

RESEARCH ARTICLE

Chemical, molecular and structural studies of *Boswellia* species: β -Boswellic Aldehyde and 3-*epi*-11 β -Dihydroxy BA as precursors in biosynthesis of boswellic acids

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Abstract

The distribution and biosynthesis of boswellic acids (BAs) is scarce in current literature. Present study aims to elucidate the BAs biosynthetic and its diversity in the resins of *Boswellia sacra* and *Boswellia papyrifera*. Results revealed the isolation of new (3 β , 11 β -dihydroxy BA) and recently known (as new source, β -boswellic aldehyde) precursors from *B. sacra* resin along with α -amyrin. Following this, a detailed nomenclature of BAs was elucidated. The quantification and distribution of amyryns (3-*epi*- α -amyrin, β -amyrin and α -amyrin) and BAs in different *Boswellia* resins showed highest amyrin and BAs in *B. sacra* as compared with *B. serrata* and *B. papyrifera*. Distribution of BAs significantly varied in the resin of *B. sacra* collected from dry mountains than coastal trees. In *B. sacra*, high content of α -amyrin was found in the roots but it lacked β -amyrin and BAs. The leaf part showed traces of β -ABA and AKBA but was deficient in amyryns. This was further confirmed by lack of transcript accumulation of amyrin-related biosynthesis gene in leaf part. In contrast, the stem showed presence of all six BAs which are attributed to existence of resin-secretory canals. In conclusion, the boswellic acids are genus-specific chemical constituents for *Boswellia* species albeit the variation of the amounts among different *Boswellia* species and grades.

Introduction

Over the centuries, humanity has known and utilized frankincense since ancient times for religious, social and therapeutic purposes [1]. *Boswellia sacra* Flueck (Burseraceae) is an endemic tree to Oman and an economically important species of genus *Boswellia* [2, 3]. *B. sacra* is a good source of high quality frankincense and bioactive compounds having a wide-range of vital biological activities. The frankincense, a yellowish-brown oleo-gum resin and its essential oil have been well-known for their ameliorative effects against human ailments

such as analgesic [4], hepato-protective [5], antioxidant [6], Alzheimer's [7], diuretic [8], anti-coagulant [9], tumor suppressive [10], anti-inflammatory [11], cardio-protective [12], gastric, hepatic, and skin disorders [13–16]. In addition, the frankincense from *Boswellia sacra* is highly demanded for various commercial products such as cosmetic ingredients (soaps, lotions, and ointment formulation), food flavors, and perfumes [17]. Frankincense exudes from the bark of the tree after a series of man-made incisions about surface area of 10 cm², at a depth of ~5 mm and 6–8 incisions in a season [18]. The frankincense oozes out from bark in the form of milky substance and gets solidified by exposure to high wind and heat (Fig 1). With the increase in demand for frankincense, the number of wounding sites can be increased, thereby affecting tree growth, physiology and regeneration capacity [19, 20]. Unsustainable wounding can further lead to pathogenic infection and insect attacks causing tree die back. The seasonal yield of frankincense per tree depends on tree size and age, as well as seasonal conditions. For example, each season an estimated 3,000 kg of resin is obtained from approximately 500,000 trees [21]. Dhofar province in Oman is the location of the highest quality of frankincense and has been involved in trading of frankincense since ancient times. It has been reported that ca. 3,000 tons were shipped to the Mediterranean countries [22, 23]. Therefore, *B. sacra* tree is considered one of the economic resources of Oman [24].

The frankincense resin is composed of essential oil (5–15%), mucus-like cluster (12–23%) and a lipophilic part (55–66%), however, this varies across different species and different grades [25]. The lipophilic part comprises of rich terpenoids among which are the medicinally important group of boswellic acids (BAs) [26–29]. The chemistry of BAs was commenced through a cluster of compounds isolated by Winterstein and Stein [30]. This was followed by elucidation of structure relationship between the BAs and their amyryns precursors [31, 32]. The configuration of the hydroxyl group at C-3 and the carboxylic group of BAs [33]. Using organic synthesis approaches, several studies were performed to assess the structure of BAs analogs [34, 35]. Furthermore, the chemistry and mechanism of inhibition [36–39] of BAs have helped to understand the composition and nature of this resin [40–42]. Numerous studies on the chemistry and biology of frankincense were carried out [7, 43–47]. In a recent study, we proposed robust method for quantification of BAs from *B. sacra* resin [44, 45]. However, the biosynthesis of triterpenes via the enzymatically driven cyclization of oxidosqualene [48] has not been fully elucidated and least has been known till now. The *B. sacra* tree and its physiological mechanism behind the production of resin and its essential constituents of resin have not been fully understood. In the current study, it was aimed to isolate and characterize pentacyclic triterpenes that support and further elucidate the biosynthetic pathway of BAs and to understand the variations in BAs in different parts of the *B. sacra* tree. In addition, molecular and anatomical analyses were used to further understand the resin production by *B. sacra*.

Materials and methods

Plant and resin collection

The ole gum resins and different parts of *B. sacra* were collected from the various locations in Oman (Dowkah valley- N19°07.76' E054°25.43; Adonab—N17°20.47' E054°04.51). The fresh plant samples were transported to lab in liquid nitrogen, whereas, the resins were brought to lab in air tight zip-bags. The resin and collected plant samples were identified by taxonomist, Saif Al-Hathmi, Royal Botanical Garden, Oman. A voucher specimen (No: BSHR-01/2012) was deposited with the Herbarium of the Chair of Oman's Medicinal Plants and Marine Natural Products, University of Nizwa, Nizwa, Oman.

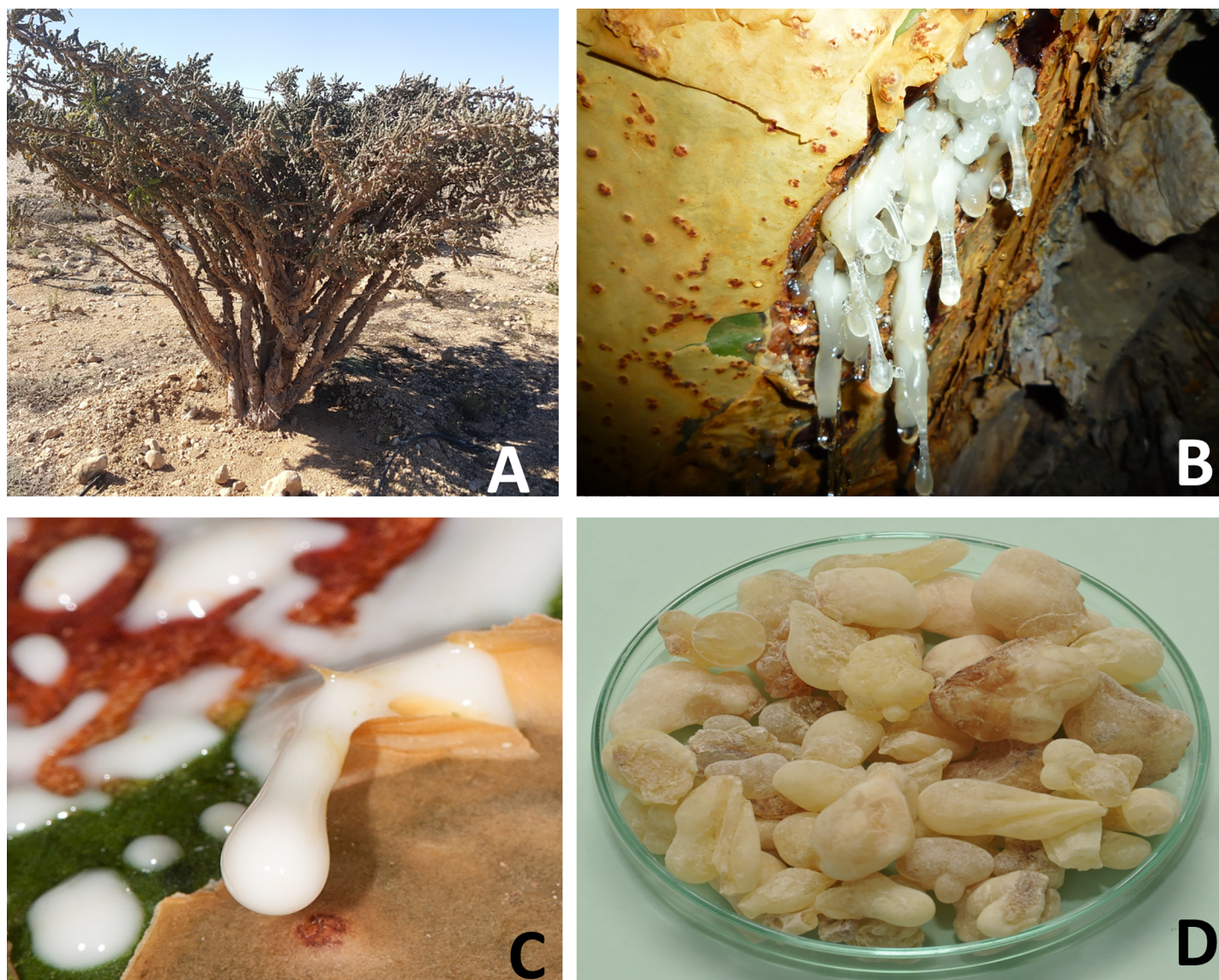


Fig 1. Resin extraction from *B. sacra* tree. a) *B. sacra* tree, Dhofar, Oman, b) milky substance is oozing out after the first incision, c) frankincense after a few days of incision, d) the final product of frankincense. All photos are from the authors' lab.

