World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Available online at: http://www.wjpsonline.org/
Original Article

Microbial transformation of the labdane diterpene 13-*epi*-cupressic acid

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Received: 15-03-2018 / Revised Accepted: 30-04-2018 / Published: 01-05-2018

ABSTRACT

The labdane diterpene, 13-*epi*-cupressic acid **1**, was isolated from the *n*-hexane-chloroform extract of *Araucaria heterophylla* resin. Of the 20 fungi screened for possible transformation, only four showed to produce metabolites including 13, 14, 15-trihydroxy-8(17)-labden-19-oic acid **2**, 7α,13-dihydroxy-8(17),14-labdadien-19-oic acid **3**,13-*epi*-cupressic acid β-glucosyl ester **4** and *iso*-communic acid **5** by *Cunninghamilla echiulata* NRRL 1382 , *Aspergillus restrictus* NRRL 2869, *Coriolus hirsutus* ATCC MYA-828 and *Cordyceps sinclairii* ATCC 24400, respectively. The identity of the isolated compounds was confirmed using 1D and 2D NMR and MS spectroscopies. Compound **4** is a new metabolite while **5** is first time being produced by biotransformation technique. Compound **3** was proved to be a potent anti-inflammatory agent through selective inhibition of COX-2 enzyme assay.

Keywords: *Araucaria heterophylla*, 13-*epi*-cupressic acid, biotransformation, COX inhibitors.

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How to Cite this Article: Amal F. Soliman, Zein M. Naeem, Ashraf T. Khalil, Kuniyoshi Shimizu, Saleh H. El-Sharkawy. Microbial transformation of the labdane diterpene 13-*epi*-cupressic acid. World J Pharm Sci 2018; 6(5): 61-69.

INTRODUCTION

Species of the genus *Araucaria* are rich in labdane diterpenes [1-4]. The nature of these compounds thought to possess anti-ulcerogenic effect in experimental animals [5, 6]. 13-*Epi*-cupressic acid **1** is a labdane diterpene found in *Araucaria heterophylla* resin as a major compound [6]. Microbial transformation can selectively introduce functional groups to the carbon skeleton of compound **1** resulting in more bioactive metabolites which are difficult to be obtained chemically [7].

Prolonged use of non-selective non-steroidal anti-inflammatory drugs (NSAIDs) resulted in severe side effects such as gastrointestinal hemorrhage due to inhibition of COX-1 enzyme [8], while most of the COX-2 selective drugs have been found to cause cardiovascular problems [9]. Consequently, there is a strong need for natural anti-inflammatory products with minimum side effects. This work describes the utilization of microorganisms for production of new metabolites from compound **1.** The obtained metabolites were evaluated for potential anti-inflammatory activity using selective COX-2 inhibitory assay.

MATERIALS AND METHODS

General: ^{1}H-NMR and ^{13}C-NMR spectra were recorded on BRUKER Ascend^{TM}400 spectrometer, BRUKER DRX 600 NMR spectrometer or Joel 500 MHz ^{TM}spectrometer using $CDCl_3$, CD_3OD or DMSO-*d6*solvents and TMS as internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with reference to TMS resonance. Infrared (IR) spectra were obtained by using Mattson 5000 FTIR (England). FAB-MS and HR-FAB-MS data were determined using LC-MS-IT-TOF (Shimadzu, Tokyo, Japan). Normal phase chromatography was carried out using silica gel 60-230 mesh (Merck, Germany) packed by the wet method in the specific solvents. The solvents used for extraction and chromatographic separation were purchased from El-Nasr Company for Pharmaceutical Chemicals, Egypt. Analytical thin layer chromatography was performed on pre-coated silica gel 60 GF_{254} on aluminum sheets (Merck, Germany). Plates were developed in different solvent mixtures and the developed chromatograms were visualized under UV light 254 and 366 and the spots were made visible by spraying with *p*-anisaldehyde spray reagent (composed of 0.5 ml *p*-anisaldehyde in 50 ml glacial acetic acid and 1 ml 97% H_2SO_4) after warming in an oven preheated to 105 ^{0}C for 1 min. Enzymes α- and β- glucosidase (Sigma-Aldrich, USA) were used for hydrolysis of compound **4**.

