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Contributed Paper

# Chemical Characterization of Polyphenols of Egyptian *Achillea fragrantissima* with *In vitro* Antioxidant Study

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## ABSTRACT

*Achillea fragrantissima* (Forssk.) Sch. Bip. is one of the most popular and important medicinal plants extensively used in Egyptian folk medicine for healing different sufferings and diseases. The antioxidant properties of hydroalcoholic extract obtained from the aerial parts of *A. fragrantissima* were *in vitro* studied. The total phenolic content was 56.6 mg gallic acid equivalents/g dry weight, and the oxygen radical absorbance capacity (ORAC) was 1.127 μmol Trolox equivalents/mg sample. The HPLC-MS identification of polyphenols revealed mainly the presence of flavones, in addition to benzoic acids, cinnamic acids, and flavonols. The hydroalcoholic extract protected human astrocytoma U373-MG cells against hydrogen peroxide-induced formation of reactive oxygen species and loss of cell viability. These findings suggest that the hydroalcoholic extract from *A. fragrantissima* is a natural source of antioxidant compounds that may be useful for counteracting the oxidative stress damage in central nervous system cells.

**Keywords:** *Achillea fragrantissima*, antioxidant properties, polyphenols

## 1. INTRODUCTION

Oxidative stress plays a key role in mediating hydrogen peroxide-induced neurotoxic damage in the origination and development of many disorders of the central nervous system (CNS) such as Alzheimer and Parkinson's diseases [1]. Hydrogen peroxide is the major reactive oxygen species (ROS) in the human body, and it can be a precursor of hydroxyl radical which can directly oxidize and damage cellular components such as lipids, proteins, and DNA [2]. The use of exogenous antioxidants represents one of the most promising therapeutic strategies for the prevention and treatment of these CNS diseases associated with oxidative stress. In this context, medicinal plants are a source of a great variety and diversity of bioactive compounds with potential antioxidant properties [3].

*Achillea fragrantissima* (Forssk.) Sch. Bip. belongs to the Asteraceae family and it is one of the most used medicinal plants in the

Egyptian folk medicine [4]. This dominant plant, found in the limestone wadis of the Sinai desert, is being traditionally employed for the treatment of cough, digestive problems, and infections [5]. The extensive use of *A. fragrantissima* for medical purposes is such that the demand exceeds its natural regrowth [6].

Searching for traditional Bedouin herbal medicines with antioxidant activity, we evaluated the antioxidant activity of hydroalcoholic extract obtained from the aerial parts of *A. fragrantissima* by determining its ROS scavenging activity, and identified and quantified its major polyphenols.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and gentamicin were obtained from Invitrogen (Carlsberg, CA, USA). Hydrogen peroxide, dimethyl sulfoxide (DMSO), 2, 7-dichlorofluorescein diacetate (DCFH-DA), 3-(3, 4-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), 2, 2'-azobis(2-amidino-propane) dihydrochloride (AAPH), fluorescein, and Trolox were purchased in Sigma - Aldrich (St Louis, MO, USA).

### 2.2 Plant Collection

The flowering aerial parts of *A. fragrantissima* were harvested in May 2008 in the mountains of Saint Katherine in Sinai (Egypt), following the recommendations in the International Code of Conduct for Plant Germplasm Collecting and Transfer from the Food and Agriculture Organization of the United Nations (FAO). Samples were identified in the Department of Plant Biology (MAF) of the Faculty of Pharmacy of the University Complutense of Madrid, Madrid, Spain. A voucher specimen is there available with the identification number MAF 169940. Plant samples were dried at room temperature in a dry place in the shade. Then, samples were milling and stored in amber flasks until extraction.

### 2.3 Preparation of Extracts

Five hundred g of dried plant material were extracted using 70% aqueous ethanol by extraction methods of shaking maceration at room temperature. The hydroalcoholic extract was filtered through Whatman filter paper (No. 1), and the filtrate was dried by rotary evaporation at 55 °C. The residue was kept at 4 °C.

