BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, August 2015. Vol. **12**(2), 1209-1222

Azadirachta indica as a Source for Antioxidant and Cytotoxic Polyphenolic Compounds

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DOI: http://dx.doi.org/10.13005/bbra/1774

(Received: 24 April 2015; accepted: 05 May 2015)

Cancer is a life threatening malignant tumor, caused by many factors including the oxidative stress. Medicinal plants containing phenolic compounds represent an important source of antioxidant and anticancer drugs. *Azadirachta indica* Family: Meliaceae contains variety of bioactive components of numerous biological and pharmacological properties. This study explored the constitutive polyphenols of *Azadirachta indica (A. indica)* growing in KSA and evaluated its antioxidant and cytotoxic activity. Chemical structures of the isolated compounds from the leaves of *A. indica* were established by spectral techniques (UV, MS, 1H, and ^{13}C NMR, and two dimentional NMR). The colourimetric assay (SRB) used to evaluate the cytotoxicity against HCT_{116}, MCF_7 and Hep-G_2 cell lines. Chromatographic separation of 80% Ethanol extract of the leaves of *A. indica* have resulted in seven polyphenolic compounds three of them isolated for the first time from this species (2,3-(*S)*-hexahydroxydiphenoyl-(α/β)-D-glucopyranose, Avicularin and Castalagin) and four known previously isolated compounds (Gallic acid, Ellagic acid, Quercetin and Quercetin-3-*O*-glucoside). The 80% Ethanol extract exhibited higher antioxidant activity than the ethyl acetate and butanol extracts, which is correlated with its phenolic content. The ethanolic extract, compounds 4 and 6 exhibited cytotoxic activity against HCT_{116}, MCF_7 and Hep-G_2. These findings revealed that the leaves of *A. indica* contains a considerable amount of polyphenolic compounds with significant antioxidant and cytotoxic activity, consequently it could be considered as a great potential source for natural health products.

Key words: Neem, polyphenolic compounds, cytotoxic and antioxidant activities.

Neem (*Azadirachta indica* A. Juss.), a member of the Meliaceae family, is a fast growing tropical evergreen tree. The neem tree (*A. indica*) is native to the Indian subcontinent and grows in many countries of the world like Egypt and Kingdom of Saudi Arabia (KSA). A massive cultivation of this tree was occurred in the plains of Arafat, Makkah Almokaramah, KSA. In 1971, Approximately 50,000 Neem trees were cultivated to provide shade for the millions of Muslim pilgrims (Maridha and Suhaibani, 2014). *A. indica* contains various active constituents with several medicinal properties. It used for treatment of fever, diabetes, and disorders that relate to arthritis and rheumatism, skin diseases and inflammation. It was practiced by different type of vaidyas and traditional healer in almost all the countries in the world, like KSA, China, India, Egypt, Rome and Greek and its proximity with

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human culture, hence neem has been called "nature's drug store" and "the wonder tree". (Dholi *et al*., 2011; Sonia Bajaj, and Srinivasan B.P 1999; El-Mahmood *et al*., 2010; Maragathavalli *et al*., 2012; Amer *et al*., 2010; Van der Nat *et al*., 1991; Chatterjee and Pakrashi 1994; Shibata, 1955; Clayton *et al*., 1996; Van der Nat *et al*., 1991; Chopra *et al*., 1956; Murthy and Sirsi, 1958; Patel *et al*., 2011; Bailey, 1953; Engler, 1964; Chopra *et al*., 1958; Koul *et al*., 1990; Haque *et al*., 2006; Habila *et al*., 2011; Sharma and Bhattacharyya, 2005; Bandyopadhyay *et al*., 2002; Sathiyaraj *et al*., 2010; Boadu *et al*., 2011; Patel *et al*., 2011; Kirtikar and Basu, 1975; Bansal *et al*., 2010; Santra and Manna , 2009; Denardi *et al*., 2010; Baral and Chattopadhyay, 2004; Habila *et al*., 2011; Schmutterer, 1995).
From the above mentioned information, it was concluded that *A. indica* have various pharmacological activities and used in the various marketed formulation, so it is necessary to throw a light on the phenolic contents and biological activities of *A. indica* growing in KSA

MATERIALS AND METHODS

Instruments and materials

NMR (^{1}H- and ^{13}C NMR) spectra were recorded at 300 MHz for 1H and 75 MHz for ^{13}C on a Varian Mercury 300. The δ-values are reported as ppm relative to TMS (tetramethyl silane) in DMSO-d_6 and *J*-values recorded in Hz. ESI-MS spectra were measured using mass spectrometer connected to an ESI-II ion source (Finnigan, Lc-MSLCQdeca. Advantage MAX, Finnigan Surveyor LC pump) (Department of Biological Genetics, National Research Center, Cairo, Egypt), the analysis was performed by the help of Dr. Amel Kamal, faculty of pharmacy, Helwan university, cairo, Egypt and as illustrated in previous study (Haggag *et al*., 2011). Purity of sample were recorded on a Shimadzu UV 240 spectrophotometer, separately as solutions in methanol and diagnosed by different UV shift reagents (Mabry *et al*., 1970) and with sprayed Naturstoff reagent (Brasseur & Angenot, 1986).

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma-Aldrich Co. (St Louis, MO). Sodium phosphate, ammonium molybdate, Folin-Ciocalteu's reagent, ascorbic acid, gallic acid were purchased from Merck Chemical Co (Darmstadt, Germany). All other chemicals, solvents and reagents used in chromatography were of analytical grade.

Cell line and culture medium

HCT_{116} and MCF_7 and Hep-G_2 cells were preserved in RPMI-1640 medium containing 10% (v/v) heat inactivated fetal bovine serum supplemented mixture of 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C under 5% CO_2 in air.

Preliminary Phytochemical Screening

Phytochemical screening methods were used for detection of various phytochemicals by qualitative chemical tests to give general idea regarding the nature of constituents present in crude drug (Trease and Evanse 1996; Parekh J and Chanda SV, 2007).

