)] from *E. matabelensis*, nine diterpenes (**4**–**12**), among them five ingols [ingol 3,12-diacetate 7-tigliate (**4**), 8-*O*-methyl-ingol 3,12-diacetate 7-tigliate (**5**), 8-*O*-methyl-ingol 3,12-diacetate 7-benzoate (**6**), ingol 3,7,12-triacetate 8-benzoate (**7**), and ingol 3,7,12-triacetate 8-tigliate (**8**)] and four ingenols [17-acetoxyingenol 3-angelate 20-acetate (**9**), 17-acetoxyingenol 3-angelate 5,20-diacetate (**10**), 17-acetoxy-20-deoxyingenol 3-angelate (**11**), and 17-acetoxy-20-deoxyingenol 5-angelate (**12**)] from *E. trigona*, and eight new pregnane glycosides (euphogossypins A–H, **13**–**20**), two new lignans (gossypilignans A and B, **21** and **22**), one known pregnane (12-*O*-benzoyldeacylmetaplexigenin, **23**), one known lignan (9*α*-hydroxypinoresinol, **24**), and two known flavonoids [naringenin (**2**) and quercitrin (**25**)] from *E. gossypina* var. *coccinea*. 10 Compounds are diterpenes and except ingenol (**1**) they are polyesters, substituted with acetyl, tigloyl, benzoyl and angeloyl groups. Interestingly, ingenols are substituted with tigloyl, while ingenanes with angeloyl groups. Compounds **7**, **8** and **10** are the most highly esterified diterpenoids, with 4 ester groups. The pregnane glycosides (**13**–**20**) are cynanforidine or metaplexigenin derivatives substituted with deoxy sugars, cymarose, digitoxose and thevetose. In case of compounds **16**–**18**, glucose is the terminal sugar. Moreover, all pregnanes but **20** are esterified by benzoyl or acetyl group at C-12. The lignans isolated from *E. gossypina* var. *coccinea* belong to different subgroups, compound **21** is a new 7,7-diarylbutanol *seco*-lignan, compound **22** is a new tetrahydrofuran lignan derivative, while the known **24** is a furofuran-type lignan.

Based on the diterpene composition, *E. trigona* displays a close relationship with *E. hermentiana*, *E. antiquorum*, *E. canariensis*, *E. candelabrum E. royleana,* and *E. kamerunica* as all

species belong to the same Section and Subsection of genus *Euphorbia*, all of them are succulents, and they accumulate similar diterpenes.

All compounds (**1**–**3**) from *E. matabelensis*, and all ingol derivatives (**4**–**8**) and the ingenane diterpenes **9** and **12** from *E. trigona* were detected for the first time from the plants. The chemical constituents of *E. gossypina* var. *coccinea* have not been investigated previously.

The *in vitro* pharmacological activities of the isolated compounds were tested in different test systems. Among them, the ingenol-type 17-acetoxyingenol 3-angelate 20-acetate (**9**) and 17-acetoxyingenol 3 angelate 5,20-diacetate (**10**), differing from ingenol mebutate only in the esterification pattern, showed higher cytotoxic activity on keratinocytes after 24 and 48 h of administration than the positive control ingenol mebutate.

Compounds isolated in our experiments enlarge the natural compound library and verify the botanical relationship of some of the *Euphorbia* species. Our investigations open up new opportunities for natural product-based drug discovery and development; especially ingenol esters **9** and **10** are promising for design new drugs for the treatment of actinic keratosis.

