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Anti-oxidant and cytotoxic activities of *Cassia nodosa* Buch.-Ham. ex Roxb. and some of its pure constituents

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Cassia nodosa Buch.-Ham. ex Roxb. is an ornamental plant which belongs to the family Fabaceae. It does not have the appropriate phytochemical and biological investigation. This was the first time to investigate anti-oxidant and cytotoxic activity of _Cassia nodosa_ flowers, leaves, stem bark methanolic extracts and their fractions (petroleum ether, methylene chloride, ethyl acetate and n-butanol). Besides, some pure constituents isolated from _C. nodosa_ were assessed as anti-oxidant and cytotoxic agents. Remarkable results were obtained specially for stem bark methanolic extract as a strong cytotoxic agent against MCF-7 and VERO cell lines. Chrysophanol (IV) displayed the highest activity as anti-oxidant (anti-hemolytic and DNA protective agent). Anti-oxidant activity of it was higher than ascorbic acid which was the positive control. This was the first time to isolate Kaempferol-3-_O_-α-L-rhamnopyranosyl (1→2)-β-D-glucoside (I) from ethyl acetate fraction of leaves. Isolation of 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein) (III) and 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) (IV) was achieved from methylene chloride fraction of flowers and stem bark, respectively. Kaempferol 3-_O_-α-L- rhamnoside (II) was isolated from ethyl acetate fraction of leaves besides compound (I).

Key words: *Cassia nodosa*, erythrocyte hemolysis, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, bleomycin, cytotoxic, kaempferol rhamnosyl glucoside, kaempferol rhamnoside, rhein, chrysophanol.

INTRODUCTION

In our research we contributed to the other world researchers in using the vast potential offered by natural resources for discovery and development of new therapeutics. This study focused on *Cassia nodosa* Buch.-Ham. ex Roxb. which is known also as *Cassia javanica* L. var. indochinensis Gagnepain (Quattrocchi, 2000). It is commonly called pink Cassia which is a common ornamental tree belonging to the family Leguminosae (Hickey and King, 1997). It is a perennial tree, 3 to 5 m height and scattered in the India, Pakistan,

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Bangladesh and Burma (Yadav et al., 2012). Traditionally, it is useful in the indigenous medicine, as its pods and leaves showed purgative activity (Rizk and Heiba, 1990). It is used for the treatment of cheloid tumor, ring worms, insect bite and rheumatism (Wealth of India, 1963; Nadkarni and Nadkarni, 1976). Bark of *C. nodosa* is used as one of ingredients in anti-diabetic ayurvedic formulation (Joshi et al., 2007). Leaves are proved to be active against *Herpes simplex* infection (Cheng et al., 2006). The presence of anti-diabetic phytochemicals of *C. nodosa* had been noticed. It showed hypoglycemic activity (Kumavat et al., 2012).

The present study has traced detailed biological assessment of variant organs extracts, fractions and some pure constituents to discover if this plant has a significant activity or not. Anti-oxidant, anti-hemolytic, DNA protective and cytotoxic activity against different cancer cell lines types were investigated for the first time. Pure compounds were isolated form leaves, stem bark and flowers. Kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucoside (I) was isolated for the first time, beside that three known constituents; kaempferol rhamnoside (II), 4, 5-dihydroxyanthraqiunone-2-carboxylic acid (rhein) (III) and 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) (IV) were identified spectrophotometrically (Scheme 1).

MATERIALS AND METHODS

Plant

Leaves, flowers and stem bark were obtained from a house garden in a village in El-Dakahlia governorate. *C. nodosa* was kindly identified by Prof. Dr. Mohammed Ibrahim Fotoh, Professor of Ornamental Horticulture and Landscape Design, Faculty of Agriculture, Tanta University, Tanta, Egypt.

Extraction

The different plant organs (leaves, flowers and stem bark) were air dried and powdered. Cold maceration at room temperature of each powdered organ (1 kg) was carried out in methanol 95% till exhaustion. Different extracts were concentrated under vacuum. Fractionation of methanolic extracts was done using petroleum ether, methylene chloride, ethyl acetate and n-butanol.

