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Original Article

Constituents of *Corynaea crassa* "Peruvian Viagra"

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ABSTRACT

A phytochemical investigation of methanol and *n*-hexane extracts of tuber/roots of *Corynaea crassa* Hook. f., Balanophoraceae, led to the isolation and characterization of β-sitosterol, lupenone, β-amyrone, lupeol, and β-amyrine. Unusual complex 1:1 mixtures of lupenone/β-amyrone and lupeol/β-amyrine obtained from the extracts were identified by NMR and HR-MS experiments. The structure of the 1:1 lupenone/β-amyrone mixture was confirmed by X-ray analysis. These triterpene ketone derivatives, only distinguished either by 5- or 6-membered E ring, co-crystallize in one common unit cell in the solid state.

Introduction

Medicinal plants with aphrodisiac activity are used for the treatment of erectile dysfunction all over the world. A variety of botanicals such as *Tribulus terrestris* L., Zygophyllaceae, *Aframomum melegueta* (Roscoe) K. Schum., Zingiberaceae, *Eurycoma longifolia* Jack, Simaroubaceae, *Cnidium monnieri* (L.) Cusson, Apiaceae, *Ferula hermonis* Boiss., Apiaceae, *Mucuna pruriens* (L.) DC., Fabaceae, *Lepidium meyenii* Walp., Brassicaceae, *Passiflora incarnata* L., Passifloraceae, and some compounds like yohimbine were reported to have beneficial effects on sexual function, supporting older claims and offering new hope (Hosseinzadeh et al., 2008).

Likewise, in contemporary Peruvian folk medicine a very large number of medicinal plants are used for aphrodisiacal purposes, a few hundred of them to modulate fertility, or to induce sterility (Pachacuti-Yamqui, 1992). Plant mixtures and magic are commonly applied to increase or to decrease libido (Elferink, 2000). One of the plants used in these aphrodisiac mixtures is commonly known as Chutarpo (or huanarpo), for which there is a female and male designation. *Jatropha macrantha* Müll. Arg., Euphorbiaceae (Bussmann and Sharon, 2007; Pardo, 2002; De Feo, 1992; Brack Egg, 1999; Desmarchelier et al., 1996a,b; Oshima et al., 2003; Tinco et al., 2011; Benavides et al., 2006; Okuyama et al., 1996; Schultes, 1980) and *Corynaea crassa* Hook. f., Balanophoraceae (Bussmann and Sharon, 2006, 2007; Bussmann and Glenn, 2010) are both under the male designation "Huanarpo macho". "Huanarpo macho" is traditionally used strictly as a male aphrodisiac, while the "Huanarpo hembra" *Cnidoscolus peruvianus* (Müll. Arg.) J.F. Macbr., Euphorbiaceae, the female designation, is used strictly as a female aphrodisiac. The use of any of these by the opposite sex is thought to have anti-aphrodisiac effect (Pachacuti-Yamqui, 1992).

A detailed description of preparation and use of *C. crassa* "Huanarpo macho" in an alcoholic beverage is given in (Bussmann et al., 2011a,b). In Peru this plant can be found in the regions of Cajamarca (San Ignacio, Chota and Jaén), Amazonas (Bagua), Lambayeque (Chiclayo), La Libertad (Trujillo), Pasco (Oxapampa) and Cusco (La Convención) (Brako and Zarucchi, 1993; Tropicos).

C. crassa is a hemi-parasitic plant, which grows on roots of other species. Likewise Balanophoraceae family consists of 17 genera and approximately 50 species (Tupac Otero et al., 2009). The natural distribution reaches from Costa Rica to Bolivia and the plant grows at altitudes from 1250 to 3600 m (Tupac Otero et al., 2009). *C. crassa* is not only an aphrodisiac plant there are also reports from ethanolic extracts which have shown biological activity against *Staphylococcus aureus* (Bussmann et al., 2010, 2011a,b). The ethanolic extract exhibit a toxicity of 116 μg/ml using a brine-shrimp lethality assay however, the water extract was found to be non-toxic (<10,000 μg/ml) for the same lethality assay (Bussmann et al., 2011a,b).