Plant material: The resin exudates from the stems of *Araucaria heterophylla* Salisb were collected from Mansoura University Gardens, Mansoura, Egypt in February 2015. The plant identity was kindly confirmed by staff members at Department of Horticulture, Faculty of Agriculture, Mansoura University, Egypt. A representative specimen was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University (AH-2-2015).

Isolation of 13-epi-cupressic acid: One kilogram of the resin was extracted with chloroform: *n*-hexane 1:1,v/v. The extract was evaporated under reduced pressure to give 500 g of a sticky brown translucent residue. About 50 g of the residue was chromatographed on a silica gel G 60 column (4.5 × 100 cm) and eluted first with *n*-hexane, then *n*-hexane/EtOAc mixtures (1% EtOAc step) . Fractions of 250 ml each were collected and monitored by silica gel GF_{254} TLC plates sprayed with *p*-anisaldehyde reagent. Compound **1** was obtained as yellow viscous liquid in 15% yield, R_f 0.59 in EtOAc- *n*-hexane (3: 7) solvent system using a normal phase TLC.

Microorganisms: Microorganisms were obtained from ATCC, NRRL and NBRC and were stored in Potato Dextrose Agar (PDA) medium at 4^{o}C. Screening procedures to transform compound **1**, were carried out on the following 20 microorganisms; *Cunnighamella echinulata* (NRRL 1382), *Aspergilus alliaceous* (NRRL 315), *Aspergilusniger* (NRRL 328), *Aspergilus ochraceous* (NRRL 398), *Cunninghamella blackesleeana* (NRRL1369), *Cunninghamella elegans* (NNRL 1392), *Penicillium vermiculatum* (NRRL 1009), *Aspergillus restrictus* **(**NRRL 2869**),** *Gymnascella citrina* (NRRL 6050), *Rhodotorula rubra* (NRRL 1592; Northern Regional Research Laboratory**,** Assuit University Mycological Centre, Egypt), *Aspergilus flavipes* (ATCC 11013), *Rhizopus species* (ATCC 36060), *Coriolus hirsutus* **(**ATCC MYA-828)**,** *Cordyceps sinclairii* **(**ATCC 24400**),** *Coriolus versicolor* (ATCC 48242), *Phlebia firma* (ATCC 64378), *Cordyceps gracilis* (ATCC 34498), *Hericium coralloides* (ATCC 52796), *Cordyceps ophioglossoides* (ATCC 36865; American Type Culture Collection, Tokyo, Japan)and *Daedalea malicola* (NBRC 4978; Biological Resource Centre, Tokyo, Japan).

Cunnighamella echinulata (NRRL 1382), *Aspergillus restrictus* **(**NRRL 2869**)**, *Coriolus hirsutus* **(**ATCC MYA-828) and *Cordyceps sinclairii* **(**ATCC 24400**)** were selected for large scale fermentation as they reproducibly form metabolites.

Screening procedures: The screening process was carried out by using two stages fermentation protocol [10] in liquid medium composed of: Dextrose 20g , peptone 5g, yeast extract 5g, NaCl 5g, K_2HPO_4 5g and distilled water to 1000 ml. The pH adjusted at 6.8 using 6N HCl [11] before autoclaving for 20 min. at 121^{0}C and 15 psi.

Stage I cultures were initiated by transferring different microbial cells from fresh slants into 125 ml Erlenmeyer flasks containing 25 ml sterile liquid medium and allow to grow for 72 h. at 27^{0}C on a gyratory shaker (New Brunswick Scientific Co., INC. Edison, U.S.A.) operating at 200 rpm.

