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# New Neuroprotective Derivatives of Cinnamic Acid by Biotransformation

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**Abstract:** Microbial transformation is extensively utilized to generate new metabolites in bulk amounts with more specificity and improved activity. As cinnamic acid was reported to exhibit several important pharmacological properties, microbial transformation was used to obtain new derivatives with enhanced biological activities. Manipulating the 2-stage fermentation protocol of biotransformation, five metabolites were produced from cinnamic acid. Two of them were new derivatives; N-propyl cinnamamide **2** and 2-methyl heptyl benzoate **3** produced by *Alternaria alternata*. The other 3 metabolites; *P*-hydroxy benzoic acid **4**, cinnamyl alcohol **5** and methyl cinnamate **6** were produced by *Rhodotorula rubra*, *Rhizopus* species and *Penicillium chrysogeneum*, respectively. Cinnamic acid and its metabolites were evaluated for their cyclooxygenase (COX) and acetylcholinesterase (AChE) inhibitory activities. Protections against $H_2O_2$ and A$\beta_{1\text{-}42}$ induced-neurotoxicity in human neuroblastoma (SH-SY5Y) cells were also monitored. Metabolite **4** was more potent as COX-2 inhibitor than the parent compound with $IC_{50}$ value of 1.85 ± 0.07 μM. Out of the tested compounds, only metabolite **2** showed AChE inhibitory activity with $IC_{50}$ value of 8.27 μM. These results were further correlated to *in silico* study of the binding interactions of the active metabolites with the active sites of the studied enzymes. Metabolite **3** was more potent as neuroprotective against $H_2O_2$ and A$\beta_{1\text{-}42}$ induced-neurotoxicity than catechin and epigallocatechin-3-gallate as positive controls. This study suggested the two new metabolites **2** and **3** along with metabolite **4** as potential leads for neurodegenerative diseases associated with cholinergic deficiency, neurotoxicity or neuroinflammation.

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**Keywords**: Cinnamic acid, biotransformation, COX inhibitors, Cholinesterase inhibitors.

## Supporting information

### *1. Extraction and isolation of cinnamic acid (1)*

Cinnamon bark powder (3.5 kg) was extracted by 95% ethanol (30 L) until exhaustion in cool dark place. The extract was evaporated under vacuum using rotatory evaporator, leaving a residue of 400 mg which was hydrolyzed by hydrochloric acid and subjected to liquid–liquid fractionation using chloroform. The chloroform fraction was dried by rotary evaporator at 30°C. The chloroform residue (30 g) was chromatographed using open glass column packaged by silica gel F60 (3.5 i.d. x 60 cm) using gradient elution with solvent system chloroform-methanol. Column effluents were monitored using TLC aluminum sheet silica gel (60F254). Cinnamic acid was eluted by chloroform-methanol (9.5:0.5). About 250 mg of pure crystals of cinnamic acid were obtained after repeated crystallization with methanol and its chemical identity was confirmed by co-chromatography, mp (133-135) °C [1,2] and different spectroscopic technique. Commercial cinnamic acid 97% was purchased from (SIGMA-ALDRICH, Missouri, USA).

### *2. α -medium composition*

The screening process was carried out by adapting two stages aerobic fermentation protocol [2, 3] using α -medium [4] which consisted of: Dextrose 20 g, yeast extract 5 g, peptone 5 g, NaCl 5 g, $K_2HPO_4$ 5 g and distilled water to 1000 mL, with pH adjusted at 6.8 using 6N HCl.

### *3. Isolation of metabolites*

a. ***Isolation of metabolite 2***

After incubation of cinnamic acid with *A. alternata* (AUMC 150207) for 48 h, the fermentation broth was extracted with ethyl acetate. EtOAc extract was dried and evaporated under reduced pressure to yield 250 mg of brown residue. This residue was column chromatographed on silica gel (15 x 1 cm i.d., 15 g). Gradient elution was used, starting with chloroform then increasing proportions of methanol in chloroform. Fractions eluted by 4% $CH_3OH$ in $CH_2CL_2$, collected, pooled, and evaporated to give a pure metabolite **2** (65 mg). Silica gel GF254 TLC plate showed quenching at $R_f$ = 0.23 using 5% $CH_3OH$ in $CH_2CL_2$ by UV light (λmax254).

b. ***Isolation of metabolite 3***

After incubation of cinnamic acid with *A. alternata* (AUMC 150207) for 48 h, the fermentation broth was extracted with ethyl acetate. EtOAc extract was dried and evaporated under reduced pressure to yield 250 mg of brown residue. This residue was column chromatographed on silica gel (15 x 1 cm i.d., 15 g). Gradient elution was used, starting with petroleum ether then increasing proportions of methylene chloride in petroleum ether. Fractions eluted by 20% $CH_2CL_2$ in petroleum ether , collected, pooled, and evaporated to give a pure metabolite **3** (20 mg). Silica gel GF254 TLC plate showed quenching at $R_f$ = 0.88 using 5% $CH_3OH$ in $CH_2CL_2$ by UV light (λmax254).

c. ***Isolation of metabolite 4***

After incubation of cinnamic acid with *R. rubra* (NRRL 1592) for 24h, the fermentation broth was extracted with ethyl acetate. EtOAc extract was dried and evaporated under reduced pressure to yield 250 mg of brown residue. This residue was column chromatographed on silica gel (15 x 1 cm i.d., 15 g). Gradient elution was used, starting with chloroform then increasing proportions of methanol in chloroform. Fractions eluted by 5% $CH_3OH$ in $CH_2CL_2$, collected, pooled, and evaporated to give a pure metabolite **4** (50 mg). Silica gel GF254 TLC plate showed quenching at $R_f$ = 0.32 using 5% $CH_3OH$ in $CH_2CL_2$ by UV light (λmax254).

d. ***Isolation of metabolite 5***

After incubation of cinnamic acid with *Rhizopus* species (ATCC 36060)for 6 h, the fermentation broth was extracted with ethyl acetate. EtOAc extract was dried and evaporated under reduced

pressure to yield 250 mg of brown residue. This residue was column chromatographed on silica gel (15 x 1 cm i.d., 15 g). Gradient elution was used, starting with petroleum ether then increasing proportions of methylene chloride in petroleum ether. Fractions eluted by 10% petroleum ether in $CH_2CL_2$, collected, pooled, and evaporated to give a pure metabolite **5** (67 mg). Silica gel GF254 TLC plate showed quenching at $R_f$ = 0.54 using 5% $CH_3OH$ in $CH_2CL_2$ by UV light (λmax254).

e. ***Isolation of metabolite 6***

After incubation of cinnamic acid with *P. chrysogeneum* (ATCC 9480)for 48h, the fermentation broth was extracted with ethyl acetate. EtOAc extract was dried and evaporated under reduced pressure to yield 250 mg of brown residue. This residue was column chromatographed on silica gel (15 x 1 cm i.d., 15 g). Gradient elution was used, starting with petroleum ether then increasing proportions of methylene chloride in petroleum ether. Fractions eluted by 20% $CH_2CL_2$ in petroleum ether , collected, pooled, and evaporated to give a pure metabolite **6** (55 mg). Silica gel GF254 TLC plate showed quenching at $R_f$ = 0.9 using 5% $CH_3OH$ in $CH_2CL_2$ by UV light (λmax254).

4. ***Identification of metabolites***

a. ***Identification of 2***

*A. alternata* (AUMC 150207) produced metabolite **2** **(**65 mg) in 21.6% yield after 48 hrs. of preparative scale incubation. The fermentation broth was extracted, concentrated and purified on silica gel column chromatography and the isolated pure compound was subjected to NMR spectral analyses. The obtained spectral data of **2** were found to be consistent with the corresponding signals of cinnamic acid **(Table S1and Fig. S2)** except the appearance of 3 new signals at $\delta_H$ 2.2 (H-1"), 1.6 (H-2") and 0.87 (H-3"). APT spectrum of **2** **(Fig. S3)** displayed 3 additional peaks. Two $CH_2$ groups appear at $\delta_c$ 48.6 (C-1") and $\delta_c$ 29.3(C-2") and $CH_3$ group at $\delta_c$ 17 (C-3"). Downfielded shifted carbon at $\delta_c$ 48 indicates C-N attachment. Two upfield shift were observed, at δc 166.3 (C-1) and δc 142 (C-3) from the corresponding carbon of the cinnamic acid at δc 168.9 and δc 144, respectively and downfield shift was observed at $\delta_c$ 119.5 (C-2) corresponding to $\delta_c$ 117 in cinnamic acid. This might confirm the presence of amide group in **2**. HR-FAB-MS spectrum showed $[2M+2Na+2H]^+$ at m/z 426.29983, corresponding to a molecular formula $C_{12}H_{15}NO$ consistent with $[M]^+$ at m/z 189 . The previously presented data showed unambiguously that **2** is N-propyl cinnamamide. This is the first report to indicate the isolation of this metabolite from microbial transformation.

