Bioactivity guided fractionation and the antioxidant evaluation of *Mimosa pigra* active constituents

Lee Tze Jiun & Shaida Fariza Sulaiman

School of Biological Sciences, Universiti Sains Malaysia, 11800, Minden, Penang

ABSTRACT

The leaves of *Mimosa pigra* were extracted by boiling in 80% methanol. The FTC (Ferric thiocyanate) and TBA (Thiobarbituric acid) methods were used to determine the antioxidant activities. 2,6-ditert-butylphenol 99.0% (BHT) and α–tocopherol were used as positive controls. After several times of fractionations using paper chromatography with 15% acetic acid and Butanol: Acetic Acid: Water (4:1:5; upper layer) as solvent systems, B2c fraction was found to exhibit the best antioxidant activity. The major phenolic compound in B2c fraction was then tentatively identified using the TLC (Thin Layer Chromatography) methods, UV spectrum and mass spectrum. Prior to that, the glycosylation of sugars that are attached to phenolic molecules in the B2c fraction was broken down via acid hydrolysis method. The aglycones were then determined. A new antioxidant compound had been isolated from the *Mimosa pigra* extract and was tentatively identified as the derivation of *O*-coumaric acid. The potential value of *Mimosa pigra* extract as antioxidant was discovered. The results can possibly change its current position from weed to economic important crop.

INTRODUCTION

Over the past several years, antioxidant compounds are of interest to biologists and clinicians because of their properties to inhibit the oxidation of other molecules by inhibiting the initiation of oxidizing chain reactions (Frankel 1996). The reactive oxygen species (ROS) such as O_2^-, OH^- and H_2O_2, together with unstable

intermediates in the autoperoxides of lipids have a potential for bringing extensive damages, including lipid peroxidation, DNA lesions and protein fragmentation within the cells of biological macromolecules (Vaughan 1997). Nutrients such as flavonoids, beta-carotene, vitamin C and E and zinc have the ability to neutralize the damaging effects of free radicals. Each of these nutrients can block the conversion of free radicals into damaging chemical compounds within the body, preventing oxidative damage to biomolecules. Among the sources of antioxidants are fruits, vegetables and legumes (Ganthavorn & Hughes 1997).

Mimosa pigra, a woody shrub from the Leguminosae family is selected in this study since it contains some types of antioxidant compounds that had been reported before. Sulaiman (1997), has isolated three types of quercetin, *i.e.* quercetin glucoside, quercetin acetylgalactoside and quercetin acetylarabinoside from *Mimosa pigra*. This plant also contains other types of flavonoids, *i.e.* myricetin glucoside, myricetin arabinoside, kaempferol rutinoside and luteolin.

The objective of this study is to conduct bioactivity guided fractionation of the *M. pigra* polar extracts using paper chromatography. The bioactive constituents found in the best antioxidant fraction were identified.

MATERIALS AND METHODS

Plant materials

The whole plant of *Mimosa pigra* was collected from various places at University Sains Malaysia campus.

Flavonoids extraction

The whole plant of *Mimosa pigra* was boiled in 80% methanol for 1-2 hour and left it overnight. The extract was then filtered to remove the debris and concentrated using the rotary evaporator. The concentrated extract was applied as a streak on 12-15 sheets of Whatman no 3 paper (46 x 57) and run in solvent BAW (Butanol : Acid acetic : Water; 4:1:5; upper layer) overnight. The dried papers were viewed under UV and all the flavonoid glycoside bands were cut out and eluted in 80% methanol overnight. A total of 7 bands were screened for their antioxidant activities. The

fraction which shows the best antioxidant activities was then again streaked and rerun on Whatman no 3 with the solvent 15% HOAc. Another 7 fractions were then screened for the antioxidant activity.