General experimental procedures

Concentrated methanolic extracts and their different fractions were obtained at a temperature not exceeding 50°C, using rotary flask evaporator.

Chromatographic materials

Thin layered chromatography (TLC) screening was carried out via pre-coated TLC sheets of 20 × 20 cm GF_{254} Merck. Observation was achieved using Camag UV lamp at 254 and 366 nm. Column chromatography was performed on silica gel 60, E Merck and reversed phase silica C-18, Sigma Chemical Co. Methanol and water for high performance liquid chromatography (HPLC) were purchased from Fischer Scientific UK limited. They were used for ODS column chromatography.

Antioxidant assays

These were performed using different reagents: 2,2`-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) ABTS, $MnO_2$, phosphate buffer solution, DNA (Calf Thymus type1), bleomycin sulphate, thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, 2,2`-azo-bis-(2-amidinopropane) dihyrochloride (AAPH), $FeCl_3$ and HCl. All reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Cytotoxic assay

Four cell lines (WI-38, VERO, MCF-7 and HepG2) were obtained from the American Type Culture Collection (ATTC), RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO®, dimethyl sulfoxide (DMSO), (4,5- Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) (MTT), sodium dodecyl- sulfonate, 5-Fluorouracil (5-FU), Sigma Chemical Co. St. Louis, MO.

Apparatus

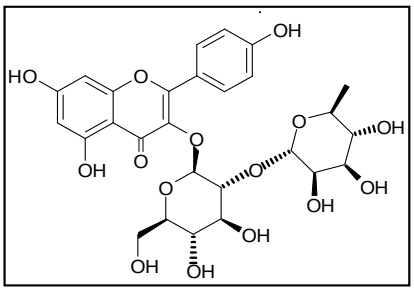
Infrared (IR) spectra were recorded on Nexus 670 fourier transform infrared (FTIR) spectrophotometer. UV spectra were recorded on Shimadzu Spectrophotometer. ^{1}H-(500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on NMR Jeol ECA Spectrophotometer. ELISA Processor II Microplate Reader was used in cytotoxic assessment.

Biological assessment of different extracts and some pure isolated compounds

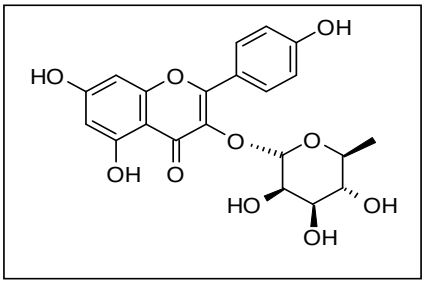
Different methanolic extracts of flowers, leaves and stem bark and their fractions (petroleum ether, methylene chloride, ethyl acetate, n-butanol) were assessed as anti-oxidant, anti-hemolytic, DNA protective and cytotoxic agent. Some pure compounds were also evaluated biologically.

Anti-oxidant assays

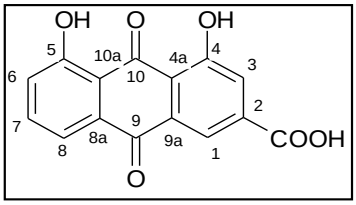
2,2`-azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) screening anti-oxidant assay: The method of Lissi et al. (1999) was adopted for the determination of ABTS activity of pure compounds and different extracts. Breifely, the method was performed as follows: For each of the investigated sample, 2 ml of ABTS solution (60 µM) was added to 3 ml MnO_2 solution (25 mg/ml), all prepared in 5 ml aqueous phosphate buffer solution (pH = 7; 0.1 M). The mixture was shaken, centrifuged, filtered and the absorbance of the resulting green-blue solution (ABTS radical solution) at λ_{max} 734 nm was adjusted to approximately 0.5. Then, 50 µl of 2 mM solution of the tested compound in spectroscopic grade MeOH/phosphate buffer (1:1) was added. The absorbance was measured and the reduction in colour intensity was expressed as inhibition percentage. L-ascorbic acid was used as standard antioxidant (positive control). Blank sample was run without ABTS and using MeOH/phosphate buffer (1:1) instead of tested compounds. Negative control was run with ABTS and MeOH/phosphate



