ORIGINAL ARTICLE

Action mode of ursolic acid as a natural antioxidant and inhibitor of superoxide dismutase: *In vitro* and *in silico* study

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Recently, the antioxidant is applied for the teeth bleaching treatment as an alternative of toxic material of hydrogen peroxide that is used in teeth bleaching. One of natural sources antioxidant is *Uncaria gambir* those containing active antioxidant agents. To be applied as a new bioactive constituent in teeth bleaching treatment, a preexperimental study is performed. The aim of the study is to identify the antioxidant constituent of *U. gambir* and predict their activity including action mode as an inhibitor of enzyme superoxide dismutase (SOD) through *in vitro* and *in silico* method. Combination of chromatography methods and spectroscopic analysis is used for isolated bioactive antioxidant constituent. The antioxidant activity was evaluated by *in vitro* assay against diphenylpicrylhydrazyl (DPPH) and SOD, respectively, while prediction of action mode of the active compounds as SOD-mutant enzyme inhibitor was conducted by *in silico* study using AutoDock 4.2 program. Antioxidant of ursolic acid was isolated from *U. gambir* with inhibitory concentration$_{50}$ values 1721 ± 30.6 and 392 ± 53.57 μg/mL, respectively, against DPPH and SOD. By *in silico* study presented that ursolic acid inhibited SOD enzyme with a binding affinity of − 5.4 kcal/mol those higher than a quercetin as a positive control. The ursolic acid was identified as a potential natural antioxidant with potentially activity to inhibit SOD mutant.

Key words: DPPH, superoxide dismutase enzyme, *Uncaria gambir,* ursolic acid

INTRODUCTION

The most popular dental care for the public is teeth whitening.[1] One of the methods used to whiten the teeth is bleaching.[2] The study showed that antioxidants play a role in the effect of tooth bond strength caused by bleaching method those can produce free radicals that are left in the tooth structure; therefore, antioxidants are needed as free radical scavengers to increase the strength of dental restoration materials bonding to the substrate.[3] An antioxidant is a substrate or molecule that can inhibit free radical reactions and is available in synthetic or natural forms since natural antioxidant is hardly safer than a synthetic antioxidant.[4,5]

Natural antioxidant agents found in medicinal plants including *Uncaria gambir* Roxb. In Indonesia, those classified as herbal were originally used as a treatment for inflammation, oral problems, diarrhea, and as a

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Submitted: 09-Apr-2021 **Revised:** 26-Jun-2021
Accepted: 19-Jul-2021 **Published:** 20-Oct-2021

Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/japtr.japtr_90_21

How to cite this article: Somantri AD, Kurnia D, Zainuddin A, Dharsono HD, Satari MH. Action mode of ursolic acid as a natural antioxidant and inhibitor of superoxide dismutase: *In vitro* and *in silico* study. J Adv Pharm Technol Res 2021;12:389-94.

component in betel chewing. The extracts of *U. gambir* were reported active as antioxidants against DPPH, as well as against superoxide anion radicals using the phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) method.[6-8] Some antibacterial, antidiabetic, and anti-inflammatory actions against damage produced by the process of mediating free radicals have been documented, but activity against the enzyme superoxide dismutase (SOD) has not yet been identified.[9]

The study focuses on the isolation and bioactivity assessment of antioxidant constituents from *U. gambir* using an *in vitro* technique against DPPH and superoxide radicals using nonenzymatic SOD. The mechanism of the antioxidant compound's molecular interaction with the SOD enzyme is then hypothesized using an *in silico* approach.

MATERIALS AND METHODS

Materials

The leaves of *U. gambir* were planted in Sumatra Barat, Indonesia, in May 2019. The voucher specimen (NP-0153) was identified and deposited in the Laboratory Taxonomy, Department of Biology, Universitas Padjadjaran, Bandung, Indonesia. Distilled organic solvents and water were utilized for isolation, whereas proanalyzed solvents from Sigma-Aldrich were used for spectroscopic analysis. The chromatography was used Silica G 60 from Merck and ODS RP-18 from Nacalai Tesque while for thin-layer chromatography (TLC) was used a plate of ODS RP-18 F_{254} and Silica G 60 F_{254} from Merck those visualized by spraying 10% of H_2SO_4 (v/v) in ethanol.

For antioxidant assay, DPPH from Wako was utilized, and for SOD assay, tetramethylethylenediamine (TEMED) 99% MB 026–100 ml and nitro blue tetrazolium (NBT) 98% MB 107–250 mg from HiMedia, riboflavin 98% from Sigma Aldrich, the solution of 1.0 M phosphate buffer pH 7.4 (VWR, E404–100 TABS), and water injection from Generic were employed.[10]

The AutoDock 4.2 software was used for molecular docking.[11] The protein data bank of the Research Collaboratory for Structural Bioinformatics (www.rcsb.org/) included the crystal structure of human SOD 1 complexed with naphthalene-catechol (protein data bank ID: 5YTO) and the chemical formulas of naphthalene-catechol (CID 134828057), quercetin (CID 5280343), and ursolic acid (CID 64945). The chemicals were found in the PubChem database (http://www.ncbi.nlm.nih.gov/pccompound). The Open Babel program was used to create the 3D structure in PDB format using SMILE notation.[12] Docking results were analyzed in Discovery Studio to establish the kind of interaction on residues.