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Currently, there is little information about constituents of *C. crassa* (Duff and Nickrent, 1997; Nickrent et al., 1997). A phytochemical investigation of the whole plants of *C. crassa* was thus performed as part of our work on the discovery of bioactive compounds from Peruvian herbal medicines. This led to isolation and characterization of β-sitosterol, lupenone, β-amyrone, lupeol, and β-amyrine.

In this work, the isolation and structure elucidation of these constituents are described.

Materials and methods

Used NMR, MS and HPLC machines and methods

NMR spectra (^{1}H, ^{13}C, APT, H,H-COSY, HSQC, and HMBC) were recorded on a Varian Mercury 400 plus (400 MHz for ^{1}H, 100 MHz for ^{13}C) and a Varian Mercury 300 plus (300 MHz for ^{1}H, 75 MHz for ^{13}C) spectrometer, respectively, at 26 °C and the use of $CDCl_3$ as a solvent. The chemical shifts are reported relative to the residual solvent peak, which was used as an internal reference (^{1}H: 7.26 ppm, ^{13}C: 77.16 ppm). Chemical shifts are given in δ values, coupling constants *J* in Hz; HR-MS spectra were performed with a Bruker Daltonics ESI-FT-ICR-MS APEX II [7 T] and HPLC Varian Pro star 360, vp 250/2 Nucleosil column 50-7.

Preparation of extracts

Isolation and structural elucidation of steroids

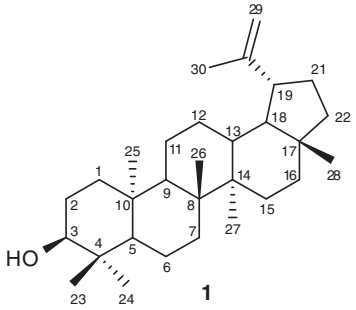
The tubers/roots of *Corynaea crassa* Hook. f., Balanophoraceae, were bought at the Mayorista Market in Trujillo and the Modelo Market in Chiclayo in Northern Peru. Dried and powdered roots (200 g) of *C. crassa* were extracted for 4 days at room temperature, using solvents of increasing polarity, namely, 1.5 L *n*-hexane and 1.5 l methanol. The solvent was then evaporated under reduced pressure, the *n*-hexane extract yielded 1.062 g and the methanol extract 4.240 g of crude product. Liebermann's reagent enabled us to determine remaining steroids and triterpenes in the methanolic extract, which was re-dissolved in water and extracted with dichloromethane yielding 906 mg. Both extracts were combined for a saponification process with 5% NaOH and we obtained in total 209 mg of steroids and triterpenes (TS1).

The plant material was taken from Bussmann and Sharon's collection (Voucher: Supporting information, p. 19).

Isolation by chromatography

TS1 (209 mg) was fractionated by column chromatography on silica gel eluting with *n*-hexane/dichloromethane gradient system (10:0.5 → 10:1 → 10:1.5 → 10:2 → 10.2.5 → 10:3) to give five fractions. Fraction 1 contained traces of two steroids (5.3 mg) seemed on the TLC plate and ^{1}H NMR but they were not possible to separate by CC and HPLC.

Fraction 2 (20.1 mg) was subjected to further HPLC with pure *n*-hexane (R_f: 0.81); After several purifications a lot of material was lost. Then the finally material was recrystallized from methanol/chloroform to yield β-amyrenone/lupenone (10.8 mg). Fractions 3 and 4 (62.9 mg) were further purified (Silica gel, *n*-hexane/DCM = 5:0.06 → 5:0.1 → 5:0.2 → 5:0.3 → 5:0.4) to yield 12.5 mg of β-sitosterol. And finally fraction 5 (18.8 mg) was purified by flash CC (*n*-hexane/DCM = 10:0.1 → 10:0.3 → 10:0.4 → 10.5 → 10:6 → 10:0.7) and HPLC (*n*-hexane/isopropanol = 9:0.0–9:0.02) then recrystallized from methanol/chloroform to yield 15.3 mg of β-amyrine/lupeol (R_f: 0.24). Finally, 2.8 mg of lupeol (**1**) was isolated by HPLC (*n*-hexane/isopropanol = 9:0.1).



