

Supporting Information

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Phenolics from *Phaleria nisidai* with Estrogenic Activity

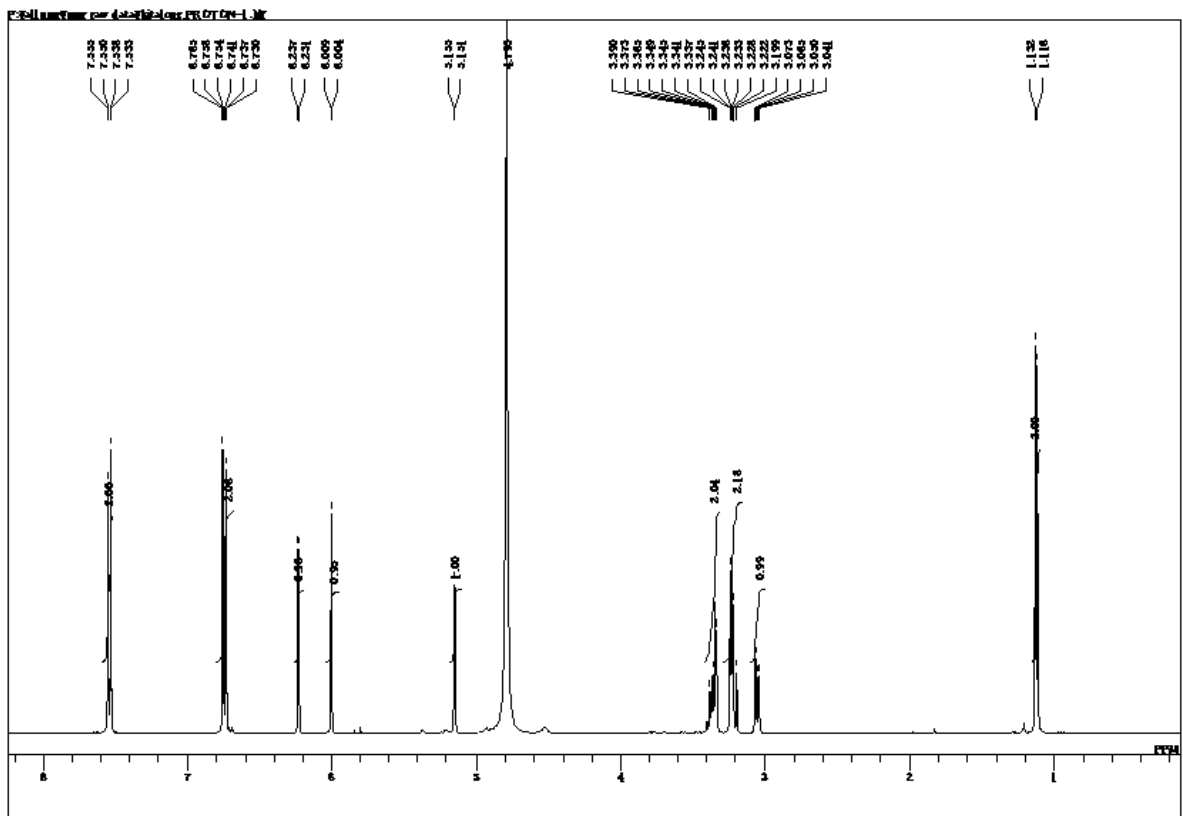
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