Instruments

The compound structure was identified by ultraviolet (UV)-Vis 8452A Diode Array, infrared (IR) by FTIR Shimadzu 8400, 1D and 2D-NMR using JEOL type ECA (500 MHz), and mass spectrometry (MS) using water acquit ultra-performance liquid chromatography type triquadrupole. UV detector lamps with maximum wavelengths of 254 and 365 nm were used to examine the TLC plates. NEST flat-bottom 96-well microplates micropipettes from Eppendorf 1.5 mL microtube (GenFollower), incubator Memmert, and microplate reader EZ 400 (Biochrom, Germany) were utilized for the antioxidant activity test.

Methods

Isolation procedure of compound 1

The leaves of *U. gambir* (1 kg) were macerated in methanol and subsequently fractionated with n-hexane, ethyl acetate, and water, yielding crude extracts weighing 298.9, 17.4, 55.7, and 44.5 g, respectively. Extracts were tested for antioxidant activity against DPPH and SOD at various doses. The active ethyl acetate extract (30 g) was purified by chromatographed on Silica G 60 eluted with n-hexane-ethyl acetate in 5% gradient resulted fractions I–VI. The purification of II (0.1812 g) by re-chromatographed on ODS RP-18 eluted with methanol-H_2O of 1% gradient to give five identical fractions of II 1–5 (0.0451 g) and then after washed with ethyl acetate resulted pure active compound 1 (0.0107 g).

Compound 1 structure determination

The chemical structure of 1 was established using spectroscopic data analysis of Uv-Vis, IR, NMR, and MS. The original spectra are available in the supplement material section [Figures S1-S10].

Antioxidant activity evaluation of the extracts and compound 1

The *U. gambir* extracts and ursolic acid were tested against DPPH assay. The assay concentrations of 5–50 µg/mL for extracts and 500–2000 µg/mL for ursolic acid were adjusted, and 60 µg/mL of DPPH solution was added in methanol in 96-well microplate and homogenized by diluting it using a micropipette, then left in a dark room for 30 min while the reaction takes place. The final reaction was measured at 517 nm by ELISA reader to determine the absorbance and inhibitory concentration $(IC)_{50}$ value, respectively.[13]

The SOD-like activity was determined according to published procedures.[10] The series concentration sample of extracts and compound 1 of 40 µL was added to 96 well microplate, and the solution was divided into two parts of solution A (aquadest, phosphate buffer pH 7.4, NBT, TEMED, and riboflavin) and solution B (blank mixture without riboflavin), and both were added to in the amount of 200 µL. The sample was diluted with a micropipette and irradiated for 10 min and their absorbance was measured at 560 nm for determine of IC_{50} values.

In silico study of the ursolic acid against superoxide dismutase

Canonical SMILES obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/64945) were used OPEN BABEL 2.4.1 software to transform the chemical structure of an ursolic acid compound into 3D in PDB format, and the RSCB (https://www.rcsb.org/structure/5YTO) was used to retrieve the 3D-structure of SOD.

AutoDock Vina software was used for docking and virtual screening of ligand-protein interaction. Naphthalene-catechol [Figure 1] was used as ligand control. Blind docking was undertaken using a box of size 40 x 40 x 40 points, covering the whole protein target, with coordinate X = 70.133, Y = 75.513, and Z = −12.173. BIOVIA/discovery studio was used to visualize the docking results. BIOVIA program showed the ligand-residue and docking position in a 3D molecule.

RESULTS

Compound 1 isolation procedures

The purity of compound 1 (10.7 mg) was evaluated by 2D-TLC analysis on ODS RP-18 eluted with methanol-water (1:9 v/v) with Rf = 0.47.

Compound 1 structure determination

Compound 1 was separated in the form of a white powder and dissolved in methanol. The IR spectrum of 1 showed absorptions at 3434, 2926, 1749, 1465, and 1059 cm^{-1} those corresponding to hydroxyl, CH sp^3, carboxyl, C = C and C-O functional group, respectively.

By NMR measurement, the ^{13}C-NMR, and DEPT 135° spectra indicated that 1 to have thirty carbon signals including carbons for the seventh methyl, nine methylene sp^3, six methine sp^3, one carbon sp^2, and seventh quaternary carbons, respectively, and were identified as six quaternary sp^3 carbons at δc 39.8, 40.7, 43.2, 48.5, and 49.9 ppm, together with one quaternary sp^2 carbon at 180.3 as carbon of carboxylate group. The ^{1}H-NMR spectrum of 1 showed proton signals for two secondary methyl, one methine at δ_H 3.14 (1H, dd), one olefinic at 5.22 (1H, s), and some overlap methylene signals at 1.2–2.2 ppm, respectively.

