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Optimal recovery of Apigenin from *Torreya grandis* by extraction, fractionation and structure elucidation

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Abstract: - Apigenin, a dietary plant derived flavone subclass of flavonoid is increasingly in demand in pharmaceutical industries due to its various biological and physiological activities including antioxidation, anti-inflammation, anti-cancer, antibacterial and antifungal etc. *Torreya grandis* Fort ex. Lindl is an indigenous medicinal plant, has a folk reputation in rural southern China as a hypoglycemic agent. The leaves and needles of this plant are major source of natural apigenin. This study developed a low-cost process encompassing the efficient extraction, isolation and fractionation to obtain high-purity apigenin from *T. grandis* and it could improve the economic utilization of this plant. The sequential separation and purification procedures established in this study involved extraction with ethanol at 80 °C for 3 hour followed by solvent-solvent extraction and then elution with aqueous ethanol at 30% and 50% (v/v) on a polyamide column. These conditions resulted in the recovery of 80% of total apigenin with over 96% purity. In the present study, high-purity apigenin was obtained from *T. grandis* through low-cost processes, the separation and purification strategy established in this study could provide valuable information to the relevant industries.

Key-Words: - Apigenin, Optimal recovery, Extraction, Isolation, Fractionation, *Torreya grandis*

1 Introduction

Apigenin is a 4′,5,7 trihydroxy flavone, belongs to a less toxic and non-mutagenic flavone subclass of flavonoids. It is derived from the Chinese medicinal plant *Torreya grandis* and many other plants. It has a variety of pharmacological activities, including antioxidant [1], anti-tumor [2], anti-inflammatory [3], anti-bacterial [4], antiproliferative [5], oxygenase inhibitor [6], induces apoptosis [7]. The monomer apigenin is fit into a pharmacophore model for ligands binding to the GABAA receptor benzodiazepine site [8]. It also act as inhibitors of IL-4 synthesis and CD40 ligand expression by basophils [9]. It has protective effect on radiation-induced chromosomal damage in human lymphocytes [10].

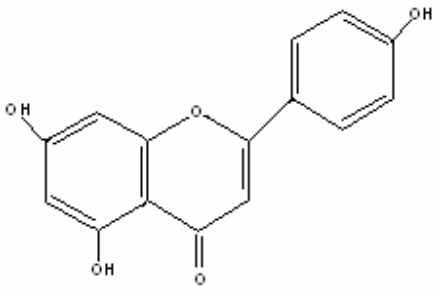


Fig-1 Structure of Apigenin

Torreya grandis Fort ex. Lindl (Taxaceae), common name Nutmeg yew tree, ornamental plant, common in China and Japan is a large sized ever green coniferous tree with dioecious flowers and drup-like fruits with nut seeds [11]. It is an indigenous medicinal plant due to its anthelmintic, antitussive, carminative, laxative [12], antifungal, antibacterial and antitumer

activity [13]. The object of this study was to extraction, isolation, fractionation and structure elucidation of apigenin and to developed an effective and industrially feasible method for efficiently obtaining high-purity apigenin from *Torreya grandis.*

2 Materials and Methods

2.1 Plant material

The leaves of *Torreya grandis* plant was selected for study, which has been collected from southern area of China. The identity was made with the help of botanist using taxonomic rules (Voucher specimen no. P-96/7) and specimen was kept for future references.

2.2 Extraction and Isolation

The 100g leaves of *T. grandis* were washed well to remove the dust, dried at room temperature and were extracted with 1000 ml of one of the following aqueous (aq.) solvents: acetone, water, methanol, ethanol, 80% ethanol, 70% ethanol and 60% ethanol. The above procedures were repeated three time and the extracts obtained from extractions were combined. The extracts were filtered and allowed to stand for 72 h in refrigerator and then concentrated under reduced pressure. After HPLC analysis of the extracts, the most appropriate extraction solvent was chosen.

The extraction temperature and time were chosen in the ranges 40–100°C and 1–5 h, respectively. The crude extract of 80% ethanol was dissolved in water and partitioned by using different suitable solvents like petroleum ether, chloroform, ethyl acetate and n-butanol. The yield of apigenin was measured.