### 2.4 Analysis of the Total Polyphenolic Content

The total polyphenol content was determined by the Folin-Ciocalteau method. Briefly, extract samples (0.5 mL) were mixed with the Folin-Ciocalteau reagent (0.5 mL). After 3 min of reaction, 10 mL of sodium carbonate (75 g/L) and distilled water (14 mL) were added. After 1 h of incubation, absorbance was read at 750 nm. The content in polyphenols was determined from a linear regression equation using the standard gallic acid. Results were expressed as gallic acid equivalents in mg/mL hydroalcoholic extract [7].

### 2.5 ORAC Assay

The antioxidant activity of the hydroalcoholic extract was determined by the oxygen radical absorbance capacity (ORAC) assay, which was performed as previously reported [9]. Briefly, 120 µL of fluorescein (70 nM final concentration) and 20 µL of different concentrations of extract samples (from 0.5 mg/mL to 1 µg/mL) and Trolox were added in a 96-well plate and preincubated for 10 min at 37 °C. Following, 60 µL of the free radical generator AAPH (12 mM final concentration) was added to the mixture.

The fluorescence was measured for 98 min on a FLUOstar Optima (BMG Labtech, Offenburg, Germany) fluorometer. The ORAC value was calculated by the area under curve (AUC) and was expressed as µM of Trolox equivalents (TE)/mg of sample.

### 2.6 Analysis of Polyphenols by HPLC-MS

Polyphenols were analyzed on a HPLC-PAD Waters system (Milford. MA, USA), connected to a mass selective Hewlett Packard 1100MS chromatograph detector (Palo Alto, CA, USA) with an electrospray ionization (ESI) interface. Twenty-five mL of the extract samples were injected onto a Nova-Pak C18 column (300 × 3.9 mm, 4 mm) using the solvents water/acetic acid (98:2 v/v; named as A) and water/acetonitrile/acetic acid (78:20:2 v/v/v; named as B). Chromatographic separation was carried out with a flow rate of 1 mL/min (from 0 to 55 min) and 1.2 mL/min (from 55 min to 100 min), as follows: 0 - 55 min, 100% - 20% B in A; 55 - 70 min, 20% - 10% B in A; 70 - 80 min, 10% - 5% B in A; 80 - 100 min, 100% B. Eluates were scanned from 210 to 400 nm with an acquisition speed of 1 s. Mass spectra were run in the negative - ion mode of analysis, $N_2$ as the nebulizing gas at 275 kPa, drying gas flow rate and temperature of 10 L/min and 340 °C, respectively, voltage at the capillary entrance set at 4000 V and variable fragmentation voltage at 100 V ($m/z$ 200-1000) and 250 V ($m/z$ 1000-2500). Mass spectra were recorded from $m/z$ 100 to 2500. Polyphenols were identified on the basis of retention times of authentic standards [8].

### 2.7 Cell Culture

The human astrocytoma U373-MG cell line was maintained in DMEM supplemented with 10% FBS and 0.5% gentamicine. The cells were cultivated in a humidified incubator of 5% $CO_2$ / 95% air at 37 °C and they were used only until passage 30. U373-MG cells were seeded in 96-well plates (50,000 cells per well). Cells were treated with different concentrations of hydroalcoholic extract samples (from 250 to 0.025 µg/mL) for 24 h, prior to hydrogen peroxide exposure (1 mM, 30 min).

### 2.8 Cell Viability

The MTT assay was used to measure cell viability as previously reported [10]. After cell treatments (see section 2.7), 100 µL of MTT [2 mg/mL in phosphate-buffered saline (PBS)] was added to cells and they were incubated for 1 h at 37 °C. After incubation, the formazan crystals were dissolved in DMSO (100 µL), and the absorbance was then read at 550 nm. Cell viability was expressed as a percentage relative to the untreated cells (control, 100%).

### 2.9 Measurement of ROS Production

Using the DCFH - DA method, the intracellular ROS production was measured. After cell treatments (see section 2.7), U373-MG cells were treated with 100 µL of DCFH-DA (0.01 M in PBS/ glucose) for 30 min at 37 °C. After incubation, cells were twice washed with PBS/glucose. Fluorescence was measured with an excitation wavelength of 485 nm and emission wavelength of 530 nm for 2 h using a Microplate fluorescence reader (FLx800; Bio-Tek Instruments, Winooski, VT, USA). The fluorescence intensity was proportional to the intracellular ROS level [11].