Signals identification by ^{1}H-^{1}H-COSY of 1 presented correlations between H-11 with H-9, H-2 with H-1, H-6 with H-5, and H-19 with H-29 (δ_H 0.87), respectively. Another signals in heteronuclear Multiple Bond Correlation spectrum of 1 showed correlation of methine proton H3 at δ_H 3.14 to methane carbon C3 at 79.6, indicating a hydroxyl group attached at C3, and a carboxyl group (δc 180.3; C30) attached to quaternary carbon C28. For structural confirmation, the molecular mass of 1 was measured and showed of m/z 455.54 $[M-H]^-$ corresponding to the molecular formula of $C_{30}H_{47}O_3$ or m/z 456.54 for $C_{30}H_{48}O_3$, respectively. Based on the spectral analysis together comparison data with published report, compound 1 was suggested to have a triterpenoid skeleton derivative and identified as ursolic acid as seen in Figure 1.[14]

Antioxidant activity of extracts and compound 1

The data in Table 1 presented that *U. gambir* extracts were active as antioxidant with IC_{50} values ≤50 ppm, and especially the ethyl acetate extract, were very active as seen in Figure 2a, for DPPH; in Figure 2b, for positive control; in Figure 3 for SOD, respectively.[10]

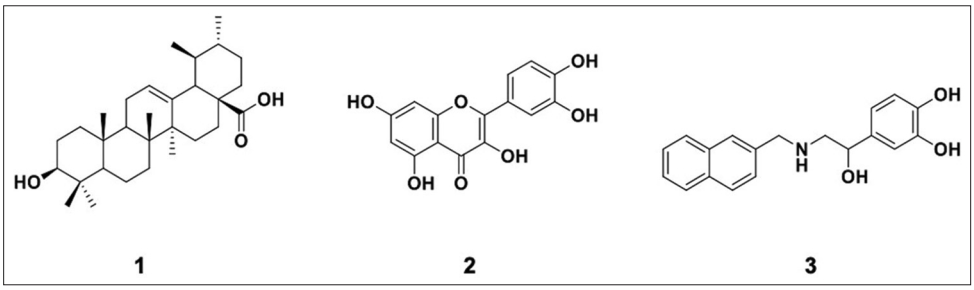

Figure 1: Structure of ursolic acid (1); quercetin (2); naphthalene-catechol (3)

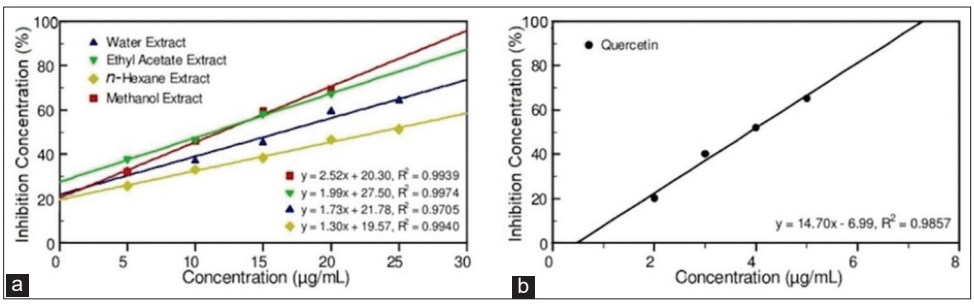

Figure 2: Graph of antioxidant activity against DPPH; *Uncaria gambir* extract (a); control positive: quercetin (b)

Further evaluation of ursolic acid (1) indicates a low inhibition activity against DPPH with IC_{50} of 1721± µg/mL while for superoxide radical against SOD was more active with IC_{50} of 392 ± 53.57 µg/mL, respectively.

Antioxidant activity prediction of ursolic acid through molecular interaction with superoxide dismutase

Validation of docking parameters was done by docking on native ligands (naphthalene-catechol) and receptors (5YTO) to find whether the close-match docking pose can be predicted. For basic mode selection, the root-mean-square deviation (RMSD) value ≤2 Å is fairly good.[15] This docking parameter will be used in virtual screening of ursolic acid and quercetin. The structure with the lowest predicted free energy of binding (−7.1 kcal/mol), i.e., conformation no. 5 was selected. There were ten conformations, where the conformational differences of the ligands were also obtained. The results of this *in silico* experiment are shown in Table 2 and Figures 4, 5.

DISCUSSION

The discovery new antioxidant as an active agent for teeth whitening is an important research target in exploring new bioactive compound from medicinal plants, and recently in dentistry, the antioxidant agent is used to decrease bond strength after bleaching.[16] Many plants were reported as source of antioxidant agents with a function to prevent any free radical reaction in our body.[17]

The medicinal plant of Gambir (*U. gambir*) is a natural antioxidant sources that contain roxburghine B as a receptor adenosine diphosphate inhibitor, (+)-catechin and procyanidin B3 as antibacterial and antioxidant, (−)-epicatechine as antiviral and gambirine D as α-glycosidase inhibitor, respectively.[18]

Antioxidant-guided isolation of ethyl acetate extract resulted an antioxidant of ursolic acid, (1) which was isolated for the first time in this research from *U. gambir* Roxb.[19] The ursolic acid (1) was reported which shows pharmacological activity as anti-inflammatory, anticancer, and antioxidant, respectively, while the activity as an antioxidant against SOD is not reported yet.[20]

