American Journal of Biochemistry and Biotechnology, 9 (1): 41-46, 2013
ISSN: 1553-3468

doi:10.3844/ajbbsp.2013.41.46 Published Online 9 (1) 2013 (http://www.thescipub.com/ajbb.toc)

Phenolic Content, Antioxidant, Antimicrobial and Cytotoxic Activities of Ethanolic Extract of *Salix alba*

Ghassan Mohammad Sulaiman, Nehia Nima Hussien, Thorria Raddam Marzoog and Hussain Abood Awad

Department of Applied Science, Biotechnology Division, University of Technology, Baghdad, Iraq

Received 2012-07-23, Revised 2013-01-27; Accepted 2013-02-15

ABSTRACT

The total phenolic content, antioxidant, antimicrobial and cytotoxic activities of hot ethanolic extract of *Salix alba* bark were investigated. The antioxidant properties and the total phenolic contents of the extract were assessed by 1, 1-Diphenyl- 2-Picrylhydrazyl (DPPH) free radical scavenging and Folin-Ciocalteu methods, respectively. The extract showed significant antioxidant activity and antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The highest effect was observed on *C. albicans*, followed by *S. aureus* then *P. aeruginosa*. While, *Escherichia coli* and *Klebsiella pneumoniae* were not affected. The cytoxicity of *S. alba* extract against the Human Leukemia (HL-60) cell line was evaluated, it had been noticed that the extract significantly reduced the viability of HL-60 cells in a dose- and time-dependent response relationship. Findings from the present study indicate that extract is highly cytotoxic to HL-60 cells. The antimicrobial and cytotoxic activities of *S. alba* extract seemed to be positively correlated with their antioxidant potentials.

Keywords: Phenolics, Antioxidant, Antimicrobial, Cytotoxicity

1. INTRODUCTION

Since the beginnings of civilization, humans have used natural products for healing diseases that afflict them. Plants are biochemical labs that produce inside their cells a variety of complex substances with numerous active compounds. With the advent of the pharmaceutical chemistry at the beginning of the nineteenth century, plants became the primary source of substances for drug development (Rates, 2001).

White willow (*Salix alba L.*), is a willow belongs to the genus salix and family salicaeae. Willows range from prostrate shrubs to large tree over 30 m high, but most are shrubs or small trees. White willow which is also known as the salicin willow, has been used for its health benefits for thousands of years (Saller *et al*., 2008).

Records suggest that, as far back as 6000 years ago, white willow was used in Mesopotamia. Subsequently, ancient peoples recorded the use of white willow to cure pain and inflammation, including the Assyrian, Babylonian, Sumerian, Egyptian, Chinese, Greek and Roman civilizations. Hippocrates recommended chewing willow bark to patients suffering from fever, inflammation and pain. He also prescribed a brew of willow leaves to ease the excruciating pains of childbirth. Since that time, white willow has continued to be used to ease pain and inflammation (Mahdi *et al*., 2006).

Willow bark has been used to treat many different kinds of pain, including rheumatic pain, back pain, toothache and menstrual cramps. It is also used to relieve sore throat, fever and headache associated with upper respiratory tract infections and influenza (Schmid *et al*., 1998).

In spite of the long and compelling history of traditional use of willow bark, study of its medical properties is still needed. Thus, the objective of the present

Corresponding Author: Ghassan Mohammad Sulaiman, Department of Applied Science, Biotechnology Division, University of Technology, Baghdad, Iraq

study was to investigate the antimicrobial activity of ethanol extract of *S. alba* bark, the anti proliferative activity of Human Leukemia (HL-60) cell line and to determine its total phenolic content and the antioxidant activity.

2. MATERIALS AND METHODS

2.1. Extraction Methods

Plant barks commonly used in herbal medicine were dried and pulverized with motor and pestle or electric mill. The fine powder of plant parts were extracted with boiled ethanol by soxholet for 7 h. The solution was filtered through Whatman filter paper using a Buchner funnel under vacuum. The filtrate was then evaporated using a rotary evaporator under vacuum at 40°C to obtain the bark extract. Then, the resulting extract was stored, protected from light in a refrigerator at 4°C in a glass container until use.