Determination of total phenolic content

The total phenolic content of 80% EtOH, EtOAc, *n*-BuOH extracts of *A. indica* was determined using Folin-Ciocalteu reagent according to the method described by Kumar *et al*. (2008). Gallic acid was used as standard. All determinations were carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g extract.

Determination of total flavonoid content

The total flavonoid content of 80% EtOH, EtOAc, *n*-BuOH extracts of *A. indica* was determined using the procedure described by Kumaran and Karunakaran (2006) using Quercetin as a standard. All determinations were carried out in triplicate. The total flavonoid in each plant extract was determined as mg quercetin equivalents (QEs)/g extract.

DPPH radical scavenging activity

The ability of 80% EtOH, EtOAc, *n*-BuOH extracts of *A. indica* to scavenge DPPH radicals was evaluated according to the procedure described by Mensor *et al*. (2001).

Reducing power assay

Reducing power of 80% EtOH extract of *A. indica* as well as two of the isolated pure compounds (4&6) was determined according to the method of Oyaizu (1986) using ascorbic acid as standard. Three replicates were made for each tested sample. Increased absorbance of the

reaction mixture indicated increased reduction power.

Cytotoxic assay

The cytotoxic activity of 80% EtOH extract of *A. indica* as well as the isolated pure compounds 4 and 6 was carried out according to the method described by Skehan *et al*. (1990) using colourimetric assay (SRB) and as described in our previous study (Haggag *et al*., 2011). The IC50 values were calculated from the prism program obtained by plotting the percentage of surviving cells vs. the concentration interpolated by cubic spine (Boyed, 1997).

Plant material

Leaves samples *of* neem (*Azadirachta indica*) were freshly collected from Bahra in KSA. The samples were collected in November 2011.

Extraction and Isolation

Air-dried ground leaves (1000 g) were extracted with hot 80% aqueous ethanol (2.5×5L) under reflux (70°C). After evaporation of solvent under reduced vacuum, the residue (115 g) was defatted with pet.ether (60-80°C) under reflux (1.5 L×5L. 60°C). The residue (110 g) after evaporation of pet.ether was fractionated on a polyamide column (Ø 5.5×120 cm) and was eluted water/methanol mixtures of decreasing polarities to afford 25 fractions. Those fractions were concentrated under vacuumand purified on Sephadex LH-20 columnand or Cellulose columnsusingdifferent eluting systems. The homogenity of the fractions was tested on 2D-PC and Comp-PC using Whatmann No. 1 paper (systems S_1 & S_2); S_1: *n*-BuOH-HOAc-H_2O (4:1:5, top layer) and S_2: 15% aqueous HOAc. The compounds were visualized by spraying with naturstoff (flavonoids) and nitrous acid or KIO_3-reagents (tannins).

Data analysis

All values in this study are expressed as mean ± standard deviation of the mean (M+SD). Data were analyzed by one-way analysis of variance (ANOVA). When variation among groups was found significant, Tukey-Kramer multiple comparisons test was carried out to compare between groups. Differences were considered significant when p value was < 0.05.

RESULTS

Phytochemical Screening

Phytochemical screening tests for *A. indica* leves extract resulted in detection of alkaloids, flavonoids, reducing sugars, steroids, carbohydrates, glycosides and tannins, while Anthraquinones and cardiac glycosides were not detected from the extract.

Total phenolic, flavonoid content and antioxidant activities

Phenolic Content of the Extracts

Ethanolic (80%), Ethyl acetate and butanol extracts from the leaves of *A. indica* were standardized for their contents of phenolic compounds. As shown in **Figure 1** The calibration curve showed linearity for gallic acid in the range of 12.5-400 $\mu g \cdot ml^{-1}$, with R_2 (correlation coefficient) of 0.999. Ethanolic contained the highest content of phenolics (69.17 ±1.57 $mg \cdot g^{-1}$), followed by ethyl acetate extract (38.13 ± 1.25 $mg \cdot g^{-1}$) and Butanol extract (24.38 ± 3.13 $mg \cdot g^{-1}$).

Flavonoid content of extracts

Ethanolic (80%), Ethyl acetate and butanol extracts from the leaves of *A. indica* were standardized for their contents of phenolic compounds. The calibration curve showed linearity for Quercetin in the range of 12.5-100 $\mu g \cdot ml^{-1}$, with a correlation coefficient (R2) of 0.999 (Figure 2). Ethanolic contained the highest content of flavonoids (42.435±0.8 $mg \cdot g^{-1}$), followed by ethyl acetate extract (25.256±0.89

Table 1: Percentage of DPPH radical scavenging activity of *A. indica* extracts

µg/ml	Ascorbic acid (Mean ±SD)	Aqu. Ethanol ext. (Mean ±SD)	Ethyl acetate ext. (Mean ±SD)	Butanol ext. (Mean ±SD)
12.5	28.595± 0.254	13.306±0.8	10.69±0.63	6.995±0.733
25	37.03±0.407	25.638±1.924	20.23±0.455	15.524±2.584
50	54.267±0.21	41.331±0.613	36.69±1.552	17.843±2.083
100	87.433±0.354	55.074±1.434	47.82±2.437	29.234±1.749

Table 2. Reducing power assay for ethanolic extract and isolated compound 4 and 6 of *A. indica*

Ug/ml	Ascorbic acid (Mean ±SD)	*Ethanol extract* (Mean ±SD)	*castalagin* (Mean ±SD)	*Avicularin* (Mean ±SD)
25	0.432±0.003	0.167±0.01	0.279±0.007	0.237±0.014
50	0.612±0.002	0.309±0.009	0.425±0.008	0.385±0.008
100	0.942±0.003	0.427±0.012	0.784±0.006	0.723±0.007

mg·g^{-1}) and Butanol extract (14.87±1.35 mg·g^{-1}).

