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Evaluation of the antioxidant and antibacterial properties of various solvents extracts of *Annona squamosa* L. leaves

Ghadir A. El-Chaghaby *, Abeer F. Ahmad, Eman S. Ramis

Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), P.O. Box 588, Orman, 9 El-Gamaa St., Giza, Egypt

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Abstract The present work was conducted aiming to evaluate the effect of different solvent extracts on the antioxidant and antibacterial activities of *Annona squamosa* L. leaves. Four solvents were chosen for the study namely; methanol 80%, acetone 50%, ethanol 50% and boiling water. Acetone and boiling water gave the highest extraction yields as compared to methanol and ethanol. Total phenolic contents of the four extracts were significantly different with acetone being the most efficient solvent and water being the least efficient one. Correlation coefficient between the total antioxidant and total phenolic content was found to be $R^2 = 0.89$ suggesting the contribution of phenolic compounds of the extract by 89% to its total antioxidant activity. The extracts were capable of scavenging H_2O_2 in a range of 43–54%. Reducing power of the extracts increased by increasing their concentration. The extracts were found to exert low to moderate antibacterial activity compared to a standard antibacterial agent. The bacterial inhibition of the extracts was found to positively correlate with their phenolic contents.

1. Introduction

In the recent years, the quest for natural food additives has become an increasing concern. Consumers' demand for healthier foods has been the initiative for many researchers seeking for natural alternatives. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Synthetic antioxidants are used for industrial processing, in order to prolong the storage stability of foods. But according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms (Türkoğlu et al., 2007). Efforts to gain extensive knowledge regarding the power of antioxidants from plants and to tap their potential are, therefore, on the increase (Ali et al., 2008). Natural antioxidants can protect the human body from free radicals that may cause some chronic diseases

* Corresponding author. Tel.: +20 101235184.
E-mail address: ghadiraly@yahoo.com (G.A. El-Chaghaby).
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including cancer, cardiovascular diseases and cataract (Kinsella et al., 1993; Lai et al., 2001).

There are also new concerns about food safety due to increasing occurrence of new food-borne disease outbreaks caused by pathogenic micro-organisms. As a consequence, natural antimicrobials are receiving a good deal of attention for a number of microorganism-control issues. Reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life extension technologies to eliminate undesirable pathogens and/or delay microbial spoilage, decreasing the development of antibiotic resistant-pathogenic microorganisms or strengthening immune cells in humans are some of the benefits (Tajkarimi et al., 2010).

The presence of bioactive compounds such as glycosides, flavonoids, proanthocyanidins, tannins, mono- and sequiterpenoids, phenylpropanoids, diterpenoids, resins, lignans, alkaloids, furocoumarines and naphthodianthrones in plants makes them a safe choice for application in the food preservation process (Bernhoft, 2010). There are many published studies reporting the antioxidant and antimicrobial activities of plant extracts including Cydonia vulgaris leaves (Yildirim et al., 2001). Rosemary, sage and their combination (Abdel-Hamied et al., 2009) *Pentatropis microphylla* L. leaves (Prabha and Vasantha, 2010). Herbs like *Amaranthus paniculatus*, *Aerva lanata*, *Coccinia indica* and *Coriandrum sativum* (Ali et al., 2008) *Moringa oleifera* (Murungai), *Musa paradisiaca* (Banana), *Azardiratica indica* (Neem), *Cynodon dactylon* (Grass), *Alternanthera sessilis* (Ponnangkani), *Anisochilus carnosus* (Karpooravalli) (Valarmathy et al., 2010).

A broad range of solvents including ethanol and ethyl acetate (Valarmathy et al., 2010), acetone and methanol (Prabha and Vasantha, 2010), hexane, ethyl acetate (Et-oAc), ethanol (Et-OH), and water (Karthikumar et al., 2007) have been used for the extraction of bioactive compounds from plant materials. Studies have shown that the extraction yield is dependent on the solvent and the method of extraction. The extraction method must allow complete extraction of the compounds of interest, and it must avoid their chemical modification (Hayouni et al., 2007).