Stage II cultures were initiated by transferring 5 ml of stage I culture to other 125 ml Erlenmeyer flasks containing 25 ml of fresh liquid medium. Cultures were allowed to grow for 24 h. before the addition of substrate **1** (5 mg to each flask) which was dissolved in 50 ul of DMSO. About 1 ml samples was periodically withdrawn from each culture (12, 24, 36 and 48 hours and every day till the end of two weeks) , extracted with 1 ml EtOAc, the extracts were chromatographed on silica gel 60 GF_{254} TLC plates using different solvent systems and the spots were made visible by spraying with *p*-anisaldehyde spray reagent. Transferring of cultures and inoculations were performed in a horizontal laminar flow (Holten TL2448, Denmark).

Large scale fermentation and isolation of metabolites: About 500 mg of substrate **1** was dissolved in 400 µl DMSO and equally divided among 10 flasks each containing a 100 ml of culture media and held on a gyratory shaker at 200 rpm at 27 ^{0}C. At the end of incubation period, the reaction was stopped by EtOAc extraction (1Lx3), and the combined solvent extracts were evaporated under reduced pressure. The dried residue (300-500 mg) was chromatographed on silica gel column, eluted with *n*-hexane then EtOAc /*n*-hexane gradient elution of increasing polarity. Similar fractions were pooled (by silica gel GF_{254} TLC plates, visualized by *p*-anisaldehyde as spray reagent) and evaporated to dryness.

Metabolite **2** was obtained after 8 days of incubation with *Cunninghamella echinulate* NRRL 1382 and eluted with 40%EtOAc /*n*-hexane from the chromatographic column.
Metabolite **3** was produced after 5 days of incubation with *Aspergillus restrictus* NRRL 2869 and eluted with 30%EtOAc /*n*-hexane from the chromatographic column.
Metabolite **4** was obtained after 10 days of incubation using *Coriolus hirsutus* ATCC MYA-828 and eluted with 100% EtOAc from the chromatographic column.
Metabolite **5** was produced after 12 days incubation with *Cordyceps sinclairii* ATCC 24400 and eluted with 3% EtOAc/*n*-hexane from the chromatographic column.

In vitro COX-1 and COX-2 enzyme inhibitory assay: The abilities of the tested compounds **1-5** to inhibit the conversion of arachidonic acid to PGH2 were evaluated using COX-1 Cayman human enzyme inhibitory assay kit (No. 701070), COX-2 Cayman human enzyme inhibitory assay kit (No.701080, USA) and ROBONIK P2000 EIA reader. Evaluation of the data was performed by using Four Parameter Logistic Curve online data analysis tool of MyAssays Ltd. Procedures were carried out according to manufacturer's instructions [12, 13]. Celecoxib® (Sigma-Aldrich, USA) was used as reference drug. The selectivity indices (SI) of the tested/reference compounds (SI = IC_{50} COX-1 / IC_{50} COX-2) were calculated [14, 15].

RESULTS AND DISCUSSION

13-*Epi*-cupressic acid **1** was extracted with *n*-hexane-chloroform (1:1, v/v) from the resins of the local *Araucaria heterophylla* (Araucariaceae) trees. The obtained extract was evaporated to dryness, About 50 g was loaded on top of a silica gel column, eluted with 6% EtOAc/*n*-hexane mixture, purified by re-chromatography, and chemically identified by using different spectroscopic techniques. The obtained data were consistent with reported data [6]. Of 20 fungi screened for their abilities to catalyze the bioconversion of **1**, only four fungal species including *C. echinulata* (NRRL 1382), *A. restrictus* **(**NRRL 2869**)**, *C. hirsutus* **(**ATCC MYA-828) and *C. sinclairii* **(**ATCC 24400**)** were able to transform **1** into four metabolites. These fungal species were selected for large scale fermentation as they reproducibly form the metabolites. The incubation mixtures were terminated after 5-12 days, extracted by organic solvent, concentrated and silica gel column chromatographed to afford the pure metabolites and subjected to analysis by MS and 1D, 2D NMR spectroscopies.