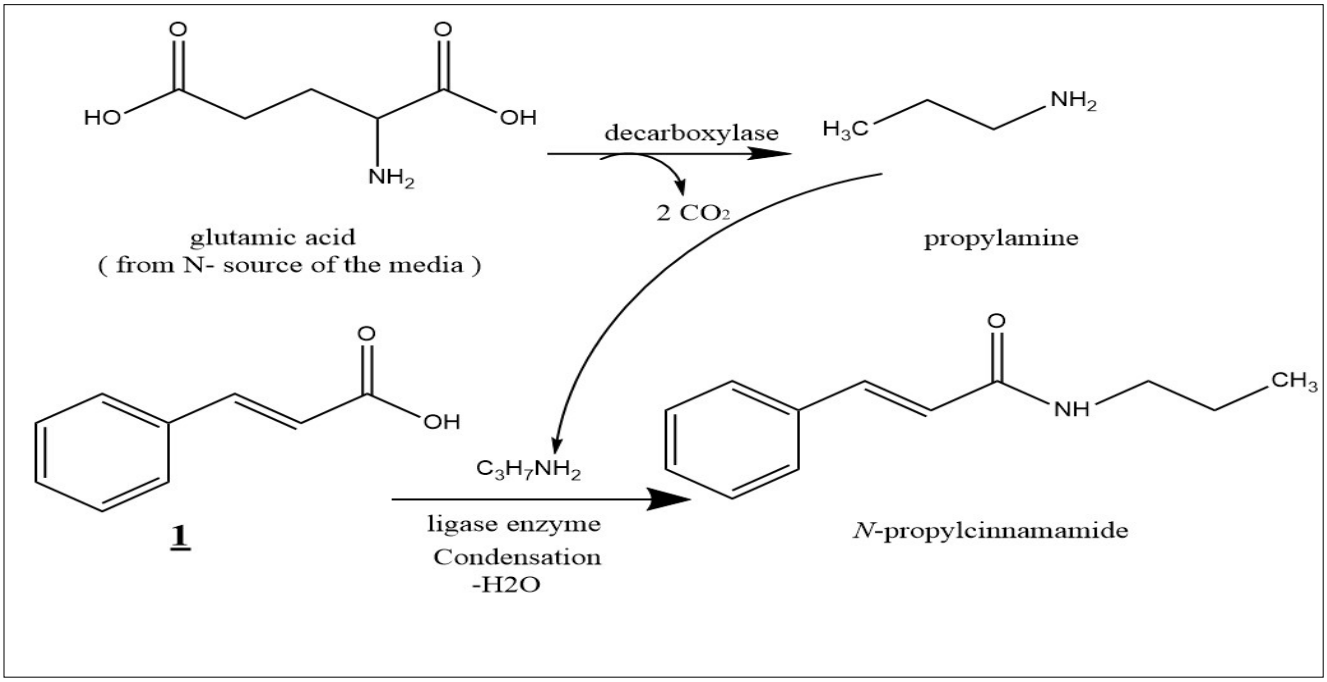


**Fig. S1. Expected mechanism of metabolite 2 formation.**

**NMR data of <u>2</u>:**

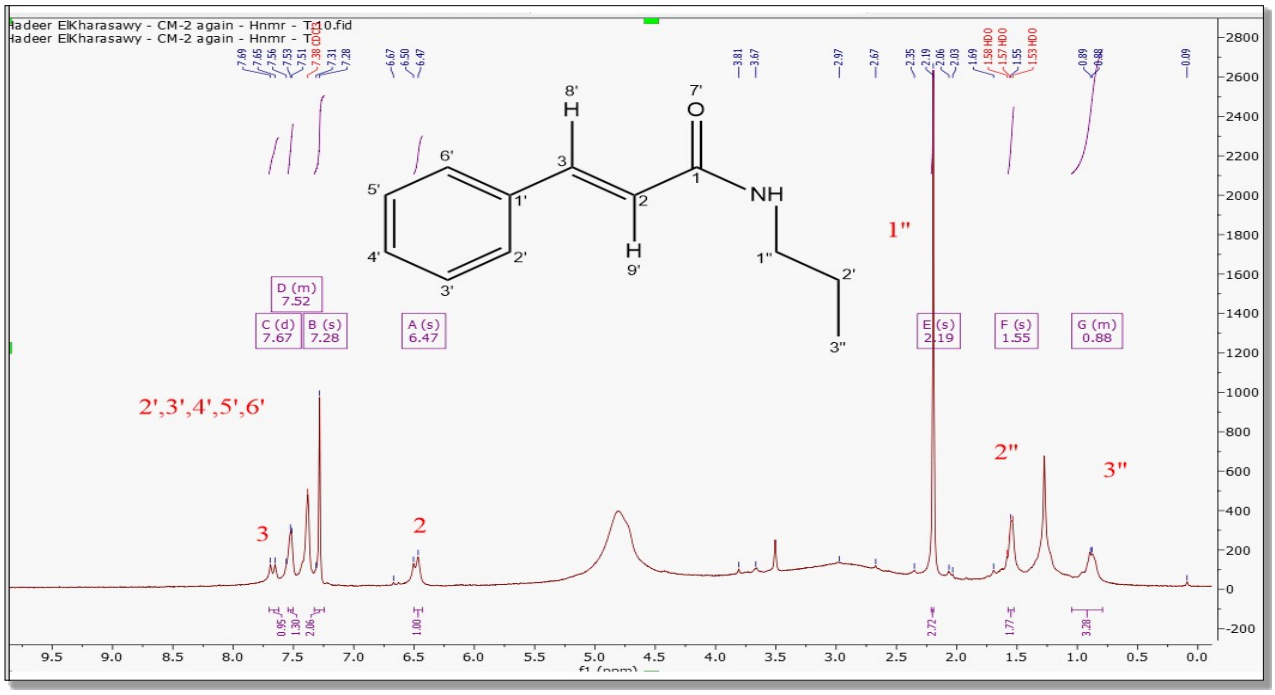


**Fig. S2. $^{1}$H NMR spectrum (400MHz in $CDCl_3$) of 2.**

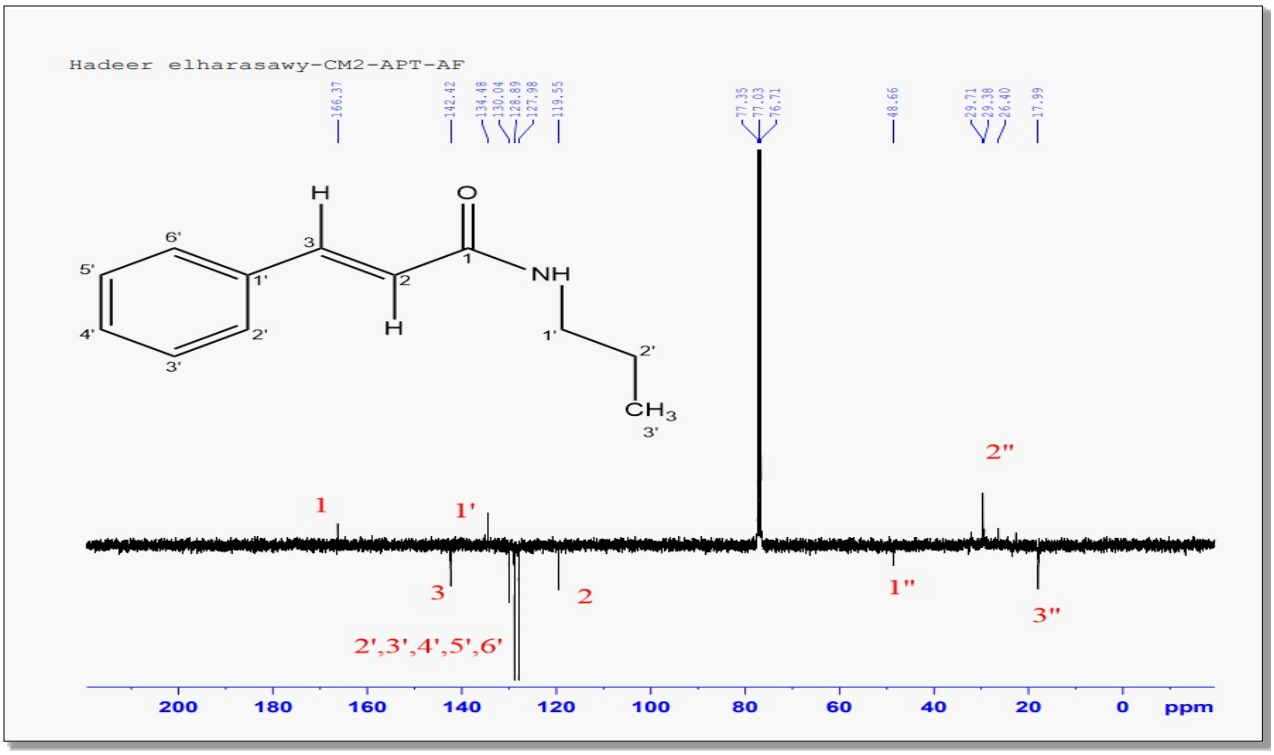


**Fig. S3. APT spectrum (400 MHz in $CDCl_3$) of 2.**

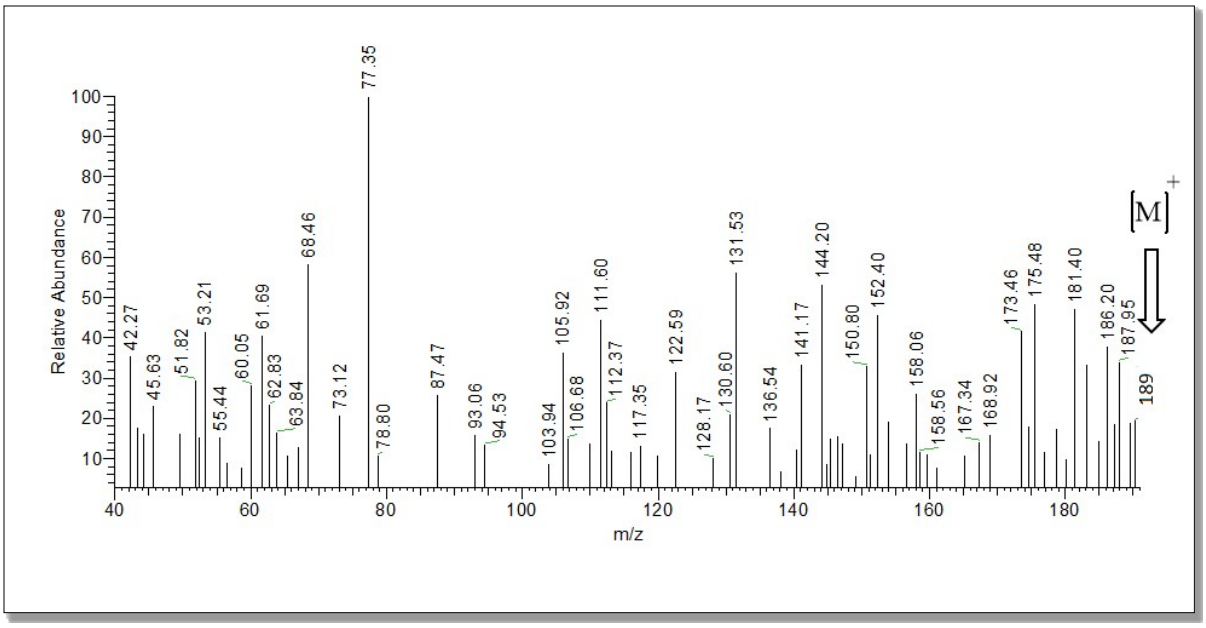


**Fig. S4. FAB-MS spectrum of 2.**

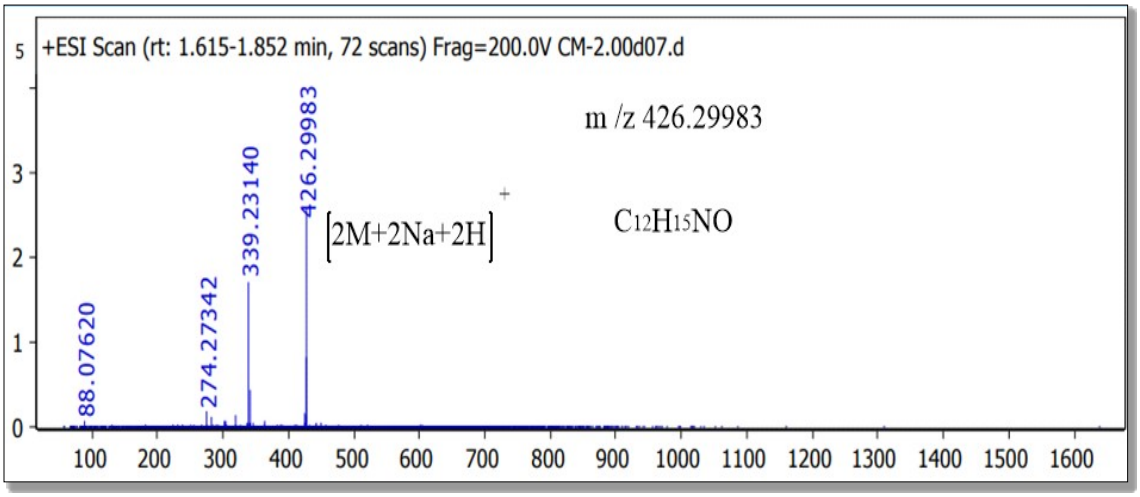


**Fig. S5. HR-FAB-MS spectrum of 2.**

### *b. Identification of 3*

*A. alternata* (AUMC 150207) produced metabolite **3** (20 mg) in 6.67% yield after 48hrs. of preparative scale incubation. The fermentation broth was extracted, concentrated and purified on silica gel column chromatography and the isolated pure compound was subjected to NMR spectral analyses. $^1$H-NMR and APT spectral data **(Table S1 and Fig. S6 and S7)** showed absence of olefinic carbon signals at $\delta_c$ 117.94(C-2) and $\delta_c$ 144.97 (C-3) and olefinic protons signals at $\delta_H$ 6.5 (H-2), 7.74 (H-3) and the appearance of new proton signals at $\delta_H$ 4.2, 1.6, 1.28, 1.25, 1.25, 1.18, 0.89, 0.80 integrated for 17 protons in the $^1$H-NMR spectrum. APT spectrum displayed appearance of 8 new signals at $\delta_c$ 68.2, 38.9, 30.5, 28.9, 23.8, 22.8, 14 ,11 assigned for 2$CH_3$ ,5$CH_2$and 1CH. One of $CH_2$ groups was oxygenated at $\delta_c$ 68.2 (C-1"). The downfield shift of C=O at $\delta_c$ 167.78 corresponding to $\delta_c$ 168.9 in cinnamic acid which might indicate esterification. HR-FAB-MS spectrum of 3 showed $[M+K+H]^+$ at m/z 274.27328, corresponding to a molecular formula $C_{15}H_{22}O_2$. The previously presented data of **3** showed that **3** is 2-methylheptyl ester of benzoic acid which wasn't previously isolated from any natural source.