Antioxidant Activity of the Extracts

The standardized ethanolic, ethyl acetate, butanol extracts and gallic acid as +ve control were assessed for their capacity to scavenge DDPH free radical. The antioxidant activity data are written as percentage of free radical inhibition in **Table 1**. The ethanolic (80%) extract of of *A. indica* levaes exhibited pronounced antioxidant activity at a concentration of 12.5-100 µg·ml^{-1}, followed by ethyl acetate and butanol extracts when compared by ascorbic acid as standard. Natural polyphenolic compounds are known to have chemopreventive and suppressive activities against cancer cells by inhibition of metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle [Newman *et al*., 2002]. Nevertheless, a compound with strong free radical scavenger activity can also contribute to DNA protection and prevent apoptosis [Rajkumar *et al*., 2011]. Further studies are therefore required to detect potential anticancer activities of the extracts reported here. So it was interesting to isolate and identify polyphenolic compounds from the highest antioxidant extract i.e. ethanolic extract which contains also the highest contents of phenolic and flavonoidal contents and estimate their reducing powers and their cytotoxic activity.

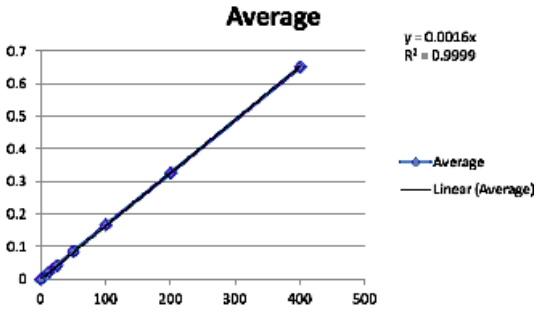

Fig. 1. Standard calibration curve of gallic acid at concentrations of 12.5, 25, 50, 100, 200 and 400 µg ·ml^{-1}. Spec- trophotometric detection was at 765 nm

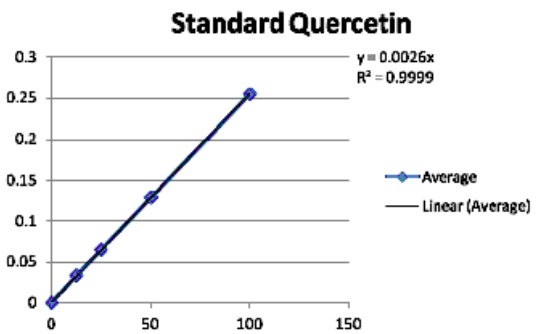

Fig. 2. Standard calibration curve of quercetin standard at concentrations of 12.5, 25, 50 and 100 µg·ml^{-1}. Spec- trophotometric detection was at 415 nm

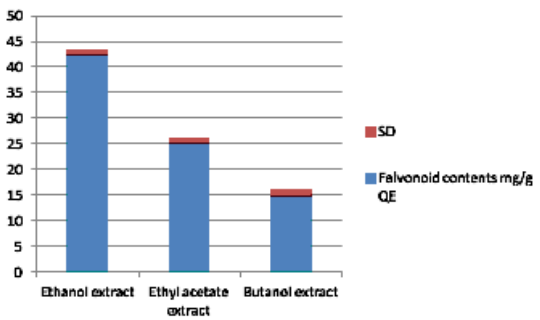

Fig. 3. Flavonoid contents of 80% ethanol, ethyl acetate and n-butanol extracts of the leaves of *A. indica* (n =3)

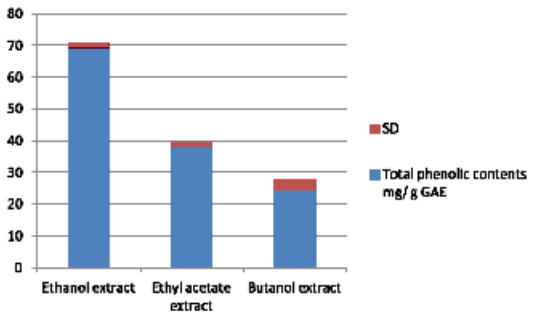

Fig. 4. Total phenolic contents of 80% ethanol, ethyl acetate and n-butanol extracts of the leaves of A. *indica* (n =3)

Identification of Phenolics isolated from *A. indica*

Compound 1

Yellowish white amorphous powder (20 mg). Chromatographic properties, R_f values: 0.65 (S_1), 0.50 (S_2); shiny violet fluorescent under UV-light turned to deep blue color with $FeCl_3$ spray reagent. This evidence was supported by the comparison with respective authentic samples and confirmed by Mass analysis. Negative HRESI-MS, 169.08[M-H]$^-$.

Compound 2

Off white amorphous powder (18 mg). Chromatographic properties: R_f-values 0.41 (S_1), 0.20 (S_2); UV/response with spray reagents: buff fluorescent spot by long and short UV light, turned to dull yellow fluorescence on exposure to ammonia vapours, greenish yellow (naturstoff), faint indigo-red colour (nitrous acid reagent) and faint blue colour ($FeCl_3$). UV λ_{max} (MeOH), nm: 216, 257, 363. Negative HRESI-MS, 300.9 [M-H]$^-$.

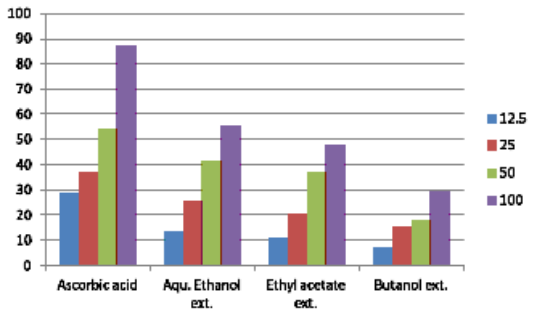

Fig. 5. Percentage of DPPH radical scavenging activity of *A. indica* extracts

Compound 3

Isolated as yellow amorphous powder (9 mg), gave chromatographic properties, UV spectral data and responses towards spray reagents similar to Quercetin. This evidence was supported by the comparison with respective authentic samples and confirmed by Mass analysis. Negative HRESI-MS: *m/z*, 301.04 [M-H]$^-$.