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Lupeol (**1**): White powder; MP. 215 °C; $[\alpha]_D$ = +26,4 (*c* = 0.4, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.69 (1H, m, H-5), 0.77 (1H, s, H-24), 0.80 (1H, s, H-28), 0.84 (1H, s, H-25), 0.94 (1H, m, H-1b), 0.96 (1H, m, H-15b), 0.96 (1H, s, H-27), 0.97 (1H, s, H-23), 1.04 (1H, s, H-26), 1.10 (1H, m, H-12b), 1.19 (1H, m, H-22), 1.27 (1H, m, H-21), 1.29 (1H, m, H-11b), 1.30 (1H, m, H-9), 1.38 (1H, m, H-7), 1.39 (1H, m, H-6b), 1.39 (1H, m, H-18), 1.43 (1H, m, H-11a), 1.48 (1H, m, H-16), 1.52 (1H, m, H-6a), 1.61 (1H, m, H-15a), 1.61 (1H, m, H-2), 1.62 (1H, m, H-13), 1.64 (1H, m, H-1a), 1.70 (1H, m, H-12a), 1.70 (1H, s, H-30), 2.38 (1H, m, H-19), 3.18 (1H, dd, *J* = 11.0; 5.3 Hz, H-3), 4.56 (1H, s, H-29b), 4.68 (1H, s, H-29a).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 14.8 (CH_3, C-27), 15.6 (CH_3, C-24), 16.1 (CH_3, C-26), 16.2 (CH_3, C-25), 18.1 (CH_3, C-28), 19.0 (CH_2, C-6), 19.8 (CH_3, C-30), 21.2 (CH_2, C-11), 25.3 (CH_2, C-12), 27.2 (CH_2, C-15), 27.5 (CH_2, C-2), 28.4 (CH_3, C-23), 30.1 (CH_2, C-21), 34.2 (CH_2, C-7), 35.9 (CH_2, C-16), 37.2 (C, C-10), 38.5 (CH, C-13), 38.7 (CH_2, C-1), 39.8 (C, C-4), 40.3 (CH_2, C-22), 41.1 (C, C-8), 42.8 (C, C-14), 43.2 (C, C-17), 47.8 (CH, C-19), 48.5 (CH, C-18), 50.9 (CH, C-9), 55.5 (CH, C-5), 79.3 (CH, C-3), 109.5 (CH_2, C-29), 151.2 (C, C-20); HR-MS: *m/z* 449.43 $[M+Na]^+$, for formula $C_{30}H_{50}O$.

β-Sitosterol HR-MS: *m/z* 437.41 $[M+Na]^+$, for formula $C_{29}H_{50}O$.

Lupenone/β-amyrone HR-MS: *m/z* 447.40 $[M+Na]^+$, for formula $C_{30}H_{48}O$.

Lupeol/β-amyrine HR-MS: *m/z* 449.43 $[M+Na]^+$, for formula $C_{30}H_{50}O$.