Compound **2** was obtained (105 mg, 21% yield, without optimization) after incubation for 8 days with *C. echinulata*. The obtained extract was concentrated and purified by silica gel column chromatography. The isolated compound was subjected to NMR analyses. It was shown that most of the ^{1}H-NMR and ^{13}C-NMR spectral data of **2** were similar to those of **1**(Table 1) except for the absence of the olefinic $\Delta^{14,15}$ signals at δ_C145.0 and 111.5and the appearance of two new signals at δ_C76.8 and 63.0 indicating hydroxylation at C-14 and C-15 positions. This resulted in upfield shifts at positions 12 and 16 by δ_C 3.2 and 6.8 ppm, respectively. FAB-MS spectrum of **2** showed a deprotonated molecular ion peak at *m/z* 353 [M-H], corresponding to a molecular formula $C_{20}H_{34}O_5$. Compound **2** was identified without any doubt as 13, 14, 15-trihydroxy-8(17)-labden-19-oic acid. This triol derivative is suggested to be produced through epoxidation of the $\Delta^{14,15}$ double bond followed by hydration of the epoxide moiety (Figure 2).

Compound **3** was obtained (72 mg, 14.4% yield, without optimization) after 5 days incubation of **1** with *A. restrictus.* The obtained extract was concentrated and purification by silica gel column chromatography. The isolated compound was subjected to NMR spectral analyses. The obtained spectral data of **3** showed that all the signals were closely consistent with the corresponding signals of **1**(Table 1) except the appearance of signals at δ_H-7 (4.29) and δ_C-7(74.8) indicating α-hydroxylation at C-7 (NOESY experiment) and consequently the downfield shifts of C-6 (δ_C 33.9), C-8 (δ_C 150.9) and C-17 (δ_C 109.7). In addition to the upfield shift of both C-5 (δ_C 48.5) and C-9 (δ_C 50.1). HMBC spectrum showed that the new oxygenated H-7 (δ_H 4.70) correlated with C-8 and C-17. FAB-MS spectral data showed a molecular ion peak at *m/z* 335 $[M-H]^-$, corresponding to a molecular formula $C_{20}H_{32}O_4$. The obtained results from spectral analyses were consistent with previously reported data[16].The chemical identity of Compound **3** was proved to be 7α,13-dihydroxy-8(17),14-labdadien-19-oic acid. It is worth to mention that compounds **2** and **3** were previously isolated by incubation of **1** with a different microorganism, *Fusarium graminearum* [16].

Compound **4** was obtained as a white amorphous powder (90 mg, 18% yield without optimization) after incubation of **1** for 10 days with *Coriolus hirsutus*. The obtained extract, was concentrated and purified on silica gel column chromatography. The isolated compound was subjected to spectral analyses.[1]H, [13]C-NMR and DEPT spectra of **4** were compared with those of **1** (Table 1). Compound **4** showed six new oxygenated carbons, 5 CHs and 1 CH_2, at δ_c 95.5 (C-1'), 72.1 (C-2'), 78.7 (C-3'), 72.2 (C-4'), 78.5 (C-5') and 62.5 (C-6') respectively, which indicated the introduction of hexapyranosyl moiety. [1]H-NMR spectra showed the distinctive doublet signal of the anomeric proton of the sugar at δ_H 5.4 (1H, *J*=7.2 Hz) and the new overlapping signals in the region 3.3-3.4 ppm which were assigned to H-2'-H-5' of the sugar. In addition to two double doubles at δ_H 3.67 (1H, *J*=12, 4.8 Hz) and 3.80 (1H, *J*=12, 1.8 Hz) assigned for the two protons at C-6' of the sugar. The hexapyranosyl moiety was identified as D-β-glucose by acid and enzymatic hydrolyses of **4** as well as co-chromatography with authentic sugars. β-configuration of the anomeric carbon was confirmed through the presence of relatively large coupling constant of the anomeric H (*J*= 7.2 Hz)[17] and by the enzymatic hydrolysis by β-glucosidase enzyme. HMBC experiment showed correlation between the anomeric proton of glucose and C-19 at δ_C 177.6. An upfield shift was observed at C-19 of compound **1**(δ_C183.0) to δ_C 177.6 in compound **4**(about 5.4 ppm) which confirmed the glucosylation of **1** at the COOH group. HR-FAB-MS showed a deprotonated molecular ion peak at *m/z* 481.2800 $[M-H]^-$, corresponding to a molecular formula $C_{26}H_{42}O_8$. Consequently, compound **4** was identified as 13-*epi*-cupressic acid β-glucosyl ester. This is the first report to indicate the isolation of this new compound from biotransformation of 13-*epi*-cupressic acid.