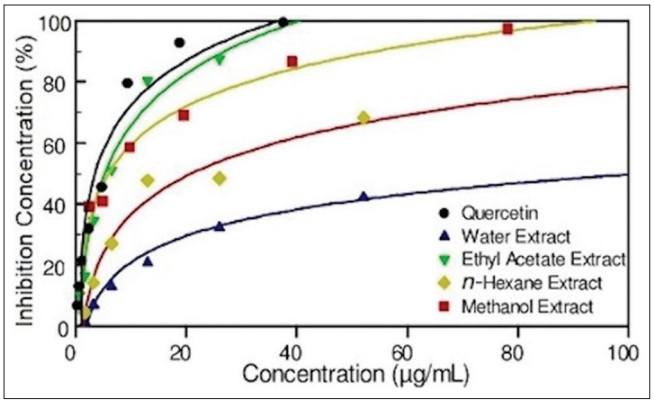

Figure 3: Graph of antioxidant activity of *Uncaria gambir* and quercetin against superoxide dismutase

According to assay data, ursolic acid has weaker antioxidant activity than quercetin, it is predicted the absence of conjugated hydroxyl groups in the structure of ursolic acid cause DPPH and superoxide radicals not being optimally scavenged. Even that activity of ursolic acid (1) is very weak, it's activity against nonenzymatic SOD is new and interesting research data and its need to further study for use of ursolic acid (1) as an alternative antioxidant agent to assist SOD enzyme in radical scavenging.

Naphthalene catechol has a binding affinity of − 7.1 kcal/mol and shows hydrogen interactions on the residues of Lys23, Glu100, and Pro28, while ursolic acid has a lower binding affinity of − 5.4 kcal/mol and has a hydrogen interaction at the same residues of Glu100 while Lys23 with hydrophobic interactions. The absence of interactions with amino acids Pro28 allows a decrease in the affinity value of ursolic acid and quercetin (−5.1 kcal/mol). However, ursolic acid has a higher binding affinity value than quercetin, and it is known that quercetin has a stronger antioxidant value than ursolic acid. Although quercetin has quite a lot of hydrogen interactions with some of the same residues such as Glu21, Glu100, and Lys23, its affinity value is lower than ursolic acid.

The more negative values of binding affinity indicate that the bond is in the best bond strength condition because it is more stable, and the bond is stronger.[21] The SOD enzyme inhibitor was known to be naphthalene catechol, which has a lower free energy than other ligands; ursolic acid's low antioxidant action is expected due to the lack of hydrogen bonding.[22,23]

Based on the location of the three complexes, all ligands are bound to SOD in the same position, it can be concluded that ursolic acid and quercetin have the same active site as naphthalene catechol competitively.[23]

Table 1: Antioxidant activity of *Uncaria gambir* extracts

Samples	IC_{50} (µg/mL)	
	SOD	DPPH
Methanol	7.1±1.78	11.8±0.26[a]
n-hexane	26.8±3.48	23.7±0.55
Ethyl acetate	6.2±0.47	11.2±0.70
Water	38±8.38	16.3±0.33
Quercetin (+)	5.26±0.27	3.8±0.31

[a]The DPPH measurements were tested in duplicate, while the SOD values were measured in pentuiplicate and were statistically expressed as mean ± standard deviation. SD: Standard deviation, SOD: Superoxide dismutase, DPPH: Diphenylpicrylhidrazyl

Table 2: Binding affinity and hydrogen bond in complex superoxide dismutase-compounds

Ligand	Binding affinity (kcal/mol)	Residues binding at ligand-protein complex	
		Hydrogen interaction	Hydrophobic interaction
Ursolic acid	−5.4	Glu100	Lys30, Lys23, Lys3
Quercetin	−5.2	Glu21, Glu100, Lys23	Thr2, Gln22, Lys30, Val29
Naphthalene-catechol	−7.1	Glu100, Pro28, Lys23	Glu21, Trp32

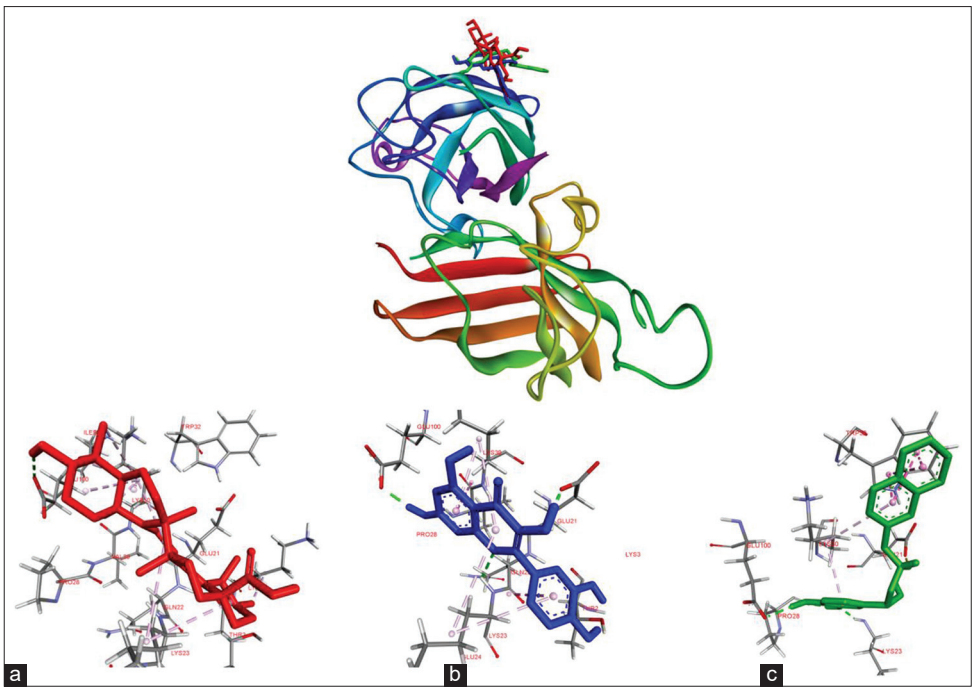

Figure 4: Ligand positions on superoxide dismutase**:** quercetin (a); ursolic acid (b); naphthalene-catechol (c)