**NMR data of 3**

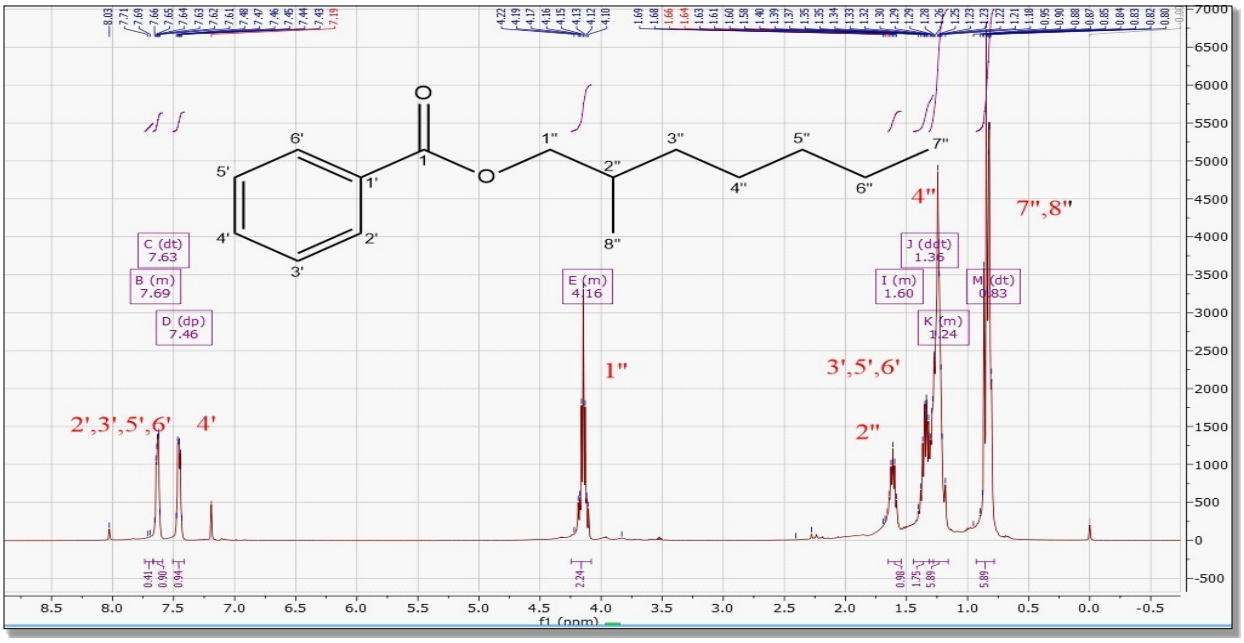


**Fig. S6. $^1$H NMR (400MHz in $CDCl_3$) spectrum of 3.**

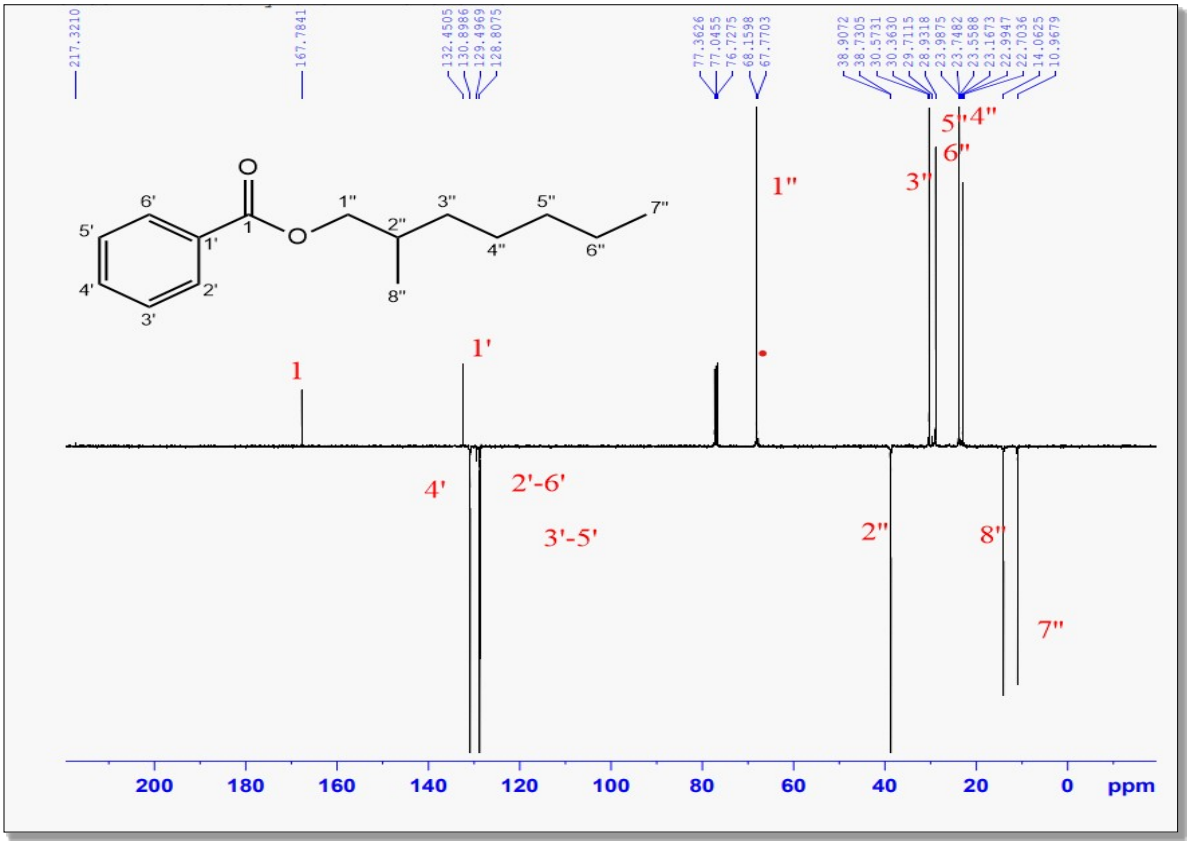


**Fig. S7. APT spectrum (400 MHz in CDCl3) of <u>3</u>.**

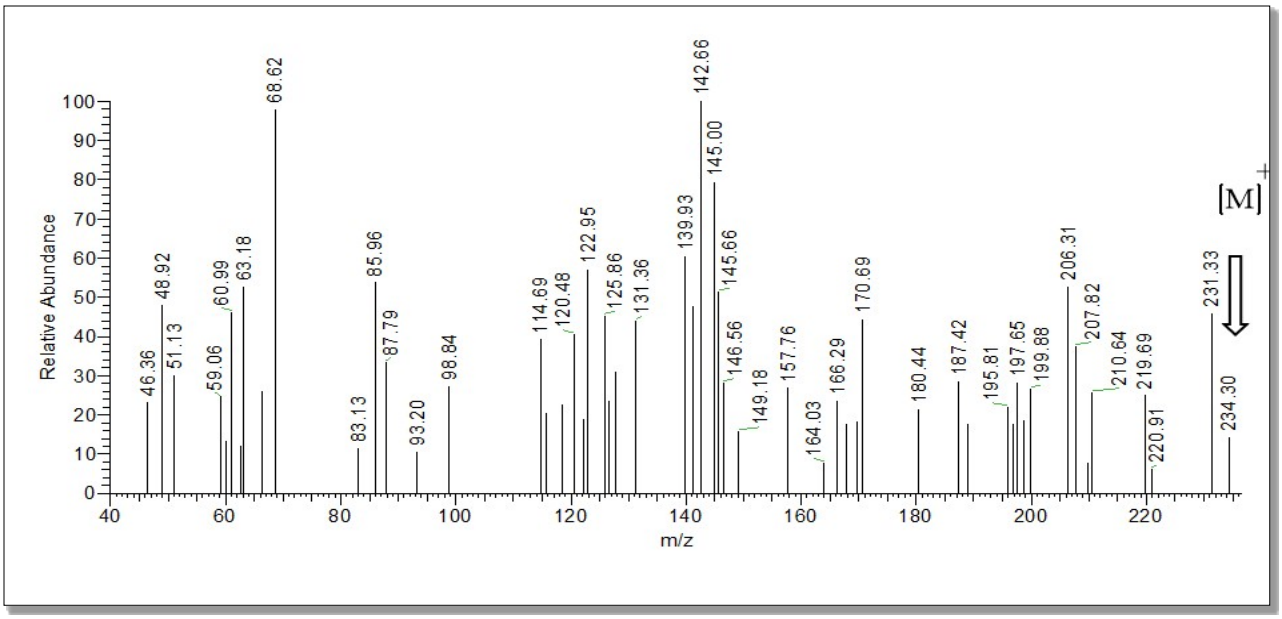


**Fig. S8.FAB-MS spectrum of 3**

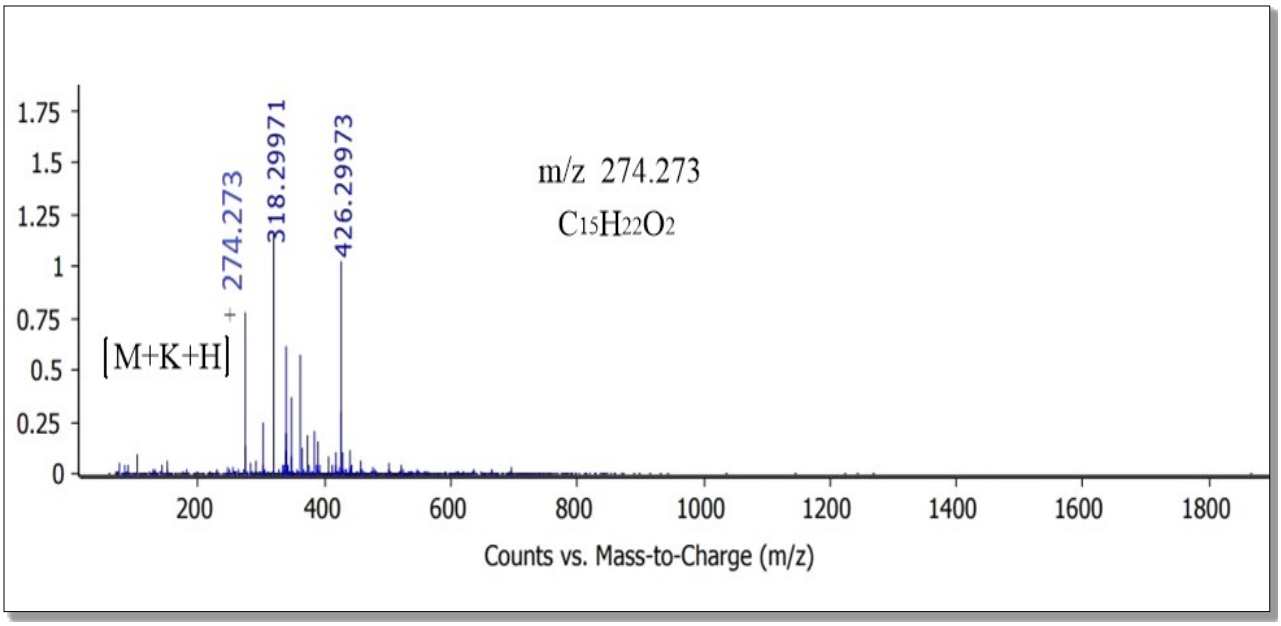


**Fig. S9.HR-FAB-MS spectrum of 3.**

### *c. Identification of 4*

*R. rubra* (NRRL 1592) produced **4** (50 mg) in 16.67% yield after 24hrs. of preparative scale incubation. The fermentation broth was extracted, concentrated and purified on silica gel column chromatography. $^{1}$H-NMR and APT spectral data of **4 (Table S1 and Fig. S11 and S12)** was compared to those of cinnamic acid, showed the absence of the two olefinic proton signals at $\delta_H$ 6.5 (H-2), 7.74 (H-3) and two olefinic carbon signals at δc 117.94(C-2) and δc 144.97 (C-3). The number of aromatic protons were decreased by one which indicating the substitution of aromatic ring. Two doublets at $\delta_H$ 7.82, 6.75 each integrated for (2H) assigned to each of the chemically equivalent H-2', H-6' and H-3', H-5', respectively. From APT, downfield shift of δc 161.6 (C-4') and upfield shift of δc 121.4 (C-1') were observed, corresponding to δc 127.8 and δc 134.4 in cinnamic acid, respectively confirm the hydroxylation at C-4. HMBC spectrum (**Fig. S13**) displayed correlations between C-1 and H-2', H-6' and between C-4' and the four aromatic proton (H-2', H-3', H-5'and H-6'). MS spectrum of **4** showed [M]+ at m/z 138.34 corresponding to $C_7H_6O_3$. Metabolite **4** was unambiguously identified as *p*-hydroxy benzoic acid. From the previously presented data, it's confirmed that metabolite **4** is *p*-hydroxy benzoic acid based on parallelism with the reported spectral data[5].