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Extraction and purification of precursors from *Boswellia sacra*

The air-dried powdered resin (1.5 kg) of *B. sacra* was extracted with methanol (MeOH; 3 L) at room temperature (27 °C; 3 × 15 days) and evaporated under reduced pressure to yield a crude MeOH extract (1.3 kg). The crude methanol extract was subjected to silica gel column chromatography (SiO₂; 500 g; 70–230 mesh; Merck) with gradient increasing polarity of *n*-hexane/ethyl acetate (EtOAc) and finally washed with pure EtOAc to afford twelve fractions (BS₁-BS₁₂). Fraction four (BS₄), 20% *n*-hexane/EtOAc, was further loaded on a column chromatography using gradient mobile phase of 10, 20, 30 and 50% EtOAc/*n*-hexane to get four sub fractions (BSS₁-BSS₅). After taking TLC, sub-fractions two (BSS₂) and three (BSS₃) were combined, eluted with 20 and 30% EtOAc/*n*-hexane, and loaded over column chromatography using isocratic mobile phase of 30% EtOAc/*n*-hexane to afford compound **10** and 40% EtOAc/

n-hexane attributed compound **19**. Both compounds were found to be new after studying detail spectroscopic techniques.

Characterization of purified compounds

Compound 1. Colorless solid; $[\alpha]_D^{25} = +83.3$ (MeOH, $c = 0.024$); **mp:** 184–186°C; **IR** (solid) $\nu_{max} = 3440$ (OH), 2940, 2870, 1730 (CHO), 1450, 1370, 1225, 1160, 1025, 970, and 880 cm^{-1} ; **HR ESI-MS** = 441.3722 [M + H; C₃₀H₄₈O₂]; **¹H NMR** (CDCl₃, 600 MHz) = δ 9.71 (1H, s, H-24), 5.12 (1H, t = 3.0 Hz, H-11), 4.11 (1H, br. s, H-3), 1.10 (3H, s, H-23), 1.08 (3H, s, H-28), 1.02 (3H, s, H-26), 0.90 (3H, d = 6.0 Hz, H-30), 0.82 (3H, s, H-25), 0.78 (3H, s, H-28), 0.77 (3H, d = 6.0 Hz, H-29); **¹³C-NMR** (CDCl₃, 125 MHz): δ 204.7 (C-24), 139.6 (C-13), 124.3 (C-12), 69.2 (C-3), 59.1 (C-18), 52.2 (C-4), 49.2 (C-5), 46.3 (C-9), 42.3 (C-8), 41.5 (C-22), 40.0 (C-14), 39.6 (C-19), 39.5 (C-20), 37.2 (C-10), 33.7 (C-17), 33.2 (C-1), 33.1 (C-7), 31.2 (C-21), 28.7 (C-28), 28.0 (C-15), 26.4 (C-16), 25.8 (C-2), 23.5 (C-11), 23.2 (C-27), 21.3 (C-30), 19.7 (C-23), 17.8 (C-6), 17.4 (C-29), 17.0 (C-26), 14.2 (C-25).

Compound 5. White solid; **UV(MeOH)** $\lambda_{max} = 220$ (3.87); **mp:** 187–189°C; **IR (solid)** $\nu_{max} \text{ cm}^{-1}$: 3420, 2965, 2864, 1702, 1668, 1632, 1467, 1319, 1264, 1228, 970 and 880; $[\alpha]_D^{25} = +34^\circ$ (MeOH, $c = 0.40$); **ESI-MS (rel. int.):** m/z 495.93 [M + H]⁺ (C₃₀H₄₈O₄); 476.97 [(M – H₂O + Na)⁺]; **¹H NMR** (CDCl₃, 600 MHz): δ 5.35 (1H, *d*, 3.0 Hz, H-12), 4.53 (1H, *dd*, 10.2, 3.0 Hz, H-11), 3.24 (1H, *dd*, 11.4, 4.8 Hz, H-3), 2.31 (1H, *m*, H-1), 2.07 (1H, *dd*, 16.0, 2.0 Hz, H-16), 2.01 (1H, *m*, H-2), 1.88 (1H, *m*, H-15), 1.81 (1H, *d*, 10.2, H-9), 1.68 (1H, *dd*, 14.1, 3.0, H-6), 1.55 ((1H, *m*, H-2), 1.54 (1H, *dd*, 11.8, 3.1 Hz, H-5), 1.46 (2H, *m*, H-7/22), 1.42 (1H, *m*, H-21), 1.39 (2H, *d*, 8.9 Hz, H-18/1), 1.37 (1H, *m*, H-21), 1.32 (1H, *m*, H-7), 1.30 (1H, *m*, H-6), 1.28 (2H, *m*, H-22), 1.24 (3H, *s*, H-23), 1.21 (1H, *m*, H-19), 1.18 (1H, *m*, H-15), 1.14 (3H, *s*, H-26), 1.13 (1H, *dd*, 16.0, 2.0 Hz, H-16), 1.05 (3H, *s*, H-27), 1.03 (3H, *s*, H-25), 0.98 (3H, *d*, 6.6 Hz, H-30), 0.91 (1H, *m*, H-20), 0.88 (3H, *d*, 6.5 Hz, H-29), 0.78 (3H, *s*, H-28); **¹³C-NMR** (CDCl₃, 125 MHz): δ 177.5 (C-24), 146.3 (C-13), 124.5 (C-12), 81.8 (C-11), 78.8 (C-3), 58.4 (C-18), 55.7 (C-5), 49.0 (C-9), 43.1 (C-4), 42.0 (C-14), 41.3 (C-22), 40.0 (C-8), 39.4 (C-19), 39.3 (C-20), 37.8 (C-10), 35.5 (C-1), 33.6 (C-17), 33.4 (C-7), 31.1 (C-21), 28.1 (C-28), 27.8 (C-15), 27.2 (C-2), 26.7 (C-16), 22.0 (C-23), 21.3 (C-30), 18.2 (C-6), 18.1 (C-27), 16.6 (C-26), 15.6 (C-29), 13.5 (C-25).

HPLC-DAD analysis

The BAs amount was quantified by a parallel liquid chromatography (1260 HPLC, Agilent Technologies; Japan) connected with a reverse phase C18 column (3.9 × 150 mm; Waters; Nova-Pak, USA). The samples were dissolved (10 mg·mL⁻¹) in HPLC-grade methanol, and then properly diluted. The chromatographic separation was carried out at a constant flow rate of 1 mL min⁻¹ with the following conditions: Mobile phase A = 99.9% water with 0.1% acetic acid; Mobile phase B = 99.9% acetonitrile with 0.1% acetic acid; Total running time (stop time) = 30 mins for BAs and 35 mins for amyryns; Washing time (post time) = 1 minute; Injection volume = 20 μL ; Column type = C18, Waters; Column temperature = 40°C; DAD Signal = wave length of 254 for BAs and 210 for amyryns; Band width = 4.0; Reference wave length = 254 nm, Reference bandwidth = 100 nm.

RNA extraction and RT-PCR

RNA was extracted from the leaf parts of the *B. sacra* tree according to the protocol of Chen et al. [49] with some modifications. Briefly, the leaf (1.0 g) was ground to powder in chilled mortar and pestle in liquid nitrogen and was immediately transferred to RNase and DNase

free falcon tubes having extraction buffer (NaCl-0.25 M, Tris-HCl—0.05M, pH 7.5; EDTA -20 mM, sodium dodecyl sulphate-1% and PVP-4% as described by Chan et al. [49]. RNA with good integrity and purity was used to synthesize cDNA through DiaStar™ RT kit (SolGent, Korea) according to the provided manufacturer's standard protocol. The gene expression of α -amyrin synthase (Forward 5' -CTTTGTGGTCCTGCTGGTAA-3'; Reverse 3' -TGGCTTCACATTTGGAAGAG-5') and *Squalene-synthase* (Forward 5' -GAGGCACCCAGATGATCTTT-3'; Reverse 3' -GAGCGAAACTTCTGGAGACC-5') was further evaluated through RT-PCR. A 2x Real-time PCR Kit (BioFACT™ Korea) including 10nM of each gene specific primer with 100 ng of template cDNA in a final volume of 20 μ L reaction mixture was used. The whole reaction was carried out according to manufacturer's standard protocol using Eco™ Real-Time PCR (Illumina™ USA) with a "no template control" (NTC) as a negative control. The expression of each gene was compared with relative expression of Actin as internal control and the experiment was repeated three times.