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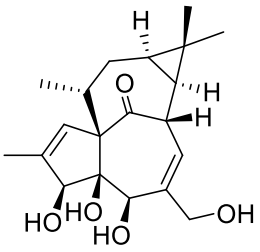
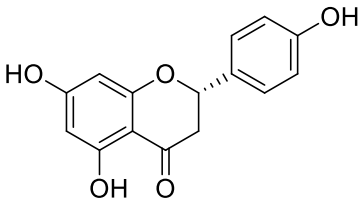
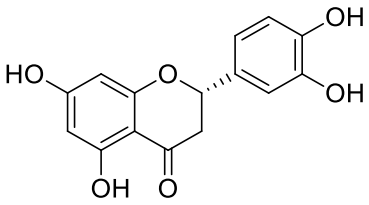
ANNEX



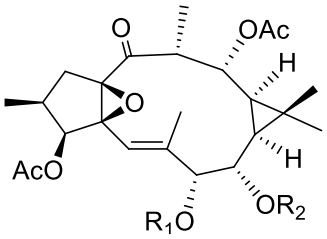
1



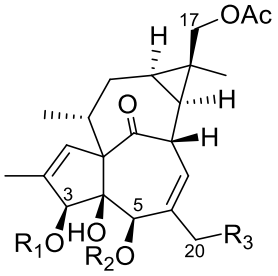
2



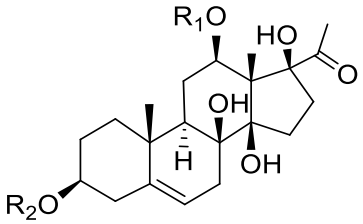
3



Compound	R_1	R_2
4	Tig	H
5	Tig	Me
6	Bz	Me
7	Ac	Bz
8	Ac	Tig

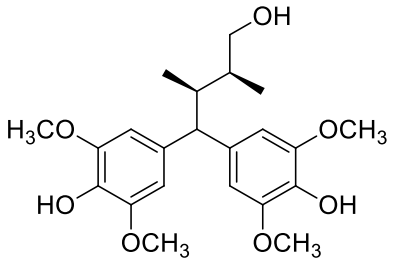


Compound	R_1	R_2	R_3
9	Ang	H	OAc
10	Ang	Ac	OAc
11	Ang	H	H
12	H	Ang	H

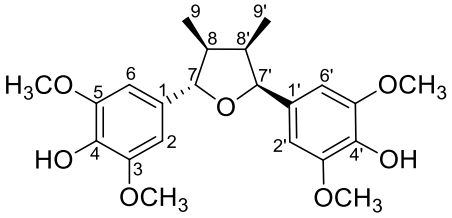


Compound	R_1	R_2 sugar chain			
		Sugar 1	Sugar 2	Sugar 3	Sugar 4
13	Bz	Cym	Cym	Thv	
14	Bz	Dig	Dig	Thv	
15	Bz	Cym	Dig	Thv	
16	Bz	Cym	Cym	Thv	Glc
17	Bz	Dig	Dig	Thv	Glc
18	Bz	Cym	Dig	Thv	Glc
19	Ac	Dig	Dig	Thv	-
20	H	Cym	Dig	Thv	
23	Bz				

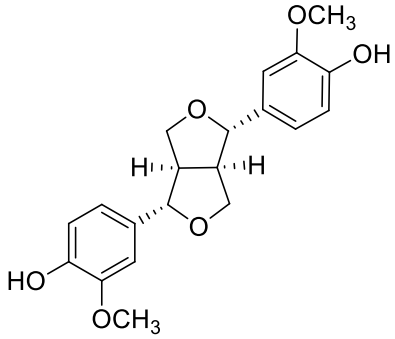
Sugar 3 (**13**-**15**, **19**, **20**) and sugar 4 (**16**-**18**) are in terminal position.



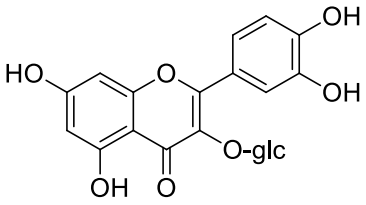
21



22



24



25

Figure A1. Structure of the isolated compounds (**1**–**25**)

Table A1. ^{1}H and ^{13}C NMR data of compounds **13**–**15**.