2.3 Fractionation of *T. grandis* extract

For fractionation 5 g of ethyl acetate extract containing apigenin were loaded onto a polyamide column (mesh size 100-200 μm) and washed with 1000 ml of 95% (v/v) aqueous ethanol and water (both at a rate of 40 ml/min). Then eluted with 500 ml of 10%, 30%, 50%, 70% and 95% (v/v) aq. ethanol. The eluted fractions were concentrated to dryness at 70°C under a reduced pressure. Chemical reagents used in this study were purchased from Sigma Chemical (St. Louis, MO, USA) unless specified. Now concentrated 50% aq ethanol extract were loaded onto Sephadex LH-20 (pharmacia) column and eluted with 250 ml of 80% methanol. Pure apigenin was collected and the residue was dissolved in methanol for HPLC analysis [14].

2.4 Instruments and conditions

HPLC analysis was performed using a BFRL-HPLC PUMP SY-8100 coupled to a BFRL-UV/VIS Detector SY 8200. Separation was performed on a Scienhome kromasil C18, 5μ column (250mm× 4.6mm. The mobile phase was acetonitrile–water (5-100%, gradient, 40 min) and the flow rate was 1.0 ml/min. The column temperature was set at 25 ◦C. The injection volume was 20 μm. It was monitored by UV absorbance at 254 nm.

MS experiments were performed on a Agilent 1100 LC-MSD-Trap-SL, mass spectrometer equipped with an ESI interface. Mass spectrometric conditions were optimized in order to achieve maximum sensitivity. The ESI conditions were as follows: HV Capillary Voltage 3500 V; Dry temperature 325 °C ; Dry Gas 5.00 l/min; Nebulizer Pressure 15.00 psi; Target Mass 540m/z; Scan Begin 100 m/z; Scan End 800 m/z; trap Drive 40.1.

1H (400 MHz) spectra were recorded on NMR spectrometer ARX-400 in DMSO. Chemical shifts were given in ppm and coupling constants (*J*) in Hz.

2.5 Statistical analysis

Means and standard errors of experimental data were calculated and plotted by using Graphpad Prism software (Graphpad Software, San Diego, CA, USA).

3 Results and Discussion

3.1 Effects of extraction solvent

For finding an effective solvent for the extraction of apigenin various solvents were tested as shown in Fig. 2-3. The use of 80% ethanol produced the highest yield of extract 34.5g, containing highest yield of apigenin 1235mg from 100 g of dry *Torreya grandis*

plant. While yields of extract from acetone 12g, water 20g, methanol 28g, ethanol 22g, 70% ethanol 33.5g and 60% ethanol 33g, containing apigenin 133mg, 228mg, 741mg, 608mg, 976mg and 807mg, respectively from 100 g of dry *Torreya grandis* plant. These results are first time reported. In our experiments, 80% ethanol was used due to high yield and the lower toxicity of ethanol compared to the other solvents tested in this study (e.g., acetone). When it was partitioned by using different suitable solvents like petroleum ether, chloroform, ethyl acetateand n-butanol, affording apigenin 0, 160, 570 and 480 mg/100g of dry plant. Ethylacetate and n-butanol contain more apigenin as compared to other solvents. Fig-4