2.2. Determination of Total Phenolics Spectrophotometrically

Total phenolic compound concentrations were determined spectrophotometrically (Mohammadzadeh *et al*., 2007). The PE (0.1 mL) was diluted with ultra pure water (7.9 mL) at a concentration of 10 mg mL^{-1}. Folin-Ciocalteu reagent (0.5 mL) was then added and the contents were mixed thoroughly. After 1 min, 0.2 mL of sodium carbonate solution was added and the mixture was mixed thoroughly. The absorbance of blue color produced solution was measured at 765 nm. Total phenolic content (mg g^{-1}) was estimated using a standard curve for Gallic acid (GAE) concentration. Three measurements were performed and the mean was adopted.

2.3. Free Radical Scavenging Activity

The free radical scavenging activity of *S. alba* extract was measured with DPPH assay (Chen *et al*., 2004). Based on this assay, equal volumes (0.5 mL) of DPPH (60 μM) and each PE (10, 50 or 100 μg mL^{-1}) were mixed in a cuvette and allowed to stand for 30 min at room temperature. Then, the absorbance was read at 517 nm in a UV/VIS Lambda 19 spectrophotometer. The absorbance of control (DPPH solution) was also read. The percentage of DPPH discoloration of the sample was calculated according to the following formula:

$$\text{Percentage of Decolouration} = \left(\frac{\text{Control Absorbance - Sample Absorbance}}{\text{Control Absorbance}}\right)\times 100$$

2.4. Antimicrobial Assay

Antimicrobial activity of *S. alba* extract was determined by the agar-well diffusion method. Four bacterial strains and one yeast were used in present study, they were *Staphylococcus aureus* (Gram positive), *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (Gram negative) and *Candida albicans* (yeast). The antimicrobial activity was performed using nutrient agar for bacteria and YEPD Agar for yeast. The cell culture suspension was adjusted by comparing against 0.4-0.5 McFarland scale standard. These suspensions (100 mL) of each target strain were spread on the plates. For the investigation of the antimicrobial activity, the extract of *S. alba* were weighed and dissolved with distilled water to obtain 10, 20, 40, 60 or 80 mg mL^{-1} extract concentration. Each sample (100 mL) was filled into the wells of agar plates directly. The diameter of the inhibition zone (mm) was measured after overnight incubation. All samples were tested in triplicate. Controls included solvent without plant extract.

2.5. Viability of Tumor Cells

The study was performed on cells human promyeloid leukemia (HL-60). The cells at density of 1.5×10^5 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 10% (v/v) inactivated fetal bovine serum, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and incubated in a humidified atmosphere of 5% CO2 at 37°C in 24-well flat-bottom culture plates. After 24 h, the six concentrations (1, 2, 4, 6, 8 or 10 μg mL^{-1}) of *S. alba* extract were added in triplicates of each. Culture medium was used instead of the extract for the negative control. The plates were incubated at 37°C in the presence of 5% (v/v) CO_2 for 6, 12 and 24 h. The cells were tested for their viability using the trypan blue exclusion test (Bennett *et al*., 1976). Two hundred cells were counted and the percentage of viable cells was estimated.

2.6. Statistical Analysis

Data were statistically analyzed using SPSS statistical software. Level of significant was assessed by using the Analysis of Variance (ANOVA) test. The level of significance was shown using the Least Significant Difference (LSD) test. Values are given as mean ± standard deviation. "P" values<0.05 were considered statistically significant.

3. RESULTS

The concentration of phenolic content in ethanolic extract of the *S. alba* bark is presented in **Table 1** and showed 162.00±14.90 mg g^{-1} of GAE. The free radical scavenging activities of different concentrations of *S. alba* bark extract are also shown in **Table 1**. In this study the pattern of DPPH radicals inhibition showed a concentration-dependent manner for *S. alba* extract.