Compound 4

Brown amorphous powder (36 mg). Chromatographic properties: R_f-values on PC; 0.1(S_1), 0.67 (S_2); UV/response with spray reagents: dark purple fluorescent spot by short UV-light and dull brown under long UV-light, turned to indigo red ($NaNO_2$/glacial AcOH) and deep blue colour ($FeCl_3$). UV λ_{max} (MeOH), nm: 248, 289 sh, 300. -ve HRESI-MS: *m/z* 933.067[M-H]$^-$, (MS^2) 914.89[M-H-H_2O]$^-$,

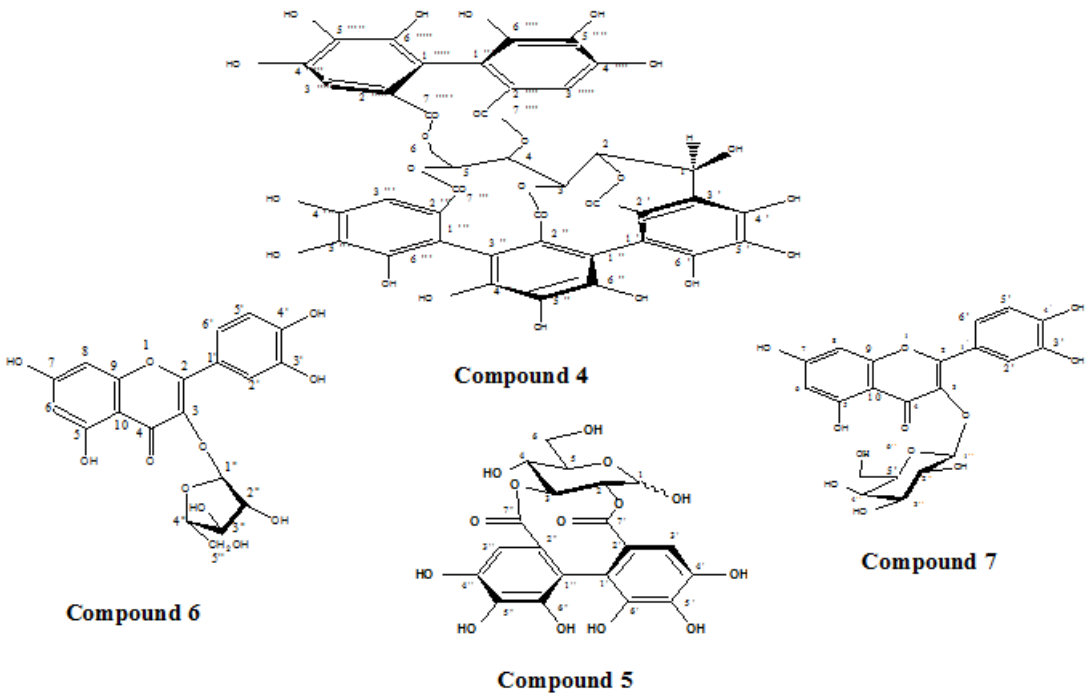

Fig. 6. Structures of compounds isolated from the leaves of *Azadirachta indica*

631.063[M-H-ellagic acid]$^-$, (MS^3) 896.95[M-H-$2H_2O$]$^-$, (MS^4) 878.96[M-H-$3H_2O$]$^-$, 301.08[Ellagic acid-H]$^-$. ^{1}H NMR:δ ppm 6.59(1H, s, H3‘‘ FLG), 6.52 (1H, s, H3‘‘‘ HHDP), 6.37 (1H, s, H3‘‘‘‘ HHDP), 5.48(1H, d, *J*=5.6 Hz, H1), 5.42(1H, br d, *J*=8.1 Hz, H5), 4.94(1H, t-like, *J* = 8.1 Hz, H4), 4.9(1H, dd, *J*=11.8, 3 Hz, H6), 4.79(2H, m, H3/2), 3.98(1H, br d, *J*=11.8 Hz, H6‘). ^{1}H, ^{1}H-COSY spectrum, Short range coupling HMQC spectrum, Long range coupling HMBC spectrum. ^{13}C NMR: ´ ppm 169.14, 165.21, 165.86, 165.80, 162.87(C7‘‘/C7‘‘‘‘HHDP, C7‘‘/C7‘‘/C7‘FLG), 146.67, 144.91, 144.85, 144.73, 144.46(C4‘‘FLG, C6‘‘/6‘‘‘‘/4‘‘/4‘‘‘‘ HHDP, 4‘/4‘‘FLG), 144.12, 143.72, 142.47(C6‘/6‘‘/6‘‘FLG), 136.98, 135.80, 135.58(C5‘‘FLG, C5‘‘/5‘‘‘‘HHDP), 135.10(C5‘‘ FLG), 133.26(C5‘‘‘ FLG), 125.90(C2‘‘ FLG), 124.79, 123.52(C2‘‘‘/2‘‘‘‘HHDP), 123.43(C2‘‘‘ FLG), 121.14(C2‘ FLG), 115.26, 114.59, 114.47, 114.36, 114.3(C1‘/1‘‘/1‘‘FLG, C1‘‘‘/1‘‘‘‘HHDP and C3‘FLG), 111.69(C3‘‘FLG), 106.73, 105.66, 105.59(C3‘‘‘FLG, C3‘‘‘/3‘‘‘‘HHDP), 72.69(C2), 69.79(C5), 68.07(C4), 66.12(C1), 65.74(C3), 65.58(C6).