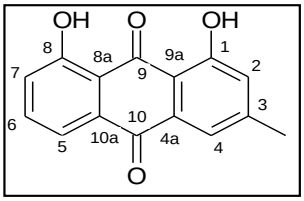
Compound (I)



Compound (II)



Compound (III)



Compound (IV)

Scheme 1. Structures of compounds I to IV.

buffer (1: 1) only. The activities of the samples were evaluated by comparison with a control of ABTS solution and positive control (L-ascorbic acid). Each sample was measured in triplicate and averaged. This activity is given as percentage $ABTS^+$ scavenging that is calculated by the following formula:

$ABTS^+$ scavenging activity (%) = [Ac – As / Ac] × 100

Where A_C is the absorbance value of the control and A_S is the absorbance value of the added samples test solution. Values are means of 3 replicates ± standard deviation (SD) and significant difference at $P < 0.05$ by Student's test.

Erythrocyte hemolysis screening assay: In this method (Malagoli, 2007) blood was obtained from rats by cardiac puncture and collected in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 10% suspension of erythrocytes in phosphate buffered solution pH 7.4 (PBS) was added to the same volume of 200 mM AAPH solution in PBS containing samples to be tested at different concentrations (stock solution of 1 mg/ml). The reaction mixture was shaken gently while being incubated at 37°C for 2 h. The reaction mixture was then removed, diluted with eight volumes of PBS and centrifuged for 10 min. The absorbance of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis and the absorbance of the supernatant obtained after centrifugation was measured at 540 nm. The data percentage hemolysis was expressed as mean of three replicates. Results were compared to L-ascorbic acid as a positive control.

Bleomycin dependent DNA damage: The reaction mixtures contained in a final volume of 1 ml, with the following reagents at the final concentrations stated: DNA (0.2 mg/ml), bleomycin (0.05 mg/ml), $FeCl_3$ (0.025 mM), magnesium chloride (5 mM), KH_2PO_4-KOH buffer (pH 7.0, 30 mM), L-ascorbic acid (0.24 mM) and the fractions tested compound diluted in MeOH to give a concentration of 0.1 mg/ml. The reaction mixtures were incubated in a water-bath at 37°C for 1 h. At the end of the incubation period, 0.1 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA) was added to stop the reaction (the iron-EDTA complex is unreactive in the bleomycin assay). DNA damage was assessed by adding 1 ml 1% (w/v) thiobarbituric acid (TBA) and 1 ml 25% (v/v) hydrochloric acid (HCl) followed by heating in a water-bath maintained at 80°C for 15 min. The chromogen formed was extracted into butan-l-ol and the absorbance was measured at 532 nm (Aeschbach et al., 1994).

2-Cytotoxic anti-tumor activity against four different cell lines: Four cell lines (WI-38, VERO, MCF-7 and HepG2) were obtained from the American Type Culture Collection (ATTC) and cultured in RPMI1640, supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin, 100 units/ml; streptomycin sulfate, 100 µg/mL) at 37°C, in an atmosphere of 95% air and 5% CO_2 under humidified condition. A stock solution (10 µM) of samples was prepared in dimethylsulfoxide (DMSO) and diluted with various concentrations with serum-free culture medium. The *in vitro* antitumor activity of different extracts, pure compounds and 5-Fluorouracil (5-FU) were determined by MTT assay method. Exponentially growing different cell lines (4000/well) were seeded in 96-well plates and treated with different concentrations of samples for 48 h and then MTT (1 mg/ml, 100 µl) was added. After incubation for 4 h at 37°C, the crystals of viable cells were dissolved overnight with SDS (sodium dodecylsulfonate, 10%, 100 µl) in each well. The absorbance spectra were measured on an enzyme linked immunosorbent assay (ELISA) Processor II Microplate Reader at a wavelength of 570 nm. The percentage of cytotoxicity was defined with treated and untreated cell lines (Block

et al., 2004; Mosmann, 1983; Wilson, 2000). The 50% antitumor activity dose (IC_{50}) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50%. Classification according to IC_{50} (µg/ml) demonstrate that from 1 to 10 (very strong activity), 11 to 25 (strong activity), 26 to 50 (moderate activity), 51 to 100 (weak activity), 100 to 200 (very weak activity) and above 200 (non cytotoxic) (Ayyad et al., 2012).