Table 1. Total phenolics and DPPH free radical scavenging activity (FRSA) of *S. alba* bark extract (parameters as Mean ± SD)

		DPPH free radical scavenging activity					
		10.0 µg mL^{-1}		50.0 µg mL^{-1}		100.0 µg mL^{-1}	
S. alba Extract	Total phenolics concentration mg g^{-1}	Absorbance*	FRSA	Absorbance*	FRSA	Absorbance*	FRSA
Bark	162.00±14.90	0.35±0.10	12.50	0.25±0.08	37.50	0.08±0.05	80.00

* Control absorbance: 0.40 ± 0.04

Table 2. Antimicrobial activity of the *S. alba* bark extract (parameters as Mean ± SD)

	Concentration mg mL^{-1}					
Micro-organisms	Control	10	20	40	60	80
S. aureus	6.00±0.00	14.66±0.88	16.33±0.88	19.00±0.57	21.33±0.66	22.33±0.33
P. aeruginosa	6.00±0.00	NA	11.66±0.66	13.66±2.18	16.33±2.02	17.00±2.51
E. coli	6.00±0.00	NA	NA	NA	NA	NA
K. pneumoniae	6.00±0.00	NA	NA	NA	NA	NA
C. albicans	6.00±0.00	14.66±0.88	18.66±0.33	21.66±0.33	22.66±0.33	23.66±0.33

* NA: Not Affected

Table 3. The effect of *S. alba* bark extract on the viability of HL-60 cells (parameters as Mean ± SD)

	Time of incubation (h)					
	6		12		24	
S. alba extract	Dead (%)	Viable (%)	Dead (%)	Viable (%)	Dead (%)	Viable (%)
HL-60+RPMI	2±0.16	98	3±0.22	98	2±0.16	98
HL-60+1 µg	6±0.30	96	8±0.44	94	11±0.60	93
HL-60+2 µg	10±0.22	90	15±2.00	85	17±1.40	87
HL-60+4 µg	20±1.22	80	26±1.40	74	38±3.10	38
HL-60+8 µg	36±2.20	64	44±1.87	36	50±2.30	50
HL-60+10 µg	68±3.23	42	72±3.20	28	84±4.21	16

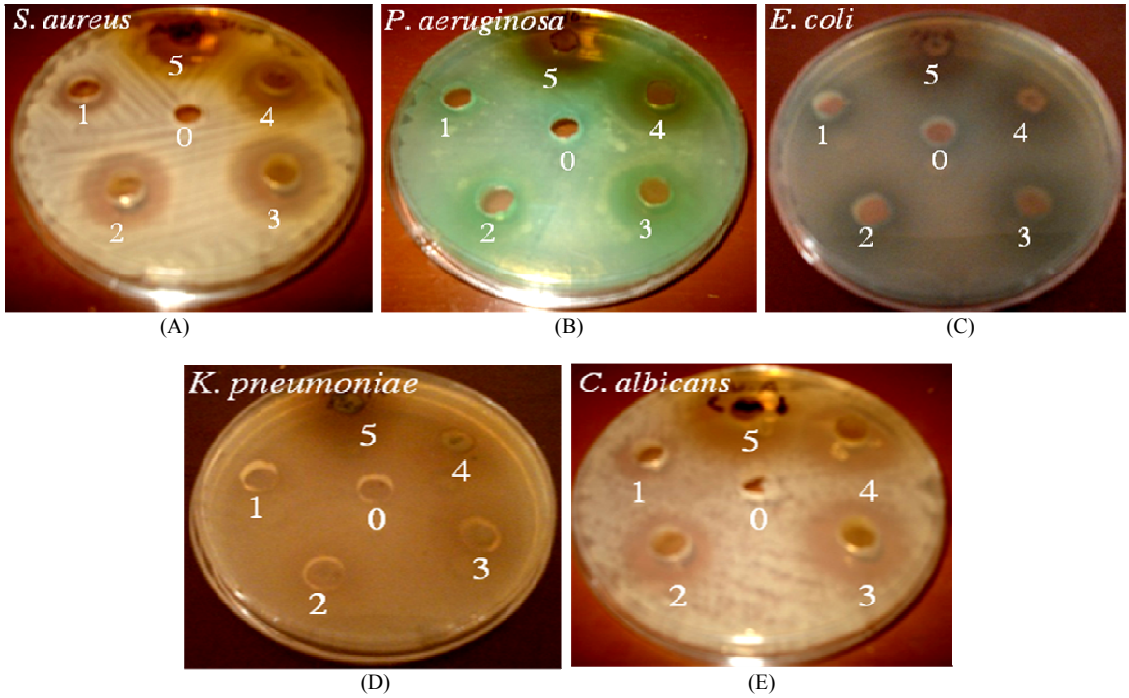

Fig. 1. Inhibition zone effects of *S. alba* bark extract. Numbers (0,1, 2, 3, 4 and 5) are control, 10, 20, 40, 60 and 80 mg mL^{-1}, respectively. (A) *Staphylococcus aureus* (B) *Pseudomonas aeruginosa* (C) *Escherichia coli* (D) *Klebsiella pneumoniae* (E) *Candida albicans*