Compound 5

Brownish amorphous powder (16 mg). Chromatographic properties: R_f-values on PC; 0.21 (S_1), 0.9 (S_2); UV/response with spray reagents: dark purple fluorescent spot by short UV-light, turned to indigo-red ($NaNO_2$-glacial AcOH) and deep blue colour ($FeCl_3$). UV λ_{max} (MeOH), nm: 246 with 286 sh and 300. Negative ESI-MS/MS: *m/z* 481.31[M-H]$^-$, (MS^2) 301.19[Ellagic acid-H]$^-$, (MS^3) 257.08[Ellagic acid-H-CO_2]$^-$, 229.26[Ellagic acid-H-CO_2-CO]$^-$. ^{1}H NMR: δ ppm 6.42(1H, s, H3‘‘), 6.19(1H, s, H3‘), 5.17 and 4.84(1/2H, s, H1α and β), 5.13 and 4.88 (1H, t-like, *J*=10 Hz, H3α and ″), 4.70 and 4.50 (1H, d, *J*=10 Hz, H2α and β), 3.72-2.72(broad band, hidden by H_2O-signal represents remaining sugar protons). ^{13}C NMR: δ ppm

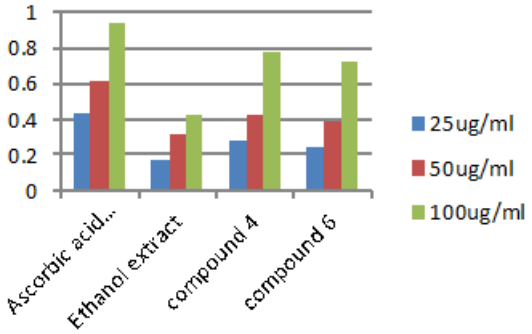

Fig. 7. Reducing power of 80% ethanol, ethyl acetate and n-butanol extracts of the leaves of *A. indica* relative to ascorbic acid (n =3)

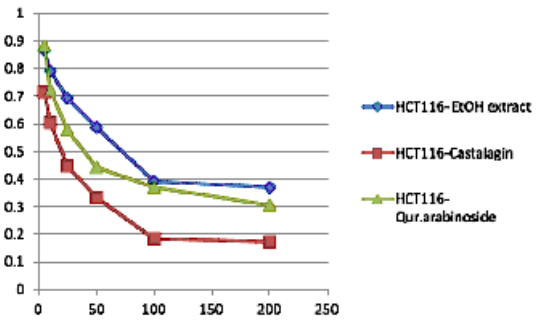

Fig. 8. Cytotoxic activity of 80% ethanol extract, compound 4 and compound 6 of *A. indica* against HCT116 cell line (n =3)

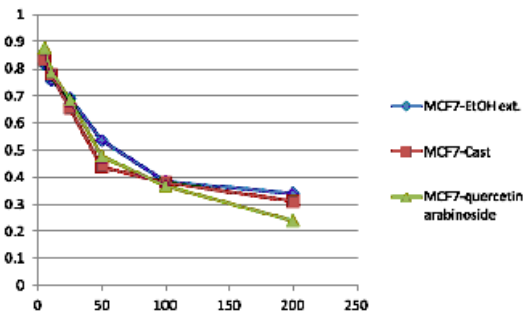

Fig. 9. Cytotoxic activity of 80% ethanol extract, compound 4 and compound 6 of *A. indica* against MCF7 cell line (n =3)

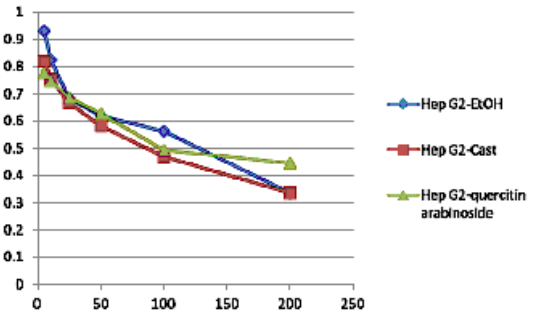

Fig. 10. Cytotoxic activity of 80% ethanol extract, compound 4 and compound 6 of *A. indica* against Hep G2 cell line (n =3)

169.24(C7“α,β), 168.63, 168.40(C7‘α,β), 144.49(C6‘/6“α,β), 144.42(C4‘/4“α,β), 134.97, 134.85(C5‘/5“α,β), 125.48, 125.29, 124.89 (C2“α,β/ C2‘α,β), 113.79, 113.71, 113.67, 113.51(C1‘‘α,β/ C1‘α,β), 105.30, 104.95(C3‘/ 3“α,β), 92.88, 88.97(C1α/ C1β), 79.61(C3α), 77.27(C3²), 76.95, 76.77(C2α/2β), 74.24(C4β), 72.17(C5α), 67.24(C4α), 66.89(C5β), 61.03(C6α,β).

Compound 6

Yellow amorphous powder (37 mg). Chromatographic properties: R_f-values 0.41 (S_1), 0.49 (S_2); UV/response with spray reagents: deep purple fluorescent spot by long UV light turned to orange (naturstoff), green ($FeCl_3$). UV λ_{max}: (MeOH), nm: 260, 297(sh), 352; (+NaOMe) 271, 329(sh), 404; (+NaOAc) 272, 318 (sh), 362; (+NaOAc/H_3BO_3) 271, 402; (+$AlCl_3$) 272, 303 (sh), 337(sh), 422; (+$AlCl_3$/HCl) 267, 300(sh), 362 (sh), 400. Negative HRESI-MS: *m/z* 433.08[M-H]$^-$, 867.32[2M-H]$^-$, 300.15[M-pentoside]$^-$=[quercetin-H]$^-$, -Ve ESI-MS: *m/z* 301.05[quercetin-H]$^-$, 179.00($C_9H_7O_4$, cinnamoyl fragment), 151.00($C_6H_3O_4$, benzoyl fragment) (MS^3). ^{1}H NMR: δ ppm 12.63(1H, s, OH5), 7.57(1H, m, Hz, H6‘), 7.48(1H, d, *J* = 2.3 Hz, H2‘), 6.86(1H, d, *J*= 8.4 Hz, H5‘), 6.41(1H, d, *J* = 2.3 Hz, H8), 6.21(1H, d, *J*=2.3 Hz, H6), 5.58(1H, d, *J* = 1.4 Hz, H1“), 4.17-3.24(remaining sugar protons). ^{13}C NMR: δ ppm 177.56 (C4), 164.15(C7), 161.18(C5), 156.95 (C2), 156.43(C9), 148.53(C4‘), 145.04 (C3‘), 133.21 (C3), 121.77(C1‘), 121.01(C6‘), 115.54 (C2‘/5‘), 108.04(C1“), 104.01(C10), 99.03(C6), 93.572 (C8), 85.57(C4“), 82.08(C2“), 77.03(C3“), 60.65(C5“).