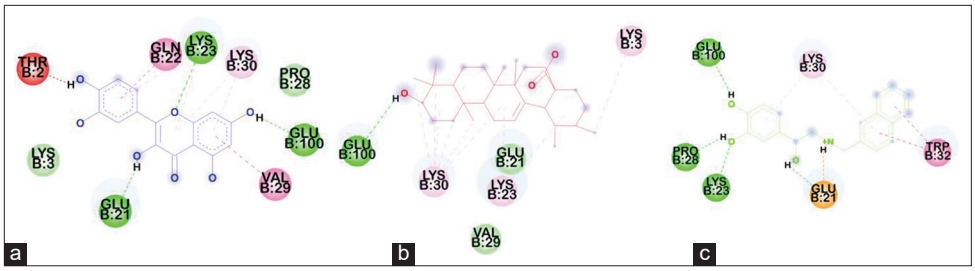

Figure 5: Interaction superoxide dismutase with ligands: quercetin (a), ursolic acid (b), and naphthalene-catechol (c)

According to *in vitro* and *in silico* studies, ursolic acid is deduced to have two "opposing faces" that mean ursolic acid which has two actions, inhibiting radicals and at the same time, attenuating the action of the SOD enzyme. In the other words, ursolic acid acts as "potentiation" (enhancement of the effects of one drug by another, but having dissimilar action) and "subtraction" agent (abolishing effect of another drug).

CONCLUSION

The herbal of *U. gambir* containing antioxidant constituents of ursolic acid (1). The *in vitro* and *in silico* study of ursolic acid against DPPH and SOD presented interesting mode action mechanism those suggested ursolic acid as new natural antioxidant through a competitive inhibitor type. This antioxidant data can be used as preliminary bioactivity of interesting drugs candidate for applied to treat oral diseases caused by the toxic whitening agent and inflammation process. However, further research such as the synthesis of lead derivatives, *in vivo* method, and clinical studies is still needed.

Acknowledgment

The authors are grateful to Central Library of Universitas

Padjadjaran for deposit of supplementary material and also grateful to Universitas Padjadjaran for all research facilities.

Financial support and sponsorship

This research was supported by Penelitian Dasar - KEMENRISTEK DIKTI/BRIN (PD: Dikdik Kurnia, Ph.D. with No. 1832/UN6.D/LT/2019) 2019 from the Indonesian Ministry of Research, Technology, and Higher Education, Jakarta-Indonesia.

Conflicts of interest

There are no conflicts of interest.

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SUPPLEMENT MATERIALS

Spectral data of compound 1 was UV: 201 nm. IR: 3434, 2926, 1749, 1465, 1375 and 1059 cm^{-1}. The MS (negative ion mode): (*m*/z) 455.54.^{1}H-NMR (CD_3OD and $CDCl_3$): δ_H 1.62 & 1.68 (2H, m, H-1), 1.64 (2H, m, H-2), 3.14 (2H, dd, 4.5 and 11.5Hz, H-3), 0.75 (1H, d, H-5), 1.43 & 1.40 (2H, m, $6H_Z$, H-6), 1.29 & 1.30 (2H, m, H-7), 1.56 (1H, m, H-9), 1.91 (2H, q, H-11), 5.22 (1H, t, H-12), 1.62 (2H, m, H-15), 1.59 & 1.63 (2H, m, H-16), 2.19 (1H, d, 11.5Hz, H-18), 1.37 (1H, m, H-19), 1.35 (1H, m, H-20), 1.48 (2H, m, H-21), 1.62 (2H, m, H-22), 0.96 (3H, s, H-23), 0.77 (3H, s, H-24), 0.97 (3H, s, H-25), 0.84 (3H, s, H-26), 1.11 (3H, s, H-27), 0.87 (3H, d, 6Hz, H-29), 0.95 (3H, d, 6Hz, H-30). ^{13}C-NMR (CD3OD): δ_C39.9 (C-1), 27.8 (C-2), 79.6 (C-3), 39.8 (C-4), 56.7 (C-5), 19.4 (C-6), 34.3 (C-7), 43.2 (C-8), 48.9 (C-9), 40.7 (C-10), 24.2 (C-11), 126.8 (C-12), 139.6 (C-13), 48.5 (C-14), 29.2 (C-15), 25.3 (C-16), 49.9 (C-17), 54.3 (C-18), 40.3 (C-19), 40.4 (C-20), 31.8 (C-21), 38.1 (C-22), 28.8 (C-23), 16.4 (C-24), 16.1 (C-25), 17.8 (C-26), 24.3 (C-27), 180.3 (C-28), 17.7 (C-29), 21.6 (C-30).