The values of the free radical scavenging activity were 12.50, 37.50 and 80.00% of 10, 50 and 100 μg mL^{-1}, respectively.

The well diffusion method was used to determine the inhibition zones of the different concentrations from *S. alba* extract as shown in **Table 2 and Fig. 1**. The four Gram-positive, Gram-negative bacterial strains and one yeast were used. The result of the inhibitory effect of the extract on the growth of bacteria showed a considerable diversity. As shown in **Table 2 and Fig. 1C and D** *E. coli* and *K. pneumoniae* were not affected after treatment with *S. alba* extract, while others were affected to various degrees. *C. albicans* showed the largest inhibition zone with (23.66±0.33 mm) at 80 μg mL^{-1} (**Table 2 and Fig. 1E**) followed by *S. aureus* (22.33±0.33 mm) (**Table 2 and Fig. 1A**) and finally *P. aeruginosa* (17±2.51 mm) (**Table 2 and Fig. 1B**).

The antiproliferative activity of *S. alba* extract was evaluated in human promyeloid leukaemia cells (HL-60). As shown in **Table 3**, treatment with *S. alba* extract at a concentration of 1-10 μg mL^{-1} showed a dose and time dependent decrease in cell viability. The viability of tumor cells after incubation with ethanolic extract of Salix bark were greatly affected (84% cell death) compared to untreated cells (2% cell death) after 24 h of incubation (**Table 3**).

4. DISCUSSION

The results indicated that *S. alba* extract have good free radical scavenging activity and can be used as a radical inhibitor or scavenger, acting possibly as a primary antioxidant. However, the good correlation between the results from total phenolics analysis and the antioxidative assay was observed. Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extract is due to these compounds (Zheng and Wang, 2001; Chen *et al*., 2002; Luximon-Ramma *et al*., 2005; Djeridane *et al*., 2006).

The role of antioxidant is to remove free radicals. One important mechanism through which this is achieved is by donating hydrogen to free radicals in its reduction to an unreactive species. Addition of hydrogen would remove the odd electron feature which is responsible for radical reactivity. The hydrogen-donating activity, measured using DPPH radicals as hydrogen acceptor, showed that there was a significant association could be found between the concentration of extract and percentage of inhibition. The scavenging activity on DPPH radicals has been widely used to determine the free radical-scavenging activity of different matrices by several authors (Pereira *et al*., 2006; Oliveira *et al*., 2008; Sulaiman *et al*., 2011).

In this study, the antimicrobial activity was investigated and exhibited better antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria. These results are in accordance with what has been previously reported of *Ficus benghalensis* and *F. racemosa* extract. The activity was highest against *S. aureus* and lowest against *K. pneumoniae* in both the aqueous and ethanolic extracts (Murti and Kumar, 2011).While, our results contradict the effect of *Woodfordia fruticosa* extract that showes more activity against Gram negative bacteria than Gram positive bacteria (Parekh and Chanda, 2007). However, it is not surprising that there are differences in the antibacterial effects of plant groups, due to the phytochemical differences between species (Murti and Kumar, 2011).

The explanation of Gram-positive bacteria is more susceptible than Gram-negative bacteria may attribute to the differences in their cell wall structure. Gram-negative organisms are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances, including antibiotics (Kaye *et al*., 2004). However, the results of this study reveals that the crude ethanolic extract of *S. alba* contain certain constituents like tannins and glycosides with significant antibacterial property which enables the extract to overcome the barrier in bacterial cell wall (Scalbert, 1991; Senthamilselvi *et al*., 2012). There were no significant difference between the bacterial species and the yeast regarding the effect of the extract on their growth (**Table 2**). It was found that taninns have antifugal activity (Otshudi *et al*., 2005). Their activity is probably due to their ability to combine with extracellular and soluble proteins and to combine with fungal cell walls. Nature of these compounds may also disrupt fungal membranes (Tsuchiya *et al*., 1996).