Atom	13 ($CDCl_3$)		14 (methanol-d_4)		15 (pyridine-d_5)	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	1.13, m, *α*/1.89, m, *β*	38.9, CH_2	1.14, m, *α*/1.83 m, *β*	39.8, CH_2	1.14, m, *α*/1.83, m, *β*	39.4, CH_2
2	1.62, m, *β*/1.92, m, *α*	29.1, CH_2	1.58, m, *β*/1.87 m, *α*	30.2, CH_2	1.82, m, *β*/2.11, m, *α*	30.3, CH_2
3	3.57, m	78.0, CH	3.54, m	79.3, CH	3.89, m	78.1, CH
4	2.29, m, *β*/2.40, m, *α*	38.9, CH_2	2.22, m; 2.38 dd (3.4, 12.7)	39.8, CH_2	2.43, m,* *β* /2.55, m, *α*	39.7, CH_2
5	-	140.8, C	-	140.3, C	-	139.9, C
6	5.38, br s	117.8, CH	5.36, br d (4.7)	119.7, CH	5.31, br s	119.6, CH
7	2.22, m	34.4, CH_2	2.12–2.22 m	35.2, CH_2	2.38, m/2.50, m	35.2, CH_2
8	-	74.4, C	-	75.2, C	-	74.8, C
9	1.59, m	43.8, CH	1.59 m	45.1, CH	1.80, m	44.9, CH
10	-	37.3, C	-	38.2, C	-	37.9, C
11	1.94, m	24.3, CH_2	1.81, m, *α*/2.00, m, *β*	25.5, CH_2	2.21, m, *α*/2.36, m, *β*	25.5, CH_2
12	4.83,dd (4.2, 11.9)	73.3, CH	4.83 dd (4.3, 11.9)	74.7, CH	5.36, dd (4.3, 11.5)	77.5, CH
13	-	58.5, C	-	59.1, C	-	58.8, C
14	-	88.1, C	-	90.0, C	-	90.0, C
15	2.03, m	33.4, CH_2	1.92, m; 2.06,* m	34.3, CH_2	2.14–2.21, m	34.3, CH_2
16	1.92, m, *β*/2.85, m, *α*	32.1, CH_2	1.73, m, *β*/2.87, m, *α*	33.5, CH_2	2.07, m/3.27, m	33.7, CH_2
17	-	91.6, C	-	93.1, C	-	93.0, C
18	1.54, s	9.7, CH_3	1.67, s	10.6, CH_3	2.10, s	11.3, CH_3
19	1.12, s	18.7, CH_3	1.16, s	18.6, CH_3	1.34, s	18.6, CH_3
20	-	209.5, C	-	212.2, C	-	210.6, C
21	2.06, s	27.5, CH_3	2.05, s	27.8, CH_3	2.37, s	28.2, CH_3
Bz						
1′	-	165.5, C	-	166.7, C	-	165.8, C
2′	-	130.1, C	-	131.6, C	-	131.8, C
3′,7′	7.93, dd (1.1, 8.2)	129.7, CH	7.95, d (7.9)	130.5, CH	8.31, d (7.8)	130.4, CH