Microscopic analysis of stem

The bark samples collected from *B. sacra* tree (epidermal and cambium region; 20 mm²) were fixed in Karnovsky solution (Karnovsky = 2.5% gluteraldehyde in sodium cacodylate buffer and Gluteraldehyde) dehydrated in increasing ethanol series [30, 50, 70, 90, and 100% (three times)] and then infiltrated with resin ethanol for polymerization [acrylic resin glycolmethacrylate and 100% ethanol at a ratio of 1:1 and then pure resin]. The blocks were cut on a microtome into 10 μ m size. Similarly, samples for SEM were fixed, dried to the critical point with CO₂ and gold sputtered prior to observation. Details about light microscopy and scanning electron microscopy (SEM) are described by Peckys et al. [50].

Statistical analysis

The quantification of BAs and RT-PCR related analysis were performed in triplicate and represented with standard deviation. The Non-metric multidimensional scaling (NMDS) which analyses the quantities of BAs in different parts of the tree and different kinds of resin, was performed by PAST (v3.01; University of Auckland, New Zealand). One-Way ANOVA analysis was performed using Graph Pad prism (v6.01) to identify the significant samples.

Results and discussion

Isolation and characterization of β , 11 β -dihydroxy boswellic acid

We have isolated 3 β , 11 β -dihydroxy boswellic acid **1** (Fig 2) for the first time from the resin of *B. papyrifera*. It analogue (**15**) was previously isolated for the first time from *B. sacra* [51]. Compound **1** was isolated as white amorphous powder having molecular formula of C₃₀H₄₈O₄ and evidenced by ESI-MS which exhibited molecular ion peaks at 495 [M + Na]⁺ and 477 [M⁺-H₂O + Na] (7° of unsaturation). The IR spectrum of **1** showed characteristic absorptions bands for hydroxyl (3410 cm⁻¹), carboxylic acid (1702 cm⁻¹), and a double bond (1632 cm⁻¹) functional groups. The ¹H NMR spectrum of **1** displayed five tertiary methyls (δ_{H} 1.24, 1.14, 1.05, 1.03, 1.06 and 0.78, each single), two secondary methyls (δ 0.98, d, J = 6.6 Hz and 0.88, d, J = 6.5 Hz) and a trisubstituted olefinic proton (δ 5.35, d, J = 3.0 Hz), which are characteristic of ursane-type triterpenes related to boswellic acids [52, 53]. The ¹³C NMR spectrum (experimental part) of compound **1** displayed 30 distinct peaks accounted for by seven methyls, eight methines, eight methylenes and seven quaternary carbons. The ¹³C-NMR spectrum of **1** (experimental part) also showed the presence of an olefinic group at δ_{C} 124.5 and 146.3 (C-12, C-13), a carboxylic group at δ_{C} 177.5 (C-24), and two hydroxylated carbons at δ_{C} 78.8 and 81.8

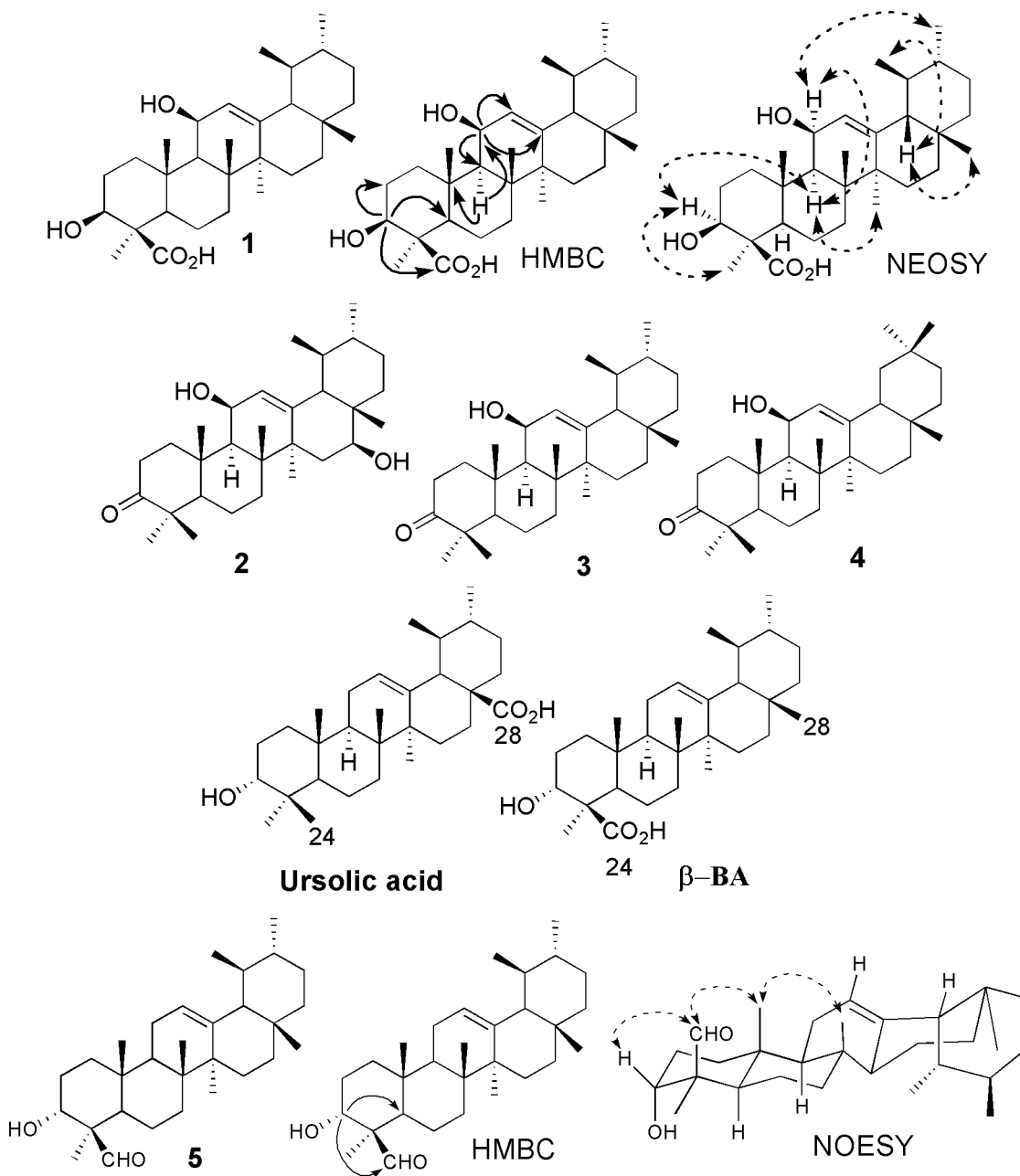


Fig 2. Chemical compounds and their NMR configuration compound 19 and its HMBC, COSY and NOESY correlations; Compounds 20–22 isolated from *Protium kleinii* [54]; Ursolic acid and β -BA; Compound 10 and its HMBC and NOESY correlations.

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(C-3, C-11). All the positions of the substitutions were deduced using the COSY and HMBC 2D NMR techniques (Fig 2). Compound 1 is direct precursor of the 3-*epi*- β -Boswellic acid and its known diastereomeric analogue 3 α , 11 α -dihydroxy boswellic acid 15.

The ^1H NMR spectrum confirmed the presence of hydroxyl group at C-3 and was in β -orientation as evidenced by the doublet of doublet (11.4, 4.8 Hz) of the α -positioned geminal proton which appeared at δ_{H} 3.24, an interpretation and β -orientation further substantiated by HMBC correlation between H-5 (δ_{H} 1.54) and C-3 (δ_{C} 78.8) and NOESY correlation between

H-3 and CH₃-23 position. On the other hand, the singlet peak at δ 5.52 (H-12) correlated with C-11 (δ c 81.8), C-9 (δ c 49.0), C-13 (δ c 146.3) and C-14 (δ c 42.0) in the HMBC spectrum allowed its assignment to olefinic double bond between C-12 and C-13.

Besides olefinic double bond, the ¹H NMR spectrum of **1** displayed a peak at δ _H 4.53 previously observed in boswellic acid derivatives [51]. The three bond long range correlations from this signal to C-9 (δ c 49.0), C-12 (δ c 124.5), and C-13 (δ c 146.3) in the HMBC spectrum allowed its assignment to H-11. This proton was associated with a carbon signal at δ 81.8 in the HSQC spectrum and showed COSY correlation with a signal at δ _H 1.81 (H-9) and 5.52 indicating the location of hydroxyl group at C-11. The configuration of H-11 in **1** was determined to be axial on the basis of coupling constants with H-9 and H-12 and further supported by NOESY correlation between H-11 and CH₃-25 which is in close agreement with the reported compounds **2–4** (Fig 2) [51]. One known analogue **15** has also been isolated from the same species having ¹³C-NMR at δ 70.6, and ¹H-NMR at δ 4.24 (dd, J = 9.6, 3.0 Hz) corresponded to the C-11 position. However, the downfield shift observed in the ¹³C-NMR of compound **1** at δ c 81.8 further strengthens the β -orientation of hydroxyl group at C-11 position [54].