X-ray crystallography

Recrystallization of the lupenone/β-amyrone mixture from methanol/chloroform (1:0.3) gives colorless prisms. A crystal of the dimensions 0.3 mm × 0.15 mm × 0.15 mm was used for analysis. The intensities were measured on an IPDS1 diffractometer (Fa. STOE) and were corrected for Lorentz and polarization effects. The structure was solved by direct methods, and the refinement was performed with SHELX-97 (Sheldrick, 1997). Crystal data: *a* = 11.203 Å, *b* = 14.956(2) Å, *c* = 30.513(6) Å, *V* = 5112.5 Å^3, space group P2(1)2(1)2(1), *Z* = 8, D_{calc} = 1.103 mg/m^3, λ = 0.71073 Å, μ (Mo*K*α) = 0.064 mm^{-1}, *F*(000) = 1888, and *T* = 213(2) K, the total of 38,852 reflections (9556 independent, R_{int} = 0.082) in the range of $2.2^\circ \leq \theta \leq 26.1^\circ$ and 559 refined parameters. The final parameters were R_1 = 0.0442, R_2 = 0.0948 for 3195 observed reflections with $I > 2\sigma(I)$ and a goodness-of-fit = 0.558.

Crystallographic data for the structure determined in this paper have been deposited at Cambridge Crystallographic Data center (CCDC reference number is CCDC 1008248) and can be obtained free of charge from Cambridge Crystallographic Data Centre CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 01223 336033; deposit@ccdc.cam.ac.uk.

Results and discussion

Spot tests were used for the qualitative determination of secondary metabolites present in the crude extract of *C. crassa*, as it was