Annona squamosa L. (Annonaceae), commonly known as sugar apple, is a tree growing in dry climate and has been widely adopted in Egypt (Morton, 1987). The plant is cultivated mainly for its edible fruits and is reputed to possess several medicinal properties (Asolkar et al., 1992). Several bioactive compounds have been isolated from the leaves of *A. squamosa* (Wagner et al., 1980; Seetharaman, 1986).The hot water extract of *A. squamosa* leaves has been reported to possess hypoglycemic and antidiabetic activity (Gupta et al., 2005); aqueous leaves extract has also been reported to ameliorate hyperthyroidism (Sunanda and Anand, 2003). Methanolic leaves extract was tested for mosquitocidal effect against *Culex quinquefasciatus* (Jaswanth et al., 2002), and the ethanolic leaves and stem extract are reported to have an anti-cancerous activity (Bhakuni et al., 1969).

Thus, the present study was conducted to investigate the effects of different extracting solvents on the total phenolic contents, antioxidant and antibacterial activities of *A. squamosa* L. extracts for their possible use as source of antioxidants and also as antibacterial agents that can be used to prevent food spoilage.

2. Materials and methods

2.1. Collection and preparation of plant material

A. squamos leaves were gathered from local gardens in Egypt. Plant leaves were washed with running tap water to cleanness. Leaves were then shade dried for 48 h, then crushed into small pieces and finally powdered using an electric blender. Plant leaves powder was then stored in plastic bags for further utilization.

2.2. Chemicals

All chemicals and solvents were purchased from Sigma–Aldrich, USA, Ltd.

2.3. Preparation of plant extracts

A. squamosa L. plant extracts were obtained by different procedures as follows: (1) 6 g of powdered *A. squamosa* L. leaves was extracted using 20 mL of methanol: distilled water (8:2), the process was repeated three times, (2) 6 g of plant powder was extracted using acetone: distilled water (1:1) in a shaking water bath at room temperature for 30 min, (3) water plant extract was prepared using boiling distilled water by the same procedure employed for acetone extraction. (4) Ethanolic extract was obtained by incubating 6 g of powdered *A. squamosa* L. leaves with 100 mL of ethanol: distilled water (1:1) for 72 h in a shaking water bath at room temperature. In all cases the extracts were centrifuged (3000 rpm) for 15 min and the supernatants were harvested and filtered using Whatman paper No. 1. The used solvents were finally evaporated through incubation at room temperature. The extraction yields were then calculated as a percent of the used powder.

2.4. Determination of total antioxidant activity (TAA)

The total antioxidant activity of *A. squamosa* L. extracts was determined using the phosphomolybdenum method according to the procedure described by Prieto et al. (1999). Each sample solution (0.1 mL, 0.5 mg/mL) was combined with 0.3 mL of reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank using a spectrophotometer UVD-3500. The antioxidant activity was expressed as (μmol/g) of ascorbic acid. Ascorbic acid and Butylated hydroxytoluene (BHT), (0.5 mg/mL) were used as reference compounds. All tests were performed in triplicate and means were calculated.

2.5. Determination of total phenolic contents (TPC)

Contents of total phenolics of *A. squamosa* L. leaves were estimated spectrophotometrically using the Folin–Ciocalteu assay (Singleton and Rossi, 1965). 0.3 mL (1 mg/mL) of sample was combined with Folin–Ciocalteu reagent (1.5 mL; diluted 10 times) and sodium carbonate (1.2 mL; 7.5% w/v). Samples were incubated for 30 min at room temperature, and then

the absorbance was measured at 765 nm. A standard curve was plotted using different concentrations of Gallic acid. The absorbance obtained was converted to gallic acid equivalent in mg per gm of dry material (mg GAE/g) using gallic acid standard curve.

2.6. Reducing power assay

In order to determine the reducing power of the different *A. squamosa* L. extracts, one milliliter of each sample solution (0.2, 0.5, 0.8 and 1.0 mg/mL) and from BHT was mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 10 g/L). After the mixture was incubated at 50 °C for 20 min, trichloroacetic acid (2.5 mL, 10%) was added, and the mixture was centrifuged at 3000*g* for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, g/L), then the absorbance was measured at 700 nm against a blank (Barros et al., 2007). Increasing absorbance of the reaction mixture indicates increasing reducing power. All tests were performed in triplicate and means were calculated.