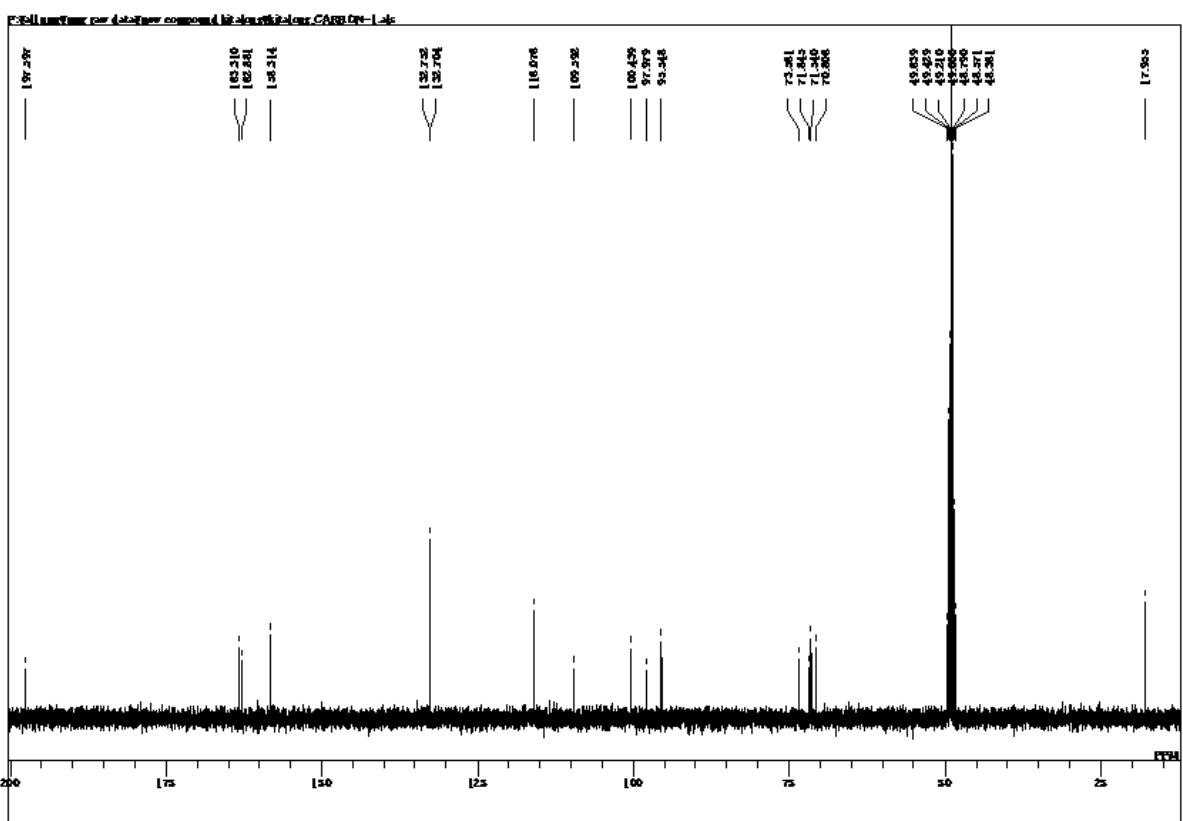
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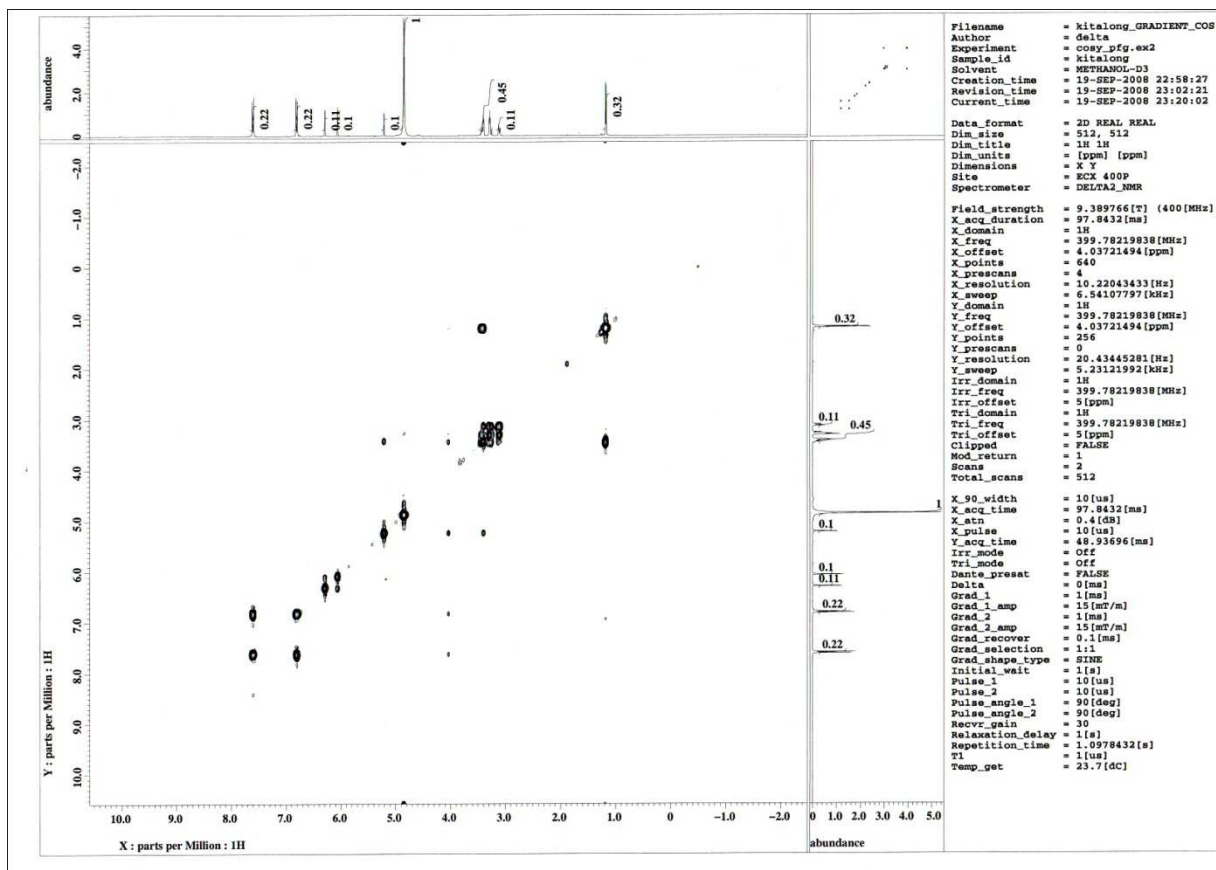
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¹H-NMR of compound 1