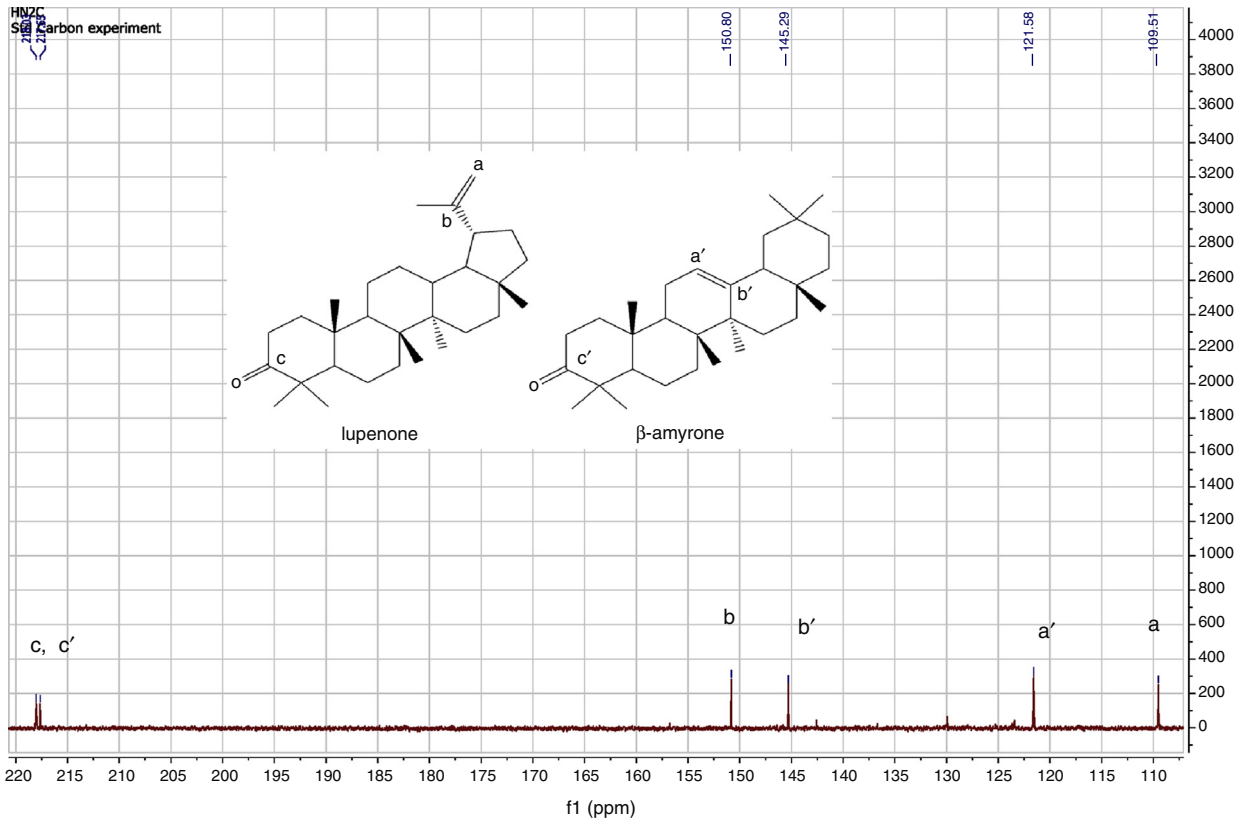

Fig. 1. Characteristical part of the ^{13}C NMR spectrum of the 1:1 lupenone/β-amyrone mixture.