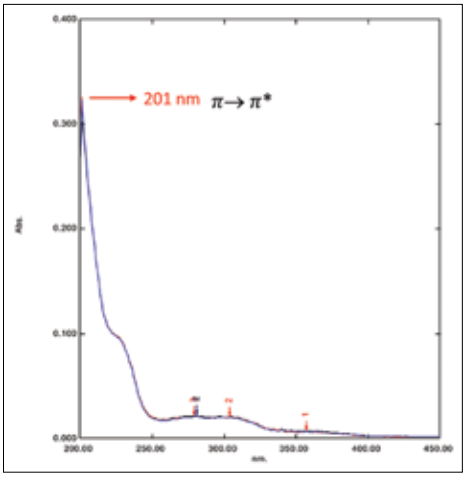

Figure S1: The UV-Vis spectrum of compound 1

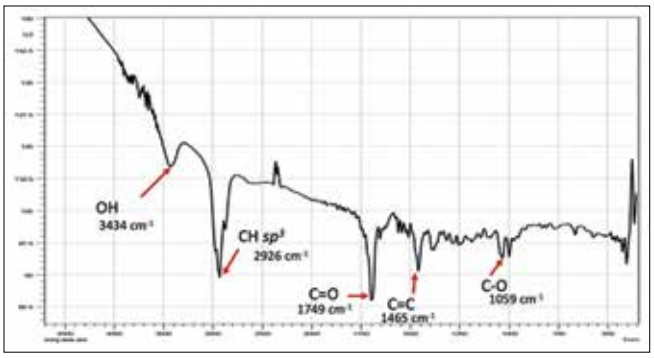

Figure S2: Infrared spectrum of compound 1

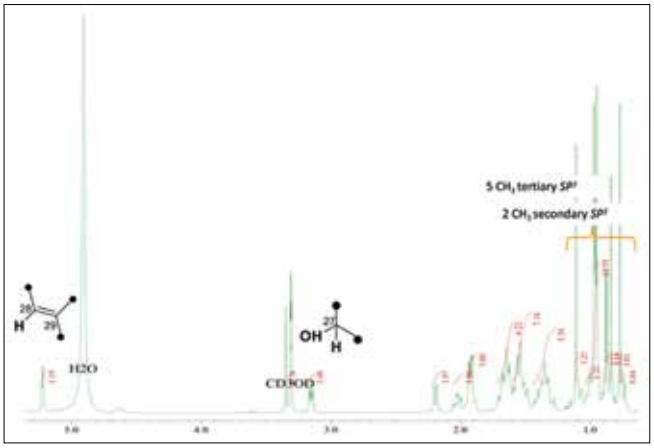

Figure S3: ^{1}H-NMR spectrum of compound 1 (in methanol)

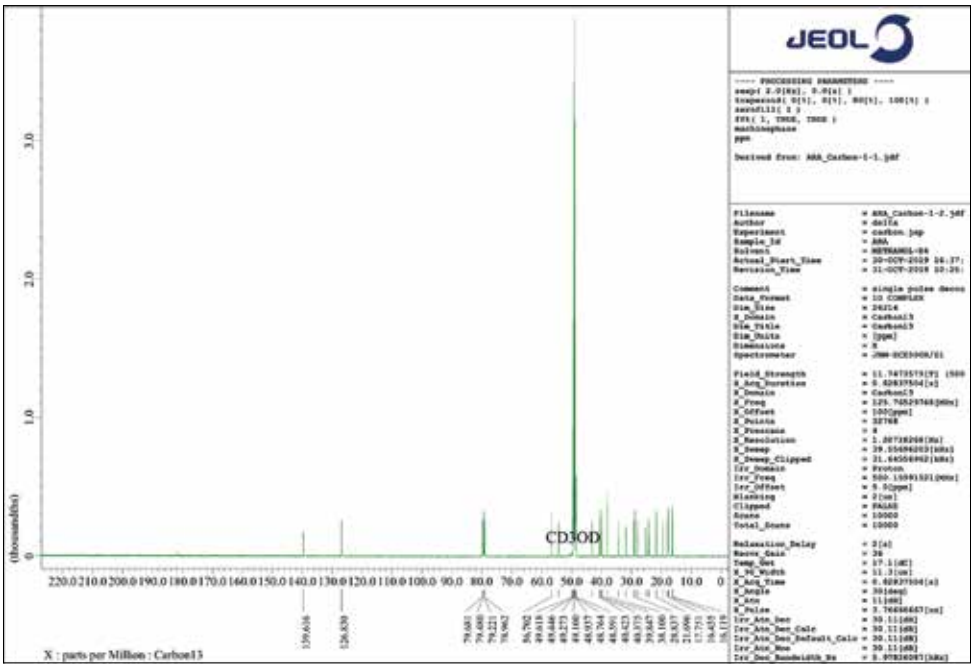

Figure S4: ^{13}C-NMR spectrum of compound 1 (in CH^3OD and $CDCl^3$)