Chromatographic separation of pure flavonoids from leaves ethyl acetate fraction: Ethyl acetate dried fraction of leaves (2 g) was chromatographed on a silica gel column (40 × 2 cm) starting with methylene chloride and increasing polarity with methanol. Fractions obtained with (methylene chloride-methanol) (90:10) were rechromatographed on ODS column starting with 100% water for HPLC and decreasing polarity with methanol for HPLC. Compound (I) was eluted using (water:methanol) (80:20) as pure yellow powder. Fractions obtained at eluent ratio (methylene chloride-methanol) (93:7) were pooled, evaporated and rechromatographed on silica gel column (15 × 1.3 cm) to yield yellow powder of compound (II). Identification of pure compounds was achieved by comparing IR, UV, ^{1}H-NMR and ^{13}C-NMR spectral data with those published in the literature.

Chromatographic separation of main anthraquinones from flowers and stem bark methylene chloride fraction: Methylene chloride dried extract of flowers (2 g) was chromatographed on silica gel column (40 × 2 cm) starting with methylene chloride and increasing polarity with methanol. Fractions obtained at eluent ratio (methylene chloride:methanol) (95:5) were pooled, concentrated and rechromatographed on another column of silica gel (15 × 1.3 cm) to provide pure orange powder of compound (III). The previous method of separation was also carried out using 1 g of methylene chloride dried fraction of stem bark. Fractions obtained at eluent ratio (methylene chloride:methanol) (98:2) were pooled and rechromatographed to give pure yellow powder of compound (IV). Identification of pure compounds was also achieved by comparing spectral data with the published data (EI-mass, UV, ^{1}H-NMR and ^{13}C-NMR spectra).

Compound (I): Yellow powder, m.p. 219 to 221°C, freely soluble in methanol, insoluble in petroleum ether, R_f = 0.18 (CH_2Cl_2:CH_3OH) (8:2). IR (KBr) spectrum showed bands at υ_{max} (cm^{-1}): 3464, 2937, 1695, 1608, 1504, 1431, 1371, 1292, 1207. UV λ_{max} (nm): MeOH 266, 348; +NaOMe 274, 325, 395; +$AlCl_3$ 274, 304, 351, 395; $AlCl_3$/HCl 275, 302, 347, 395; +NaOAc 273, 379; +NaOAc/H_3BO_3 266, 347. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR data (125 MHz, CD_3OD) are listed in Tables 1 and 2, respectively.

Compound (II): Yellow powder, m.p. 152 to 153°C, freely soluble in methanol and ethyl acetate, insoluble in petroleum ether, R_f = 0.31 (CH_2Cl_2:CH_3OH:formic acid) (8.5:1.5:0.1) IR (KBr) spectrum showed bands at υ_{max} (cm^{-1}): 3433, 2926, 1655, 1609, 1501, 1454, 1367, 1269, 1209. UV λ_{max} (nm): MeOH 265, 341; +NaOMe 273, 324, 389; +$AlCl_3$ 273, 303, 346, 393; $AlCl_3$/HCl 274, 302, 342, 391; +NaOAc 273, 375; +NaOAc/H_3BO_3 265, 342. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) data are shown in Tables 1 and 2.

Compound (III): Orange powder, m.p. 320 to 322°C, soluble in methanol and chloroform, R_f = 0.25 (CH_2Cl_2:CH_3OH) (9:1). EIMS m/z (rel. int.): 283 $[M]^+$ (9.2), 98 (11), 81 (19.3), 78 (74), 63 (100), 45 (25.5), 28 (62), 18 (45.6). UV λ_{max} (nm): MeOH 430, 232; +NaOH504, 484. ^{1}H-NMR (500 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6) data are listed in Tables 3 and 4.