2.7. Hydrogen peroxide scavenging assay

The ability of *A. squamosa* L. to scavenge H_2O_2 was determined using the method of Ruch et al. (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically by absorption at 230 nm. One mL of the extract or standard antioxidant was added to H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The percentage scavenging of H_2O_2 by *A. squamosa* L. extracts and standard compounds was determined as follows: Hydrogen peroxide scavenging activity, $[\%] = [A_o - A_1]/A_o \times 100]$ where A_o is the absorbance of the control, and A_1 is the absorbance in the presence of the sample *A. squamosa* or standards (Ascorbic acid and BHT).

2.8. Determination of antibacterial activity of A. squamosa leaves extracts

2.8.1. Test micro-organisms

A total of six bacterial species were tested including *Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus faecalis* have been used.

2.8.2. Disc diffusion assay

Antimicrobial activity of the tested *A. squamosa* extracts was determined using a modified Kirby–Bauer disc diffusion method (Bauer et al., 1966). Briefly, 100 µl of the bacteria grown in 10 mL of fresh media until they reached a count of approximately 108 cells/mL for bacteria (Pfaller et al., 1988). 100 µl of microbial suspension was spread onto Mueller Hinton (MH) agar (NCCLS, 1993, 1997) in Petri plates corresponding to the broth in which they were maintained. An aliquot (10 µl) of each of the *A. squamosa* L. leaves extracts was pipetted on a sterile paper disc (Whatman No. 1, 5.5 mm paper disc) on the agar surface. Standard discs of Tetracycline (Antibacterial agent) served as positive control for antimicrobial activity and filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as a negative control. The plates were inverted and incubated for 18 h at 37 °C. Microbial inhibition was determined by measuring the diameter of the clear zone of inhibition of growth around each disc and recorded as diameter of inhibition zone in millimeter. The zone diameters were measured with slipping calipers of the National committee for clinical Laboratory Standards (NCCLS, 1993).

2.9. Statistical analysis

The data were expressed as means ± standard error (SE) and analyzed using 11.0 for windows. One-way analysis of variance (ANOVA) and Turkey's multiple comparisons were carried out to test any significant differences between the means. Differences between the means at the 5% confidence level were considered significant. Correlations between variables were computed using the regression model in SPSS 11.0 for windows.

3. Results and discussion

3.1. Extraction yields

The extraction yield depends on solvents, time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature conditions, the solvent used and the chemical property of the sample are the two most important factors (Shimada et al., 1992). The recommended effective extracting solvents are aqueous mixtures of methanol, ethanol and acetone (Waterman and Mole, 1994).

In the present study the obtained extraction yields for the different tested solvents (Table 1) ranked in the following order: acetone 50% > water > ethanol 50% > methanol 80%.

3.2. Total phenolic Contents (TPC)

It is well known that plants contain many phenolic compounds which contain a hydroxyl group on an aromatic ring. These phenolic compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. So they act as reducing agents and antioxidants (Bursal and Köksal, 2010).

The total phenolic contents of acetone, ethanol, methanol and water extracts of *A. squamosa* L. leaves were determined by Folin–Ciocalteu method. The assays were performed in the whole extracts, as that could be more beneficial than isolated constituents because of the additive and synergistic effects, and considering that a bioactive individual component can change its properties in the presence of other compounds present in the extracts (Liu, 2003). A calibration curve was first plotted using different concentrations of gallic acid. The results

Table 1 Extracts characteristics and yields obtained for the different extraction solvents.

Solvent	Extract characteristics	Extract yield (%)
Methanol:water (8:2)	Brownish green semisolid	5.76
Acetone:water (1:1)	Dark brownish solid	10.36
Water (boiling)	Light brownish solid	10.34
Ethanol:water (1:1)	Brownish semisolid	6.97

were determined from the regression equation of the calibration curve ($A = 10.97 \times [GAE] - 0.4098$, $R^2 = 0.998$) as gallic acid equivalents in milligram per gram of extract (mgGAE/g extract).