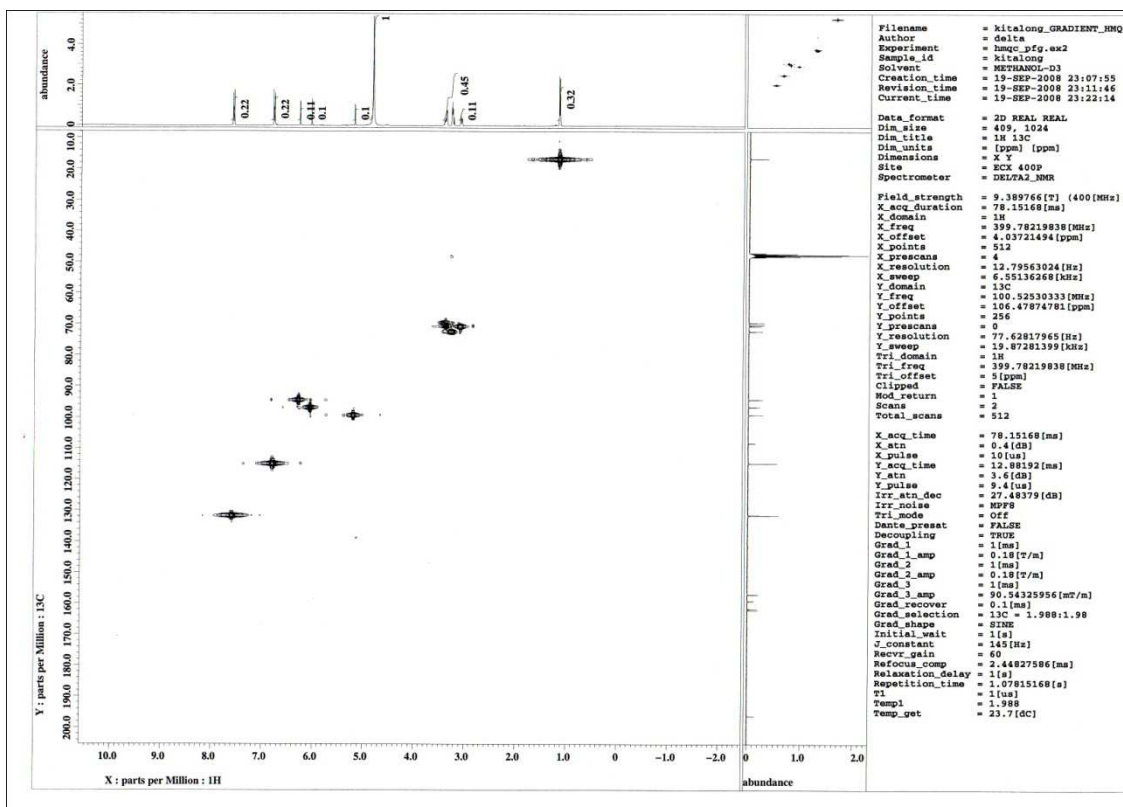


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