Compound (IV): Yellow powder, m.p. 198 to 200°C, freely soluble in chloroform and ether, insoluble in petroleum ether, R_f = 0.91(CH_2Cl_2:CH_3OH) (9:1). EIMS m/z (rel. int.): 254 $[M]^+$ (100), 226 (16.5), 197 (11.2), 181 (3.8), 169 (5), 153 (2.7), 141 (6.1), 127 (4.5), 115 (7.4), 78 (54.7), 63 (71.4). UV λ_{max} (nm): MeOH 429, 287, 256, 224; +NaOH 505, 285. ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data are shown in Tables 3 and 4.

RESULTS

The $ABTS^+$ scavenging activity of different extracts and pure compounds are demonstrated in Figure 1. Rhein showed the best activity by 74.8% inhibition. Chrysophanol and kaempferol rhamnoside showed activity with average percentage of inhibition of 61.28 and 51.21%, respectively. The anti-hemolytic antioxidant assay results are shown in Figure 2. It was found that chrysophanol exhibited the best anti-hemolytic activity by 3.28% of hemolysis at 0.1 mg/ml, which was better than L-ascorbic acid (3.75%). Kaempferol rhamnoside and rhein also showed anti-hemolytic activity by 4.21 and 5% of hemolysis. The DNA protective activity results are displayed in Figure 3. The absorbance of chrysophanol was 0.069 for sample concentration of 0.1 mg/ml. It showed best protective activity which exceeded the positive control itself (absorbance = 0.47). Kaempferol rhamnoside and rhein exhibited high activity at absorbance of 0.38 and 0.433, respectively. The different extracts unfortunately were inactive in $ABTS^+$ scavenging, antihemolytic, DNA protection assays. This needs more investigation to have the answer. The cytotoxic activity against the different cell lines of VERO, MCF-7, WI-38 and HepG2 are displayed in Figures 4 to 7. It was found that the methanolic extract of stem bark reduced the viability of VERO and MCF-7 cells with IC_{50} = 20.5 and 20.2 µg/ml, respectively. This indicated a strong activity according to the classification. Also, petroleum ether fraction of leaves and flowers with IC_{50} = 25.8 and 29.1 µg/ml showed strong and moderate activity, respectively. Rhein displayed moderate activity with IC_{50} = 26.3 and 28.3 µg/ml, respectively. The petroleum ether fraction of leaves with IC_{50} = 27.3 and 22.4 µg/ml exhibited moderate and strong activity against WI-38 and HepG2 cells, respectively. Rhein showed moderate and strong activity with IC_{50} = 28.4 and 23.7 µg/ml, respectively. Also methylene chloride fraction of leaves showed moderate activity against VERO, MCF-7 and WI-38 cell lines while displayed strong activity against HepG2 cell line.

DISCUSSION

Cancer is a leading cause of death all over the world. Although the etiology of the cancer are many, free radicals play a major role for the pathophysiological processes (Soobrattee et al., 2006). $ABTS^+$ scavenging,

Table 1. ^{1}H-NMR of compound I and II.

#Hydrogen atom	Compound (I) 500 MHz - CD_3OD	#Hydrogen atom	Compound (II) 500 MHz - CD_3OD
OH-5	12.59	H-2`,6`	7.74 (2H, d, *J*=8.4 Hz)
H-2`,6`	8.03 (2H, d, *J*= 8.4 Hz)	H- 3`,5`	6.90 (2H, d, *J*=8.4 Hz)
H-3`,5`	6.86 (2H, d, *J*= 8.4 Hz))	H-6	6.36 (1H, d, *J*=1.5 Hz)
H-6	6. 36 (1H, d, *J*= 1.5 Hz)	H-8	6.18 (1H, d, *J*=2.3 Hz)
H-8	6.16 (1H, d, *J*= 1.55 Hz)	Rh-H-1	5.35 (1H, s)
(H-1) glucose	5.71 (1H, d, *J*= 7.65 Hz)	Rh-CH_3	0.90 (3H, d, *J*=5.53 Hz)
(H-1) rhamnose	4.43 (1H, d, *J*=1.1 Hz)	-	-
CH_3 rhamnose	0.93 (3H, d, *J*= 6.15 Hz)	-	-

Table 2. ^{13}C-NMR of compounds I and II.