Antioxidant assay

Ferric Thiocyanate (FTC) method

The autoxidation assay was performed based on the method of Osawa and Namiki (1981) under slight modification. A sample solution containing 4 mg plant extract in 4 ml 99.5% ethanol, 4.1 ml 2.5% linoleic acid in 99.5% ethanol, 8 ml 0.02 M phosphate buffer (pH 7.0) and 3.9 distilled water was placed in a columnar vial with a screw cap and incubated in the dark at 40° C for 11 days. To 0.1 ml of this sample solution, 9.7 ml 75% ethanol and 0.1 ml 30% ammonium thiocyanate were added. Precisely 3 min after the addition of 0.1 ml 2 X 10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, absorbance of the red colour was measured at 500 nm. BHT (4 mg) was used as a positive control.

Thiobarbituric acid (TBA) method

The sample solution was prepared and incubated as described above. The assay was based upon the reaction of TBA with Malonaldehyde, one of the aldehyde products of lipid peroxidation. The sample was heated with TBA under acidic conditions (add 2.0 ml 0.67% trichloroacetic acid), and the formation of malonaldehyde was measured by reading the absorbance at 532 nm (Ottolenghi 1959), one day after the final day of the FTC assay.

Identification of the phenolic compounds

The extract which showed the best antioxidant activity was then tested for the determination of phenolic compounds by using the TLC (Thin Layer Chromatography) methods. For the phenolic test, cellulose plates had been used and were run in the three solvents: BAW, 15% acetic acid and distilled water. The plates were viewed under UV light to detect the colour of the spots and to determine the R_f values.

Acid hydrolysis for aglycones

An aliquot of the extract was hydrolyzed with 2M HCl for 30-40 minutes in the boiling water. Hydrolyzed sample was allowed to cool and ethyl acetate was used for extracting the flavonoid aglycones. The mixture was vortexed and the aglycones were extracted from the upper layer. The extracts were run on cellulose TLC plate with different solvents *i.e.* 15% acetic acid, BAW, distilled water, BN and BEW. The plates were viewed under UV light to detect the colour of the spots and the R_f value were determined.

UV-Visible Spectrophotometry

A CARY 50 Conc-UV Visible Spectrophotometer was used to determine the type of flavonoid. The spectrum of a flavonoid usually consisting of two major absorption maxima in the range of 200-400nm and 200-700nm.

Liquid Chromatography-Mass Spectrophotometer (LC-MS)

The sample was analyzed using a Spectra System UV600LP in the range 220nm to determine the molecular weight of the compound and to confirm the identification.

RESULTS AND DISCUSSION

Seven fractions namely B1-B7, which were fractionated using BAW as solvent system from the paper chromatography were screened for their antioxidant activity using FTC and TBA method. Figure 1 shows the changes of the absorbance values of different fractions during the 10 days of incubation in the FTC methodology. The individual activity of samples with low absorbance value indicated high levels of antioxidant activity. B1, B2 and B3 were most successfully inhibited the oxidation of linoleic acid, better than α-tocopherol. The results were supported by the TBA method where it is used for measuring the extent of lipid peroxidation (Figure 2). Those three fractions were again fractionated using 15% acetic acid as solvent system.

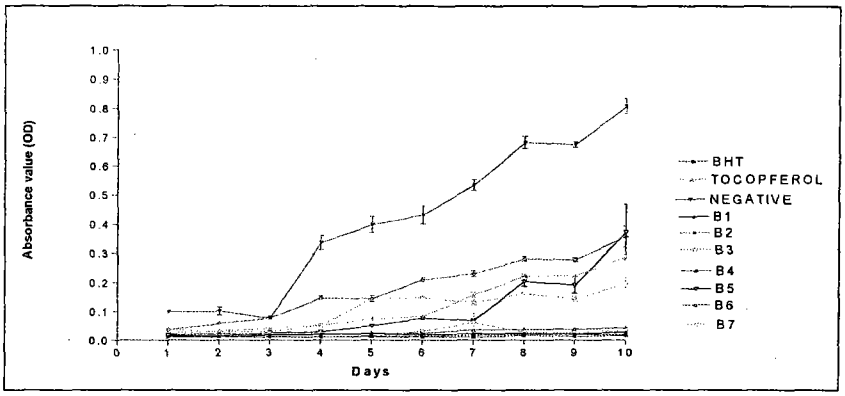

Fig. 1. Absorbance values of different fractions using FTC method. The data are expressed as mean ± S.E.M. in nine replicates. BHT and α-tocopherol were used as a positive control.