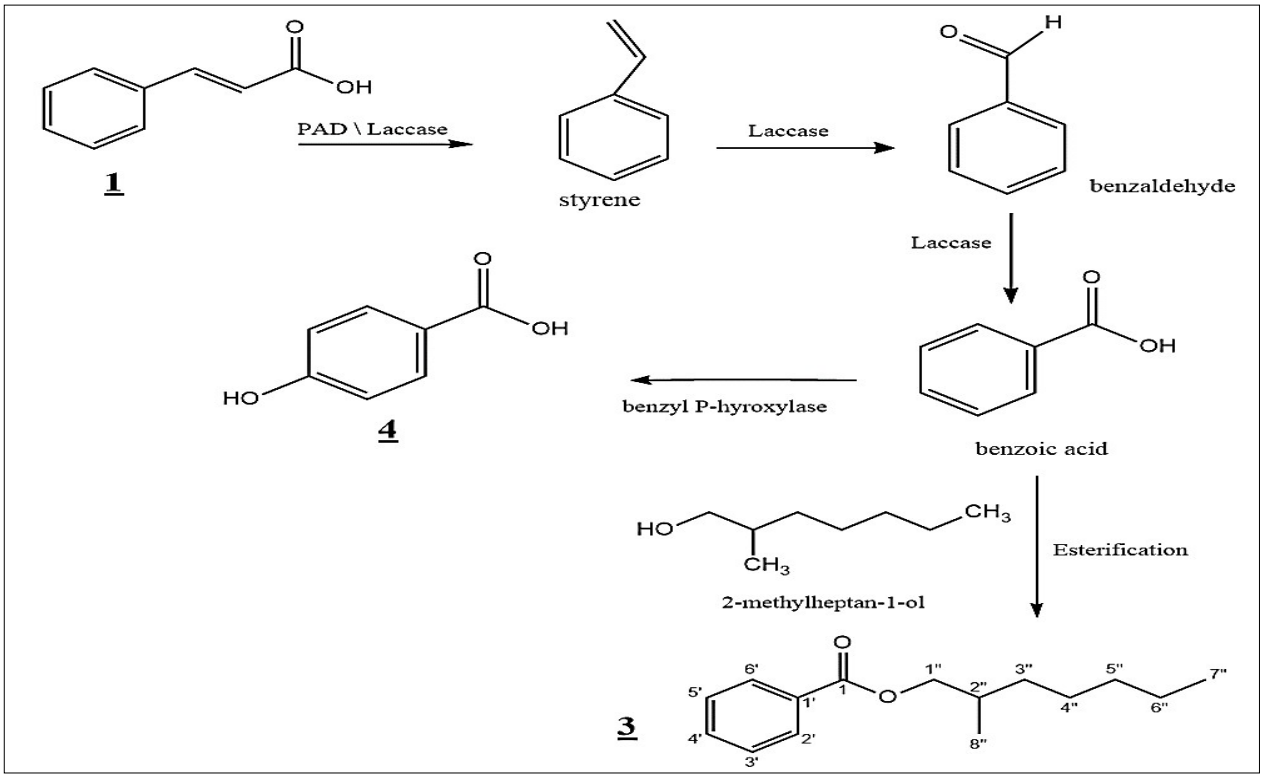


**Fig. S10. proposed mechanism of metabolites 3 and 4 formation.**

**NMR data of 4**

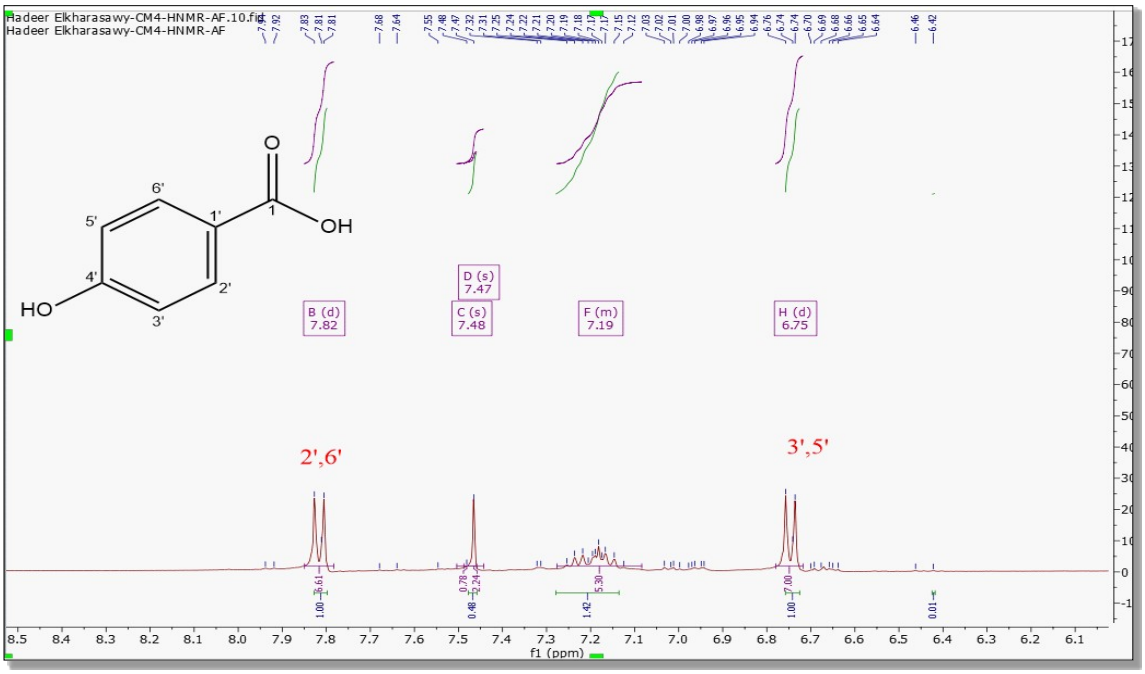


**Fig. S11.$^{1}$H NMR spectrum of 4.**

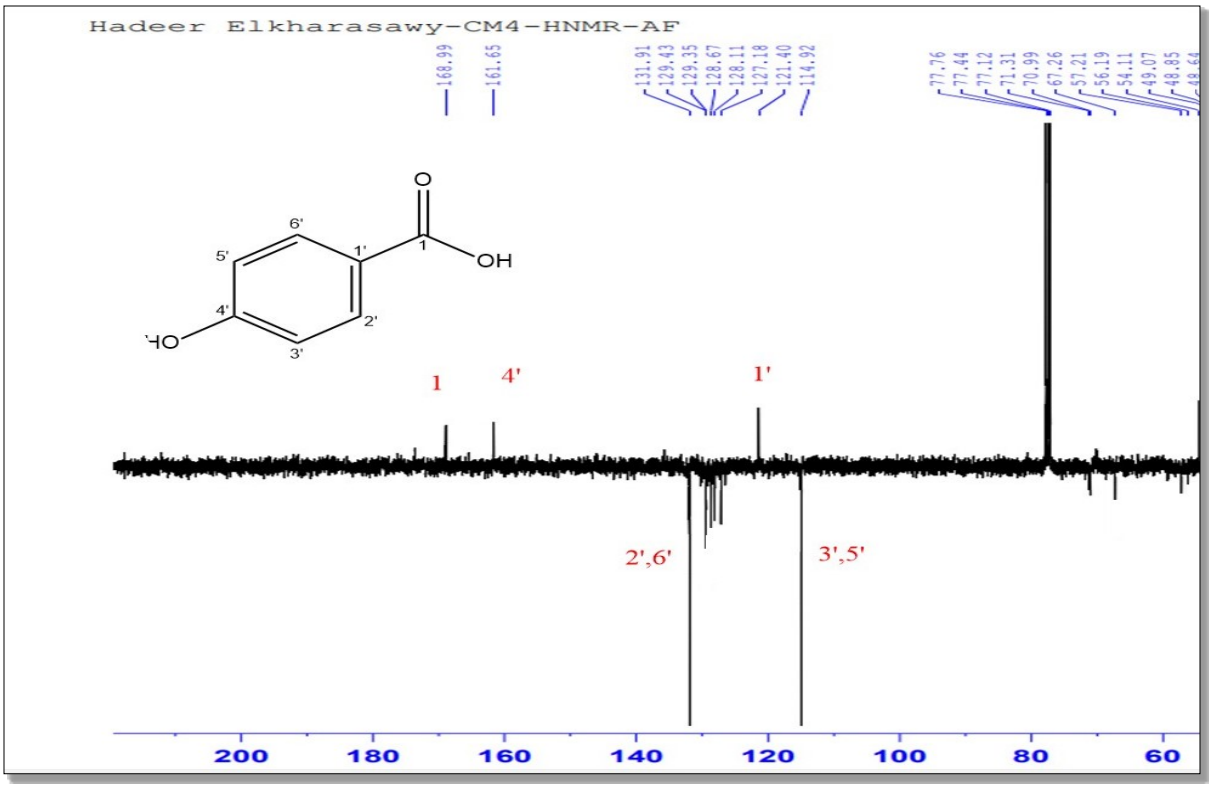


**Fig. S12.APT spectrum (400MHz in $CD_3OD$) of <u>4</u>.**

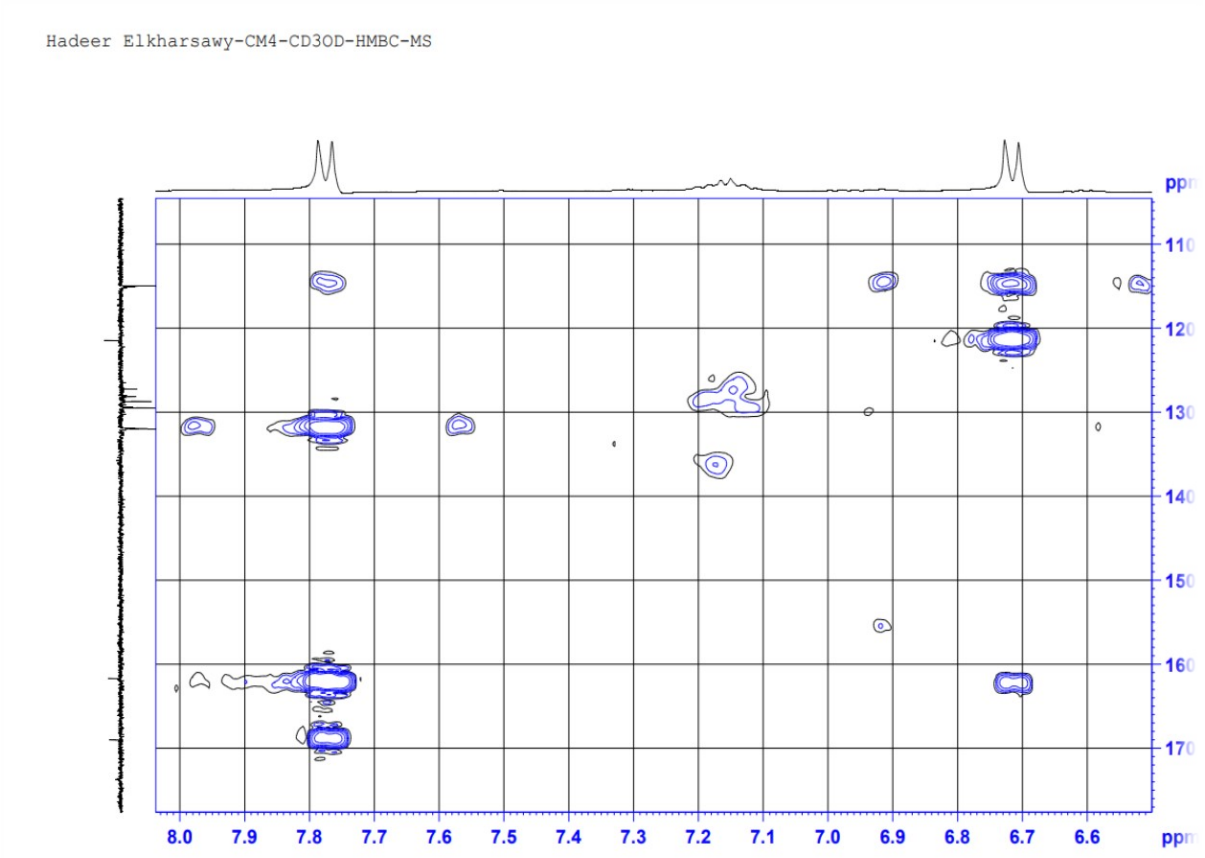


**Fig. S13. HMBC spectrum of 4.**

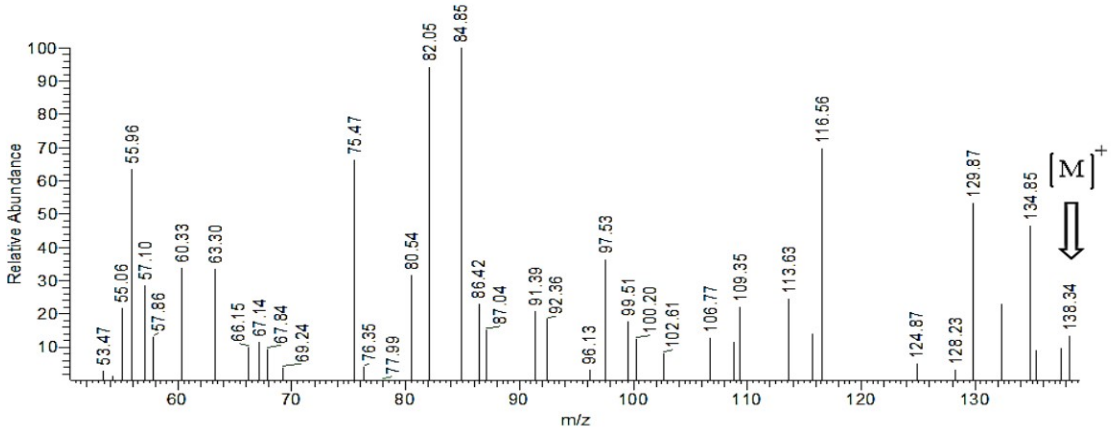


**Fig. S14. FAB-MS spectrum of 4.**

### *d. Identification of* <u>5</u>

Metabolite **5** was produced **(**67 mg) in 22.3% yield after 6 hrs. of preparative scale incubation of *Rhizopus species (*ATCC 36060). Metabolite **5** was identified as cinnamyl alcohol. Its physical and spectral data were consistent with those of reported data[6].