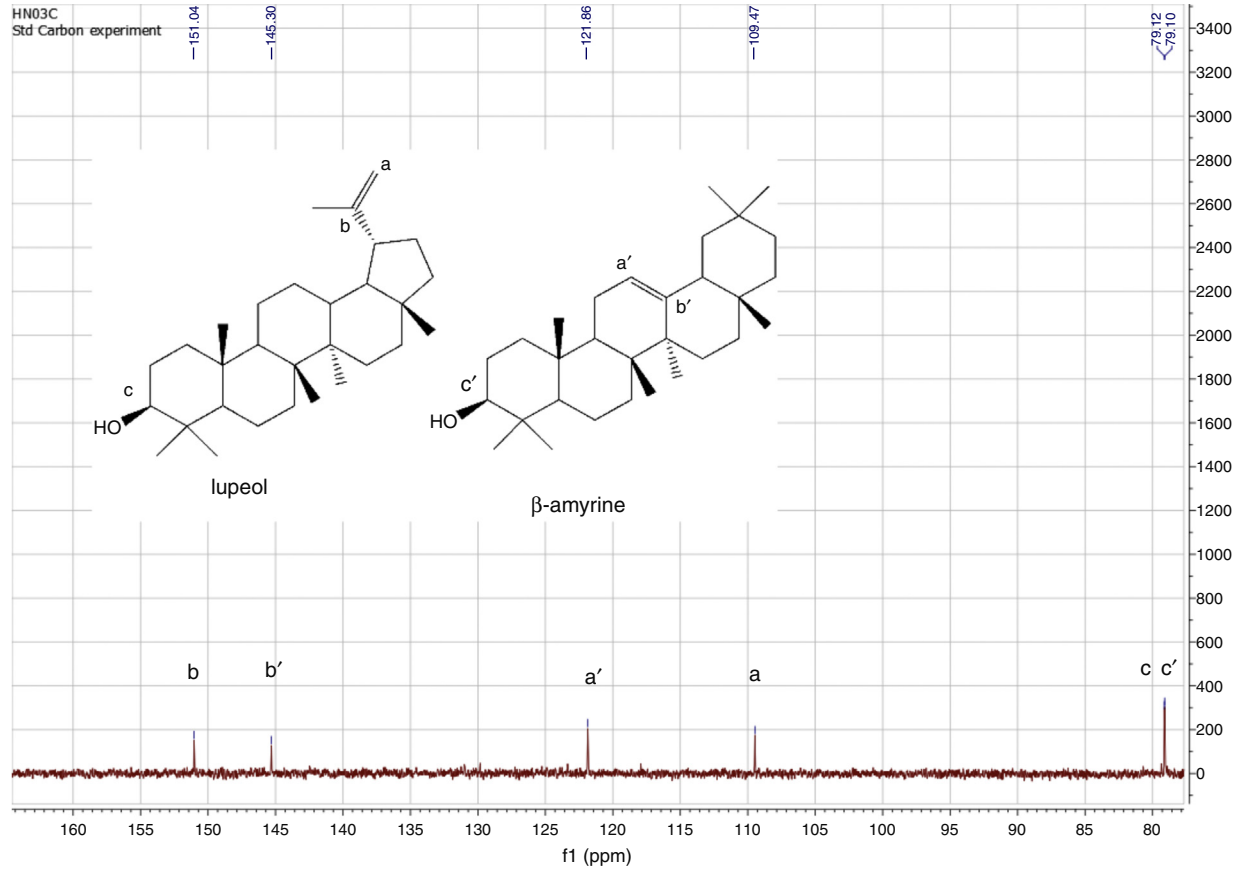

Fig. 2. Characteristical part of the ^{13}C NMR spectrum of the 1:1 lupeol/β-amyrine mixture.

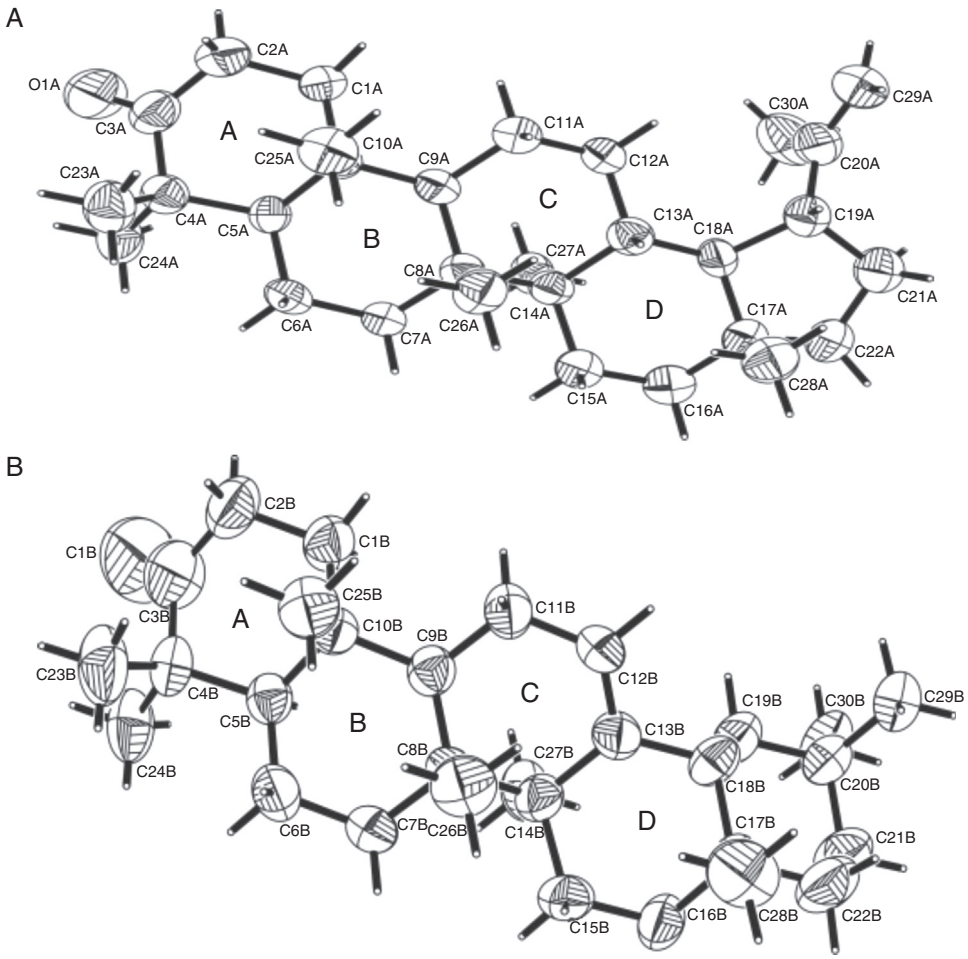

Fig. 3. Molecular structures of lupenone (A) and β-amyrone (B) (ellipsoids with 50% probability).

described in (Dominguez, 1973; Harborne, 1984). We have identified steroids, triterpenes, flavonoids, cardiotonics, tannins and anthocyanines, whereas alkaloids have not been detected. According to Liebermann–Burchard's tests, the experiments also have shown an intense green dark coloration for steroids and triterpenes, indicating that these secondary metabolites are in the crude extract.