4′,6′	7.43, t (8.0)	128.6, CH	7.48, dd (7.9, 7.4)	129.6, CH	7.49, t (7.7)	129.4, CH
5′	7.55, t (8.1)	133.3, CH	7.61, t (7.4)	134.3, CH	7.59, t (7.7)	133.7, CH
	Cym I		**Cym**		**Dig I**	
1	4.85, dd (2.0, 9.0)	96.2, CH	4.87, dd (1.9, 8.9)	97.2, CH	5.48, d (9.4)	96.9, CH
2	1.58, m, *a*/2.08, m, *e*	35.7, CH_2	1.54, m, *a*/2.06, m,* *e*	36.7, CH_2	2.05, m; 2.43, m*	39.5, CH_2
3	3.80, m	77.2,* CH	3.85, m	78.6, CH	4.65, m	68.0, CH
4	3.21, dd (3.0, 9.6)	82.7, CH	3.24, m*	83.9, CH	3.53, m	83.9, CH
5	3.84, m	68.7, CH	3.81, m	70.0, CH	4.31, m	69.1, CH
6	1.22, d (6.3)	18.3, CH_3	1.20, d (6.2)	18.5, CH_3	1.45, d (6.0)	19.1, CH_3
3-OMe	3.42, s*	58.1,# CH_3	3.44, s	58.5, CH_3		
	Cym II		**Dig**		**Dig II**	
1	4.76, dd (1.8, 9.5)	99.7, CH	4.89, dd (1.7, 9.1)	101.0, CH	5.41, d (9.6)	100.3, CH
2	1.65, m, *a*/2.16, m, *e*	35.3, CH_2	1.71, m, *a*/2.02, m, *e*	38.8, CH_2	2.00, m, *a*/ 2.43, m,* *e*	39.4, CH_2
3	3.78, m	77.1,* CH	4.21, m	68.6, CH	4.70, m	68.3, CH
4	3.26, dd (2.9, 9.6)	82.8, CH	3.24,* m	83.8, CH	3.60, m	84.1, CH
5	3.90, m	68.4, CH	3.87, m	69.5, CH	4.37, m	69.5, CH
6	1.27, d (6.2)	18.6, CH_3	1.31, d (6.2)	18.6, CH_3	1.59, d (6.1)	19.0, CH_3
3-OMe	3.44, s*	58.2,# CH_3				
	Thv		**Thv**		**Thv**	
	4.30, d (7.8)	104.5, CH	4.35, d (7.9)	105.5, CH	4.82, d (7.9)	106.3, CH
	3.51, m	74.8, CH	3.28, m	75.2, CH	3.85, m	75.3, CH
	3.10, t (9.0)	85.4, CH	3.01, m	87.5, CH	3.61, m	88.3, CH
	3.18, t (9.2)	74.8, CH	3.04, m	76.5, CH	3.58, m	76.3, CH
	3.36, dd (6.2, 9.2)	71.8, CH	3.31,+ m	73.2, CH	3.73, m	73.2, CH
	1.31, d (6.2)	17.9, CH_3	1.25, d (6.1)	18.1, CH_3	1.53, d (6.0)	18.9, CH_3
3-OMe	3.65, s	60.8, CH_3	3.63, s	61.1, CH_3	3.91, s	61.4, CH_3