### 2.10 Statistical Analysis

All experiments were done at least three times independently. Results were expressed as mean ± standard deviation (S.D.). The statistical evaluation was determined by

one-way analysis of variance followed by Tukey's test. *P* value < 0.05 was considered as statistically significant.

## 3. Results and Discussion

### 3.1 Antioxidant Activity

The present study aimed to assess the potential antioxidant properties of the hydroalcoholic extract of the aerial parts of *A. fragrantissima* from Saint Katherine, Egypt.

Among the different types of natural products, polyphenols have significant antioxidant potential [12]. The hydroxyl group and the benzene ring that make up the polyphenolic structure are responsible for their antioxidant capacities. Reactive oxygen species (ROS) are neutralized by polyphenols through H - atom and electron transfer processes[13].

### 3.1.1 Total phenol content

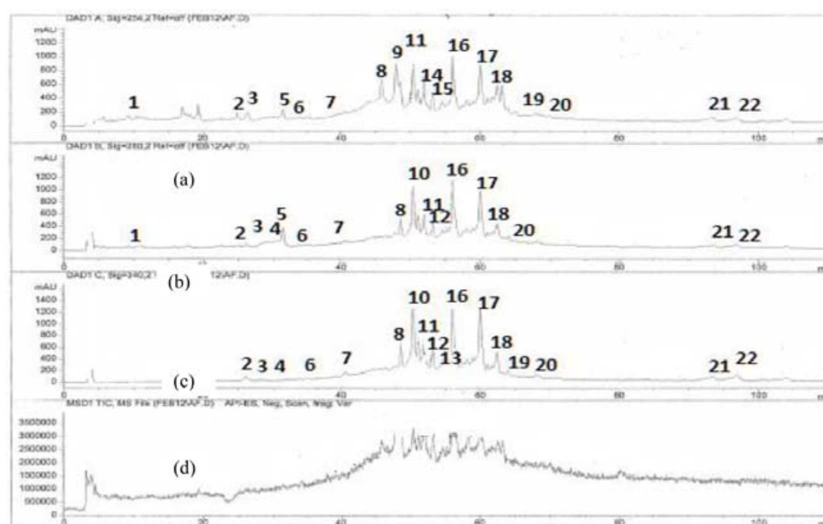
Medicinal herbs are a source of polyphenolic compounds [12]. Given the therapeutic value of natural products with phenol structure to neutralize excess ROS molecules [13], the total phenol content in the ethanolic extract of *A. fragrantissima* was first measured using the Folin-Ciocalteau method. This is a very common colorimetric method for the phenolic content determination. Compounds with a phenol group in their structure react with the Folin-Ciocalteu reagent in a basic medium and this is determined spectrophotometrically by a change of the solution colour to blue [7]. The results of the present study demonstrated that the hydroalcoholic extract of *A. fragrantissima* is rich in total phenols (56.6 mg gallic acid/g sample).

### 3.1.2 ORAC assay

The key characteristic of an antioxidant, it is its capacity to neutralize the damage induced by free radicals [13]. Therefore, the antioxidant action of the hydroalcoholic extract of *A. fragrantissima* was investigated by employing the analytical method of oxygen radical absorbance capacity (ORAC). The ORAC method is widely employed for quantifying the peroxyl radical-trapping antioxidant capacity [9]. Peroxyl free radicals are involved in the peroxidation of lipids in biological membranes, one of the main cellular mechanisms of oxidative stress-derived injury [2]. The ORAC value for the ethanolic extract of *A. fragrantissima* was 1.13 μmol TE/mg sample. The antioxidant activity of *Achillea* species using the ORAC assay has been previously determined for the ethanolic, acetone, and water extract from *Achillea millefolium*. The types of the extraction solvent determine the varying conditions extraction of compounds with antioxidant activity. The results of these studies revealed that the extract of *Achillea millefolium* possess the capacity to scavenge free radicals [14]. The present work showed that another *Achillea* sp., *A. fragrantissima*, has also good scavenging properties.