Fig-2 Effect of extraction on yield of extract

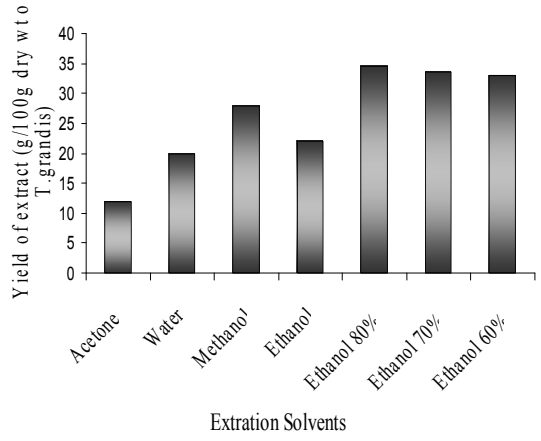


Fig-3 Effect of extration solvents on yield of Apigenin

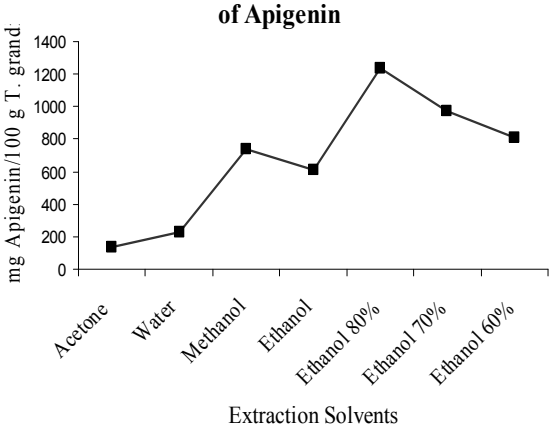
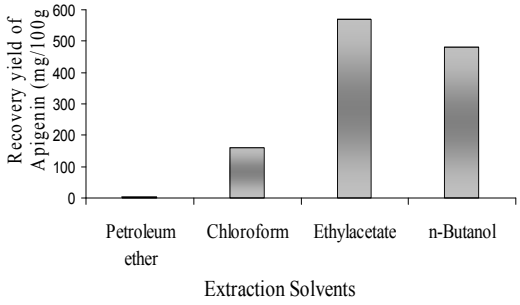


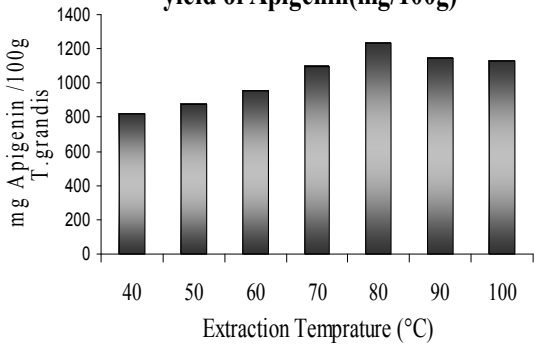
Fig-4 Effect of solvent-solvent extration on recovery yield of Apigenin (mg/100g)



3.2 Effects of extraction temperature and time

The extractions were carried out at various temperatures under the conditions described in Section 2.2. The recovery yield of extract and the apigenin content generally increased with an increase in extraction temperature up to 80°C and these results are probably due to a higher solubility and diffusivity of solute in liquid at a higher temperature. Since the increase in the extraction yield was very large from 70–90°C, extraction temperatures. So 80°C was considered optimal temperature for achieving a high recovery yield of extract containing apigenin. Fig-5

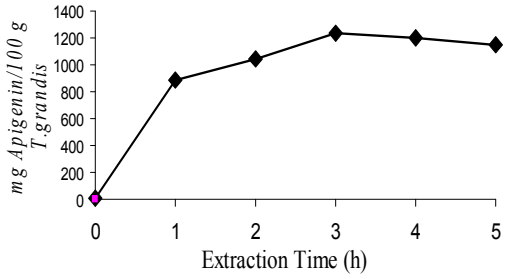
Fig- 5 Effect of temprature on recovery yield of Apigenin(mg/100g)



Using the optimal extraction conditions selected in sections 2.2, extractions were conducted for various extraction time lengths. The extract recovery yield appeared to increase by increasing extraction time up to 4 h, but the further extending of extraction time did not result in an increase of extract recovery yield.

The polyphenol content were not much affected by varying extraction time, as described by Kima *et., al*. 2004 [15]. Therefore, the optimal extraction time was considered to be 3 h. Fig 6

Fig-6 Effects of time on recovery yield of Apigenin (mg/100g)



3.3. Effects of fractionation of extract using a polyamide resin

The leaves of *Torreya grandis* were extracted under optimal conditions (80% aq. ethanol, 80 ◦C, and 3 h) determined in the preceding sections. Results are listed in Table 1. To increase the concentration apigenin extract obtained from ethylacetate fraction under the optimal conditions were fractionated using the polyamide resin column, the column was washed with ethanol and water then eluted with 10%, 30%, 50%, 70% and 95% (v/v) aq. ethanol, respectively, at a flow rate of 40 ml/min. When the elution flow rate exceeded 100 ml/min, the yield of final product decreased, probably due to insufficient adsorption time. The results in Table-1 show that 10% aq. ethanol preferentially dissolved the highest proportion of hydrophilic fraction relative to apigenin, eluting with 30% and 50% aq. ethanol yielded 18 and 80% (w/w) of total apigenin with over 96% of purity, while the elution of apigenin by 70% ethanol gave only 0.55% and elution with 95% ethanol gave no apigenin in this plant.