Table A2. ^{1}H and ^{13}C NMR data of compounds **16** and **17** in $CDCl_3$.

Atom	16		17	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	1.08, m, *α*/1.87, m, *β*	39.0,* CH_2	1.10, m, *α*/1.86, m, *β*	39.0,* CH_2
2	1.66, m, *β*/1.94, m, *α*	29.2, CH_2	1.64, m, *β*/1.93, m,* *α*	29.0, CH_2
3	3.55, m	78.1, CH	3.57, m	78.1, CH
4	2.30, m, *β* /2.38, m, *α*	39.1,* CH_2	2.29, m, *β*/2.41, m, *α*	39.0,* CH_2
5	-	140.8, C	-	141.4, C
6	5.36, br s	117.9, CH	5.35, br s	117.5, CH
7	2.18, m	34.5, CH_2	2.20, m	34.3, CH_2
8	-	74.5, C	-	74.7, C
9	1.46, dd (3.2, 13.1)	44.4, CH	1.52, m	43.9, CH
10	-	37.3, C	-	37.4, C
11	1.60, m, *α*/1.90, m, *β*	28.2, CH_2	1.78, m	24.4, CH_2
12	5.68, m	69.7, CH	4.51,dd (5.8, 10.3)	72.7, CH
13	-	61.1, C	-	57.8, C
14	-	88.0, C	-	88.3, C
15	1.94, m*	34.3, CH_2	1.93, m*	32.8, CH_2
16	1.92, m, *β* /2.75, m, *α*	33.7, CH_2	1.83, m, *β*/2.87, m, *α*	32.4, CH_2
17	-	92.1, C	-	91.9, C
18	1.27, s	7.9, CH_3	1.42, s	9.4, CH_3
19	1.16, s	18.9, CH_3	1.12, s	18.9, CH_3
20	-	213.9, C	-	209.4, C
21	2.34, s	28.4, CH_3	2.24, s	27.4, CH_3
12-OAc				170.0, C
			1.95, s	20.8, CH_3
14-OH	4.12, br s		3.94, s	
17-OH	4.61, br s		4.42, s	
	Cym		**Dig I**	
1	4.85, br d (9.5)*	96.3, CH	4.92, dd (1.7, 9.3)	96.1, CH
2	1.59, m; 2.09, m	35.9, CH_2	1.72, m, *a*/2.08, m, *e*	37.3, CH_2
3	3.81, m	77.3, CH	4.24, m*	66.7, CH
4	3.24, dd (2.9, 9.6)	82.9, CH	3.23, dd (3.0, 9.4)	82.8, CH
5	3.85, m#	68.7, CH	3.79, dq (6.3, 9.4)	68.3, CH
6	1.22, d (6.2)	18.4, CH_3	1.23, d (6.3)	18.4, CH_3
3-OMe	3.45, s	58.2, CH_3		
	Dig		**Dig II**	
1	4.85, br d (9.5)*	99.6, CH	4.91, dd (1.7, 9.3)	98.5, CH
2	1.77, m, *a*/ 2.15, m,* *e*	37.1, CH_2	1.75, m, *a*/2.14, m, *e*	37.0, CH_2
3	4.21, m	66.9, CH	4.23, m*	66.8, CH
4	3.27, dd (3.0, 9.4)	83.4, CH	3.26, dd (2.9, 9.4)	83.2, CH
5	3.86, m#	67.9, CH	3.90, dq (6.2, 9.4)	68.2, CH
6	1.29, d (6.2)	18.5, CH_3	1.29, d (6.2)	18.5, CH_3
3-OMe				
	Thv		**Thv**	
	4.34, d (7.7)	103.5, CH	4.35, d (7.7)	103.5, CH
	3.45, m	74.7, CH	3.47, m	74.7, CH
	3.10, t (9.0)	88.3, CH	3.11, t (9.0)	85.4, CH
	3.19, t (9.0)	74.8, CH	3.19, t (9.0)	74.8, CH
	3.39, dd (6.1, 9.0)	72.2, CH	3.40, m	72.2, CH
	1.31, d (6.1)	17.9, CH_3	1.32, d (6.1)	17.9, CH_3
3-OMe	3.66, s	60.8, CH_3	3.66, s	60.9, CH_3

Table A3. ^{1}H and ^{13}C NMR data of compounds **18**–**20** in CD_3OD.