**NMR data of <u>5</u>**

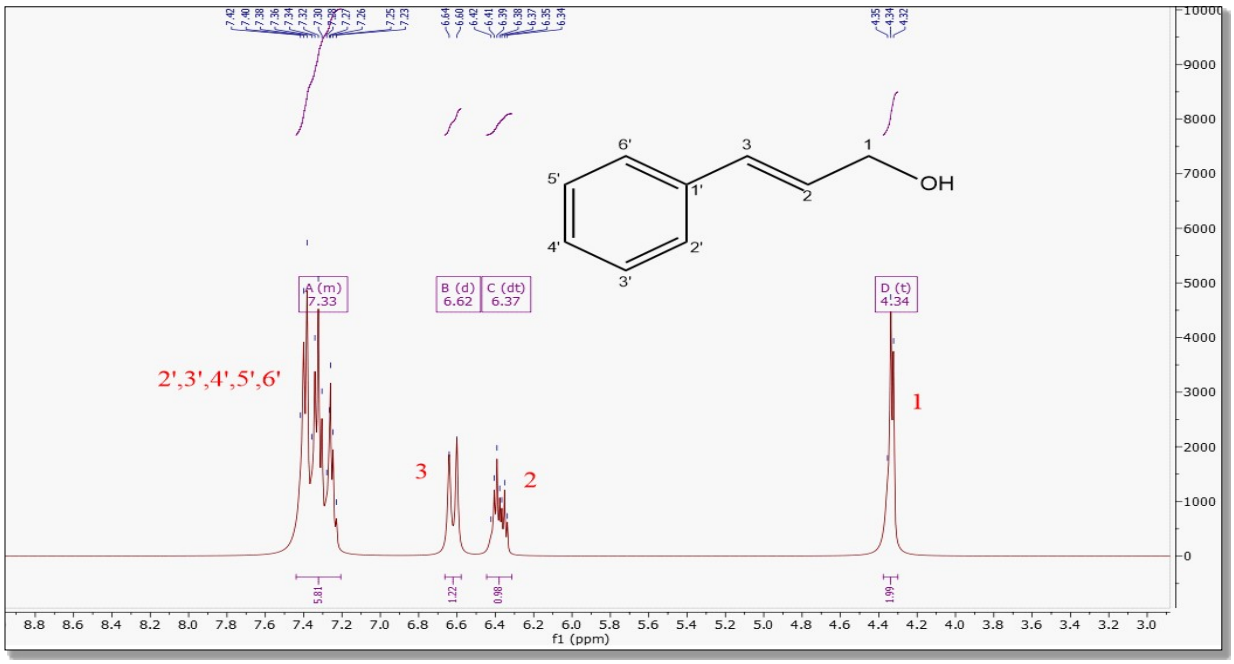


**Fig. S15.[1]H NMR spectrum (400MHz in $CDCl_3$) of <u>5</u>.**

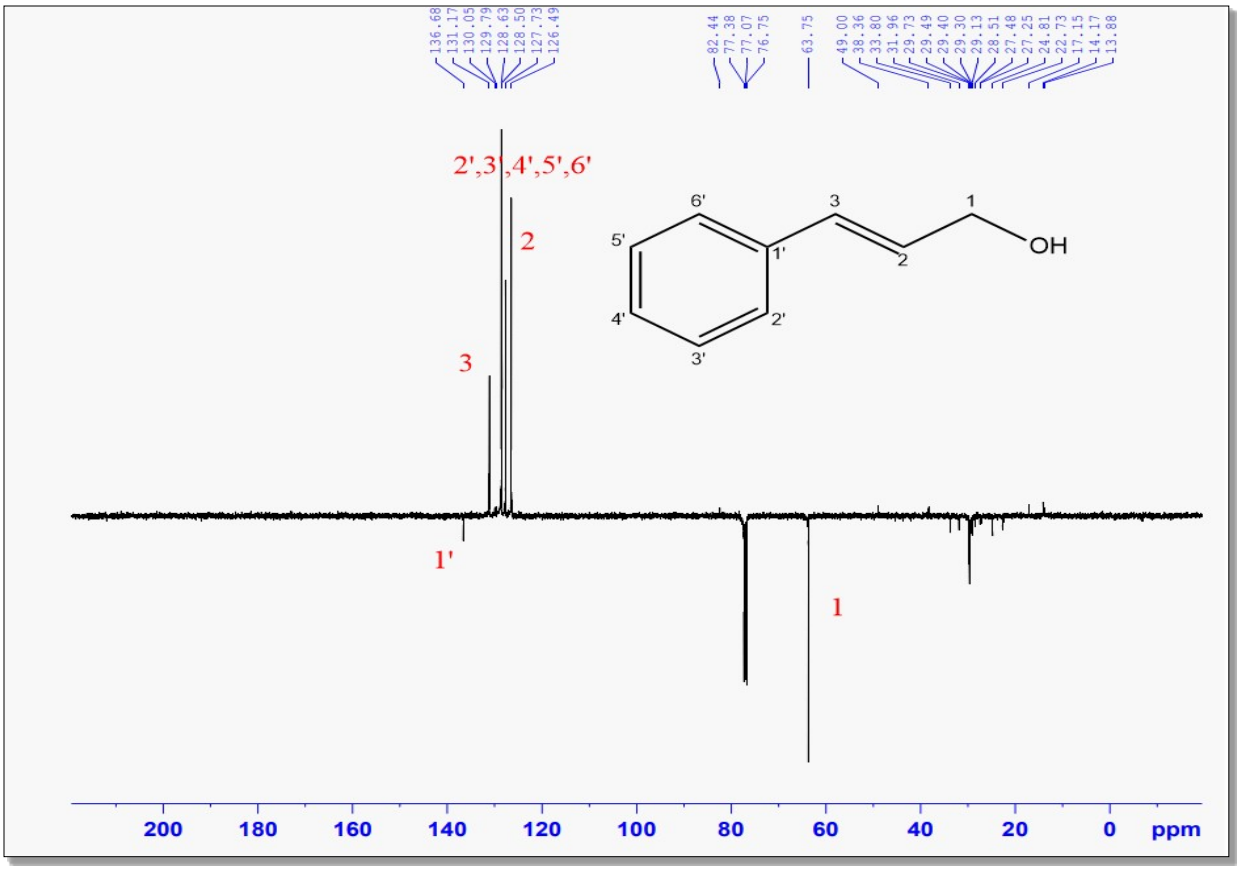


**Fig. S16. APT spectrum (400 MHz in $CDCl_3$) of <u>5</u>.**

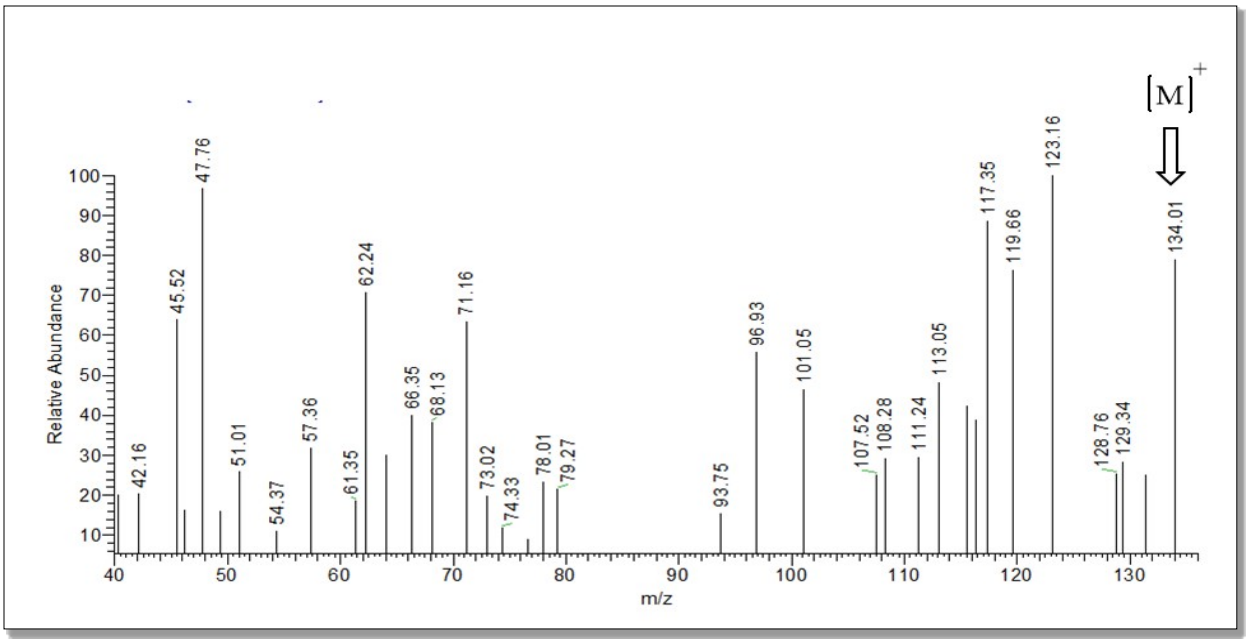


**Fig. S17. FAB-MS spectrum of 5.**

### *e. Identification of* <u>6</u>

Metabolite **<u>6</u>** was produced **(**55 mg) in 18.3% yield after 48hrs. of preparative scale incubation of *P. chrysogeneum* (ATCC 9480). Metabolite **<u>6</u>** is methyl cinnamat**e** based on parallelism with the reported spectral data[7].