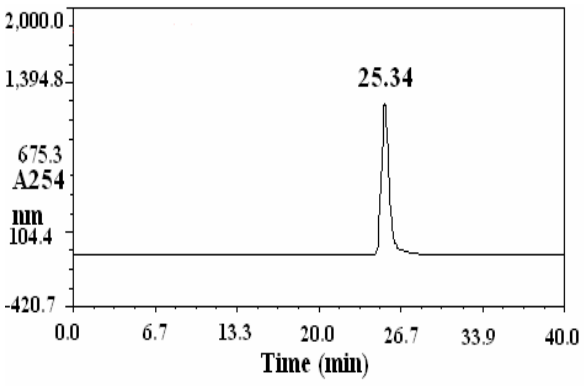
Table-1 **Fractionation of Apigenin on Polyamide column with aqueous Ethanol**

Solvents	Total dry solids		Apigenin Recovery yield (%, w/w)
	Weight (mg)	Recovery yield (%, w/w)	
Water	2810±42	56.2± 1.7	0.0 ± 0.0
20% Ethanol	250 ± 3	5.0 ± 0.3	0.87± 0.2
30% Ethanol	420 ± 5	8.4 ± 0.6	18.0± 0.8
50% Ethanol	1230 ± 8	24.6± 1.5	80.0± 2.0
70% Ethanol	180 ± 2	3.6 ± 0.2	0.55± 0.1
95% Ethanol	110 ± 1	-	-

3.4 Identification of isolated compound

HPLC analysis Fig. 7 revealed that the purity of the apigenin crystals was 96%, with the final recovery yield of apigenin for the overall separation and purification processes involving extraction, fractionation steps being as high as 80%.

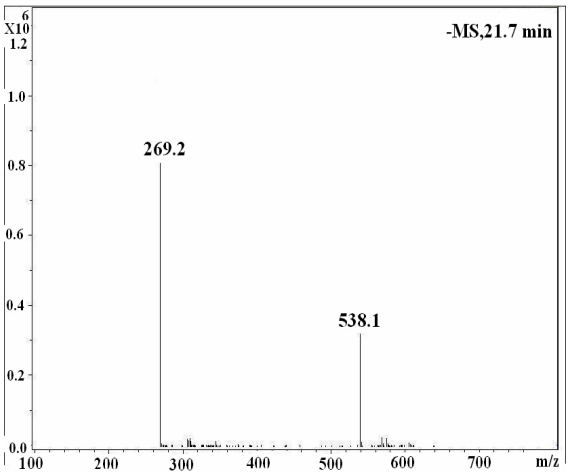
Fig-7 HPLC chromatogram of Apigenin purified from Ethylacetate extract of *Torreya grandis*



Apigenin pale yellow crystals identified as 4′,5,7 trihydroxy flavone MP 232 °C, MF: $C_{15}H_{10}O_5$. Practically insoluble in water, moderately soluble in hot alcohol, soluble in dilute KOH and Incompatible with strong oxidizing agents. ESI spectra shown [M-H] 269, [2M-H] 538 (by-MS spectra, Rt-21.7 min),Fig-8.

1H NMR (DMSO, 400 MHz): δ 7.83 (2H, *d*, *J* = 8.8 Hz, H-2*0* and H-6*0*), 6.92 (2H, *d*, *J* = 8.8 Hz, H-3*0* and H-5*0*), 6.83 (1H, *d*, *J* = 2.1 Hz, H-6), 6.71 (1H, *d*, *J* = 2.1 Hz, H-8), 6.58 (1H, *s*,H-3). By comparison of 1H NMR data with those given in the literature, the structure of this compound was identied as apigenin [16-17].

Fig-8 ESI Mass spectra of Apigenin



4. Conclusion

This study demonstrates to an effective process for separating and fractionating of apigenin. Optimal conditions for the recovery high content of apigenin was determined to be extraction with 80% aq. ethanol at 80 ◦C for 3 h, followed by fractionation with 50% aq. ethanol using polyamide column. Apigenin was obtained in crystal form with over 96% purity through relatively low-cost sequential processes only requiring water, ethanol, and polyamide resin. This economically-feasible process can be readily applied in those industries demanding high-purity apigenin, with high value due to its various biological and physiological activities.

In further studies its antioxidant activity *(in vitro and in vivo)* should, however, be performed in order to understand the mechanisms of action, as well as to evaluate the toxicity, looking toward a clinical employment of this bioactive compound, and also required to elucidate the broader implication of apigenin.

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