As can be seen from Table 2, the extraction solvents significantly ($P < 0.05$) affected the amount of total *A. squamosa* L. leaves phenolic compounds. Acetone and methanol followed by ethanol were found to be more efficient in the extraction of phenolic compounds compared to water.

Whereas water gave high amount of yield, it was not a good solvent for the extraction of polyphenols. This could be explained by the fact that water extracts only the water-soluble bioactive compounds; moreover much other residual substances and impurities are present in the aqueous extracts (Mohammedi and Atik, 2011).

The obtained results are very consistent with many previously reported results indicating that phenolic compounds are generally more soluble in polar organic solvents than in water (Wanga et al., 2009). Our results are also in agreement with the results of Zhou and Yu (2004) who reported that among solvents tested, 50% acetone extracts contained greatest level of total phenolics from wheat; and with those reported by Yu et al. (2005) indicating that ethanol and methanol were found to be more efficient than water for extracting total phenolics from peanut skin.

3.3. Total antioxidant activity (TAA)

The total antioxidant activities of *A. squamosa* leaves extracts were determined using the phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. High absorbance values indicate that the sample has a significant antioxidant activity.

In the present work, the total antioxidant activities of *A. squamosa* leaves extracts were measured and compared with that of butylated hydroxytoluene (BHT). A standard curve using ascorbic acid at different concentrations was first plotted. The antioxidant activity of the tested samples was determined using the obtained calibration equation (Absorbance = $0.0049 \times$ concentration + 0.0409; $R^2 = 0.998$).

The antioxidant activities of *A. squamosa* L. leaves extracts and BHT expressed as (μmol of ascorbic acid/g extract) are given in Table 2. The different extracts exhibited significantly different ($P < 0.05$) antioxidant activities that were found to be in the following order: BHT > acetone extract > methanol extract > ethanol extract > water extract. The results of total phenolic contents and total antioxidant activity indicate that most of the potent antioxidant and phenolic compounds in *A. squamosa* L. leaves were soluble in acetone.

The variation of the total antioxidant activity as affected by the extraction solvent used has been reported in many previous studies (Hayouni et al., 2007; Pinelo et al., 2004; Mohdaly et al., 2010).

Table 2 Total antioxidant activity (TAA), total phenolic contents (TPC) and hydrogen peroxide scavenging activity of *A. squamosa* leaves extracts.

	TAA[1]	TPC[2]	H_2O_2 scavenged[3]
Acetone	$1625.38^{a} \pm 68.55$	$395^{a} \pm 31.40$	$44.48^{b} \pm 0.09$
Ethanol	$917.44^{c} \pm 34.03$	$316^{b} \pm 38.16$	$45.02^{b} \pm 1.62$
Methanol	$1409.15^{b} \pm 58.83$	$353^{ab} \pm 20.88$	$42.96^{b} \pm 0.52$
Water	$639.65^{d} \pm 22.17$	$210^{c} \pm 59.03$	$54.06^{a} \pm 0.14$
BHT	2202.50 ± 82.17	–	50.71 ± 0.10
Ascorbic acid	–	–	53.76 ± 0.10

Values are expressed as mean ± standard deviation ($n = 3$).
[1] Expressed as μmol of ascorbic acid/g of extract.
[2] Expressed as mg gallic acid equivalent/g of extract (mgGAE/g).
[3] H_2O_2 scavenging activity (%) of *A. squamosa* L. extracts of at a concentration of 25 μg/mL. Different letters in the same column indicate significant difference ($P < 0.05$).

3.4. Correlation between TAA and TPC

The total phenolic contents could be regarded as an important indication of antioxidant properties of plant extracts (Wang et al., 2010). In the present study, the correlation coefficient (R^2) between the total antioxidant activity and total phenolic contents of the four studied *A. squamosa* extracts (Fig. 1) was found to be 0.8965. This correlation coefficient suggests that the phenolic compounds of *A. squamosa* extracts contributed by 89% to their antioxidant activities. Also, it can be concluded that antioxidant activity of plant extracts is not limited to phenolics. Activity may also come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins, among others (Javanmardi et al., 2003).