Atom	18		19		20	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	1.16, m, *α*/1.84, m, *β*	39.8, CH_2	1.16, m, *α*/1.85 m, *β*	39.8, CH_2	1.14, m, *α*/1.85, m, *β*	39.8,* CH_2
2	1.59, m, *β*/1.87, m, *α*	30.2, CH_2	1.61, m,* *β*/1.88 m, *α*	30.1, CH_2	1.60, m,* *β*/1.88, m, *α*	30.2, CH_2
3	3.54, m	79.3, CH	3.56, m	79.3, CH	3.55, m	79.3, CH
4	2.22, m, *β*/2.38, m, *α*	39.8, CH_2	2.23, m; 2.40 dd (3.5, 12.7)	39.8, CH_2	2.23, m, *β* /2.38, m, *α*	39.8,* CH_2
5	-	140.3, C	-	140.3, C	-	140.2, C
6	5.36, br s	119.7, CH	5.37, br d (4.6)	119.6, CH	5.37, br d (4.6)	119.7, CH
7	2.14–2.21, m	35.2, CH_2	2.14–2.22 m	35.2, CH_2	2.13–2.22, m	35.2, CH_2
8	-	75.0, C	-	75.0, C	-	75.0, C
9	1.60, m	45.1, CH	1.61 m*	45.1, CH	1.61, m*	45.1, CH
10	-	38.2, C	-	38.2, C	-	38.2, C
11	1.82, m, *α*/2.01, m, *β*	25.4 CH_2	1.83, m, *α*/2.02, m, *β*	25.4, CH_2	1.83, m, *α*/2.02, m, *β*	25.5, CH_2
12	4.82,dd (overlaps)	73.3, CH	4.82 dd (4.3, 11.9)	74.7, CH	4.83, dd+	74.7, CH
13	-	59.1, C	-	59.1, C	-	59.1, C
14	-	90.0, C	-	90.0, C	-	90.0, C
15	1.93, m; 2.07, m	34.3, CH_2	1.94, m; 2.08, m	34.3, CH_2	1.94, m; 2.07, m	34.3, CH_2
16	1.74, m, *β*/2.87, m, *α*	33.5, CH_2	1.75, m, *β*/2.87, m, *α*	33.5, CH_2	1.75, m, *β*//2.87, m, *α*	33.5, CH_2
17	-	93.1, C	-	93.1, C	-	93.1, C
18	1.67, s	10.6, CH_3	1.67, s	10.6, CH_3	1.67, s	10.6, CH_3
19	1.16, s	18.6, CH_3	1.16, s	18.6, CH_3	1.16, s	18.6, CH_3
20	-	212.2, C	-	212.2, C	-	212.3, C
21	2.05, s	27.8, CH_3	2.06, s	27.8, CH_3	2.06, s	27.8, CH_3
Bz						
1′	-	166.7, C	-	166.7, C	-	166.7, C
2′	-	131.5, C	-	131.5, C	-	131.5, C
3′,7′	7.95, d (8.2)	130.5, CH	7.95, d (7.9)	130.5, CH	7.95, d (7.9)	130.5, CH
4′,6′	7.48, t (7.9)	129.6, CH	7.48, t (7.9)	129.5, CH	7.48, t (7.8)	129.6, CH
5′	7.60, t (7.9)	134.3, CH	7.61, t (7.8)	134.3, CH	7.60, t (7.5)	134.3, CH
	Cym		**Dig I**		**Cym I**	
1	4.87, dd (1.9, 8.9)	97.2, CH	4.96, dd (1.7, 9.7)	97.0, CH	4.87, dd (1.6, 9.6)	97.2, CH
2	1.54, m, *a*/2.06, m, *e*	36.7, CH_2	1.68, m, *a*/1.96, m, *e*	38.9, CH_2	1.55, m, *a*/2.07, m,* *e*	36.6, CH_2
3	3.85, m	78.6, CH	4.24, m*	68.4, CH	3.85, m#	78.6, CH
4	3.26, m*	83.8,* CH	3.23, m*	83.7, CH	3.24, m*	83.9, CH
5	3.82, m	70.0, CH	3.81, m	69.5, CH	3.81, m	70.0, CH
6	1.20, d (6.1)	18.5, CH_3	1.21, d (6.2)	18.5,* CH_3	1.19, d (6.3)	18.5,# CH_3
3-OMe	3.44, s	58.5, CH_3			3.43, s#	58.4, CH_3
	Dig		**Dig II**		**Cym II**	
1	4.89, dd (1.7, 9.1)	101.0, CH	4.93, dd (1.6, 9.7)	100.4, CH	4.80, m+	101.1, CH
2	1.71, m, *a*/2.02, m, *e*	38.8, CH_2	1.76, m, *a*/2.03, m, *e*	38.7, CH_2	1,59, m, *a*/ 2.14, m,* *e*	36.4, CH_2
3	4.22, m	65.6, CH	4.22, m*	68.5, CH	3.84, m*	78.6, CH
4	3.26, m*	83.9,* CH	3.27, m	83.8, CH	3.28, m	84.1, CH
5	3.88, m	69.5, CH	3.91, m	69.7, CH	3.88, m*	70.1, CH
6	1.31, d (6.2)	18.6, CH_3	1.31, d (6.2)	18.5, CH_3	1.30, d (6.3)	18.7, CH_3
3-OMe					3.44, s#	58.6, CH_3
	Thv		**Thv**		**Thv**	
	4.37, d (7.8)	105.5, CH	4.37, d (7.8)	105.5, CH	4.34, d (7.8)	106.1, CH
	3.33, m	74.8, CH	3.34, m	74.7, CH	3.30, m	75.0, CH
	3.19, m	86.0, CH	3.20, m	86.0, CH	3.19, m*	86.1, CH
	3.37, m	82.8, CH	3.38, m	82.8, CH	3.37, m	82.8, CH
	3.48, m	72.6, CH	3.48, m	72.6, CH	3.47, m	72.5, CH