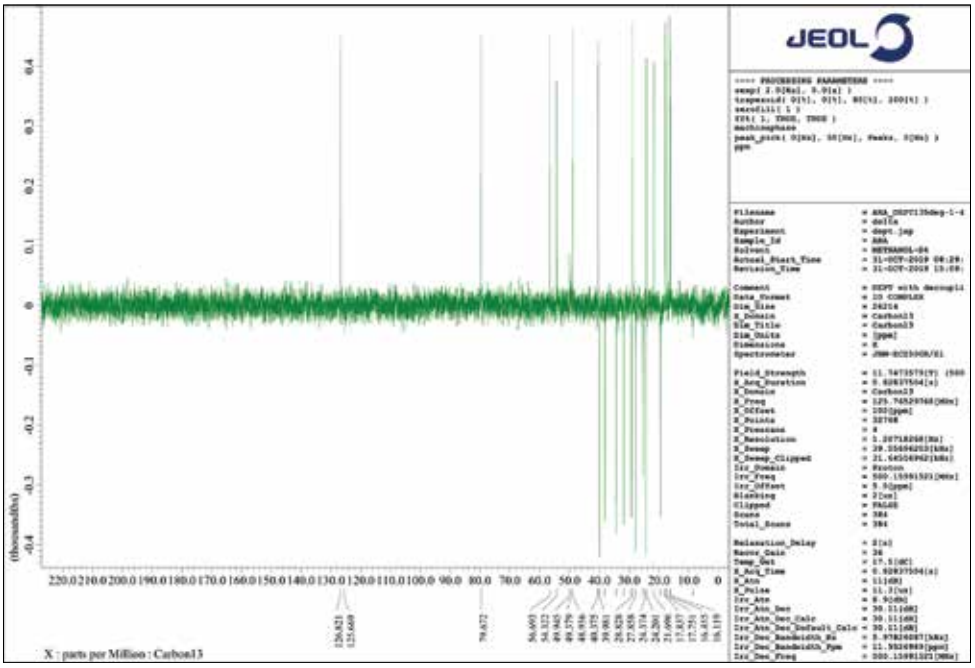

Figure S5: DEPT-NMR spectrum of compound 1 (in CH3OD and CDCl3)

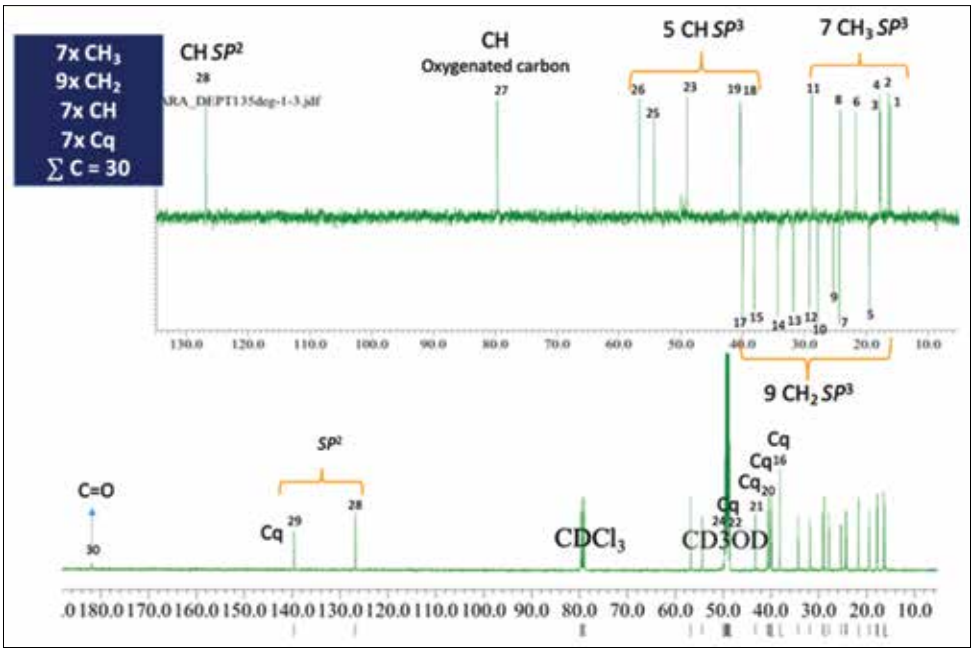

Figure S6: ^{13}C-NMR and DEPT-NMR spectrum of compound 1 (500MHz, in CH3OD and $CDCl_3$)