**NMR data of <u>6</u>**

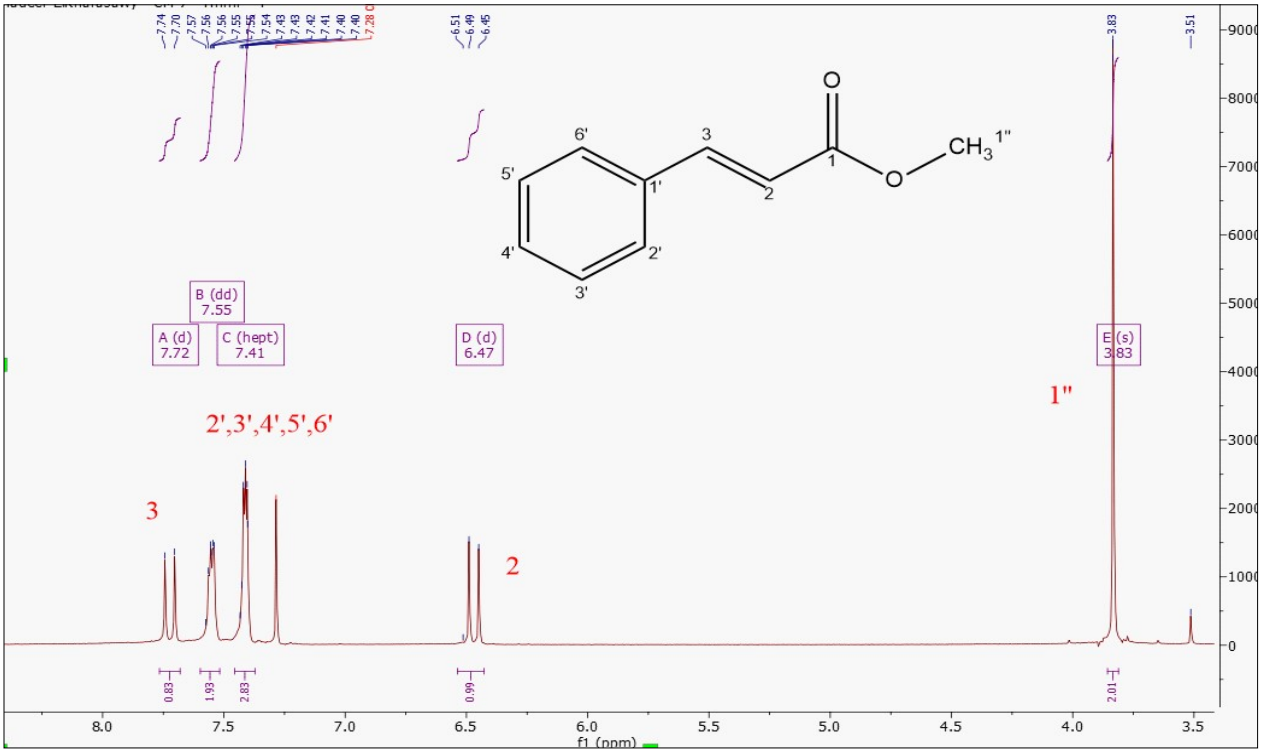


**Fig. S18. $^{1}$H NMR spectrum (400MHz in $CDCl_3$) of <u>6</u>.**

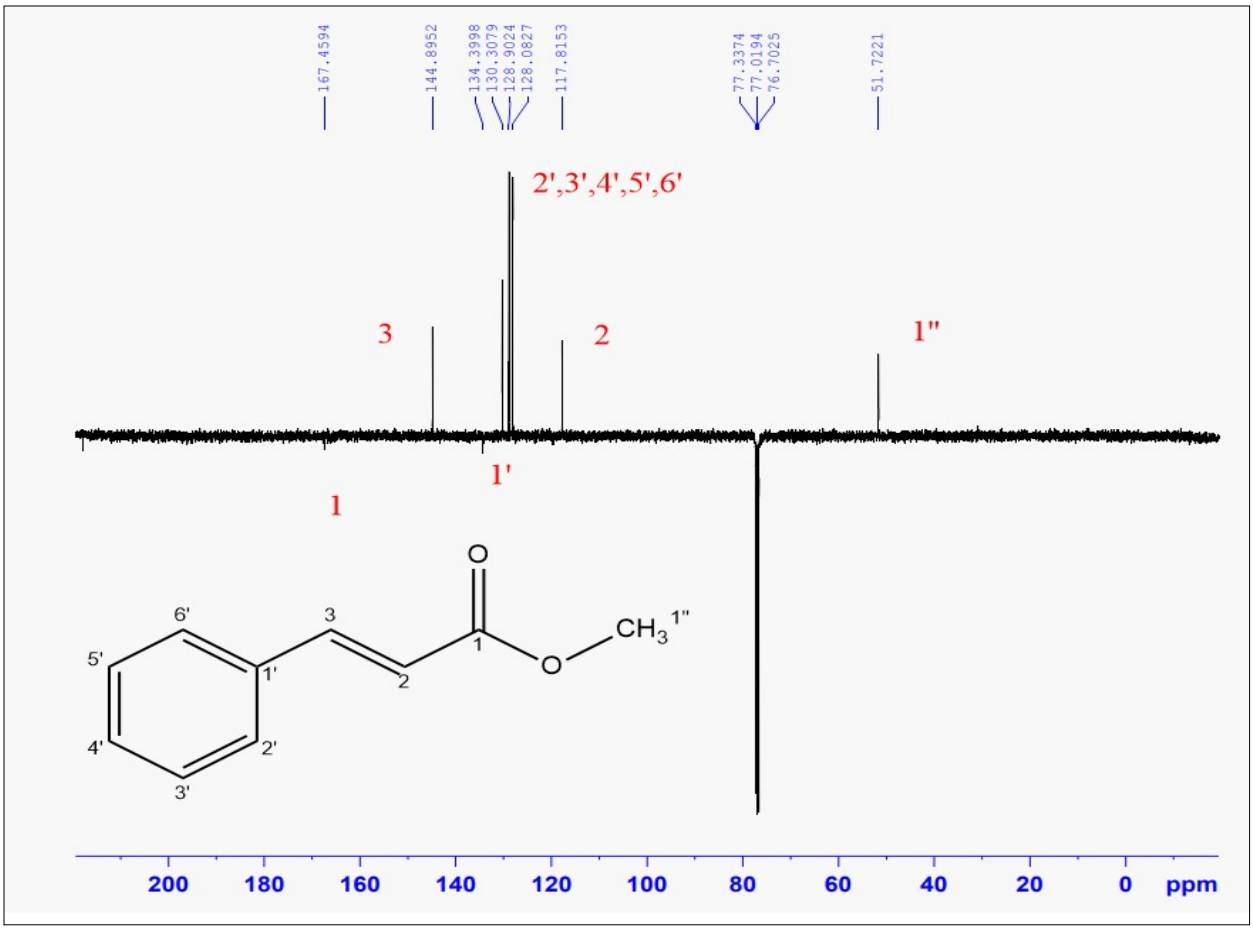


**Fig. S19. APT spectrum (400MHz in $CDCl_3$) of 6.**

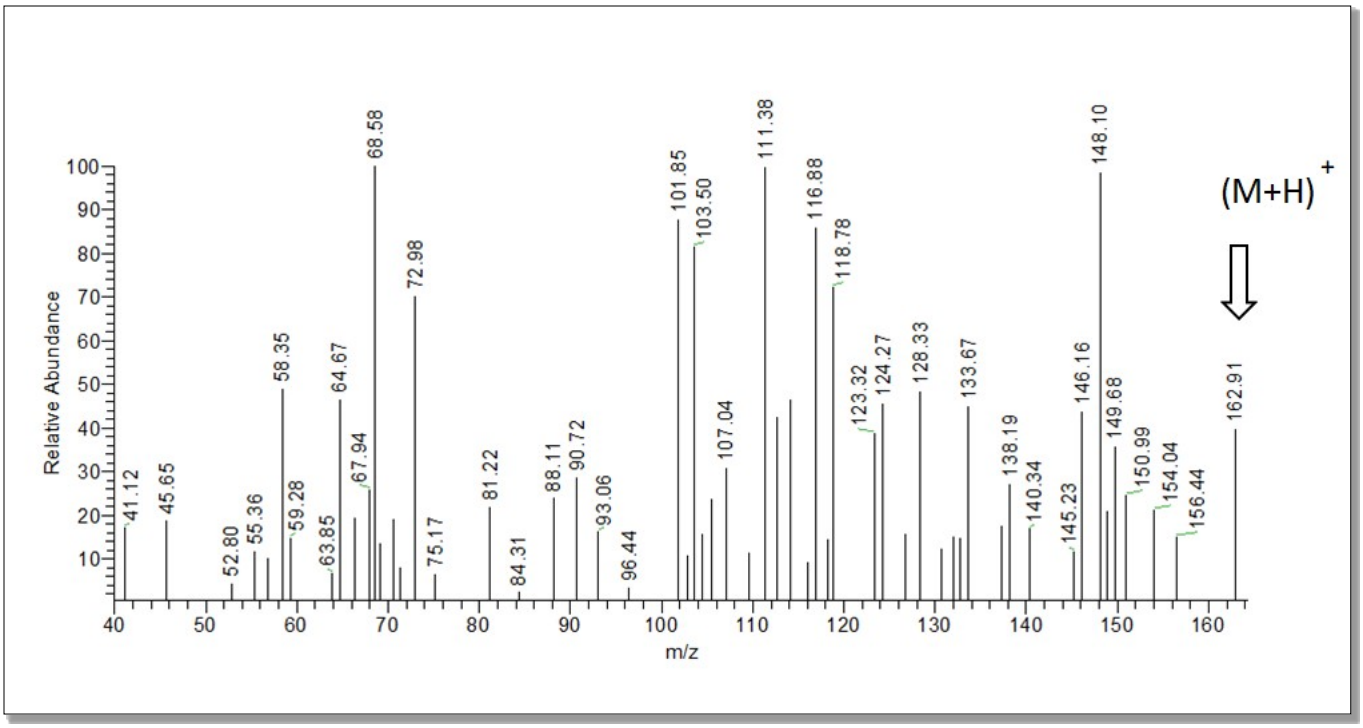


**Fig. S20. FAB-MS spectrum of <u>6</u>.**

**Table S1. $^{13}$C-NMR and $^{1}$H-NMR spectral data of cinnamic acid and its metabolites**

| C/H | 1 [a] | | 2 [a] | | 3 [a] | | 4 [b] | |
|---|---|---|---|---|---|---|---|---|
| | $^{13}$C-NMR | $^{1}$H-NMR | $^{13}$C-NMR | $^{1}$H-NMR | $^{13}$C-NMR | $^{1}$H-NMR | $^{13}$C-NMR | $^{1}$H-NMR |
| 1 | 168.99 | ---------- | 166.3 | ---------- | 167.78 | ------ | 168.9 | -------- |
| 2 | 117.94 | 6.5 (1H, d *J*=16) | 119.5 | 6.46 (1H, d *J*=16) | ------- | ------ | -------- | -------- |
| 3 | 144.97 | 7.74 (1H, m *J*=16) | 142.4 | 7.65 (1H, m *J*=16) | ------- | ------ | -------- | -------- |
| 1' | 134.4 | ---------- | 134.4 | --------- | 132.4 | ------ | 121.4 | -------- |
| 2' | 128.82 | 7. 58 (1H, m) | 128.8 | 7. 52 (1H, m) | 129.4 | 7.64 (1H, m) | 131.91 | 7.82 (1H, d J=8) |
| 3' | 130.04 | 7.40 (1H, m) | 130 | 7.38 (1H, m) | 128.8 | 7.64 (1H, m) | 114.92 | 6.75 (1H, d *J*=8) |
| 4' | 127.8 | 7.39 (1H, m) | 127.9 | 7.38 (1H, m) | 130.8 | 7.47 (1H, m) | 161.65 | ---------- |
| 5' | 130.04 | 7.40 (1H, m) | 130 | 7.38 (1H, m) | 128.8 | 7.64 (1H, m) | 114.92 | 6.75 (1H, d *J*=8) |
| 6' | 128.82 | 7. 58 (1H, m) | 128.8 | 7. 52 (1H, m) | 128.8 | 7.64 (1H, m) | 131.91 | 7.82 (1H, d *J*=8) |
| 1'' | ------- | --------- | 48.6 | 2.2 (2H, q) | 68.2 | 4.2 (2H, d) | ------- | --------- |