The steroid β-sitosterol, was one of the main products isolated from the *n*-hexane extract and purification by chromatography with *n*-hexane/dichloromethane. β-Sitosterol itself is widely distributed in the plant kingdom and used in herbal therapy, especially for benign prostatic hyperplasia (BPH) (Berges et al., 1995). The structure was confirmed by NMR, HR-MS and comparison with the literature results (Wright et al., 1978; Faizi et al., 2001).

In a next step, the *n*-hexane extract furnished a 1:1 mixture of the pentacyclic isomeric triterpenes lupenone and β-amyrone. It was not possible to separate both compounds using chromatographic methods. Finally, we were successful to identify the structure of the complex mixture by use of 1D and 2D NMR spectroscopy, X-ray and HR-MS. Our results are in agreement with the literature data (Wenkert et al., 1978; Carpenter et al., 1980). These isomeric triterpene ketone derivatives are only distinguished in its structure by their 5- and 6-membered E ring, respectively, and give the same high resolution mass spectrum. In the ^{1}H NMR spectrum of the mixture we observed three signals of olefinic protons in a ratio 1:1:1. The signal at 5.18 ppm was assigned to the methine proton in β-amyrone (Fig. 1), while the signals at 4.66 and 4.54 ppm, respectively, result from the methylene protons of lupenone.

The ^{13}C NMR spectrum shows typical signals at 218.03 and 217.65 ppm corresponding to the carbonyl groups. The olefinic carbon atoms appear at 109.51 (=CH_2) and 150.80 ppm (=C–) (lupenone), and at 121.59 (=CH–) and 145.29 ppm (=C–) (β-amyrone) (Fig. 1).

We were able to recrystallize the triterpene mixture using methanol/chloroform (1:0.2) to give crystals enough suitable for X-ray analysis. The crystal structure unambiguously confirms the interpretation of the spectra and the structure of the isolated products. In the orthorhombic unit cell there are two structural constitution isomers of the formula $C_{30}H_{48}O$: lupenone and β-amyrone (Fig. 3).

The formation of a 1:1 mixed crystal is due to the close resemblance of the conformation with the rings A, B, C and D. A calculation of the puckering parameters according to (Cremer and Pople, 1975) gives parameters which agree with a C (chair) – conformation. Only the C-ring of β-amyrone has an E (envelope) – configuration. The reason for this difference is the double bond between C12B–C13B (1.335 (5) Å). A fitting of both molecular structures shows the broad consistency between the rings A, B, C and D (Fig. 4).

Our X-ray data are in agreement with the X-ray structures of lupenone and β-amyrone obtained as separated compounds by isolation from other plants (Dampawan et al., 1977; Yan et al., 1989; Jaiswal et al., 2004).

Besides the lupenone/β-amyrone mixture a combination of lupeol/β-amyrine (also in 1:1 ratio; Fig. 2), could be isolated in lower yield (30% compared to lupenone/β-amyrone). The structure of these triterpenes was also confirmed by 1D and 2D NMR