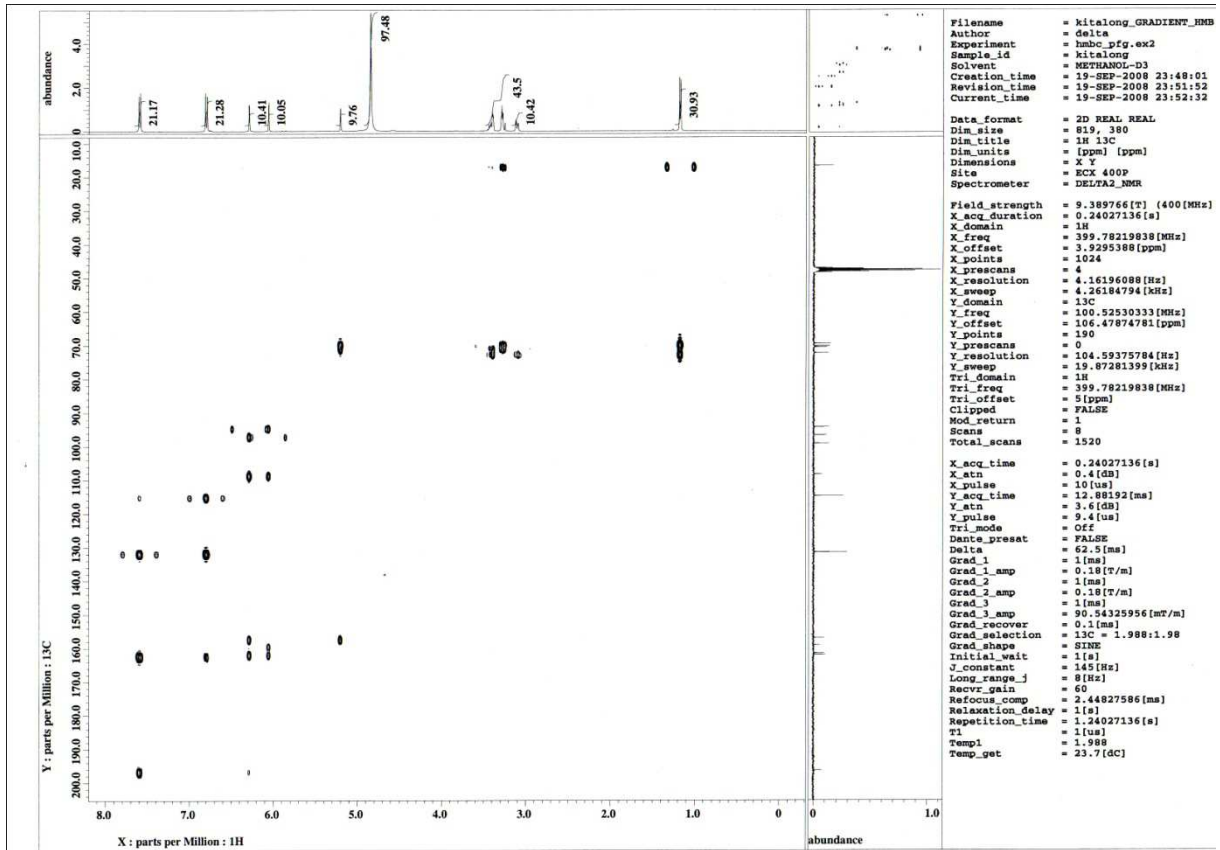
¹³C-NMR of compound 1