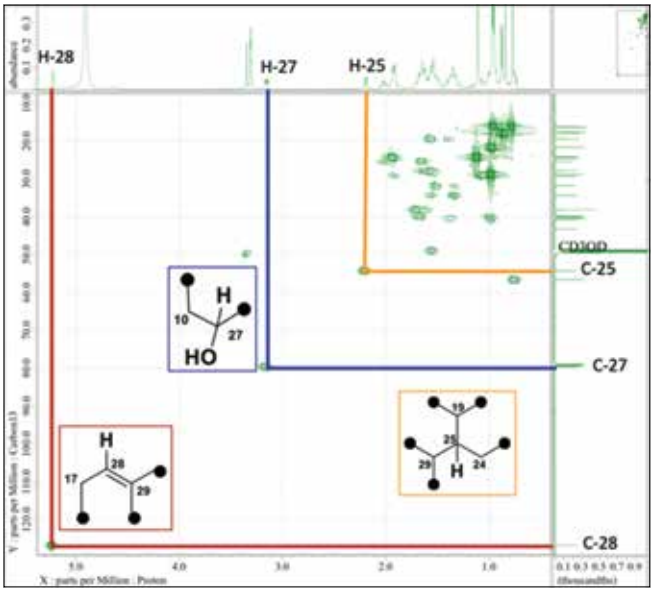

Figure S7: HMQC spectra of compound 1

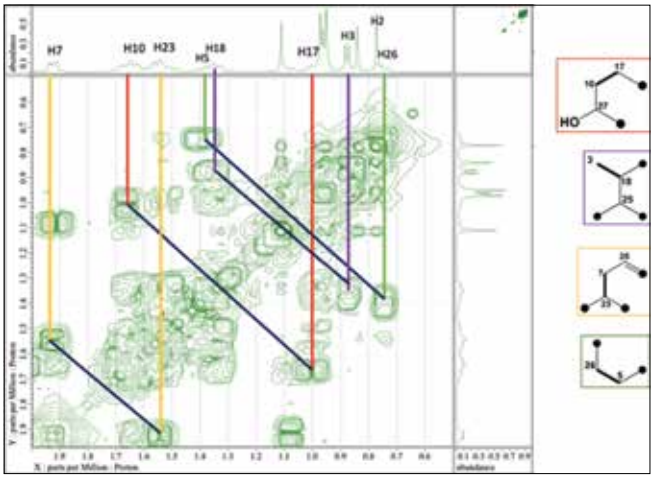

Figure S8: COSY spectra of compound 1

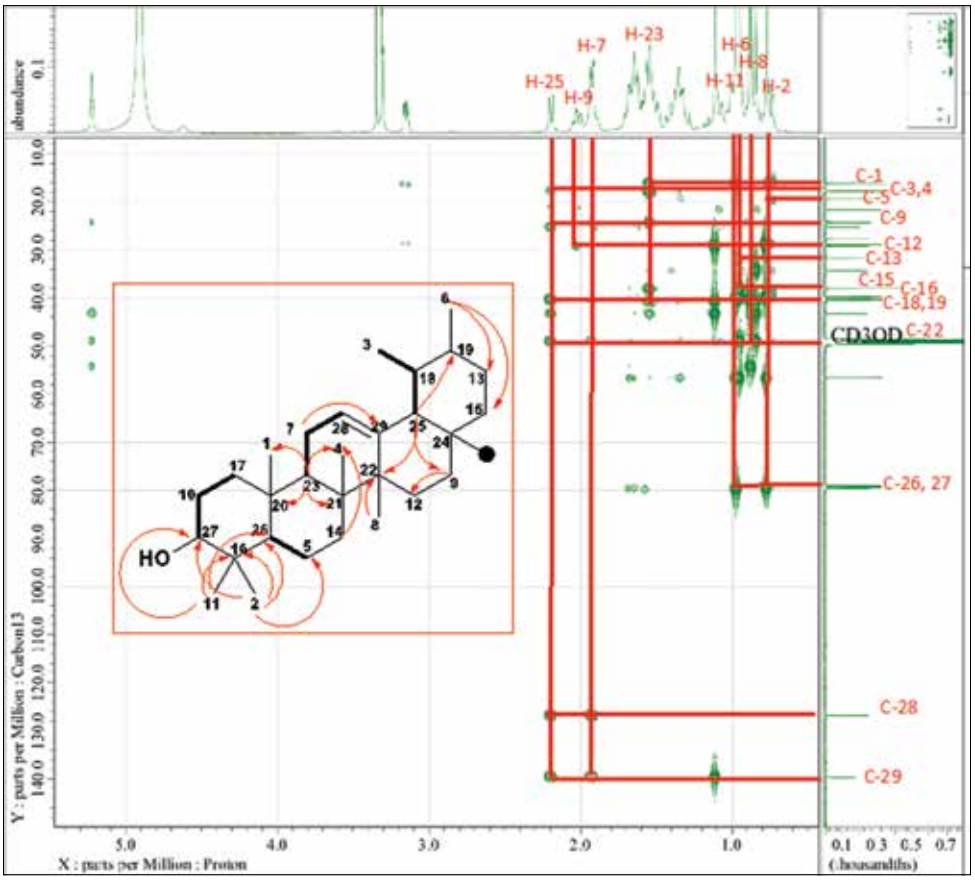

Figure S9: HMBC spectra of compound 1

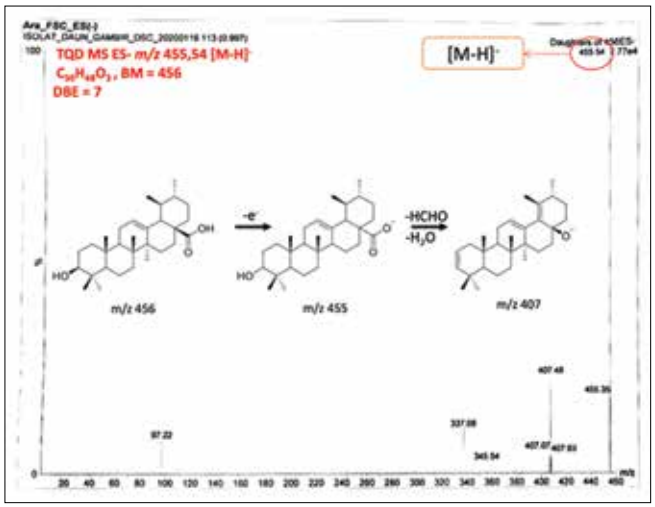

Figure S10: MS spectra of compound 1