| 2'' | ------- | --------- | 29.3 | 1.6 (2H, m) | 38.9 | 1.62 (1H, m) | ------- | --------- |
| 3'' | ------- | --------- | 17.9 | 0.87 (3H, t) | 30.5 | 1.18 (2H, m) | ------- | --------- |
| 4'' | ------- | --------- | ------- | --------- | 23.8 | 1.25 (2H, m) | ------- | --------- |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **5''** | **-------** | **---------** | **-------** | **---------** | **28.9** | **1.25 (2H, m)** | **-------** | **---------** |
| **6''** | **-------** | **---------** | **-------** | **---------** | **22.8** | **1.28 (2H, m)** | **-------** | **---------** |
| **7''** | **-------** | **---------** | **-------** | **---------** | **11** | **0.86 (3H, m)** | **-------** | **---------** |
| **8''** | **-------** | **---------** | **-------** | **---------** | **14** | **0.80 (3H, m)** | **-------** | **---------** |

δc and δH in ppm and *J* in Hz

[a] $^{1}$H-NMR and $^{13}$C-NMR in $CDCl_3$ at 400 MHz and 100 MHz respectively.

[b] $^{1}$H-NMR and $^{13}$C-NMR in $CD_3OD$ at 400 MHz and 100 MHz respectively.

**5.** *Biological activities of cinnamic acid and its metabolites*

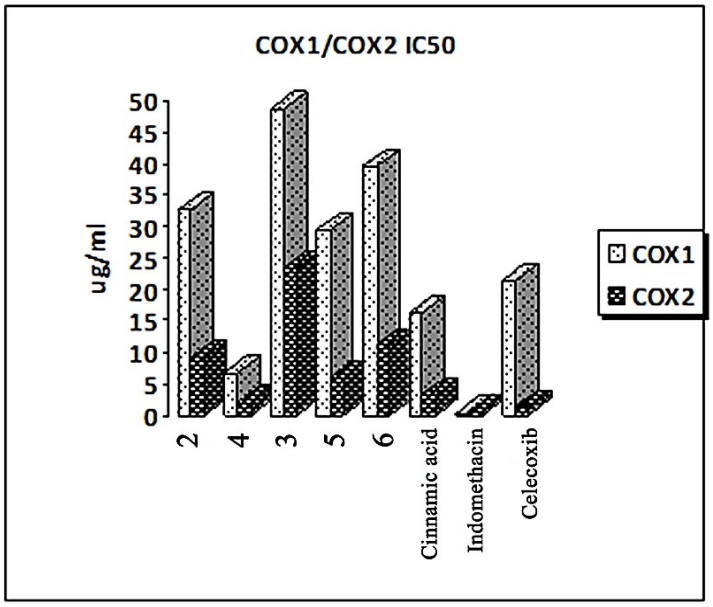


**Fig. S21. Graphical presentation of the results of the inhibitory activities of COX-1 and COX-2 enzymes.**

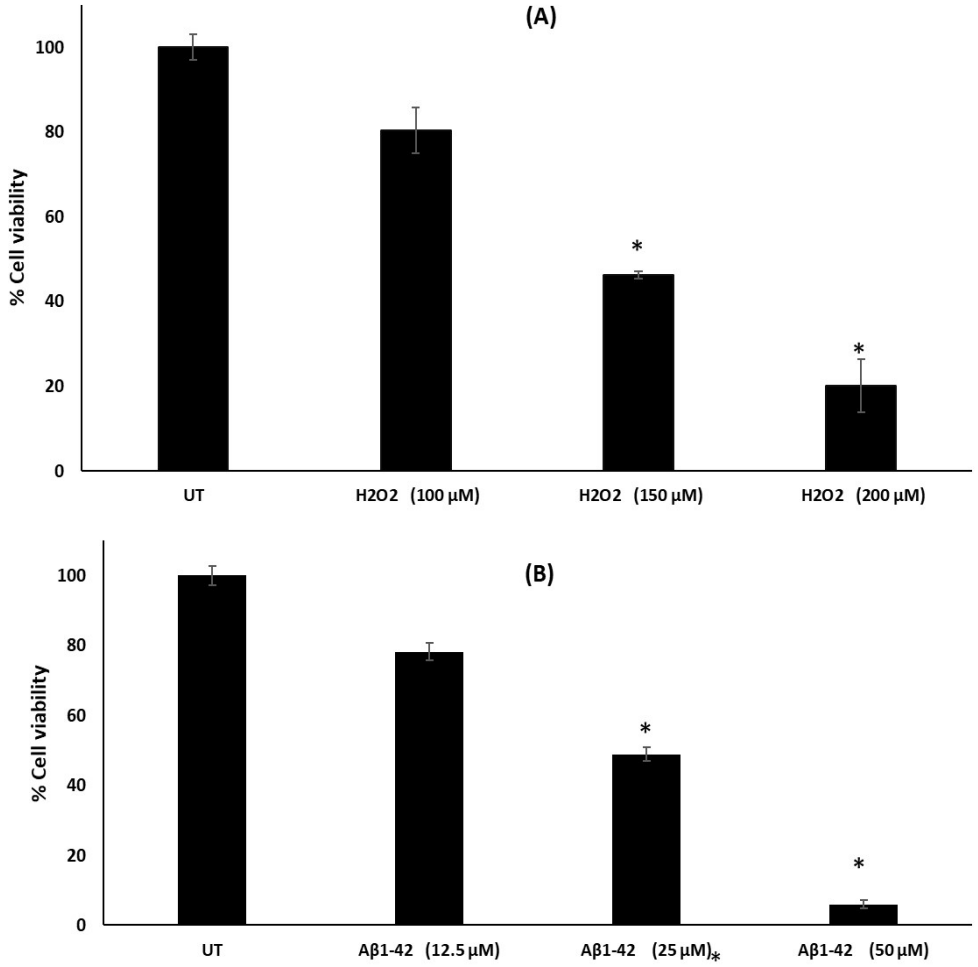


**Fig. S22: (A) Dose-dependent cytotoxic effects of $H_2O_2$ on SH-SY5Y cells. (B) Dose-dependent cytotoxic effects of $A\beta_{1-42}$ on SH-SY5Y cells. Values are represented as means ± standard deviations (SD), *n* =5. UT: is the cells treated with DMSO. * Significant difference from UT at $p < 0.01$.**

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