Similarly, the H-3 showed an HMBC cross-peak with a peak at δ c 177.7 which further supported the presence of carboxylic group in ring A at either C-23 or C-24 positions. The characteristic NOESY correlation between CH₃-23 and H-5 allowed the assignment of the functional group at C-24 as previously observed boswellic acids [55]. All the above data can be accommodated only on an urs-12-ene triterpenoid structure for compound **1** with a hydroxyl group at the C-3 β position [56] and another secondary hydroxyl group attached to the C-11 β position [54]. Thus, on the basis of spectroscopic data and also comparison with literature data, structure of compound **1** was deduced as 3 β , 11 β -dihydroxy-12-en-24-oic acid commonly known as 3 β , 11 β -dihydroxy- β -boswellic acid.

It is noteworthy mentioning that the sole source of boswellic acids is *Boswellia* species where the oxidation takes place at C-24 by Cytochrome P450 enzymes [57] whilst ursolic acid exists abundantly in plant kingdom and the oxidation takes place at C-28. Thus, the boswellic acids can be considered as genus-specific and can be specific biomarkers for *Boswellia* species albeit the variation of the amounts among different *Boswellia* species and grades (Fig 2).

Isolation and characterization of boswellic aldehyde

Compound **5** was obtained as a colorless solid having melting point of 184–186°C and assigned a molecular formula of C₃₀H₄₈O₂ as evidenced by HRESI-MS which showed an [M + H]⁺ ion peak at m/z 441.3722 (calculated: 441.3718). The ESI-MS molecular ion peak suggested seven double bond equivalents, six of which are assigned to the rings of the pentacyclic triterpene skeleton and one was attributable to the formyl group (δ _C 204.6). The IR spectrum of **5** showed characteristic peaks at 3440, 2940, 2870 and 1730 cm⁻¹, which confirmed the presence of OH and CHO groups. An equivalent, assigned to a double bond consistent with Δ^{12} -ursane skeleton, showed resonances at δ c 124.3 (C-12) and 139.6 (C-13).

In the ¹H NMR spectrum (experimental) of compound **5**, the presence of vinylic proton at 5.12 (1H, t, J = 3.0 Hz, H-12), one oxymethine proton at 4.11 (1H, br s, H-3), five tertiary methyls at 1.10, 1.08, 1.02, 0.82, and 0.78 (all singlets), and two secondary methyls at 0.90, (3H, d, J = 6.0 Hz) and 0.77 (3H, d, J = 6.0 Hz) were observed, unambiguously confirming the presence of Δ^{12} -ursane skeleton [51]. The ¹³C NMR spectrum (experimental part) exhibited a total of 30 carbon signals which were sorted out into nine CH₂, seven CH₃, seven CH including one olefinic, and one oxygen-bearing carbon as well as seven quaternary carbons. Comparison of the ¹H and ¹³C NMR spectra of compound **5** with those of β -boswellic acids [55] displayed

close similarities with notable difference being the substitution mode in ring A. The chemical shift at δ_c 69.2 in the ^{13}C -NMR spectra and HMBC correlations of H-3 with CH_3 -23 (δ_c 19.7) and formylcarbon C-24 (δ_c 204.7) confirmed the presence of hydroxyl group in ring A at C-3 position, while the alpha orientation was further confirmed by NEOSY cross peaks (Fig 2) [58].

The presence of formyl group was assumed to be located either at the C-23, C-24 or at the C-28 position. The key HMBC correlations of the formyl proton H-24 (δ_H 9.71) with C-3 (69.2) and C-4 (52.2); CH_3 -23 (19.7) with C-3 (69.2) and C-24 (204.7) proved that aldehyde group located either at C-23 or C-24 positions. Since the formyl group was assumed to be formed in the reaction by oxidation of the CH_2OH group of **14** ([59]; Fig 3), it might be expected that the -CHO group will have the same location and orientation as the CH_2OH group has in **9**. Therefore, the formyl group in compound **5** located the position at C-24 (β -orientation) which was further confirmed by NEOSY correlations [60]. On the basis of the above results, compound **5** was deduced as 3α -hydroxy-urs-12-ene-24-al commonly named as boswellic acid aldehyde. This compound, up to the best knowledge, is described for the first time from *B. sacra* and recently isolated from *B. serrata* as a first natural product [61].

Biosynthetic pathway of 3-*epi*- α -amyrin and its precursors

The biosynthesis of β -amyrin has been postulated [62] following the first proposal of the isoprene rule [63, 64]. In order to understand the biosynthesis of BA and its consecutive oxidative and acetylated derivatives, the biosynthesis of their precursor β -amyrin is depicted in Fig 4. This historic precursor is commenced at the *chair-chair-chair-boat* conformation of (3S)-2,3-oxidosqualene **A**, which cyclized to the corresponding 6.6.6.5-fused tetracyclic dammarenyl C-20 cation **B** which at this stage led to the formation of the dammaranes isolated from *Boswellia* species [62]. A concerted hydride shifts from C-13 to C-17, methyl group (C-18) shift from C-14 to C-13 and methyl group (C-30) shift from C-8 to C-14, followed by enzymatically catalyzed base abstraction of C-9 proton, generating the double bond between C-8 and C-9, afforded tirucallanes isolated from *Boswellia* species [65]. After ring D enlargement, electrophilic addition on the terminal double bond of the tetracyclic baccharenyl cation **C**, a five-membered ring E is constructed and the Lupanyl cation **D** is generated which leads to the formation of Lupanes isolated from *Boswellia* species [66]. This is followed by ring E enlargement which occurs via C-30 shift from C-20 to C-19 to afford the oleanyl cation **E** which can lead to the formation of Oleananes isolated from *Boswellia* species [67] to which β -amyrin (precursor for α -Boswellic acid) belongs. The oleanyl cation **F** then undergoes sequential 1,2-hydride shifts from C-18 to C-19 and from C-13 to C-18 followed by elimination of H-12 to afford the 6.6.6.6.6-fused pentacyclic Ursanes isolated from *Boswellia* species [68] to which α -amyrin (precursor for β -Boswellic acid) belongs.

Chemical diversity of boswellic acids

The four amyryns are known from different plant species including *Boswellia* [69], some of their corresponding boswellic acids remained unknown due to their existence in infinitesimal amounts in the resins which doesn't allow their isolation and hence characterization. It is imperative to highlight a few conventions adopted in the literature which can be confusing. In general, α -amyryns where C-29 and C-30 are in vicinal relationship at C-19 and C-20 respectively will lead to β -boswellic acids. Moreover, β -amyryns where C-29 and C-30 are in germinal relationship both at C-20 will lead to α -boswellic acids.

C-H activation and oxidation of the α -configured methyl group C-24 of the 3-*epi*- α -amyrin **6**, the 3-*epi*- β -amyrin **8** (Fig 5) afforded the β -boswellic acid **5** and α -boswellic acid **7**