Similar positive correlation between phenolic contents and antioxidant potential of various plant extracts have been well demonstrated in prior reports (Barreira et al., 2008; Zhang et al., 2010).

3.5. H_2O_2 scavenging activity

It is well established that hydrogen peroxide is not dangerous as it is, but may well be because of its ability to form the hydroxyl radical, thereby emphasizing on the importance of its elimination (Özen and Türkekul1, 2010). Table 2 shows the

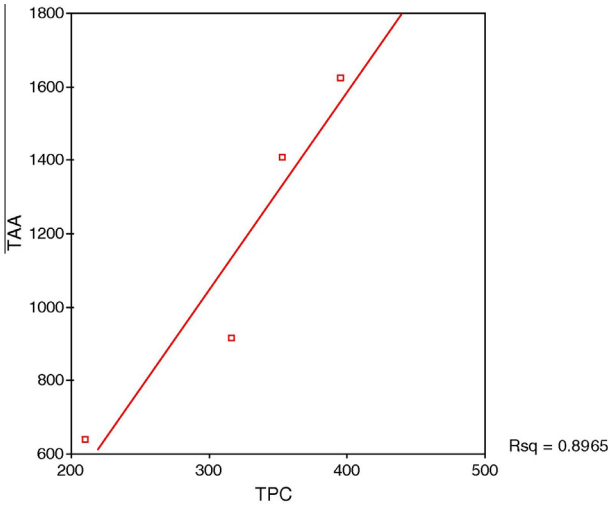

Figure 1 Linear correlation of the total antioxidant activity (Y) versus the total phenolic content (X) of the four studied *A. squamosa* extracts, using regression model in SPSS 11.0 for windows.

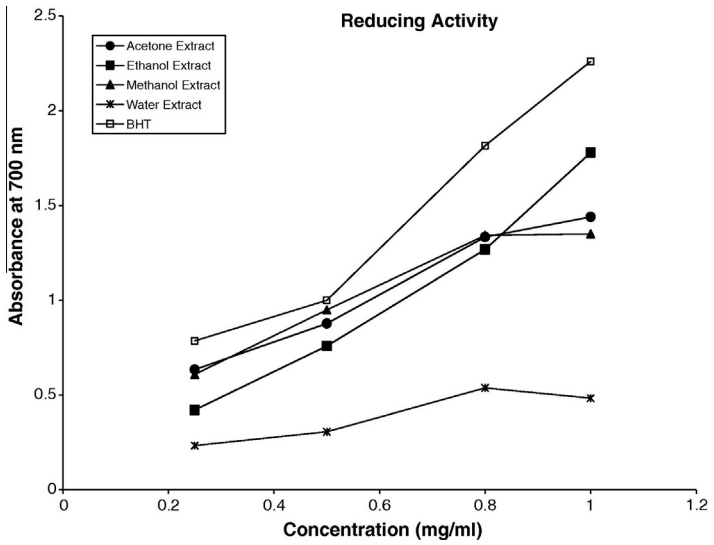

Figure 2 Total reducing power of different concentrations (0.2–1.0 mg/mL) of *A. squamosa* extracts and reference antioxidant: BHT.

hydrogen peroxide scavenging activity of *A. squamosa* L. extracts and that of ascorbic acid and BHT used as reference antioxidants. *A. squamosa* L. water extract exhibited significantly ($P < 0.05$) higher hydrogen peroxide scavenging activity than acetone, ethanol and methanol extracts. The differences in H2O2 scavenging capacities between the four extracts may be attributed to the structural features of their active components, which determine their electron donating abilities (Halliwell and Gutteridge, 1990). The measurement of hydrogen peroxide scavenging activity can be one of the useful methods determining the ability of antioxidants to decrease the level of pro-oxidants such as hydrogen peroxide (Pazdzioch-Czochra and Widenska, 2002).