Compound **5** was obtained (25 mg, 5% yield without optimization) after 12 days preparative scale incubation of **1** with *Cordyceps sinclairii*. After extraction, concentration and purification on silica gel column chromatography, the isolated compound has been subjected to NMR spectral analyses.[1]H- and [13]C-NMR spectral data of **5** was compared to those of **1** (Table 1). The obtained data revealed the presence of a new double bond $\Delta^{13,16}$ at δ_C147.0 (C-13) and 115.5(C-16). This conclusion confirmed by the disappearance of the C-16 methyl singlet at δ_H 1.30 and the appearance of two new olefinic proton signals at position 16 corresponding to δ_H 4.98 and 4.93 ppm. Furthermore,C-12 and C-14 were upfield shifted by 11.3 and 6 ppm respectively. HR-FAB-MS spectrum of compound **5** showed a protonated molecular ion peak at *m/z* 303.2310 $[M+H]^+$, corresponding to a molecular formula $C_{20}H_{30}O_2$. Compound **5** was identified as *iso/mirceo*-communic acid or 8(17), 13(16), 14-labdatriene-19-oic acid. This triene derivative is being isolated for the first time from biotransformation of **1**. It is suggested that *iso*-commuic acid may be produced through dehydration (removal of OH of C-13 and H of C-12) of 13-*epi*-cupressic acid (Figure 3) giving *trans*-communic acid, followed by isomerization to *iso*-communicacid where the C-12(13) double bond moved to C-13(16) [18-20].

The anti-inflammatory activity of 13-*epi*-cupressic acid **1**and its metabolites (compounds **2-5**) was evaluated by measuring their ability to inhibit COX-1 and COX-2 enzymes and comparing their selectivity indices (SI = IC_{50} COX-1 / IC_{50} COX-2) with that of a reference compound, celecoxib. The results (Table 2, Figure 4) showed that compound **1** presented good anti-inflammatory action (SI=9.82).

The isolated metabolites showed selectivity indices (SI) **2** (0.38), **3**(16.22), **4**(0.98) and **5**(1.24). These results showed that the terminal $\Delta^{14,15}$double bond, the free C_{13}-α-OH and the free C_{19}-COOH are essential for the anti-inflammatory activity using COX assay. The loss of $\Delta^{14,15}$double bond as in compound **2**, the esterification of C_{19}-COOH as in compound **4** and the loss of the free C_{13}-α-OH as in compound **5**, resulting in the loss of selective COX-2 inhibitory activity. However, compound **3** showed a more potent anti-inflammatory effect, as it showed about double the activity of **1**. This might be attributed to C-7 hydroxylation. It is worth to mention that *trans*- communic acid was reported to have selective COX-2 inhibitory activity [21], while in this report *iso*-communic acid showed COX-1 inhibitory activity.

CONCLUSION

Four metabolites were obtained through biotransformation of 13-*epi*-cupressic acid. The glucosylated derivative, 13-*epi*-cupressic acid β-glucosyl ester, and *iso*-communic acid were being isolated for the first time by biotransformation of 13-*epi*-cupressic. By evaluation of the anti-inflammatory activity using COX inhibitory assay, 13-*epi*-cupressic acid showed a potent activity. The structure-activity relationship of 13-*epi*-cupressic acid indicated that the terminal $\Delta^{14,15}$double bond with the adjacent free OH group at C-13 and the free COOH group (C-19) were essential for the anti-inflammatory activity of 13-*epi*-cupressic acid. In addition, the introduction of free OH group at C-7 (compound **3**) almost doubled the anti-inflammatory activity.