From antiproliferative activity values againsts HL-60, the viability of cells considerably decreased with increasing doses and time of incubation. The mortality data obtained in these results allow us to predict their potential not only because of the cytotoxic effect, but also in terms of the potential for tumor reduction. However, previous report of El-Shemy *et al*. (2007) revealed that aqueous extract from willow leaves prevented proliferation of three cancer cell types Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL) and Ehrlich Ascites Carcinoma Cells (EACC) by causing DNA fragmentation and inducing apoptosis within treated cells. Also, they found that fractions obtaind by polar organic solvents (water and 70% ethanol) has a major destractive effectes on tumor cells as copmpared with fractions of nonpolar solvents (El-Shemy *et al*., 2007). It is clear that the antitumor activity of the willow bark was

mostly due to compounds that are soluble in ethanol. The phenolic compounds, mostly glycosides and many types of tannin will dissolve in ethanol or even water (Bravo, 1998). Therefore, these groups of compounds may contain the major active components for the destruction of leukemia cells.

The willow compounds may bind with receptors on the surface of tumor cells and penetrate into the cells. The cells could be killed through denaturation of some enzymes and proteins that are induced by some phenolic compounds like salicin and saligenin (El-Shemy *et al*., 2003; 2007). Other mechanisms of HL-60 death may be associated with Willow extract's toxicity like increase of tumor P^{53} protein expression or stopping the cell cycle at the metaphase (or other phases) and then inhibits cell division (Hostanska *et al*., 2007).

5. CONCLUSION

The result of this study support the folkloric usage of the studied plant and possesses both significant antimicrobial activity and growth inhibition effects in HL-60 cells due to the antioxidant potentials. The results of cancer cell line suggest that it could be use in prevention of cancer while warranting further *in vitro* and *in vivo* investigations to understand more about cell death mechanisms.

6. REFERENCES

Bennett, J.M., D. Catovsky, M.T. Danniel, D.G. Galton and H.R. Graanlnik *et al*., 1976. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br. J. Heam., 33: 451-458. PMID: 188440

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. Nutr. Rev., 56: 317-333. DOI: 10.1111/ j.1753-4887.1998.tb01670.x

Chen, C.N., M.S. Weng, C.L. Wu and J.K. Lin, 2004. Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources. Evid. Complement Alternat Med., 1: 175-185. PMID: 15480443

Chen, H.G., Y.G. Yu and O.X. Zeng, 2002. Study on extraction of flavonoids and Alkaloids from lotus leaf. Food Sci., 23: 69-71.

Djeridane, A., M. Yousfi, B. Nadjemi, D. Boutassouma and P. Stocker *et al*., 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chem., 97: 654-660. DOI: 10.1016/j.foodchem.2005.04.028

El-Shemy, H.A., A.M. Aboul-Enein, K.M. Aboul-Enein and K. Fujita, 2007. Willow leaves' extracts contain anti-tumor agents effective against three cell types. Plos One, 2: e178-e178. DOI: 10.1371/journal.pone.0000178

El-Shemy, H.A., A.M. Aboul-Enein, M.I. Aboul-Enein and K. Fujita, 2003. The effect of willow leaf extracts on human leukemic cells *in vitro*. J. Biochem. Mol. Biol., 36: 387-389. PMID: 12895297

Hostanska, K., G. Jurgenliemk, A. Nahrstedt, G. Abel and R. Saller, 2007. Willow bark extract (BNO 1455) and its fractions promote apoptosis in human lung cancer cells irrespective their basal level of COX-2, p53 and Bcl-2 genes. Planta Med., 73: SL018- SL018. DOI: 10.1055/s-2007-986736

Kaye, K.S., J.J. Engemann, H.S. Fraimow, E. Abrutyn, 2004. Pathogens resistant to antimicrobial agents: epidemiology, molecular mechanisms and clinical management. Infect. Dis. Clin. North Am., 18: 467-511. PMID: 15308273