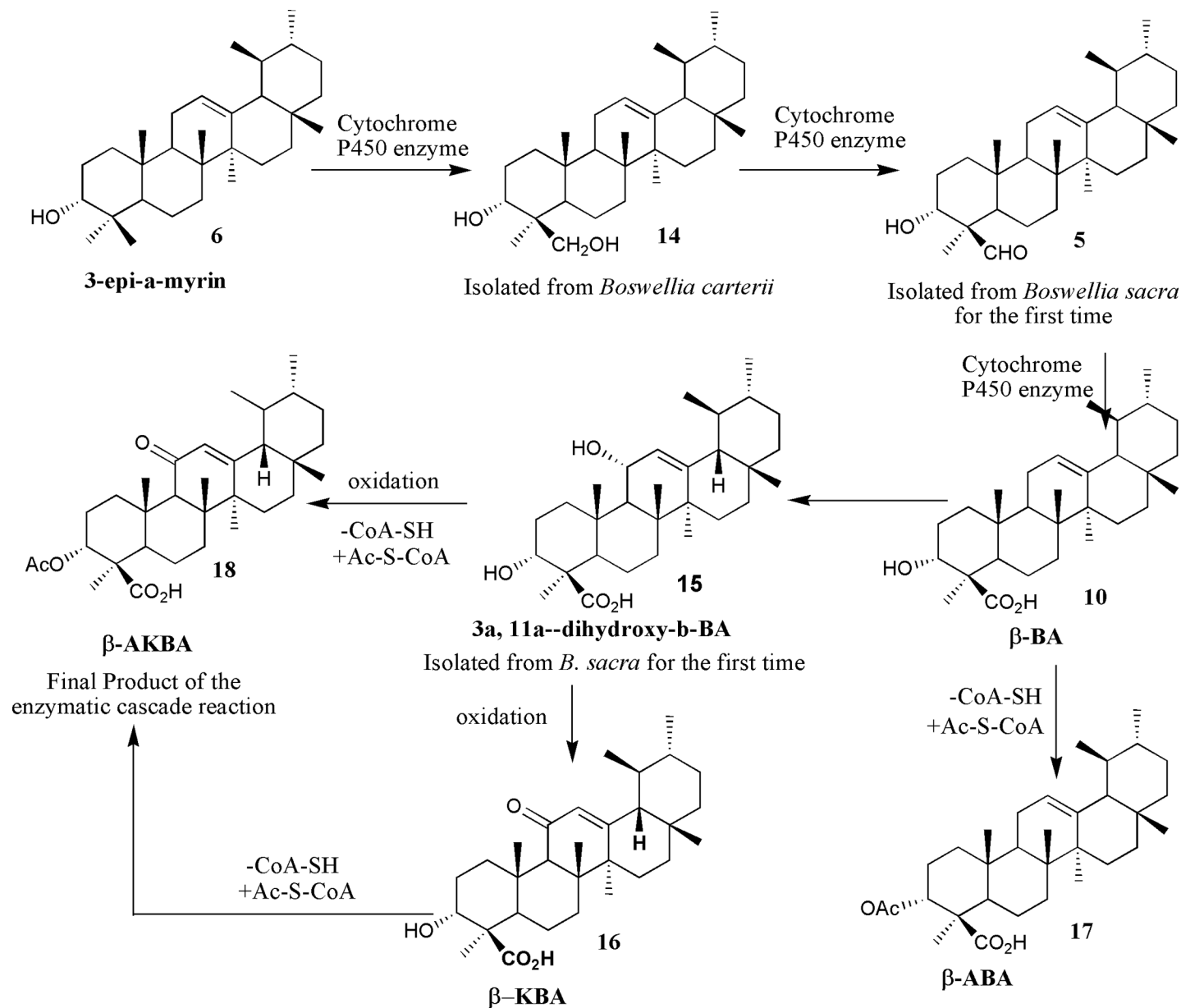


Fig 3. Formation of boswellic acid derivatives.

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respectively which were isolated from different *Boswellia* species. [70, 71] Likewise C-H activation and oxidation of the α -configured methyl group C-24 of the α -amyrin **2** furnished the 3-*epi*- β -boswellic acid **6** (where the hydroxyl group at C-3 and the methyl group at C-4 are both β -configured and the two methyl groups at C-19 and C-20 are in vicinal relationship) which was only reported once from *B. carterii* [59].

Similarly, C-H activation and oxidation of the α -configured methyl group C-24 of the β -amyrin **9** should afford 3-*epi*- α -boswellic acid **8**. However, as far as we are aware there is no report which describes the isolation of the 3-*epi*- α -boswellic acid. We found it vitally important to introduce the prefix “*epi*” to differentiate the configuration of the hydroxyl group at C-3 which has been a long-standing confusion in literature for decades. In this regard, 3 β -amyrin

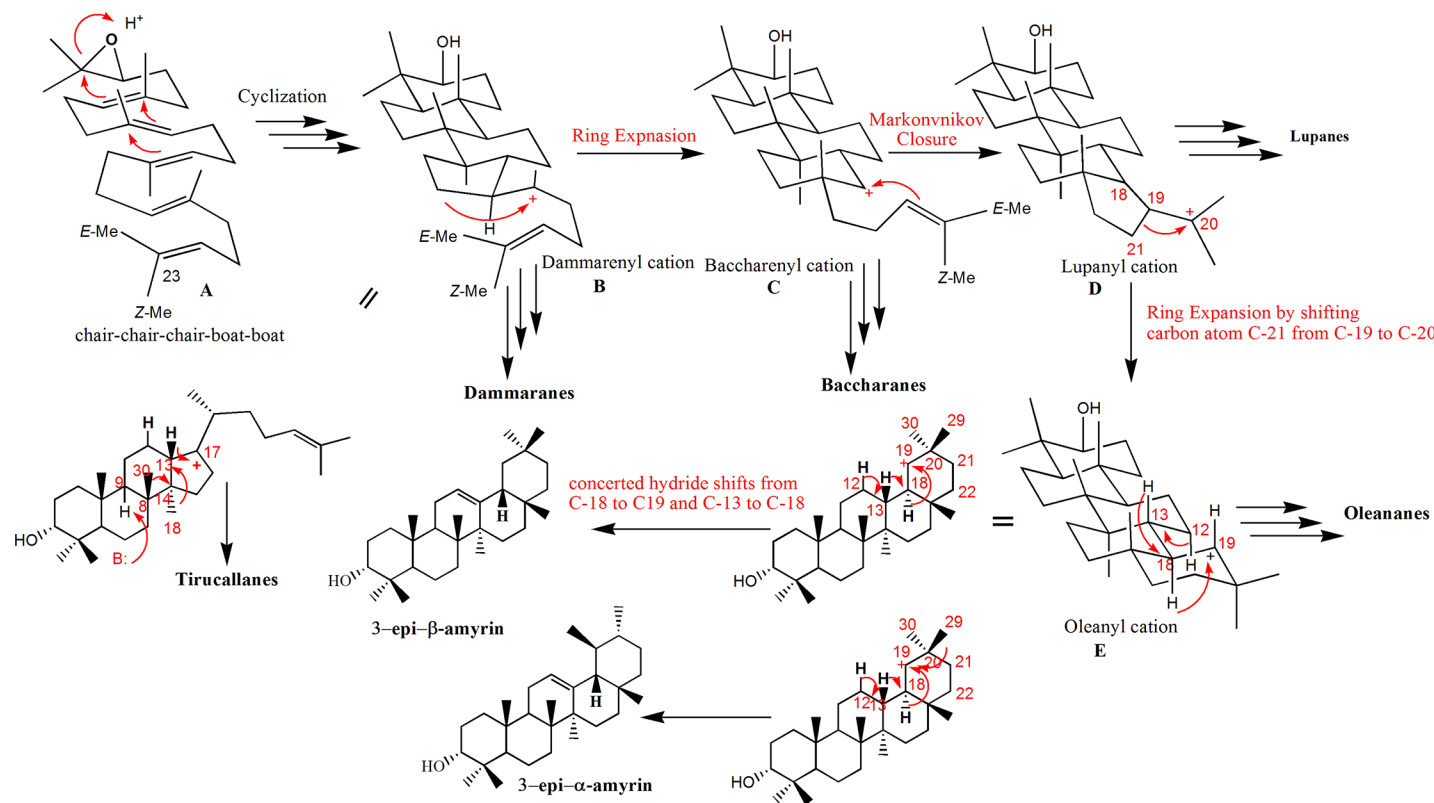


Fig 4. Proposed biosynthetic pathway of 3-*epi*- α -amyrin and its precursors.

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where the β -OH at C-3 is above the plane will lead to 3-*epi*-boswellic acid again where the β -OH at C3 is above the plane. In contrast, 3-*epi*-amyrin where the α -OH at C-3 is under the plane will lead to boswellic acid where the α -OH at C-3 is under the plane (Fig 6).

Biosynthesis of boswellic acids

A series of enzymatic oxidations take place to convert C-24 into carboxylic acid at 3-*epi*- α -amyrin **6** which undergoes a conversion to the corresponding alcohol **14** that is isolated from *B. carteri* [59]. Alcohol **14** is oxidized to the aldehyde **5** that is isolated as a new compound from *Boswellia sacra* with full spectrometric data [61]. The aldehydic group in **5** upon oxidation by Cytochrome P450 enzyme affords the carboxylic acid moiety of β -boswellic acid **10**. The OH group is introduced at C-11 of the β -boswellic acid **10** which led to the formation of diol **15** which has also been isolated from *Boswellia sacra*, as a new source, by our group and its ethoxy analogue was reported earlier from *B. neglecta* [51] which upon oxidation of the OH at C11 should lead to β -KBA **16**. Acetylation of the OH at C-3 in β -boswellic acid **5** via Ac-CoA and an acetyl-transferase will give β -ABA **17**. Oxidation of the OH at C-11 and acetylation of the OH at C-3 of diol **15** afforded the AKBA derivative **18** which appeared to be the final product of this enzymatically-driven cascade reaction. The reaction sequence of the acetylation and oxidation which led to formation of ABA and KBA is not known hitherto and lacks supported evidences. Certainly, comprehensive physiological investigations on the frankincense trees are mandatory to elucidate the exact order of steps for the biosynthesis of AKBA.