Table (1): ^{13}C-NMR and ^{1}H-NMR spectral data of compounds 1-5 (δ_C and δ_H in ppm and (*J*) in Hz).

C	1[a]		2[b]		3[c]		4[c]		5[d]	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1	39.2		39.3		40.3		40.4		39.9	
2	19.9		20.1		21.2		21.1		19.8	
3	38.0		38.7		39.4		39.2		37.9	
4	44.2		43.8		44.8		45.7		44.1	
5	56.4		55.8		49.7		57.9		56.1	
6	26.0		26.4		33.9		27.3		26.0	
7	38.4		39.2		74.8	4.29 t (3)	39.9		38.7	
8	148		148.8		150.9		149.6		147.9	
9	56.7		56.8		51.6		58.1		56.6	
10	40.7		40.7		42.1		41.8		40.4	
11	17.9		17.1		18.7		19.2		22.1	
12	41.5		38.3		41.7		42.6		30.2	
13	73.6		73.5		74.2		74.1		147.0	
14	145.0	5.95 dd (12, 16)	76.8	3.31*	146.6	5.90 dd (10.8, 17.4)	146.6	5.87 dd (10.8, 17.4)	139.0	6.34 dd (10.5, 17.5)
15	111.5	5.08 (16) 5.24d(12)	63.0	3.70* 3.30*	111.9	5.01 dd (1.8, 17.4) 5.18 dd (1.8, 10.8)	111.9	5.01dd (1.8, 17.4) 5.18 dd (1.8, 10.8)	113.2	5.04 d (17.5) 5.20 d (10.5)
16	29.2	1.30 s	22.4	0.96 s	29.3	1.17 s	29.4	1.23 s	115.5	4.98 brs 4.93 brs
17	106.6	4.56brs 4.87brs	106.8	4.54 brs 4.81 brs	109.7	4.16 brs 4.53 brs	107.2	4.54 brs 4.82 brs	106.4	4.54 s 4.85 s
18	27.6	1.26 s	29.1	1.13 brs	27.5	1.23 s	27.4	1.22 s	28.9	1.22 s
19	183.0		182.0		181.5		177.6		183.6	
20	12.7	0.63 s	13.1	0.56	12.4	0.60 s	13.8	0.60 s	12.8	0.54 s

1'							95.5	5.42 d (7.2)		
2'							72.1			
3'							78.7			
4'							72.2			
5'							78.5			
6'							62.5	3.67 dd (4.8, 12) 3.80 dd (1.8, 12)		

*overlapped.
[a]^{1}H-NMRand ^{13}C-NMRwere measured in $CDCl_3$ at 400 MHz and 100 MHz respectively.
[b]^{1}H-NMRand ^{13}C-NMRwere measured in DMSO-d_6 at 400 MHz and 100 MHz respectively .
[c]^{1}H-NMRand ^{13}C-NMRwere measured in CD_3OD at 600 MHz and 150 MHz respectively.
[d]^{1}H-NMRand ^{13}C-NMRwere measured in $CDCl_3$ at 500 MHz and 125 MHz respectively.

Table (2): Selectivity indices of compounds 1-5/ celecoxib.