3.6. Reducing power activity

The ability of *A. squamosa* L. extracts to reduce iron (III) to iron (II) was determined at different extract concentrations compared to BHT. Fig. 2 shows the data for the reducing power of the different *A. squamosa* L. extracts and BHT used as reference. The four *A. squamosa* L. extracts showed different capacities for electron donation which was found to be proportionally related to the extract concentration however, the activities were inferior to that of BHT. The results of reducing power demonstrated the electron donor properties of the different *A. squamosa* L. extracts by neutralizing free radicals and forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging (Gülçina et al., 2010).

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other (Gülçina et al., 2010). It has been previously reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. As given in Fig. 3, the results of the reducing power of different *A. squamosa* L. extracts were found to positively correlate with their corresponding antioxidant activities by a regression coefficient ($R^2 = 0.95$).

3.7. Antibacterial activity

The results of the antibacterial activity of acetone, ethanol, methanol and water extracts of *A. squamosa* L. leaves are given in Table 3.The obtained antibacterial activities were categorized as follows: (a) strong: for inhibition $\geqslant$ 70%, (b) moderate: for inhibition 50–70% or (c) weak: for inhibition $<$ 50% (Chan et al., 2007). The antimicrobial activity of the tested extracts showed different selectivity for each microorganism. The results revealed that water extract was found to have no activity against all tested organisms except *S. faecalis* and *E. coli*. Acetone extract exhibited moderate antibacterial activity against *S. aureus and S. faecalis*, whereas its activity toward the other four tested organisms was found to be weak. Ethanolic and methanolic extracts showed weak inhibition against all tested organisms.

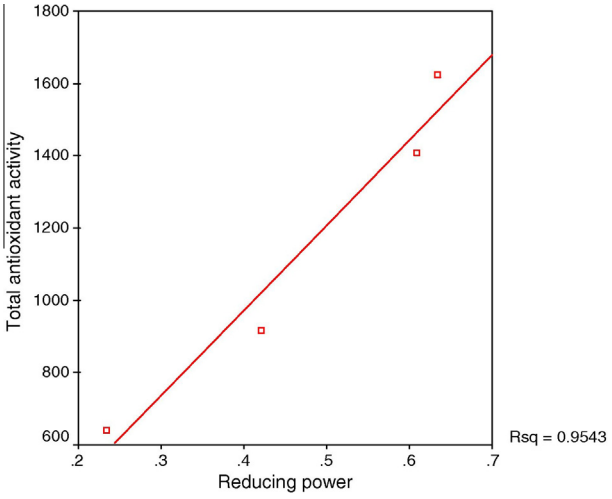

Figure 3 Linear correlation of the total antioxidant activity (Y) versus the reducing power (X) of *A. squamosa* L. extracts, using regression model in SPSS 11.0 for windows.

Table 3 Antibacterial activity of *A. squamosa* L. leaves extracts using disc-diffusion method.

Microorganism	Gram reaction	Inhibition zone diameter in mm (inhibition%)				
		Standard antibacterial agent	Acetone extract	Ethanol extract	Methanol extract	Water extract
B. subtilis	G^+	34	14 (41)$^+$	12 (35)$^+$	13 (38)$^+$	0
S. aureus	G^+	29	15 (51)$^{++}$	14 (48)$^+$	12 (41)$^+$	0
S. faecalis	G^+	30	15 (50)$^{++}$	13 (43)$^+$	13 (43)$^+$	11 (36)$^+$
N. gonorrhoeae	G^-	34	16 (47)$^+$	14 (41)$^+$	13 (38)$^+$	0
P. aeruginosa	G^-	33	14 (42)$^+$	14 (42)$^+$	13 (39)$^+$	0
E. coli	G^-	32	15 (46)$^+$	14 (43)$^+$	13 (40)$^+$	9 (28)$^+$

Values in parentheses are the inhibition percentages compared to standard antibacterial agent.
$^+$ Weak inhibition.
$^{++}$ Moderate inhibition.

It is worth noting that all of the extracts showed greater potent antibacterial activity against *Gram-positive* bacteria than *Gram-negative*. This result is supported by the fact that *Gram-negative* bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra and Greenwood, 2001). This renders the *Gram-negative* bacteria generally less susceptible to plant extracts than the Gram-positive bacteria (Chan et al., 2007).