	1.36, d (6.2)	18.5, CH_3	1.36, d (6.2)	18.6, CH_3	1.37, d (6.1)	18.5,[#] CH_3
3-OMe	3.62, s	61.3, CH_3	3.63, s	61.3, CH_3	3.63, s	61.2, CH_3
	Glc		**Glc**		**Glc**	
1	4.43 d (7.8)	104.3, CH	4.43, d (7.8)	104.3, CH	4.43, d (7.7)	104.3, CH
2	3.18, m	75.7, CH	3.18, m	75.7, CH	3.18, m[*]	75.7, CH
3	3.35, m	78.0, CH	3.35, m	78.0, CH	3.35, m	78.0, CH
4	3.22, m	71.9, CH	3.23, m[*]	71.9, CH	3.23, m[*]	71.9, CH
5	3.26, m[*]	78.4, CH	3.26, m	78.4, CH	3.26, m	78.4, CH
6	3.64, m; 3.87, m	63.2, CH_2	3.64, dd (6.4, 12.0); 3.87, dd (2.0, 12.0)	63.2, CH_2	3.64, dd (6.4, 12.0); 3.87, m	63.2, CH_2

Table A4. ^{1}H and ^{13}C NMR data of compounds **21** and **22** in CD_3OD.

Atom	**21**		**22**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	-	136.2, C	-	137.0, C
2,6	6.69, br s	104.7, CH	6.66, s	106.4, CH
3,5	-	149.3, CH	-	149.1/149.2, C
4	-	134.7, C	-	134.7, C
7	4.64, d (9.3)	87.6, CH	3.52, d (11.8)	57.9, CH
8	2.49, m[*]	48.6, C	2.62, m	37.2, C
9	1.00, d (6.4)	12.1, CH	0.68, d (6.9)	12.1, CH
3,5-OMe	3.86, s	56.7/56.8, CH_3	3.82, s/3.83, s	56.8, CH_3
1′	-	132.5, C	-	137.8, C
2′,6′	6.63, br s	104.3, CH	6.64, s	106.3, CH
3′,5′	-	149.1, C	-	149.1/149.2, C
4′	-	135.4, C	-	134.7, C
7′	5.47, d (4.4)	86.5, CH	3.35, dd (6.6, 10.7) 3.45, dd (8.3, 10.7)	67.3, C
8′	2.48, m[*]	44.6, CH	1.77, m	37.0, CH_3
9′	0.63, d (7.0)	9.8, CH_3	0.76, d (7.0)s	10.0, CH_3
3′,5′-OMe	3.84, s	56.7/56.8, CH_3	3.82, s/3.83, s	56.8, C