Compound	COX-1 IC_{50} (nM/ml)	COX-2 IC_{50} (nM/ml)	SI= (COX-1 IC_{50}/ COX-2 IC_{50})
Comp. 1	95.3	9.7	9.824
Comp. 2	27.02	71.8	0.376
Comp. 3	114.5	7.04	16.221
Comp. 4	100.47	102.4	0.981
Comp. 5	48.9	39.3	1.244
Celecoxib	78.4	27.23	2.879

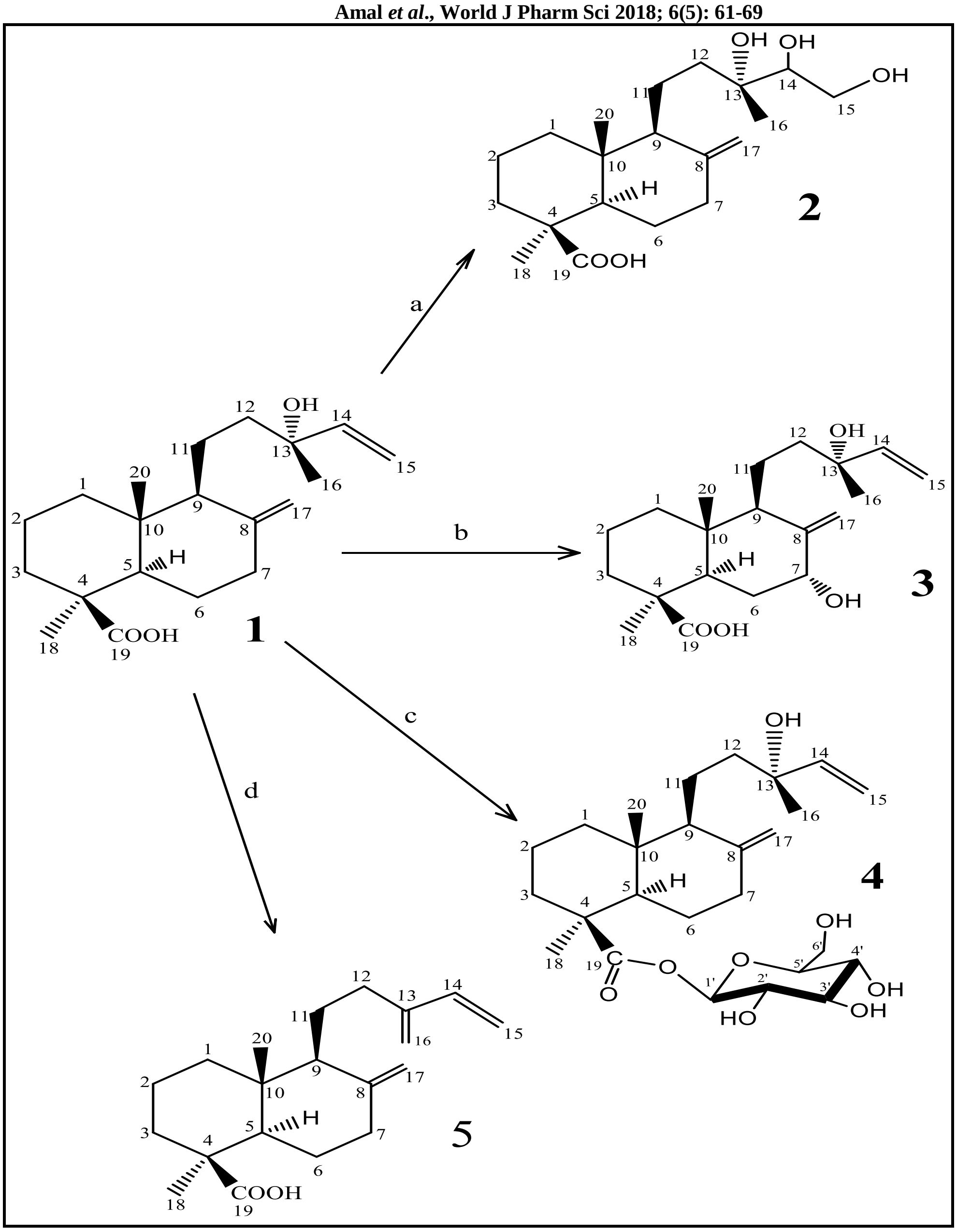

Figure (1): Schematic presentation of microbial transformation of 1.
a:*Cunnighamilla echinulata* (NRRL 1382), b:*Aspergillus restrictus* (NRRL 2869), c:*Coriolus hirsutus* (ATCC MYA- 828), d:*Cordyceps sinclairii* (ATCC 24400).