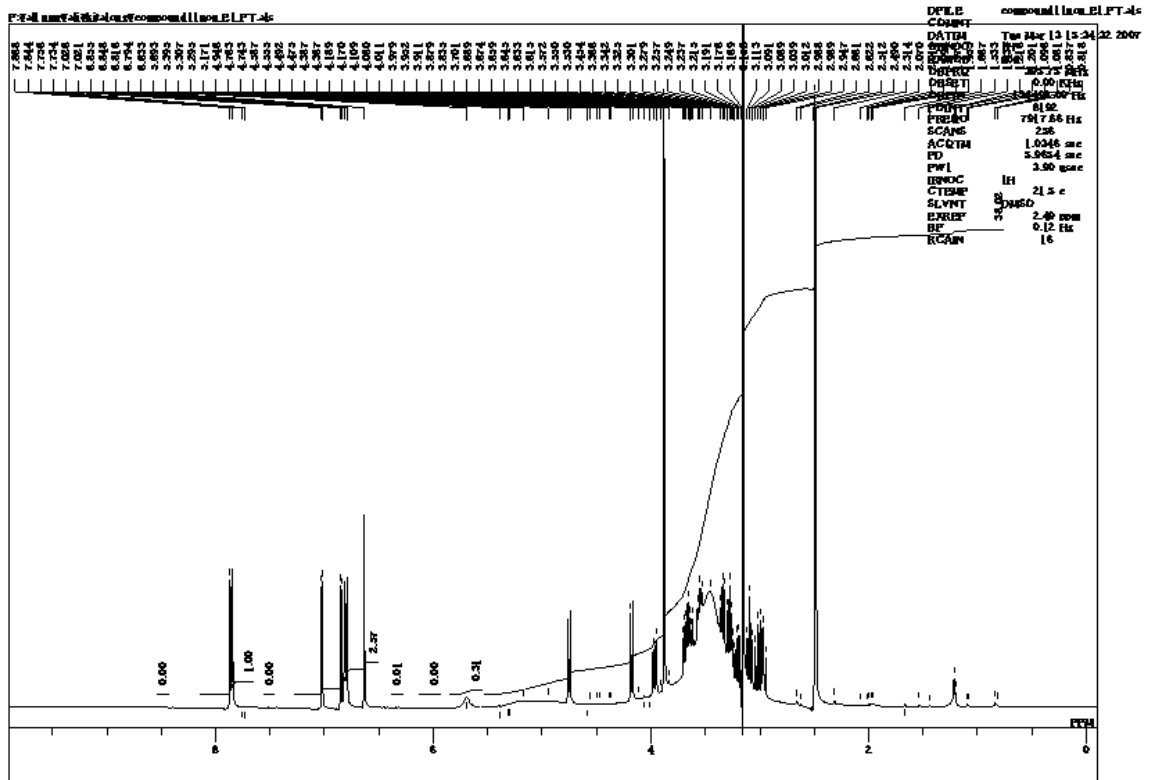
^1H - ^1H COSY of compound 1



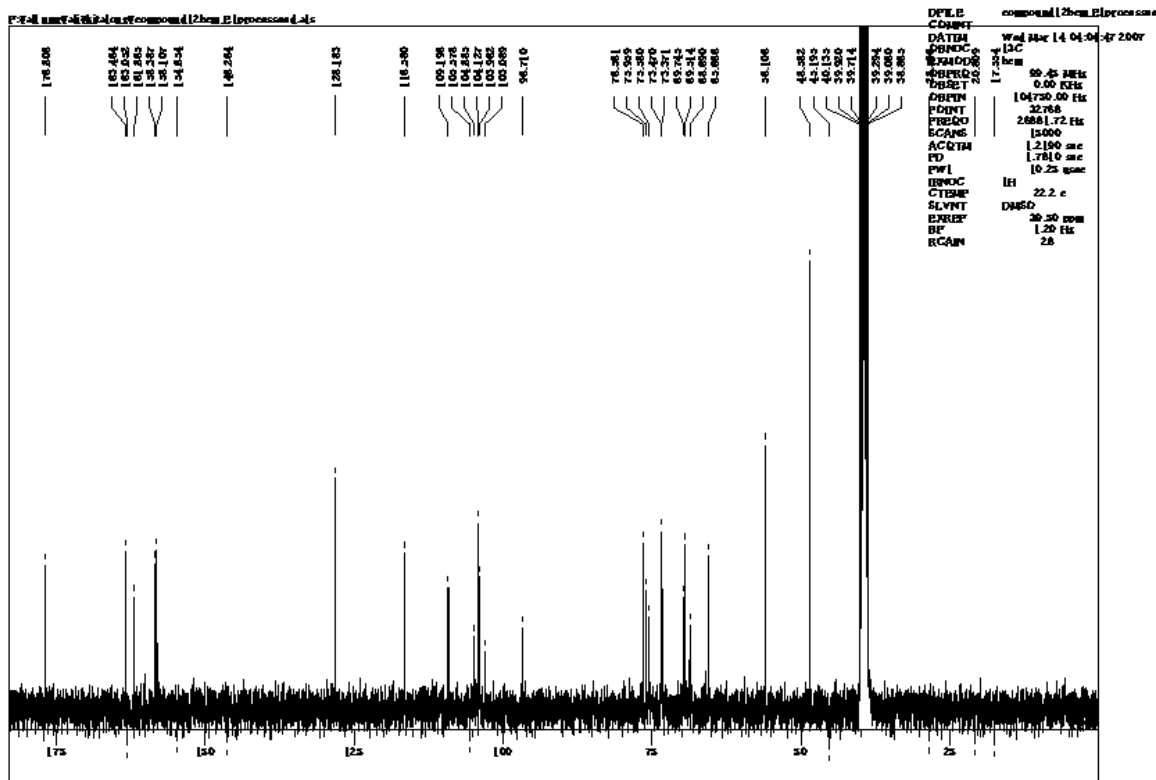
HMQC of compound 1



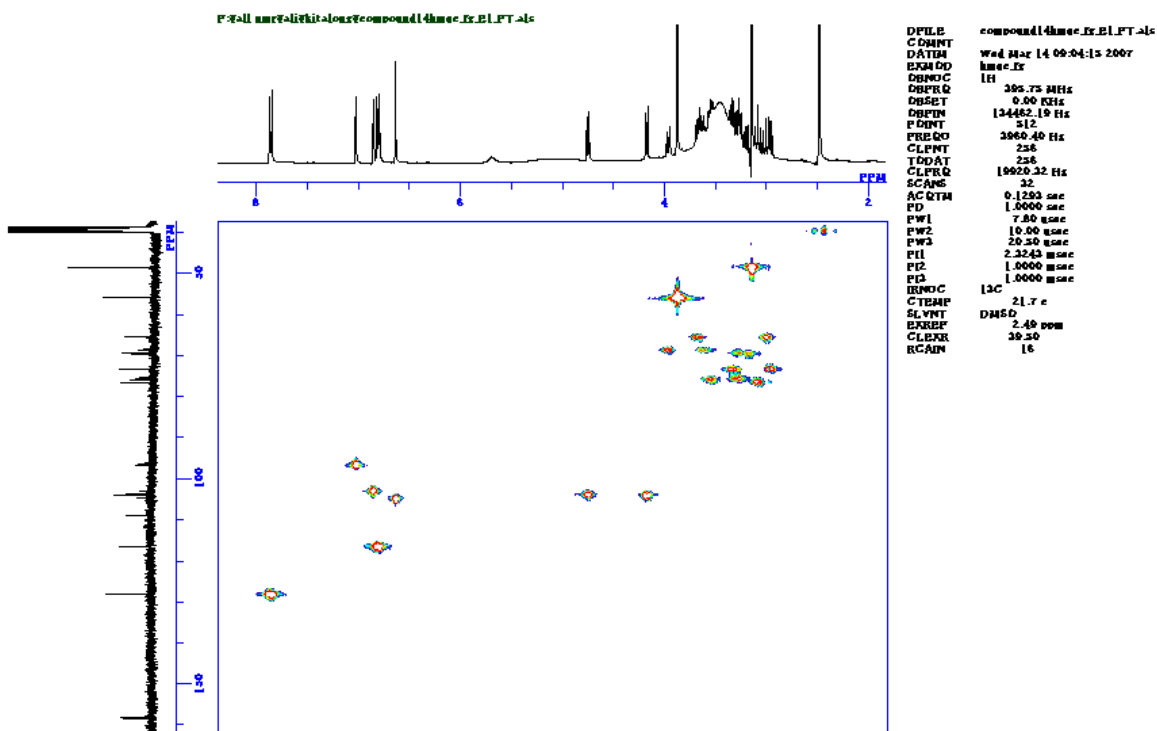
HMBC of compound 1



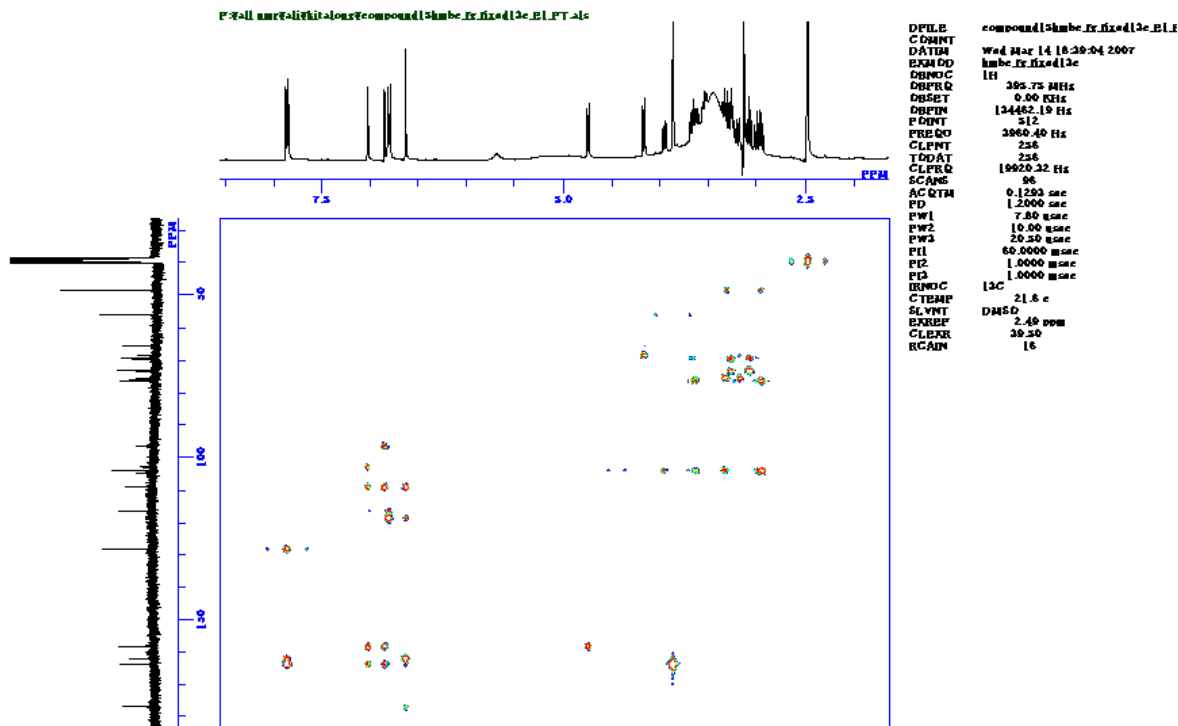
¹H-NMR of compound 2



^{13}C -NMR of compound 2



HMQC of compound 2



HMBC of compound 2

Experimental

General Experimental Procedures

^1H - and ^{13}C -NMR were measured with a JHA-LAA 400 WB-FT (^1H , 400 MHz; ^{13}C , 100 MHz; Jeol Co., Tokyo) spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. ESI-MS was carried out on an Esquire 3000 mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) system with an ESI ionization source. TLC was carried out on pre-coated silica gel 60 F₂₅₄ (0.25 mm, Merck) and RP-18 F₂₅₄S (0.25 mm, Merck Co., Darmstadt). Column chromatography (CC) was carried out on BW-820MH silica gel, Diaion HP-20 (Mitsubishi Kasei, Tokyo) and Sephadex LH-20 (Pharmacia Co.), Wakosil C-300 