* interchangeable signals

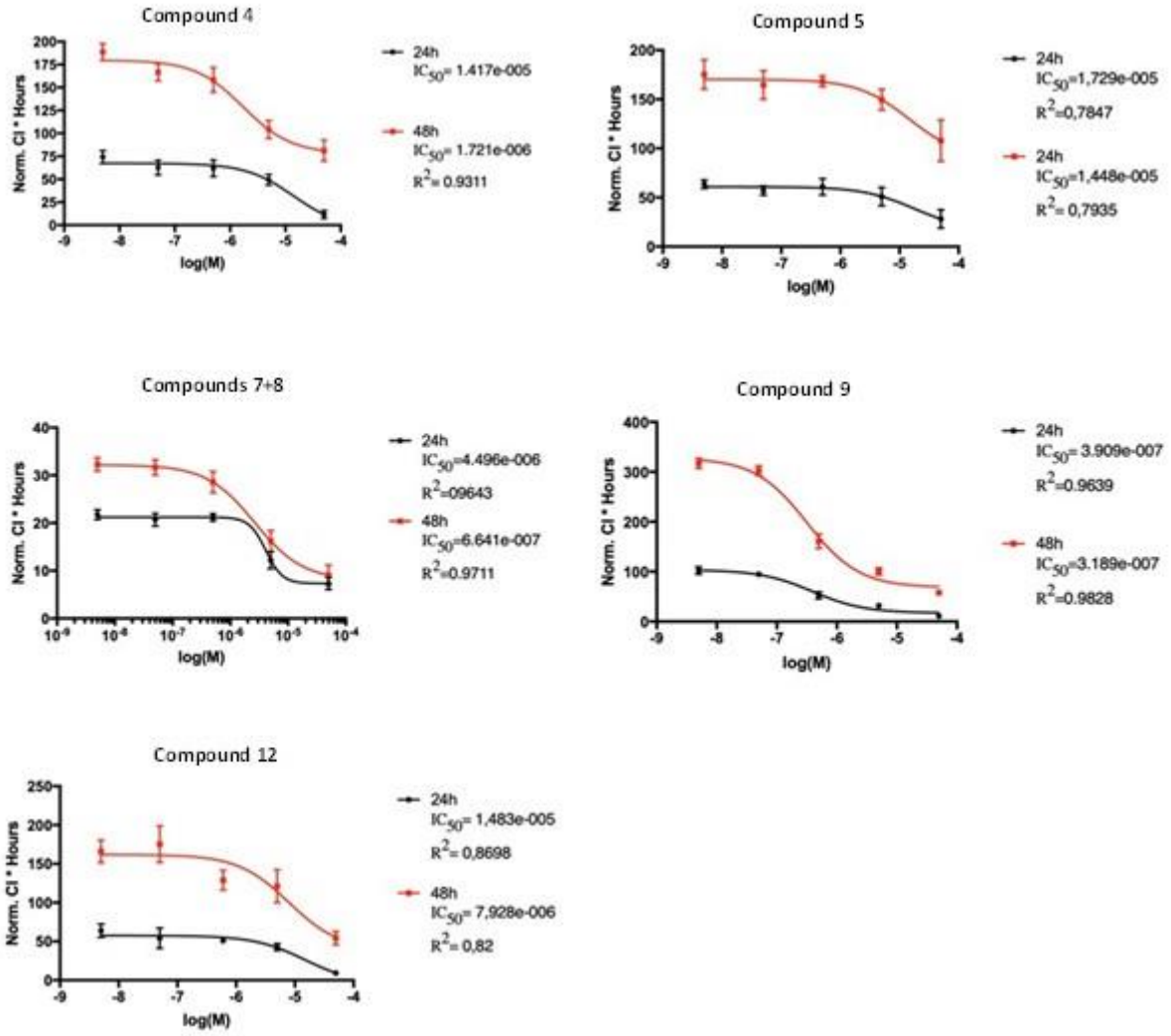


Figure A2. RTCA (real-time cell analysis) measurement of CI (cell index) values of HPV-Ker cells treated with compounds **4**, **5**, **7**+**8**, **9** and **12**. Normalized CI * hours values were plotted as a function of concentration of the indicated diterpenoid (logM).

APPENDIX

The thesis is based on the following publications:

I. **Hammadi R**, Kúsz N, Mwangi PW, Kulmány Á, Zupkó I, Orvos P, Tálosi L, Hohmann J, Vasas A. Isolation and pharmacological investigation of compounds from *Euphorbia matabelensis* *Natural Product Communications* **2019**, *14*, 1–5. DOI: 10.1177/1934578X19863509

II. **Hammadi R**, Kúsz N, Dávid CZ, Behány Z, Papp L, Kemény L, Hohmann J, Lakatos L, Vasas A. Ingol and ingenol-type diterpenes from *Euphorbia trigona* Miller with keratinocyte inhibitory activity
Plants **2021**, *10*, 1206. DOI: 10.3390/plants10061206

III. **Hammadi R**, Kúsz N, Dávid CZ, Mwangi PW, Berkecz R, Szemerédi N, Spengler G, Hohmann J, Vasas A.
Polyoxypregnane ester derivatives and lignans from *Euphorbia gossypina* var. *coccinea* Pax.
Plants **2022**, *11*, 1299. DOI: 10.3390/plants11101299