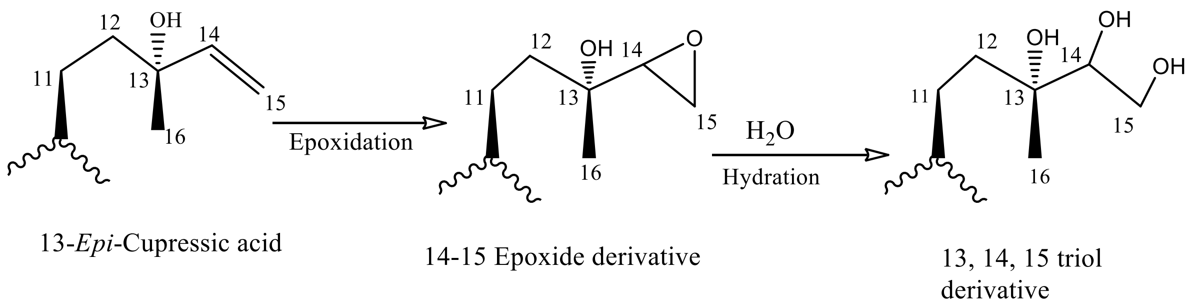

Figure (2): Proposed mechanism of microbial transformation of 1 to 2.

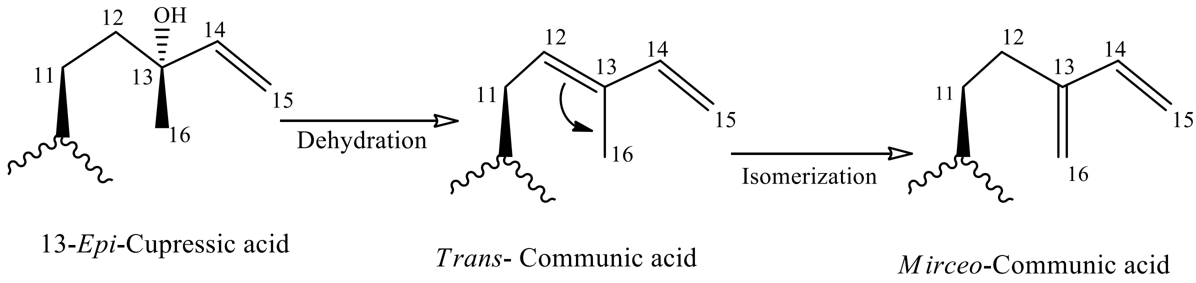

Figure (3): Proposed mechanism of microbial transformation of 1 to 5.

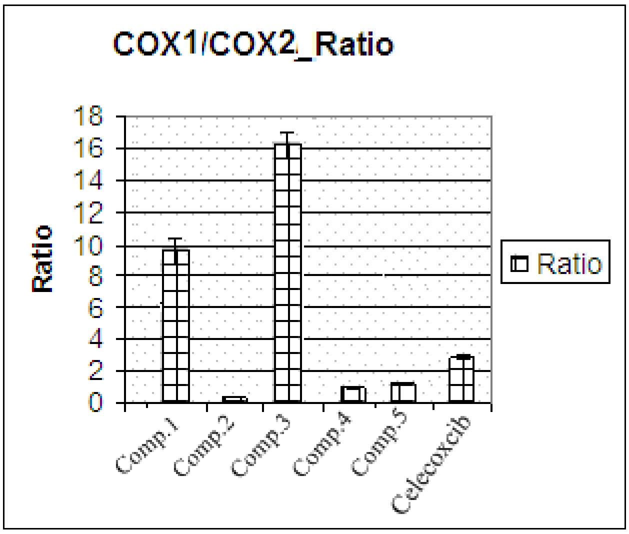

Figure (4): Selectivity indices of compounds 1-5 compared to celecoxib.

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