silica gel (40-63 μm) (Wako Chem. Co., Osaka, Japan) and ODS DM 1020T (ODS, Fuji Silysia, Nagoya, Japan). Medium pressure liquid chromatography (MPLC) was performed on LiChroprep RP-18 and LiChroprep Si 60 (size A and B, Merck Co.).

Extraction and isolation

P. nisidai dried leaves (500 g) were extracted with methanol, under reflux, for three hours, three times and dried under reduced pressure. The crude extract (53g) was then suspended in distilled water and extracted with chloroform (3 X 250ml) to yield a chloroform fraction (4 gm). The water-

soluble fraction afforded a precipitate upon concentration. The precipitate was washed repeatedly with chloroform, and subsequently washed repeatedly with methanol to obtain compound **3** (13 g). The remaining water layer was subsequently run over Diaion HP20 column (5X60 cm) yielding three fractions: H₂O, 50% MeOH and 100% MeOH.

The 50% MeOH fraction (7 g) was subjected to ODS column (2X20 cm), eluted with MeOH:H₂O 1:9 v/v increasing methanol gradiently till 80%, and 9 fractions were collected. Fraction 3 (900 mg) was dried and washed with MeOH to afford mangiferin crystals. The supernatant of fraction 3 was run over ODS column (1X15 cm), eluted with MeOH: H₂O gradiently (10% ~ 50%). Four fractions were collected: 3a-3d. Fraction 3d was then run over MPLC on a RP-18 size A column, using MeOH:H₂O (6:4 v/v) to afford compound **2** (237.2 mg). Fraction 7 (500 mg) was applied to MPLC column (RP-18, size A) and eluted with MeOH:H₂O (7:3 v/v) to obtain compound **1** (20 mg).

Acid hydrolysis of compound 1 and 2

A solution of **1** and **2** (4 mg of each) in a 1% H₂SO₄ (0.2 ml) in a sealed tube was heated on a boiling water bath for 1h^[1,2]. The solution was then extracted with EtOAc and concentrated under vacuum to get compounds **4** and **5**, respectively. The identity of the aglycones was verified by comparison with the reported data^[3].