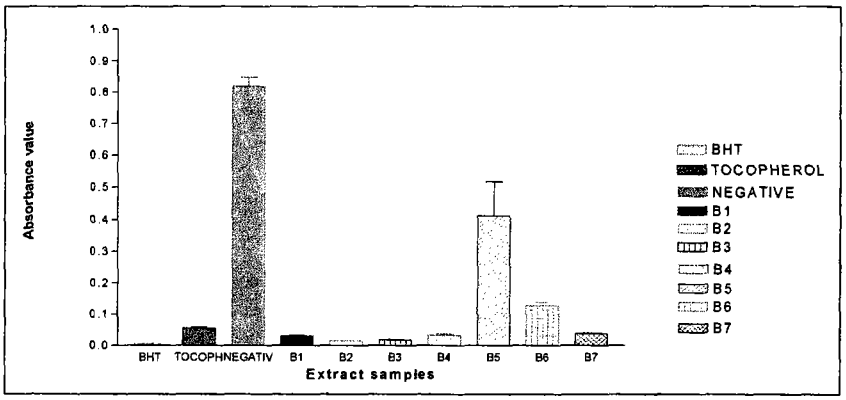

Fig. 2. Absorbance values of different fractions using TBA method (solvent BAW).

Among all the tested fractions, the B2c was found to exhibit the best antioxidant activity in the FTC method as the absorbance value lower than BHT (Figure 3). The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation. During the lipid oxidation process, peroxide decomposes to form malondialdehyde (MDA) that are measured using by the TBA method (Mackeen *et. al.* 2000). In TBA test, only B2c showed absorbance value lower than α-tocopherol, but higher than BHT (Figure 4). All other extracts showed weaker antioxidant activity than α-tocopherol and BHT.

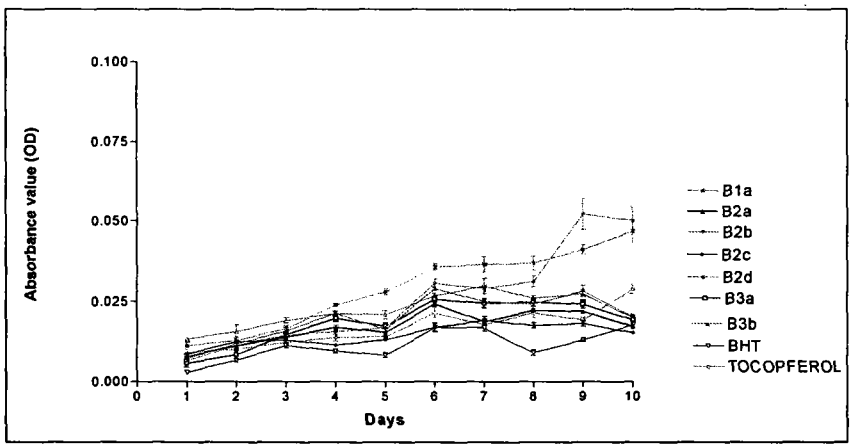

Fig. 3. Absorbance values of different fractions using FTC method. The data are expressed as mean ± S.E.M. in nine replicates. BHT and α-tocopherol were used as a positive control. The fractions were isolated using 15% acetic acid as solvent system.