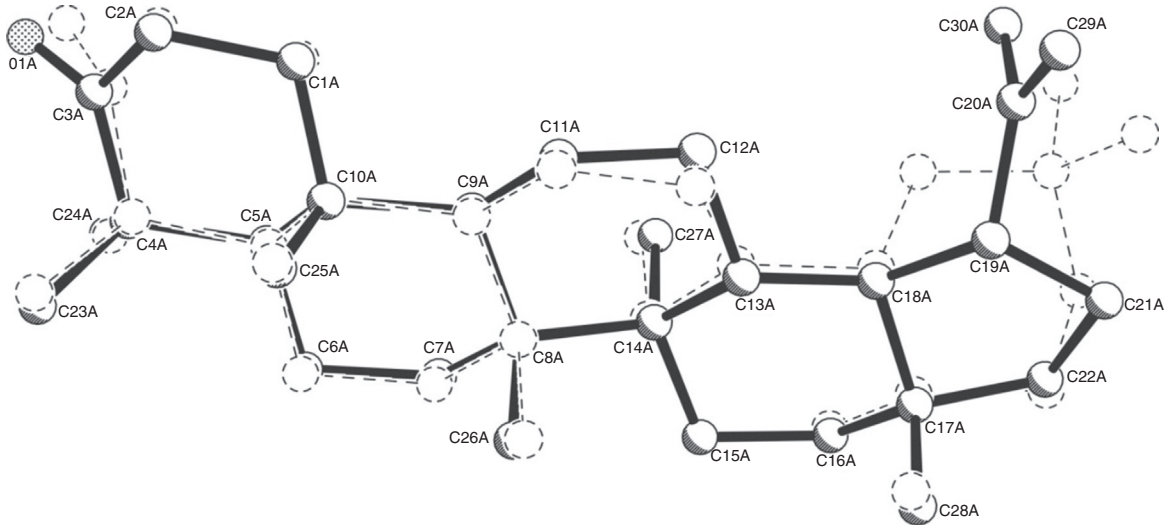

Fig. 4. Fitting of the molecular structures of lupenone and β-amyrone. In the crystal structure the two different molecules also form separate helical arrangements along the *b*-axis.

techniques, MS measurements and comparison with the literature results (Wenkert et al., 1978; Carpenter et al., 1980). In the ^{1}H NMR spectrum the olefinic protons appear at 5.18 (=CH— of β-amyrine), 4.68 and 4.56 (=CH_2 of lupeol), and additionally the typical CH—O absorptions at 3.20 ppm. In the ^{13}C spectrum the olefinic carbons appear nearly unchanged (151.03, 145.29, 121.85 and 109.45 ppm) in comparison to the spectrum of the lupenone/β-amyrone mixture. But instead of carbonyl signals now two closely related CH—O signals appear at 79.12 and 79.10 ppm.

After additional HPLC purification of this mixture it was possible to isolate lupeol as nearly pure compound for separate NMR characterization.

Interestingly, the common appearance of the combinations lupenone/β-amyrone and lupeol/β-amyrine was observed for first time. In contrast, the combination of lupanone/lupeol already was found in *Tamarindus indica* L., Fabaceae (Mathur, 2012), and the combination of β-amyrone/β-amyrine was isolated and characterized from *Diospyros morrisiana* Hance, Ebenaceae (Yan et al., 1989).

The formation of both lupenone/β-amyrone and lupeol/β-amyrine each in a 1:1 ratio was unexpected because lupenone and lupeol were formed via the same lupenyl cation, whereas β-amyrine and β-amyrone were formed via the oleanyl cation in the proposed biosynthetical pathway (Gallo and Sarachine, 2009; Seo et al., 1981).

A complex mixture of all these 4 pentacyclic triterpenes from the same plant has never been observed before. Otherwise, the individual compounds have been isolated and described as constituents in different aphrodisiac plants (Mathur, 2012; Mazumder et al., 1999; Corrêa et al., 2009; Imman et al., 2007).

Conclusion

The present paper describes the isolation and characterization of five constituents from *C. crassa*: β-sitosterol and two interesting 1:1 mixtures of triterpenes: lupenone/β-amyrone and lupeol/β-amyrine. The results may be helpful in further investigations of the biological activity of these natural compounds.

Authors' contribution

GRMG (PhD student) contributed in collecting plant sample and identification, running the laboratory work, and drafted the paper; LH did the NMR investigations; JS performed the X-ray analysis; RWB contributed in collecting plant sample and identification. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.02.007.

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