Luximon-Ramma, A., T. Bahorun, A.M. Soobrattee and O.I. Aruoma, 2005. Antioxidant activities of phenolic, proanthocyanidin and flavonoid components in extracts of cassia fistula. J. Agric. Food Chem., 50: 5042-5047. DOI: 10.1021/jf0201172

Mahdi, J.G., A.J. Mahdi, A.J. Mahdi and I.D. Bowen, 2006. The historical analysis of aspirin discovery, its relation to the willow tree and antiproliferative and anticancer potential. Cell Prolif., 39: 147-55. DOI: 10.1111/j.1365-2184.2006.00377.x

Mohammadzadeh, S., M. Sharriatpanahi, M. Hamedi, Y. Amanzadeh and S.S.E. Ebrahimi *et al*., 2007. Antioxidant power of Iranian propolis extract. Food Chem., 103: 729-733. DOI: 10.1016/j.foodchem.2006.09.014

Murti, K. and U. Kumar, 2011. Antimicrobial Activity of *Ficus benghalensis* and *Ficus racemosa* roots *L*. Am. J. Microbiol., 2: 21-24. DOI: 10.3844/ajmsp.2011.21.24

Oliveira, I., A. Sousa, I.C. Ferreira, A. Bento and L. Estevinho *et al*., 2008. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia L*.) green husks. Food Chem. Toxicol., 46: 2326-2331. DOI: 10.1016/j.fct.2008.03.017

Otshudi, A.L., S. Apers, L. Pieters, M. Claeys and C. Pannecouque *et al*., 2005. Biologically active bisbenzylisoquinoline alkaloids from the root bark of *Epinetrum villosum*. J. Ethnopharmacol., 102: 89-94. DOI: 10.1016/j.jep.2005.05.021

Parekh, J. and S. Chanda, 2007. *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz. flower (Lythraceae). Braz. J. Microbiol., 38: 204-207. DOI: 10.1590/S1517-83822007000200004

Pereira, J.A., A.P.G. Pereira, I.C.F.R. Ferreira, P. Valentao and P.B. Andrade *et al*., 2006. Table olives from Portugal: phenolic compounds, antioxidant potential and antimicrobial activity. J. Agric. Food Chem., 54: 8425-8431. PMID: 17061816

Rates, S.M.K., 2001. Plants as source of drugs. Toxicon, 39: 603-613. DOI: 10.1016/S0041-0101(00)00154-9

Saller, R., J. Melzer and M. Felder, 2008. Pain relief with a proprietary extract of Willow bark in rheumatology. An Open Trial. Schweiz. Zschr. GanzheitsMedizin, 20: 156-162. DOI: 10.5167/uzh-13538

Scalbert, A., 1991. Antimicrobial properties of tannins. Phytochemistry, 30: 3875-3883. DOI: 10.1016/0031-9422(91)83426-L

Schmid, B., B.Tschirdewahn, I. Katter, I. Gunaydin, R. Ludtke *et al*., 1998. Analgesic effects of willow bark extract in osteoarthritis: Results of a clinical double-blind trial. Focus Alternat. Complementary Therapies, 3: 186-186. DOI: 10.1111/j.2042-7166.1998.tb00927.x

Senthamilselvi, M.M., D. Kesavan and N. Sulochana, 2012. An anti-inflammatory and anti-microbial flavone glycoside from flowers of Cleome viscosa. Org. Med. Chem. Lett., 2: 19. DOI: 10.1186/2191-2858-2-19

Sulaiman, G.M., K.W. Al Sammarrae, A.H. Adhiah, M. Zucchetti and R. Frapolli *et al*., 2011. Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. Food Chem. Toxicol., 49: 2415-2421. DOI: 10.1016/j.fct.2011.06.060

Tsuchiya, H., M. Sato, T. Miyazaki, S. Fujiwara and S. Tanigaki *et al*., 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. J. Ethnopharmacol., 50: 27-34. DOI: 10.1016/0378-8741(96)85514-0

Zheng, W. and S.Y. Wang, 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem., 49: 5165-5170. DOI: 10.1021/jf010697n