### 3.2 Chemical Characterization

Following, we analyzed the individual polyphenols contained in the hydroalcoholic extract of *A. fragrantissima* by HPLC-MS. The chromatographic profile revealed the presence of diverse flavones, mainly apigenin-derived, and cinnamic acids, mainly ferulic acid-derived. Moreover, benzoic acids and flavonols have been also identified (Figure 1). Several of the polyphenolic compounds identified in *A. fragrantissima*, including apigenin-derived and ferulic acid-derived, have previously demonstrated beneficial health properties against free radicals such as the inhibition of lipid peroxidation, the mitigation of the oxidative DNA damage, and the enhancement of the enzymatic and non-enzymatic antioxidant defense [15-17].




| Compound number | Compounds | $\lambda_{max}$ (nm) | $[M-H]^-$ (*m/z*) | Fragment Ions (*m/z*) |
|---|---|---|---|---|
| 1 | Protocatechuic acid | 256, 291 | 153 | |
| 2 | Vanillic acid | 260, 290 | 167 | |
| 3 | Chlorogenic acid 3 | 326 | 353 | 191, 179, 135 |
| 4 | Ferulic acid dehydrotrimer 1 | 324 | 563 | 193 |
| 5 | Ferulic acid dehydrotrimer 2 | 326 | 563 | 193 |
| 6 | Ferulic acid dehydrotrimer 2 | 326 | 563 | 193 |
| 7 | Chlorogenic acid 4 | 326 | 353 | 191, 179,173,135 |
| 8 | Apigenin neohesperidoside | 268, 334 | 577 | 269 |
| 9 | Chlorogenic acid 5 | 326 | 353 | 191, 135 |
| 10 | Apigenin apiofuranosyl glucoside | 268, 335 | 549 | 269 |
| 11 | Apigenin arabinosyl glucoside | 268, 337 | 563 | 269 |
| 12 | Apigenin rutinoside | 268, 338 | 577 | 269 |
| 13 | Luteolin 3′,4′-dimethyl ether 7-rhamnoside | 265, 354 | 457 | 285 |
| 14 | Kaempferol xyloside rutinoside | 266, 367 | 725 | 285 |
| 15 | Apigenin trimethyl ether glucoside | 265, 325 | 471 | 269 |
| 16 | Apigenin glucuronide | 266, 332 | 445 | 269 |
| 17 | Vitexin rhamnoside | 270, 300, 333 | 571 | 269 |
| 18 | Cirsiliol derivative | 256, 371 | | 330 |
| 19 | Cirsiliol derivative | 256, 371 | | 330 |
| 20 | Caffeoil quinic derivates | 328 | 543 | 352, 191, 173, 133 |
| 21 | Luteolin | 254, 351 | 285 | |
| 22 | Diosmetin | 251, 345 | 299 | |

**Figure 1.** HPLC - MS chromatogram of the polyphenols found in *A. fragrantissima* extract (70:30, ethanol:water) at (a) 254 nm, (b) 280 nm and (c) 340 nm (d) MS profile.

Recently, Elmann *et al*. analysed the polyphenolic content of an aqueous ethanol extract of *A. fragrantissima* collected in Arava Valley (Israel) [18]. In this cited work, the authors only identified four compounds named as epicatechin - rhamnoside, acatecin - rhamnoside, acacetin - glucoside and quercetin - glucoside, which have been not detected in our work. The number of polyphenolic compounds detected was higher in our study than Elmann et al. (2011) work [18]. The qualitative differences found in polyphenols between both studies may be attributed, on one hand, to the place where plant samples have been collected and the conditions to prepare extract, and on the other hand, to the HPLC and MS conditions employed [19]. As example, in our work, plant samples were dried at room temperature whereas in work plants were dried at 40 °C for 3 days [18].