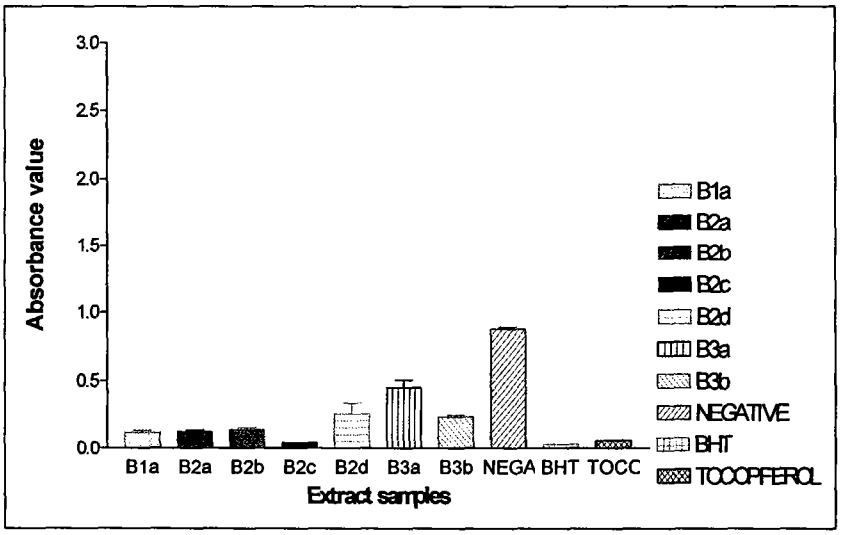

Fig. 4. Absorbance values for the bands on the 11th day for TBA method (15% acetic acid).

The phenolic compounds found in B2c were determined based on the R_f values, UV spectrum and mass spectrum.

The best method of separating and identifying the phenolic compounds is by using TLC (Thin Layer Chromatography) (Harborne 1998). They are detected after acid hydrolysis treatment where the flavonoid glycosides were separated to flavonoid aglycones and sugars. Table 1 shows the R_f value and colour of the aglycone compound of the B2c when it is developed using the BAW, 15% Acetic acid and H_2O as the solvent system; while Table 2 shows the R_f value and colour of the glycosides.

Table 1. R_f value and colour for the aglycones.

Phenolic compound	**Colour**	**Rf x 100**				
	UV	**BAW**	**15 % acetic acid**	**H_2o**	**BN**	**BEW**
A	Florescence yellow	90	90	93.75	32.5	76.25

Table 2. R_f value and colour of the phenolic compound in B2c which had been developed using different kind of solvents i.e. BAW, 15% Acetic acid and H_2O.

Phenolic compound	**Colour**		**Rf x 100**		
	UV	**Ammonia**	**BAW**	**15 % acetic acid**	**H_2O**
A	Florescence green	No changes	26.3	85	75

Table 3. This table shows the molecule weight (MS), retention time and formula molecule of the B2c.

Sample	Molecule weight (MS) at *m/z*	Retention Time	Formula molecule + glucose (weight)
B2c	$[M + H]^+$ 365.2	2.17	*o-Coumaric* acid ($C_9H_7O_3$:163) + acyl group (C_3H_4 : 40) + Glucose ($C_6H_{10}O_5$:162)

As indicated in Figure 5, UV spectrum of the sample B2c showed that it has the maximum peak at 200nm. The mass spectrum analysis gave a major molecule ion, $[M+H]^+$ at *m/z* 365.2. It had been detected at retention time 2.17 min (Figure 6). Based on all the information, the new antioxidant compound found in the *Mimosa pigra* extract is tentatively identified as a derivative of *o-Coumaric* acid. The suggested chemical structure is indicated in Figure 6.