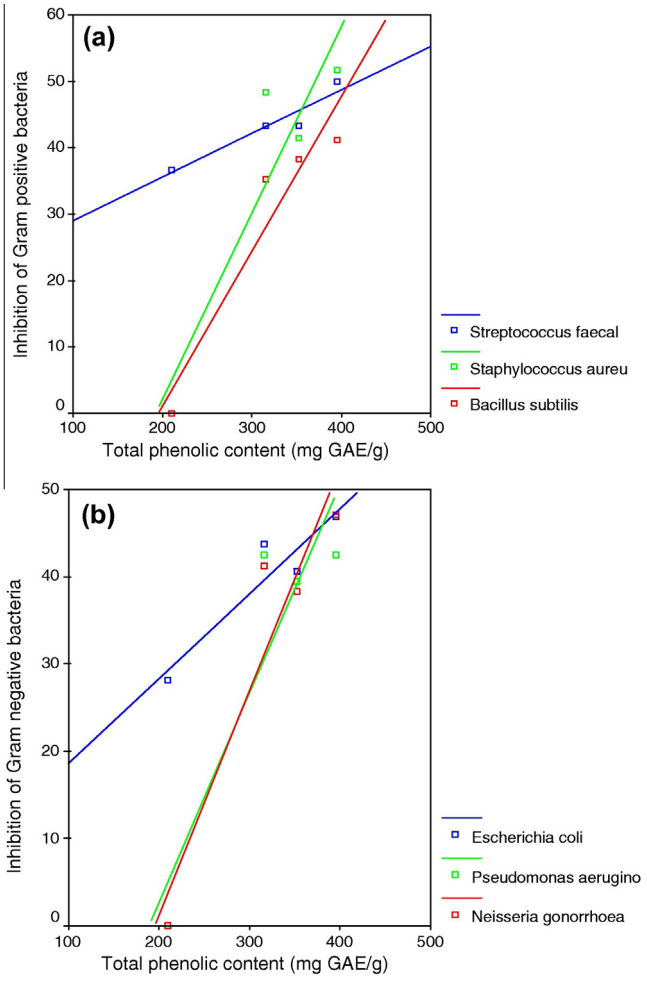

Figure 4 Linear correlation of the total phenolic content (X) versus the antibacterial effect (Y) of *A. squamosa* L. extracts against (a) Gram positive bacteria, (b) Gram negative bacteria.

The antibacterial activity of the plant extracts might be attributed to the presence bioactive plant compounds such as tannins, phenolic compounds, polyphenols and flavonoids (Ouattara et al., 2011). Among these bioactive compounds, Fernandez et al. (1996), Shoko et al. (1999) and Baydar et al. (2004) confirmed that phenolics were the most important active compounds against bacteria. Thus the results of antibacterial activities obtained in the present study for each of the *A. squamosa* L. extracts were correlated to their total phenolic contents (Fig. 4a and b).

Positive correlations were obtained between the concentrations of phenolic compounds in the different *A. squamosa* L. extracts and inhibition of all of the tested bacteria. The correlation coefficients (R^2) values were found to be: 0.91, 0.85 and 0.91 for the *Gram positive* bacteria *B. subtilis, S. aureus* and *S.* faecalis; respectively whereas for the *Gram negative* bacteria *N. gonorrhoeae, P. aeruginosa* and *E.* coli, the corresponding (R^2) values were 0.89, 0.83 and 0.87.

4. Conclusion

The present study highlights the possible use of *A. squamosa* L. leaves extracts as a source of antioxidants and as antibacterial agents that can be used to prevent food spoilage. The study showed that the results of extraction yield, total phenolic compounds and bioactivity tests varied depending on the type of solvent being used. The study revealed that the leaves of *A. squamosa* L. contain a considerable quantity of phenolic compounds that were found to be the major contributor for their antioxidant and antibacterial activities. Future research should be addressed on the application of using *A. squamosa* L. leaves as natural food preservative and to protect against peroxidative damage in living systems related to aging and carcinogenesis.

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