Compound 7

Yellowish amorphous powder (17 mg). Chromatographic properties: R_f-values 0.47 (S_1), 0.60 (S_2); UV/response with spray reagents: deep purple fluorescent spot by long UV light turned to yellowish orange (naturstoff), and green by $FeCl_3$. UV λ_{max}: (MeOH), nm: 259, 355; (+NaOMe) 273, 412; (+NaOAc) 266, 373; (+NaOAc/H_3BO_3) 261, 375; (+$AlCl_3$) 250 (sh), 250, 271, 405; (+$AlCl_3$/HCl) 250 (sh), 262, 271, 362. -Ve HRESI-MS: *m/z* 463.1[M-H]$^-$, 301.1[M-hexoside]$^-$=[quercetin-H]$^-$, (MS^3) 178.9($C_9H_7O_4$ represent cinnamoyl fragment), 150.9($C_6H_3O_4$ represent benzoyl fragment). ^{1}H NMR: δ ppm 12.61(1H, s, OH5), 7.67(1H, m, H6‘), 7.59(1H, d, *J*=2.4 Hz, H2‘), 6.84(1H, d, *J*= 8.7 Hz, H5‘), 6.41(1H, d, *J*=3 Hz, H8), 6.21(1H, d, *J*=3 Hz, H6), 5.37 (1H, d, *J*=8Hz, H1“), 3.67-3.26 (remaining sugar protons). ^{13}C NMR: δ ppm 177.33(C-4), 164.28(C-7), 161.19(C-5), 156.30(C-2), 156.26(C9), 148.43(C4‘), 144.78(C3‘), 133.51(C3), 121.23(C1‘), 121.08(C6‘), 115.80(C5‘), 115.19(C2‘), 104.01(C10), 101.19(C1“), 98.51(C6), 93.68(C8), 73.8(C3“), 73.32(C5“), 71.25(C2“), 67.53(C4“), 60.30(C6“).

Reducing power

Compounds 4 and 6 represents the major comounds have been solated from the extract so it was be interesting to evaluate their reducing power in comparison with the ethanol extract. Compound 4 exhibited highest activity followed by compound 6, then the ethanolic extract (Table 2), when compared to ascorbic acid as standard. Based on the obtained data, it is interesting to carry out the cytotoxic assay.

Cytotoxic activity

Significant antioxidant and reducing power activity of the ethanolic extract and compound 4 and 6 directed us to test their cytotoxic activity against certain cell lines such as MCF_7, Hep-G_2 and HCT_{116} using the SRB method (Skehan *et al*., 1990). The ethanolic extract exhibited cytotoxic activity against HCT_{116}, MCF_7 and Hep-G_2 (IC_{50} value 72.29, 61.86 and 128.7µg/ ml respectively). Compound **4** was the most active compound (IC_{50} =20.38, 42.52 and 86.75 µg/ml respectively). Compound **6** exhibited cytotoxic activity (IC_{50}=39.25, 47.28 and 99.24 µg/ml, respectively). Accordingly, compound 4 is the most active followed by the compound 6 and finally the ethanolic extract.

DISCUSSION

Phytochemical screening for active substances of *A. indica* resulted in detection various compounds such as glycosides, tannins, alkaloids, flavonoids, reducing sugars, steroids and carbohydrates, while anthraquinones and cardiac glycosides were absent from the extract, which are in accordance with previous studies (Imran *et al*., 2010; Ejoba, 2012). It is well known that there is a strong relationship between

phenolic content of plants and the antioxidant activity, as phenols possess strong scavenging activity for free radicals due to their phenolic hydroxyl groups (Wojdylo *et al*., 2007; Bendini *et al*., 2006; Dlugosz *et al*., 2006 Yildirim *et al*., 2000; Mohamed *et al*., 2008; Singh *et al*., 2009). This study also proved that the extracts of neem (i.e. ethanolic extract, ethyl acetate and butanol extracts) have significant free radical scavenging activity. This may be attributed to their polyphenolic and flavonoidal contents where ethanol extract of the neem showed the highest DPPH inhibition activity followed by ethyl acetate extract then butanol extract and that was parallel to their phenolic and flavonoidal contents (Figures 3 and 4). Owing to the high antioxidant activity of 80% EtOH extract (Fig. 5), it was subjected to chromatographic fractionation.

The dried residue of 80% EtOH extract, which was extracted with chloroform for defatting and extracting of aglycones (Marzouk *et al*., 2009) was chromatographed on a polyamide column followed by successive purification on Sephadex LH-20 and/or cellulose columns getting seven pure compounds, among which was **compound 1**, which show chromatographic properties of gallic acid, This evidence was supported by the comparison with respective authentic samples and confirmed by Mass analysis. Negative HRESI-MS, 169.08[M-H]$^-$. Resulted in Gallic acid (compound 1) which isolated before from *A. indica*. (Singh *et al*., 2005).

Compound 2, this compound gave more or less the same chromatographic properties (R_f-values, colour and fluorescence changes on exposure to ammonia vapours and with the different spray reagents) of the ellagic acid authentic sample. Based on its intrinsic δ-electronic conjugation over the total C-skeleton, it showed the characteristic UV-absorption bands of ellagic acid (Tanaka *et al*., 1986 a and b). Negative HRESI-MS of compound 2 exhibited a molecular ion peak at *m/z* 300.9 [M-H]$^-$, that corresponding to the MF $C_{14}H_5O_8$ of ellagic acid. Accordingly, compound 2 was established as ellagic acid (Tanaka *et al*., 1985), which is isolated before from *A. indica* (Garima *et al*., 2014).