### 3.3 Cytoprotective Properties

#### 3.3.1 Protection on cell viability

Neurodegenerative diseases have been related to a ROS overproduction. The causative role of ROS in the physiopathology of some CNS diseases (*i.e.* Alzheimer and Parkinson's diseases) implies the oxidation of biological molecules and, consequently, loss of neurons in different regions of the brain [1]. Counteracting ROS-mediated damage constitutes a useful target for the prevention of the oxidative brain injury. Since the last few years, there is an emerging interest in the investigation of polyphenolic compounds as neuroprotective compounds and in the search for new sources of this type of natural products [3]. Therefore, and based on the previous results on polyphenolic content and antioxidant activity of the hydroalcoholic extract of *A. fragrantissima*, their potential protective effect against oxidative stress induced by hydrogen peroxide in the astrocytoma U373-MG cell line was investigated. Initially, a series of increasing concentrations (from 0.025 μg/mL to 250 μg/mL) was evaluated for their effect on cell viability. None of the tested concentrations was cytotoxic for U373-MG cells (Figure 2A). Then, U373-MG cells were exposed to hydrogen peroxide (1 mM, 30 min) and a significant decrease of cell viability over 17% was observed (Figure 2B). High concentrations of hydrogen peroxide can induce death of astrocytes by alteration of membrane and cytoskeleton properties through the direct oxidation of macromolecules [20]. However, when U373-MG cells were pretreated with the hydroalcoholic extract of *A. fragrantissima* for 24 h, prior to hydrogen peroxide exposure, the cell viability was significantly increased at the concentrations of 0.025 μg/mL and 0.25 μg/mL (Figure 2B).

#### 3.1.2 Protection against ROS damage

Moreover, these two concentrations reduced hydrogen peroxide-induced intracellular ROS production in U373-MG cells, protecting cells from a stress response (Figure 3). These findings support the previous work of Elmann et al. (2011) [18] who assessed the role of the hydroalcoholic extract obtained from *A. fragrantissima* grown in Israel on the intracellular ROS production in primary glial cells. Comparing the effect of the hydroalcoholic extract of *A. fragrantissima* growing in Israel and Egypt, plant samples from Israel have stronger ability to scavenge intracellular ROS in astrocytes than those from Egypt. The quantitative differences in the antioxidant activity found between our study and that of Elmann et al. (2011) may be attributed at least in part to the differences found in the polyphenolic composition [18].

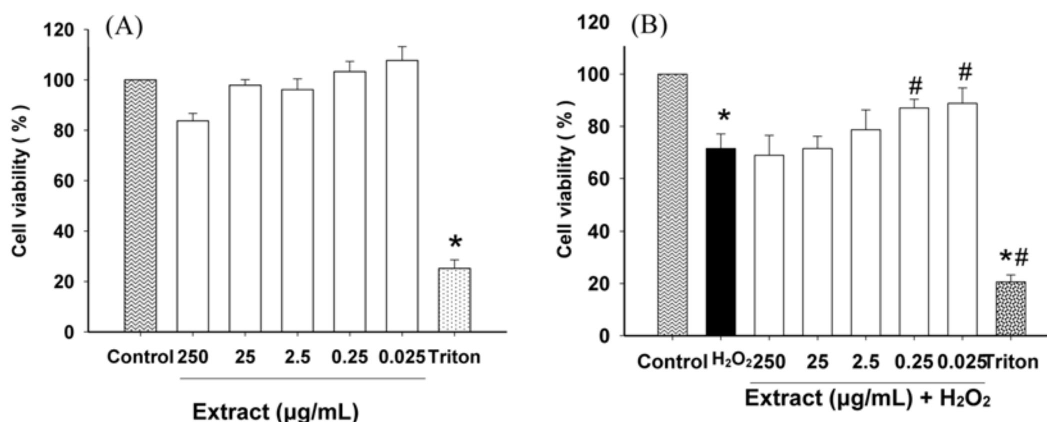



**Figure 2.** Effect of *A. fragrantissima* extract (ethanol/water, 70:30) on cell viability, measured by the MTT assay. (A) U373 MG cells were treated with plant extract (concentration range, 0.025 - 250 μg/mL) for 24 h. (B) U373 MG cells were treated with plant extract (concentration range, 0.025 - 250 μg/mL) for 24 h, prior to 1 mM $H_2O_2$ addition for 30 min. Data (mean ± SD) were expressed as percentage of control cells (100%). *$P$ < 0.05 *versus* control cells; #$P$ < 0.05 *versus* $H_2O_2$.