The boswellic acid derivative **21** (Fig 7) have resulted from the oxidation of alcohol **20**. Isomer **22** was isolated from *B. sacra* earlier [72]. In a similar analogy to alcohol **14**, alcohol **20** is a

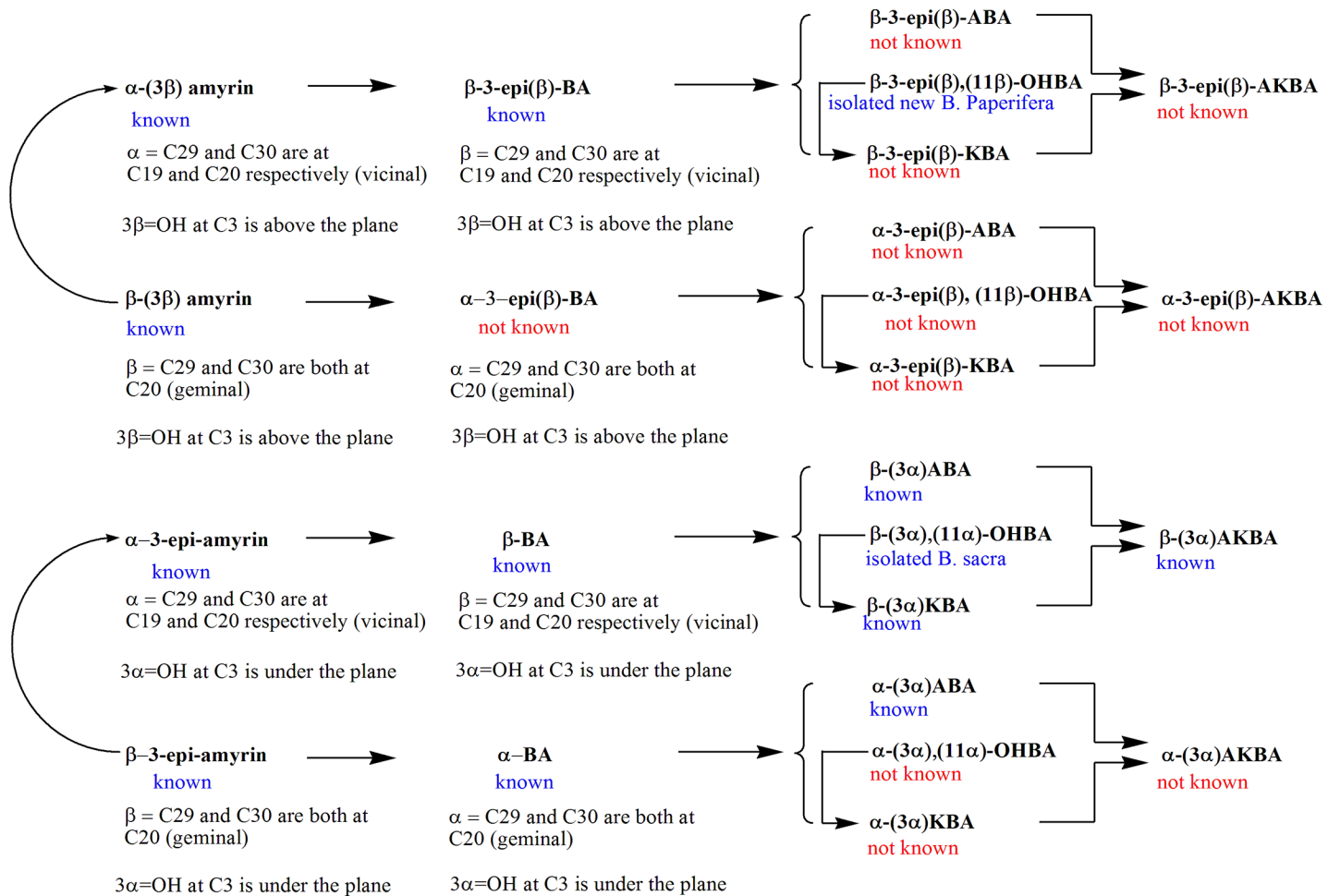


Fig 5. Conversions of α-amyrin and β-amyrin into boswellic acids.

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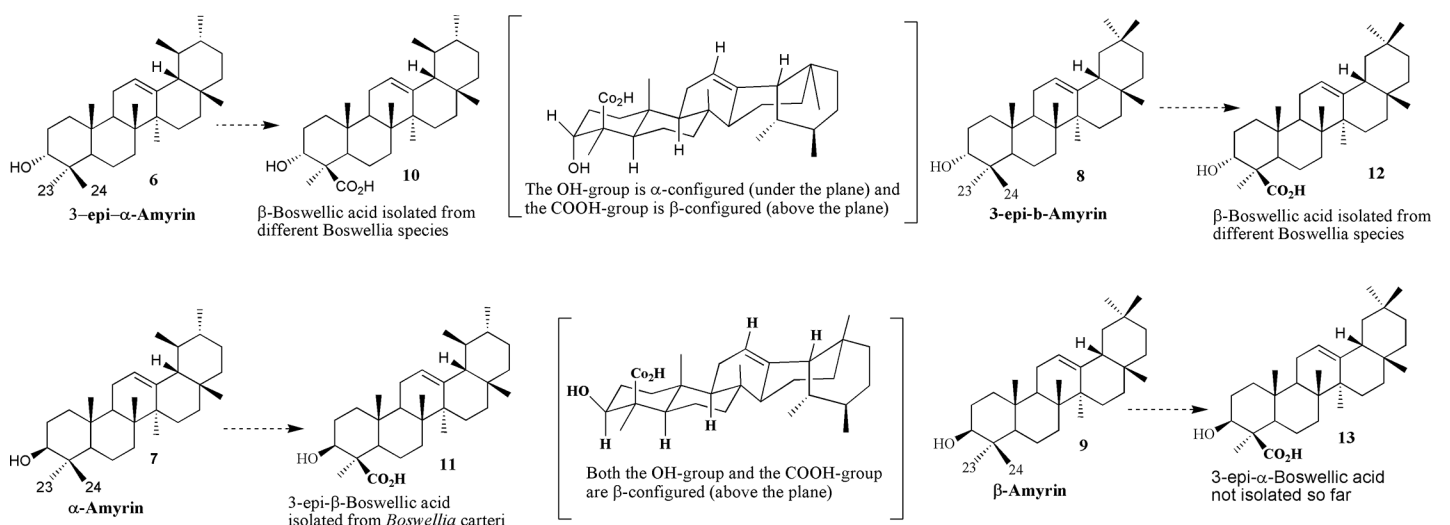
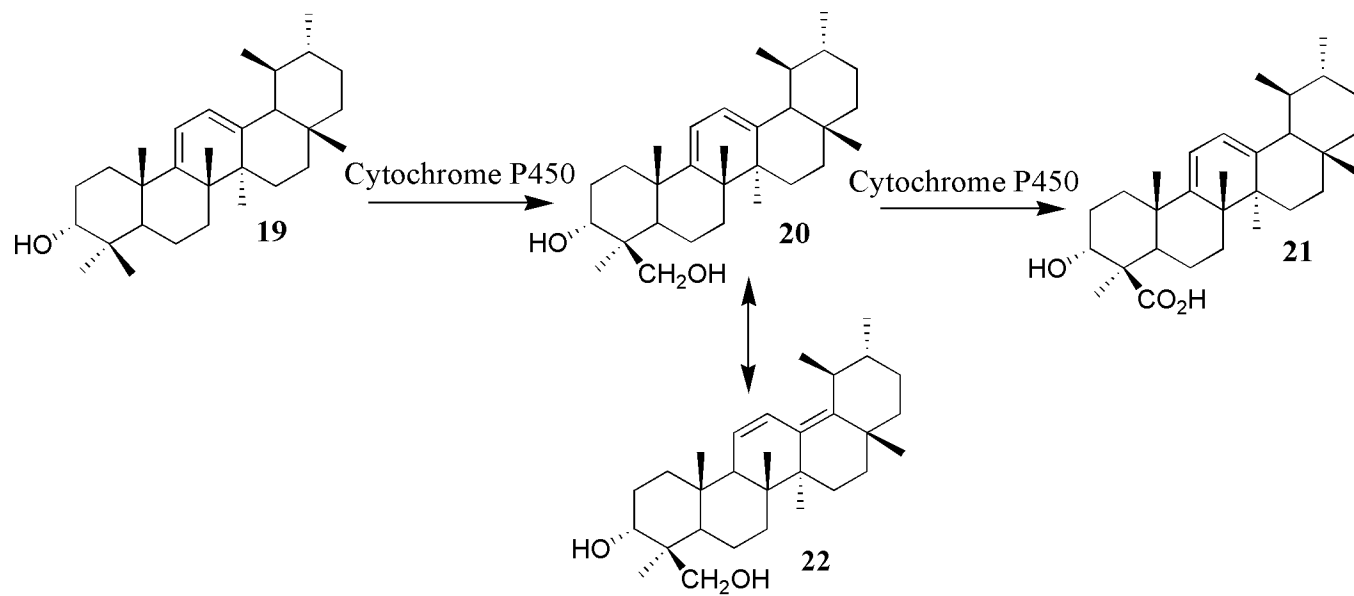


Fig 6. Configuration of amyryns and boswellic acids.