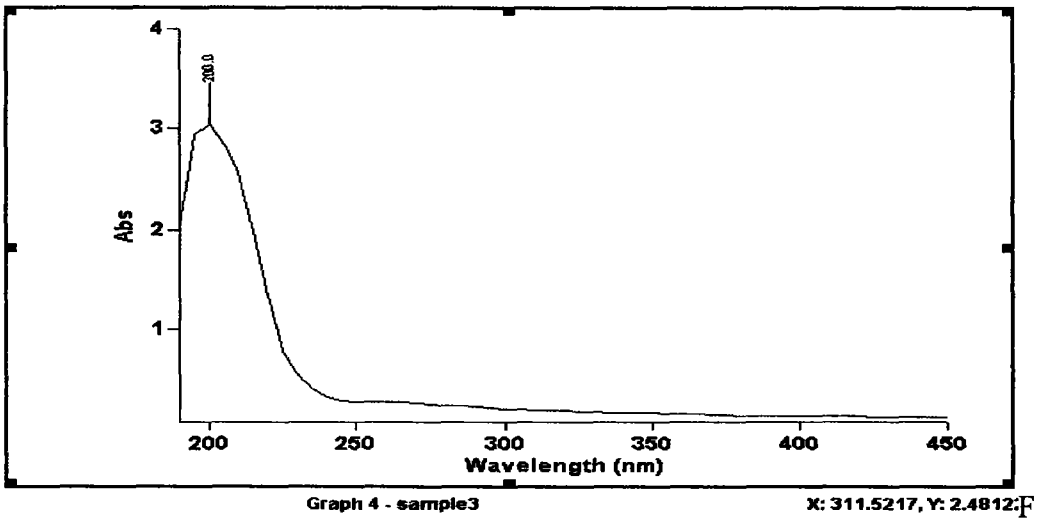

Fig.5. The peak correspond to flavonoid glycone.

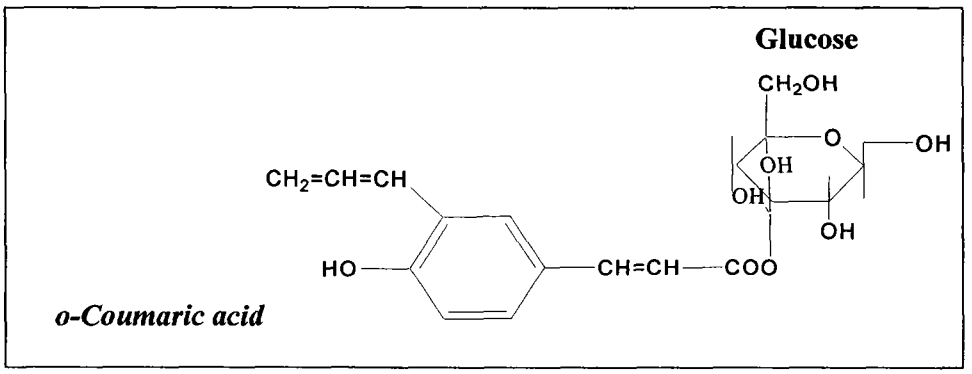

Fig. 6 Molecule structure of the flavonoid glycosides.

REFERENCE

Frankel, E.N. (1996). Antioxidants in lipid foods and their impact on food quality. *Food Chem.* **57**: 51-55

Ganthavorn, C. and Hughes, J.S. (1997). Inhibition of soybean oil oxidation by extracts of dry beans (*Phaseolus vulgaris*). *J. Am. Oil Chem. Soc.* **74**: 1030-1035.

Harborne, J.B. (1998). *Phytochemical Methods-a guide to modern tecniques of plant analysis.* 3rd edition. Chapman and Hall.

Mackeen, M.M., Ali A.M., Lajis N.H., Kawazu K., Hassan Z., Amran M., Habsah M., Mooi L.Y. and Mohamed S.M. (2000). Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. Ex T. Anders. *Journal of Ethnopharmacology* **72**: 395-402.

Ottolenghi, A. 1959. *Arch. Biochem. Biophys.* **79**: 355.

Sulaiman, S.F. (1997). Chemosystematics studies of the Tribe Mimoseae.PhD. thesis, University of Reading, Whiteknight, England.

Osawa, T. & Namiki, M. 1981. A novel type of antioxidant isolated from leaf wax of *M Eucalyptus* leaves. *Agricultural and Biological Chemistry.* **45 (3)**: 735-739.

Vaughan, 1997. Oxidative modification if macromolecules minireview series. *J. Biol. Chem.* **272**: 18513-18513.