Compond 3, UV spectral data and responses towards spray reagents similar to Quercetin. This evidence was supported by the comparison with respective authentic samples and confirmed by Mass analysis. Negative HRESI-MS: *m/z,* 301.04 [M-H]$^-$. Accordingly, this compound **was** established as quercetin. (Mabry *et al*., 1970). Which isolated before from *A. indica* (Garima *et al*., 2014).

Compound 4 showed more or less the same chromatographic behavior of C-glycosidic ellagitannin. It exhibited also, very broad strong UV absorption maxima with the characteristic shoulder at 289 nm. Upon acid hydrolysis it gave ellagic acid in the organic phase and an unknown ellagitannin. The absence of the glucose in the aqueous hydrolysate was a confirmative evidence for the C-glycosidic nature of Compound 4 (Okuda *et al*, 1983). -Ve HRESI-MS spectrum showed a molecular ion peak at m/z 933.067 [M-H]$^-$ representing $C_{41}H_{25}O_{26}$. At high fragmentation energy MS/MS spectra, several diagnostic fragment ions were recorded; the most important ones are at 631.063 and 301.08 corresponding to the loss of a HHDP moiety and the negative ellagic acid ion, which was indirect indication for the other C-glycosidic phenoyl moiety as flavogallonoyl. The ^{1}H NMR spectrum exhibited three singlets, each one proton assigned at ´ 6.59, 6.52 and 6.37 ppm for a C-glycosidic flavogallonoyl attached to C-1, C-2, C-3 and C-5 and HHDP at C-4 and C-6 of open chain glucose. The ´- and *J*-values of the glucose moiety, especially that of H-1 at δ 5.48 with J_{12} of 5.6 Hz, were confirmative for full substituted open glucose with anomeric axial OH-group (Vivas *et al*, 1995; Okuda *et al*, 1983; Moharram *et al*, 2003). Like in the previous C-glycosidic ellagitannin, ^{13}C NMR spectrum showed 6 upfield typical carbon resonances of a C-glycosidic glucose and 14 resonances of a HHDP moiety together with 21 of flavogallonoyl forming C-glycosidic linkage with C-1 glucose. Characteristic downfield shift of C-3‘ at δ 114.3 (H”+10 ppm) and upfield shift of the carbonyl carbon C-7‘ at 162.87 (H”-7 ppm) of the flavogallonoyl moiety. The δ- and J_{12}- values of H-1 glucose proved that the absolute configuration at the anomeric position should be of axial OH and equatorial H, which was completely, agree with those of castalagin (Vivas

et al., 1995; Okuda *et al*., 1983 and Moharram *et al*, 2003). All assigned ^{1}H and ^{13}C signals were confirmed by 2D NMR spectral analysis. Accordingly, Compound 4 was finally identified as 2,3,5-(*S*)-flavogalonoyl-4,6-(*S*)-hexahydroxy diphenoyl-D-glucose (Castalagin) (figure 6), (Vivas *et al.*, 1995). Compound 4, was isolated for the first time from *A. indica* leaves.

Compound 5 appeared as deep purple fluorescent spot by short UV-light changed into deep blue colour with $FeCl_3$ and indigo-red colour with HNO_2 spray reagent characteristic for hexahydroxydiphenoyl esters (ellagitannins), (Gupta *et al*., 1982). The UV-spectrum showed intrinsic broad absorption band with a shoulder at λ_{max} 286 nm characteristic for the ellagitannins. On complete acid hydrolysis, compound 5 gave ellagic acid in the organic phase and glucose in the aqueous one (CoPC with authentic phenolic acid and sugars samples). Accordingly, it was expected that the structure of compound 5 is a hexahydroxydiphenoyl ester of glucose. The -ve ESI-MS spectra gave a molecular ion peak at *m/z* 481.31[M-H]$^-$ corresponding to a mono-hexahydroxydiphenoyl-glucose. In addition at high fragmentation energy, three ellagic acid fragment ions have been detected at 301.19 [Ellagic acid-H]$^-$ (MS^2), 257.08 [Ellagic acid-H-CO_2]$^-$, 229.26 [Ellagic acid-H-CO_2-CO]$^-$ (MS^3) in MS/MS spectra. These fragments were confirmative for the presence of a hexahydroxydiphenoyl group (HHDP). The positions of the attachment of HHDP onto glucose and its stereochemistry were concluded from NMR analysis. ^{1}H NMR exhibited two singlet signals, each one proton, at δ ppm 6.42 and 6.19 of one HHDP group (H-3", 3'). In the aliphatic region, duplication of all assigned ^{1}H-resonances was indicative to a free anomeric-OH and the presence of compound 5 in the form of α/β-anomers (Okuda *et al*., 1989). The downfield assignment of H-3 and H-2 in both anomers at 5.13, 4.88 (3α/β) and 4.70, 4.50 (2α/β) indicated the attachment of the HHDP-group at C-2 and C-3. As well as, the large J-values (9-10.1Hz) for all sugar signals together with H-1α and H-1β at 5.17 and 4.84 (J = 8.3 Hz) were confirmative evidences for an α/β-$^{4}C_1$-pyranose structure of the glucose moiety (Gupta *et al*., 1982, Okuda *et al*., 1983). Therefore, compound 5 was identified as 2,3-hexahydroxydiphenoyl-(α/β)-D-$^{4}C_1$-glucopyranose. The ^{13}C NMR spectrum showed also the duplication of all aliphatic and aromatic signals to confirm the α/β-configuration of the glucose, especially those of the anomeric carbon at 92.88 and 88.97 for C-1α and C-1β. The remaining ^{13}C-signals were assigned by the comparison with published data of structural similar compounds (Okuda *et al*., 1982). Thus, compound 5 was confirmed as 2, 3-(*S)*-hexahydroxydiphenoyl-(α/β)-D-glucopyranose (figure 6). This isolated for the first time in *A. indica* leaves.