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Olean-11,13(18)-dien-3 b ,24-diol [72]

Fig 7. Formation of pentacyclic isomers.

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result of the oxidation of the amyrin 19. All these four derivatives are isolated by our group from *B. sacra* [72].

Quantification of α -amyrin and BAs in different *Boswellia* tree parts and resins

Given the fact that there are no specific enzymes isolated from *Boswellia* species to elucidate the biosynthetic pathway, we have attempted quantification of boswellic acid derivatives using HPLC. This quantification is performed on the leaf, bark, roots and resin of *B. sacra* tree. Moreover, three resins are analyzed for their amyryns and BA contents namely *B. sacra*, *B. serrata* and *B. papyrifera*. In order to verify the influence of the geographical location of the *Boswellia sacra* tree, the resins of two grades (mountainous and costal) were analyzed for their

Table 1. Quantification of amyryns and BAs in different *Boswellia* tree parts.

Part	<i>Epi</i> - α -amyrin	α -amyrin	β -amyrin	Total Amyryns	β -BA	α -BA	β -ABA	β -KBA	β -AKBA	α -ABA	Total BAs
Bark (Hypodermis) outer	ND	ND	ND	ND	ND	ND	0.02 \pm 0.00	Trace	0.32 \pm 0.01	0.66 \pm 0.02	1.0 \pm 0.01
Bark (Cambium) inner	ND	ND	ND	ND	1.34 \pm 0.08	Trace	0.98 \pm 0.02*	0.14 \pm 0.01	1.37 \pm 0.04*	1.10 \pm 0.1	4.93 \pm 0.37**
Root	-	2.63 \pm 0.02	-	2.63 \pm 0.03	ND	ND	ND	ND	ND	ND	ND
Leaf	-	ND	-	ND	ND	ND	0.28 \pm 0.01	ND	0.28 \pm 0.01	ND	0.56 \pm 0.01

The values with \pm shows the standard error of mean values of three replications. The asterisks (

* $p < 0.05$

** $p < 0.01$) show that the values are significantly different in each column using a One-Way ANOVA analysis in Graph Pad prism (v6.01) using Bonferroni test.

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boswellic acids composition. We realized that the quantities of the triterpenes vary significantly depending on the *Boswellia* species as well as on the environmental factors within the same species (Table 1). While in some species large amounts of a triterpenes exist, only infinitesimal quantities of the same compound can be detected which explains the huge chemical diversity of the triterpenes in *Boswellia* species (Table 1).

B. serrata is distinguished from the other two species by higher amounts of β -KBA (1.29%) whereas *B. papyrifera* and *B. sacra* possess 0.46% and 0.73% respectively. This suggests β -KBA as a biomarker for *B. serrata*. *B. papyrifera* proved a complete absence of β -BA and lower amounts in case of *B. serrata* whilst higher amounts on average of the two grades in *B. sacra*. Therefore, β -BA can be identified as a biomarker for *B. sacra*. Comparable quantities of α -BA in all three species are found which makes it difficult to use α -BA as a biomarker to distinguish these species. The same conclusion can be drawn for α -ABA and β -ABA. The amounts of β -AKBA are comparable in *B. serrata* and *B. papyrifera* as well as in the costal grade of the *B. sacra* whereas higher amounts are observed in the mountainous type of *B. sacra*. The precursor of all β -boswellic acids (β -BA, β -ABA, β -KBA and β -AKBA) presented in Table 1 above is *epi*- α -amyrin which was detected in higher amounts in *B. sacra* species whilst completely absent in *B. serrata* and *B. papyrifera*. The total β -Boswellic acids content is 7.04 and 9.68% in *B. papyrifera* and *B. serrata* respectively (Table 2). Significantly higher amounts of β -boswellic acids are observed for the two *B. sacra* species. It is noteworthy mentioning that the amounts of α -ABA are lower in the case of *B. sacra* when compared with the other two species.

The HPLC results reported herein are in total agreement with our recently published quantification of KBA (keto- β -boswellic acid) and AKBA (3-acetyl-11-keto- β -boswellic acid) using NIRS coupled with PLS regression [73,40]. For the first time, NIR spectroscopy coupled with PLS regression as a rapid and alternative method was developed to quantify the amount of AKBA and KBA in different plant parts of *Boswellia sacra* and the resin exudates of the trunk. The quantification of various plant parts and the resin indicated that the MeOH extract of the resin has the highest concentration of AKBA (7.0%) followed by epidermis (1.37%), and essential oil (0.1%). Thus it can be concluded that AKBA is only present in the gum-resin exudate and epidermis of the stem. The existence of moderate amounts of AKBA in the epidermis is due to the presence of resin-producing canals [40]. A similar study was conducted for KBA and obtain results indicated that the MeOH extract of resin has the highest concentration of KBA (0.6%) followed by essential oil (0.1%). The MeOH extract of the resin was subjected to column chromatography to get various sub-fractions at different polarity of organic solvents.

Table 2. Quantification of α -amyrin and BAs in different kinds of *Boswellia* resins.

Resin source	<i>Epi</i> - α -amyrin	α -amyrin	β -amyrin	Total Amyrin	β -BA	α -BA	β -ABA	β -KBA	β -AKBA	α -ABA	Total BAs
<i>B. papyrifera</i>	Trace	Trace	Trace	Trace	ND	2.36±0.03	0.57±0.04	0.46±0.02	3.65±0.11	1.84±0.01	8.88±0.23
<i>B. serrata</i>	trace	1.49±0.01	0.98±0.01	2.47±0.13	0.61±0.02	2.31±0.11	0.97±0.1	1.29±0.13*	4.42±0.21	1.92±0.16	11.52±0.27
<i>B. sacra</i> Shaabi (costal)	5.80±0.20**	1.38±0.03	1.24±0.12*	8.42±0.23	1.73±0.21	4.34±0.28**	0.12±0.01	0.73±0.01	3.82±0.11	0.60±0.01	11.34±0.62
<i>B. sacra</i> Green (mountainous)	2.67±0.21	12.10±0.7***	0.61±0.001	15.38±0.82**	3.05±0.71*	2.75±0.21	3.99±0.17**	0.19±0.01	6.90±0.18**	0.69±0.23	17.57±0.82**

The values with± shows the standard error of mean values of three replications. The asterisks (

*p<0.05

**p<0.01

***p<0.001) show that the values are significantly different in each column using a One-Way ANOVA analysis in Graph Pad prism (v6.01) using Bonferroni test

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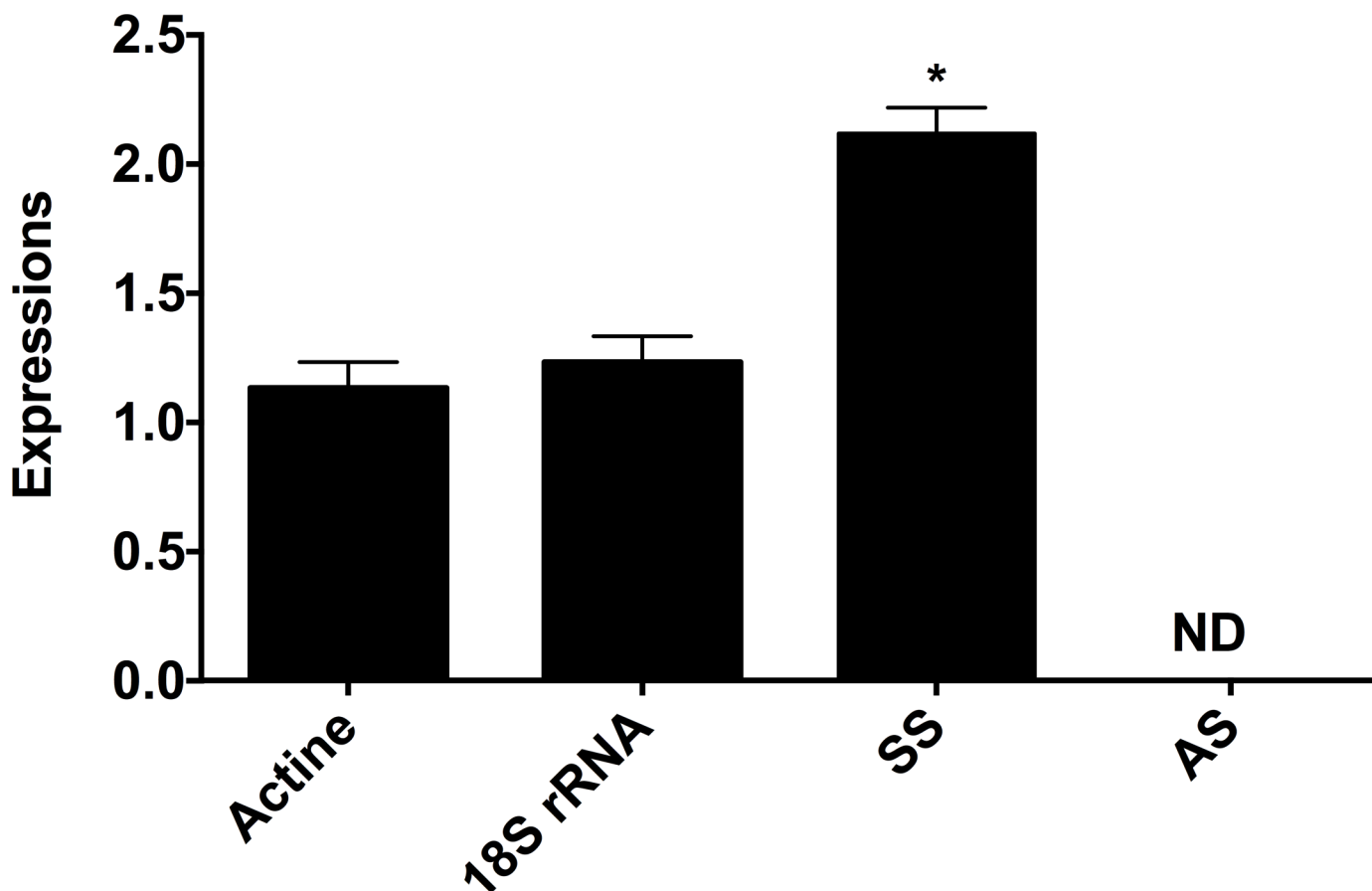