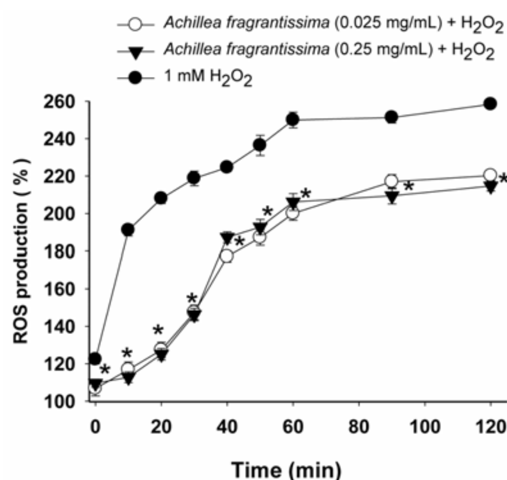



**Figure 3.** Effect of *A. fragrantissima* extract (ethanol/water, 70:30) on intracellular ROS production, measured by the dichlorofluorescein assay. U373 MG cells were treated with plant extract at the concentrations of 0.025 and 0.25 μg/mL for 24 h, prior to 1 mM $H_2O_2$ addition for 30 min. Data (mean ± S.D) were expressed as a percentage of control cells (100%). *$P$ < 0.05 *versus* $H_2O_2$.

## 4. Conclusions

In conclusion, *A. fragrantissima* has a great value in health for its antioxidant properties and its protective role under oxidative stress conditions.

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## References

[1] Kim G.H., Kim J.E., Rhie S.J. and Yoon S., *Exp. Neurobiol.*, 2015; **24**: 325.

[2] Gough D.R. and Cotter T.G., *Cell Death Dis.*, 2011; **2**: e213. DOI 10.1038/cddis. 2011.96.

[3] Krishnaiah D., Sarbatly R. and Nithyanandam R., *Food Bioprod. Process.*, 2011; **89**: 2011.

[4] Al-Qarawi A.A., Assaeed A.M. and Al-Doss A.A., *Egypt J. Appl. Sci*., 1996; **11**: 168.

[5] Saeidnia S., Gohari A., Mokhber-Dezfuli N. and Kiuchi F., *Daru*, 2011; **9**: 173-186.

[6] Al-Gaby A.M. and Allam R.F., *J. Herbs Spices Med. Plants*, 2000; **17**: 1.

[7] Saura-Calixto F., Serrano J. and Goni I., *Food Chem*., 2007; **101**: 492.

[8] Duenas M., Hernández T. and Estrella I., *Eur. Food Res. Technol*., 2004; **219**: 116.

[9] Dávalos A., Gómez-Cordovés C. and Bartolomé B., *J. Agric. Food Chem*., 2004; **52**: 48-54.

[10] Mosmann T., *J. Immunol. Methods*, 1983; **65**: 55-63.

[11] LeBel C.P., Ischiropoulos H. and Bondy S.C., *Chem. Res. Toxicol*., 1992; **5**: 227-232.

[12] Scalbert A., Johnson I.T. and Saltmarsh M., *Am. J. Clin. Nutr*., 2005; **81**: 215S.

[13] Leopoldini M., Russo N. and Toscano M., *Food Chem*., 2011; **125**: 288.

[14] Moldovan L., Gaspar A., Toma L., Craciunescu O. and Saviuc C., *Rev. Chim. (Bucharest)*, 2011; **62**: 299.

[15] Singh J.P., Selvendiran K., Banu S.M., Padmavathi R. and Sakthisekaran D., *Phytomedicine*, 2004; **11**: 309-314.

[16] Jeyabal P.V., Syed M.B., Venkataraman M., Sambandham J.K. and Sakthisekaran D., *Mol. Carcinog*., 2005; **44**: 11-20.

[17] Sultana R., Ravagna A., Mohmmad-Abdul H., Calabrese V. and Butterfield D.A., *J. Neurochem*., 2005; **92**: 749-758.

[18] Elmann A., Telerman A., Mordechay S., Erlank H., Rindner M., Ofir R. and Beit-Yannai E., Extract of *Achillea fragrantissima* downregulates ROS production and protects astrocytes from oxidative-stress-induced cell death. In book: Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring, 2011.

[19] Heck C.I., Schmalko M. and Gonzalez de Mejia E., *J. Agric. Food Chem*., 2008; **56**: 8394-403. DOI 10.1021/jf801748s.

[20] Zhu D., Tan K.S., Zhang X., Sun A.Y., Sun G.Y. and Lee J.C., *J. Cell Sci*., 2005; **118**: 3695-703.