On the basis of its chromatographic properties and UV spectral data, **compound 6** was suggested to be quercetin-3-*O*-glycoside. UV-spectrum in MeOH showed the two characteristic bands for a quercetin aglycone. The presence of a free 4'-OH group was deduced from the bathochromic shift in band I (+52 nm) with the increase in the intensity (Mabry *et al*, 1970). On addition of NaOAc, a bathochromic shift in band II (~+10 nm) was an evidence for a free 7-OH. Moreover, the remaining of the bathochromic shifts occurred in band I and II on both NaOAc and $AlCl_3$ spectra after the addition of H_3BO_3 and HCl revealed the *ortho* 3',4'-dihydroxy-function and the free OH-5. Complete acid hydrolysis produced quercetin in organic phase and arabinose in aqueous phase. -Ve HRESI-MS spectrum exhibited a molecular ion peak at *m/z* 433.08, corresponding to MF $C_{20}H_{18}O_{11}$ for a quercetin pentoside. As well as, this evidence was further supported by the fragment ions at *m/z* 301.05, 179.00 and 151.00. The ^{1}H NMR, showed in the aromatic region the two characteristic spin coupling systems i.e. ABX (H-2'd, H-6' dd, H-5'd) and AM (H-8 d; H-6 d) for 3',4'-dihydroxy B- and 5,7-dihydroxy A- ring protons. In the aliphatic region, a doublet at 5.58 with J_{12} =1.4 Hz was characteristic for α-L-arabinofuranoside moiety (Servettaz, 1984) with the other sugar protons. ^{13}C NMR spectrum showed 15 carbon resonances among which the key signals at ´ 177.56 (C-4), 148.53 (C-4'), 145.04 (C-3') and 133.21 (C-3) for a 3-*O*-glycosyl-quercetin (Agrawal, 1989). In addition, five highly strained and downfield shifted ^{13}C resonances particulary those at 108.04, 85.57 and 82.08 assignable to C-1", C-4" and C-2" of an arabinofuranoside

moiety (Agrawal, 1989). So **Compound 6** was identified as quercetin3-*O*-±-L-arabinofuranoside (Avicularin) (figure 6), which is isolated for the first time from *A. indica* leaves.

Like compound 6, **Compound 7** had chromatographic properties and UV spectral data of a quercetin-3-*O*-glycoside. As in case of compound 6, the bathochromic shift with increase in the intensity of band I upon addition of NaOMe was confirmative to a free 4'-OH. This evidence was also deduced from the remaining of the bathochromic shifts recorded in both NaOAc and $AlCl_3$ spectra after the addition of H_3BO_3 and HCl. Acid hydrolysis of compound 7 afforded quercetin as an aglycone and galactose as sugar moiety, were identified by CoPC in different convenient solvent systems (Mabry, 1970). Negative HRESI-MS spectrum showed a molecular ion peak at *m/z* 463.09 corresponding to molecular formula (MF) $C_{21}H_{20}O_{12}$ of a quercetin hexoside, together with other fragment ion peaks at 301.02, 178.9 and 150.9 confirming the presence of the quercetin as an aglycone. ^{1}H NMR, showed in its aromatic region two characteristic spin coupling systems. ABX system was recorded as a doublet of doublet at 7.67, a *meta* doublet at 7.59 and an *ortho* doublet of one proton at 6.84, which were assignable to H-6', 2' and 5' of 3', 4'- dihydroxy B-ring. The AM system was recorded as *meta*-coupled doublets at 6.41 and 6.21 for H-8 and H-6 of 5, 7-dihydroxy A-ring, respectively (Harborne, 1984). ^{13}C NMR spectra showed more or less the same 15 carbon resonances of a quercetin-3-*O*-glycoside, which were assigned in case of the previous compound **6**. The sugar moiety was finally proved to be glucoside in a β-$^{4}C_{1}$-pyranose stereostructure due to its typical six signals, particularly those of C-5" and C-3", which have a difference less than 1 ppm (Agrawal, 1989). Thus, compound 7 was identified as quercetin 3-*O*-β-D-$^{4}C_{1}$-glucopyranoside (figure 6), which is isolated before from *A. indica* (Monirul *et al*., 2012; Chakroborty *et al*., 1989; Babu *et al*., 2008).

EtOH extract and the major isolated compounds (i.e. compound 4 and comound 6) evaluated as reducing power activity. In this assay, the ability of 80% EtOH extract, compound 4 and compound 6 of the leaves of *A. indica* to reduce ferric to ferrous was evaluated at 700 nm. From Figure 7, it is evident that the tested samples have reducing power. Higher absorbance at 700 nm corresponds to higher reducing power. When compared the reducing power of the extracts to standard reference (ascorbic acid), it was appeared that the 80% EtOH extract, compound 4 and compound 6 exhibited significant reducing power.

Recent studies prove that the efficacy of chemotherapeutic agents can be enhanced and lower their toxicity to normal tissues when combined with plant derived chemopreventive agents to prevent the action of generation of free radicals (Silici *et al*., 2011; Takaki-Doi *et al*., 2009). In our study neem exhibited significant cytotoxic activity against the tested cell lines and its extract has high antioxidant activity and that was supported by the previous reported data where Neem is one of those candidate plants which have chemoprotective effect and strong antioxidant potential (Sithisarn, *et al*., 2005; Chattopadhyay, 2003; Biswas *et al*., 2002). Castalagin (compound 4), avicularin (compound 6) and ethanolic extract exhibited significant cytotoxic activity against the tested cell lines (Figures 8, 9 and 10). Castalagin represents an important compound where it supressed human promyelocytic leukemia cell line HL-60 (Yang *et al*., 2000). Castalagin exhibited marvelous cytotoxic activity against colorectal *carcinoma (*HCT_{116} *SC50= 20.38* µg/ml). As Colorectal cancer (CRC) ranks first and third in males and females, respectively among all cancers in Saudi Arabia (KSA) and high incidence of rectal cancer in Egypt [Abdul *et al*., 2011; Darlene *et al*., 2012], so Neem and castalagin could introduce a help for suppression of colorectal carcinoma.

ACKNOWLEDGMENT

The authors would like to thank the Deanship of Scientific Research and the Institute of Scientific Research and Revival of Islamic Heritage at Umm Al-Qura University, KSA (Project ID: 4331014) for the financial support.

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