Estrogenic activity

Ligand binding assay

An estrogen receptor (α) competitor assay kit (Wako Chemical Japan Inc.) was employed to determine the efficacy of test compounds to bind to the estrogen receptor (ER α). Direct comparisons were performed with a labeled estrogen mixture. The amount of the ligand that binds to the ER α coated on the microplate well was determined by a dynamic equilibrium among all the ligand concentrations in the mixture, the difference of their binding affinities to the receptor, and incubation time. A reduction in fluorescence intensities from the labeled estrogens provides a measure of the affinity of the added compounds to the estrogen receptor. The prepared derivatives were tested at concentrations of 10⁻⁵, 10⁻⁴ and 10⁻³ M. 17- β estradiol (E2) was used as a positive control. The assay was carried out according to the method reported by the company, briefly; the test compounds were prepared as stock in DMSO. 6 μ l of each dilution was added to labeled estrogen mixture (114 μ l) and mixed well in a micro tube. 100 μ l of the prepared mixture is pipetted into each well in the supplied ER α coated microplate. DMSO (5% in the estrogen labeled mixture) was used as the control. The

plate is incubated at room temperature for exactly 2 h. The microplate was washed with 100 ml of the wash solution followed by draining the residual solutions from the wells. 100 μ l of the assay solution was added to every well and the fluorescence intensity in each well was measured at excitation 485 nm and emission 535 nm. The results were calculated as percentages of the control. The fluorimetric analyses were performed on an automated TECAN GENios plate reader with excitation wavelength at 485 nm and emission at 530 nm. Results were calculated as percentages of labeled estrogen mixture binding.

Virtual Docking Study

The crystal structure of estrogen receptor α (ER α) bound to 17 β estradiol (protein data bank ID 1A52) was downloaded from www.pdb.org. The crystal structure was prepared for docking study using the Internal Coordinate Mechanics (ICM-Pro) software version 3.4-8C (MolSoft LLC, San Diego CA) ^[4]. The crystal structure was first transformed to ICM object and the water molecules were eliminated. The protein model was adjusted (regularized) so that the optimal positions of polar hydrogens were identified, missing hydrogen and heavy atoms were added, atom types and partial charges were assigned. The 3D structures of the ligand molecules were generated and energy minimized using Merck Molecular Force Field (MMFF). The active site of the regularized protein was identified and adjusted using the ICM small molecule docking procedure (MolSoft ICM manual). Receptor energy maps were constructed including energy terms for electrostatic, directional hydrogen bond, hydrophobic interactions, and two van der Waals interactions for steric repulsion and dispersion attraction. Finally, docking was carried out using interactive docking using one ligand at a time (interactive docking/Mol table ligand) and the ICM scores were calculated. Re-docking of the co-crystal structure ligand (17 β estradiol) and calculating the RMSD were carried out; the results were compared to the literature to validate the docking process.

Statistical analysis

The IC₅₀ was calculated using a non-linear regression analysis (for one site competition). Data was represented as means \pm S.E.M.

References

1. S. Hara, H. Okabe and K. Mihashi (1987). Gas-liquid chromatographic separation of aldose enantiomers as trimethylsilyl ethers of methyl 2- (polyhydroxyalkyl) -thiazolidine-4 (R) -carboxylates, *Chem. Pharm. Bull. (Tokyo)*. **35**,501-506.

2. T. Hatano, H. Uebayashi, H. Ito, S. Shiota, T. Tsuchiya and T. Yoshida (1999). Phenolic constituents of Cassia seeds and antibacterial effect of some naphthalenes and anthraquinones on methicillin-resistant *Staphylococcus aureus*, *Chem. Pharm. Bull. (Tokyo)*. **47**, 1121-7.
3. S. Rancon, A. Chaboud, N. Darbour, G. Comte, C. Bayet, P. N. Simon, J. Raynaud, A. Di Pietro, P. Cabalion and D. Barron (2001). Natural and synthetic benzophenones: interaction with the cytosolic binding domain of P-glycoprotein, *Phytochemistry*. **57**, 553-7.
4. R. A. Abagyan, M. M. Totrov and D. N. Kuznetsov (1994). ICM - a new method for protein modeling and design. Applications to docking and structure prediction from the distorted native conformation, *J. Comp. Chem.* **15** 488-506.