Fig 8. RT-PCR analysis of α -myrin synthase (AS) and Squalene-synthase (SS) in the leaf part of *B. sacra*. The values are mean of three replications and bars with * shows values are significantly ($P < 0.05$) different from the expression of Actin.

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The sub-fraction at 4% MeOH/CHCl₃ (4.1% of KBA) was found to contain the highest percentage of KBA followed by another sub-fraction at 2% MeOH/CHCl₃ (2.2% of KBA; Table 2). The results also indicated that KBA is only present in the gum-resin of the trunk and not in the plant itself [73].

Interestingly, there are clear differences with regard to the quantities of amyryns and boswellic acids of the two grades of *B. sacra* which explain the influence of the geographical location and the environmental conditions of the two grades. This is in agreement with our recent genetic diversity work, which was based on the analysis of simple sequences repeat, and random-amplified polymorphic DNA genetic markers, suggesting a clear isolation of *B. sacra* populations in the eastern coastal regions (unpublished data). Thus, the current variation in the results can also be attributed to the tree's genetic diversity and climatic conditions. This is also in correlation with the results published [74–76] where it was shown that both genetic and climatic factors can influence the resin production and composition. To further confirm expression of amyryn related transcripts, primers were designed using known available genomic sets derived from *Azadirachta indica*. Since biochemical related genomic and molecular information are not available for any of *Boswellia* species, therefore, the homologues species at order level (*Azadirachta indica*) was selected [77]. The gene expression for both α -myrin synthase was not detected in the leaf part of the *B. sacra* tree (Fig 8). However, Squalene-synthase related transcript was expressed in leaf part which suggests that BAs might be synthesized through this

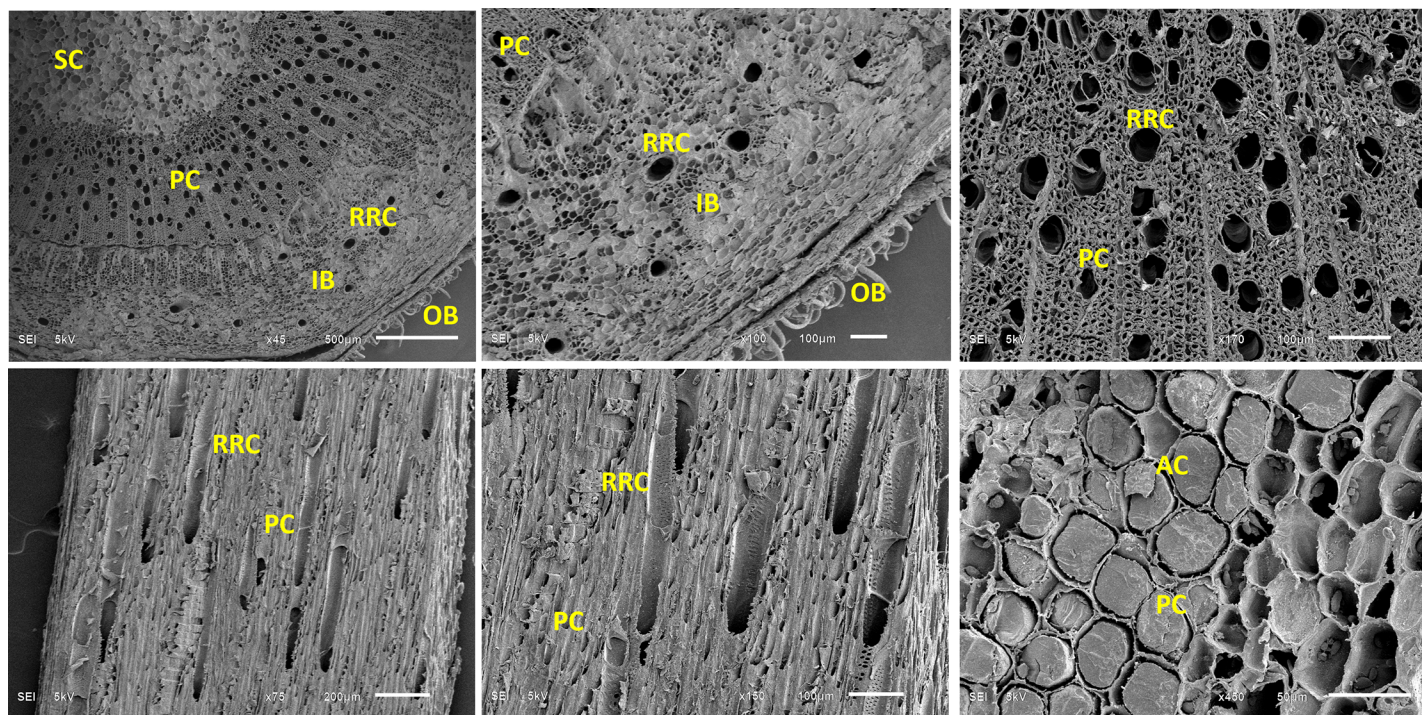


Fig 9. Transversal and radial sectioning of *B. sacra* stem part structure and analysis through Scanning Electron Micrographs. The micrographs are representative to ten individual stem parts of the tree. The Micrographs are taken on SEM using 50 to 200 μ m section and zoom from 100x to 1.2Kx. SC = Sclerenchyma; PC = Parenchyma; AC = Axial canals; RRC = Radial resin canal; IB = Inner bark; OB = Outer bark.

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precursor. However, this needs a detailed transcriptomic analysis to obtain conclusive evidences on the biosynthetic pathway.

Interestingly, the roots contain α -amyrin (2.63%) and proved a complete absence of β -amyrin and all boswellic acids. The leaves have only trace amounts of β -ABA and β -AKBA. Not unexpectedly, the stem proved to be completely free of amyryns and boswellic acids. This explains that the production of the resin is taking place at the cambium (total boswellic acids 1.0%) and epidermis (total of boswellic acids 4.93%) due to the existence of the resin-producing canals. This is supported by the scanning electron microscope images (Fig 9). The micrographs clearly demarcate the presence of radial and axial resin canals in the inner bark region (between epidermis and vascular cambium). The current results show an anatomical harmony with the presence of resinous ducts in the *B. papyrifera* [78].

Conclusion

The current study showed the isolation and characterization of new chemical constituents (β -boswellic aldehyde and 3β , 11β -dihydroxy BA) from the *B. sacra* resins along with known α -amyrin (3-*epi*- α -amyrin, β -amyrin and α -amyrin). A detailed elucidation of the BAs biosynthesis was made for the first time, providing chemical, molecular and structural details to reinforce the previously reported studies. A detailed quantification of BAs and their amyryn precursors in different parts of three *Boswellia* species (*B. sacra*, *B. serrata* and *B. papyrifera*) suggests variation of triterpenes on the basis of species, tree parts and geographical locations. However, BAs were more consistently available in the inner bark of the tree. This was also revealed by the micrographs of resin canals in the inner bark. The current study concludes that BAs are the major class of chemical constituents in *Boswellia*, where future studies should consider the characterization of enzymes responsible in its biosynthesis.

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