#Carbon	Compound (I) (125 MHz - CD_3OD)	#Carbon	Compound (II) (125 MHz - CD_3OD)
C-2	157.0	C-2	157.2
C-3	133.0	C-3	134.8
C-4	178.0	C-4	178.2
C-5	159.9	C-5	157.9
C-6	98.3	C-6	98.5
C-7	164.7	C-7	164.5
C-8	93.2	C-8	93.4
C-9	157.1	C-9	160.2
C-10	104.5	C-10	104.5
C-1`	121.7	C-1`	121.2
C-2`, 6`	130.7	C-2`,6`	130.5
C-3`, 5`	114.7	C-3`,5`	115.1
C-4`	161.8	C-4`	161.8
C-1``(glucose)	101.2	C-1``(rhamnose)	102.1
C-2``	78.7	C-2``	70.71
C-3``	77.0	C-3``	70.76
C-4``	71.0	C-4``	71.8
C-5``	77.6	C-5``	70.5
C-6``	61.2	C-6``	16.3
C-1```(rhamnose)	98.8	-	-
C-2```	70.4	-	-
C-3```	70.9	-	-
C-4```	72.6	-	-
C-5```	68.5	-	-
C-6```	16.1	-	-

erythrocyte hemolytic and bleomycin dependant DNA damage assays were performed because compounds possessing potent antioxidant and anticancer activity may not be useful in pharmacological preparations if they possess hemolytic effect or other side effects. Therefore, a search for compounds that can reduce the harmful side effects of anticancer drugs in normal tissues is necessary (Sun and Peng, 2008). In addition, the different anti-oxidant assays also may reveal some information about the mechanism of cytotoxicity. It was found that pure compounds had a significant anti-oxidant effect comparable to crude extracts and L-ascorbic acid. Rhein was the best $ABTS^{+}$ scavenging agent among the other pure compounds. Chrysophanol displayed the most significant effect. It showed the highest activity as anti-hemolytic and DNA protective agent, which exceeded L-ascorbic acid

Table 3. ^{1}H-NMR of compound III, IV

#Hydrogen atom	Compound (III) 500 MHz - DMSO,d_6	#Hydrogen atom	Compound (IV) 500 MHz - $CDCl_3$
H-3	7.78 (1H, br s)	CH_3	2.46 (3H, s)
H-6	7.33 (1H, d, *J*=8.4 Hz)	H-2	7.1 (1H, br s)
H-1	8.05 (1H, br s)	H-7	7.28 (1H, d, *J*=8.4Hz)
H-7	7.67 (1H, t, *J*=8.4 Hz)	H-4	7.62 (1H, br s)
H-8	7.75 (1H, d, *J*=8.4 Hz)	H-6	7.67 (1H, d, *J*=7.65 Hz)
OH	11.84 (1H, br s)	H-5	7.81 (1H,d, *J*=7.65 Hz)
-	-	OH-1	12.02 (s)
-	-	OH-8	12.13 (s)

Table 4. ^{13}C-NMR of compounds III and IV.

#Carbon	Compound (III) (125 MHz – DMSO,d_6)	#Carbon	Compound (IV) (125 MHz – $CDCl_3$)
C-4	161.5	C-1	162.5
C-3	124.6	C-2	124.4
C-2	134.2	C-3	149.4
C-1	119.9	C-4	121.4
C-8	125.1	C-5	124.6
C-7	138.1	C-6	137.0
C-6	119.1	C-7	120.0
C-5	161.9	C-8	162.8
C-10	191.7	C-9	192.6
C-9	181.4	C-10	182.0
C-11	165.9	(CH_3)	22.3
C-9a	133.6	C-4a	133.7
C-10a	116.6	C-8a	115.9
C-4a	119.2	C-9a	113.8
C-8a	138.6	C-10a	133.3

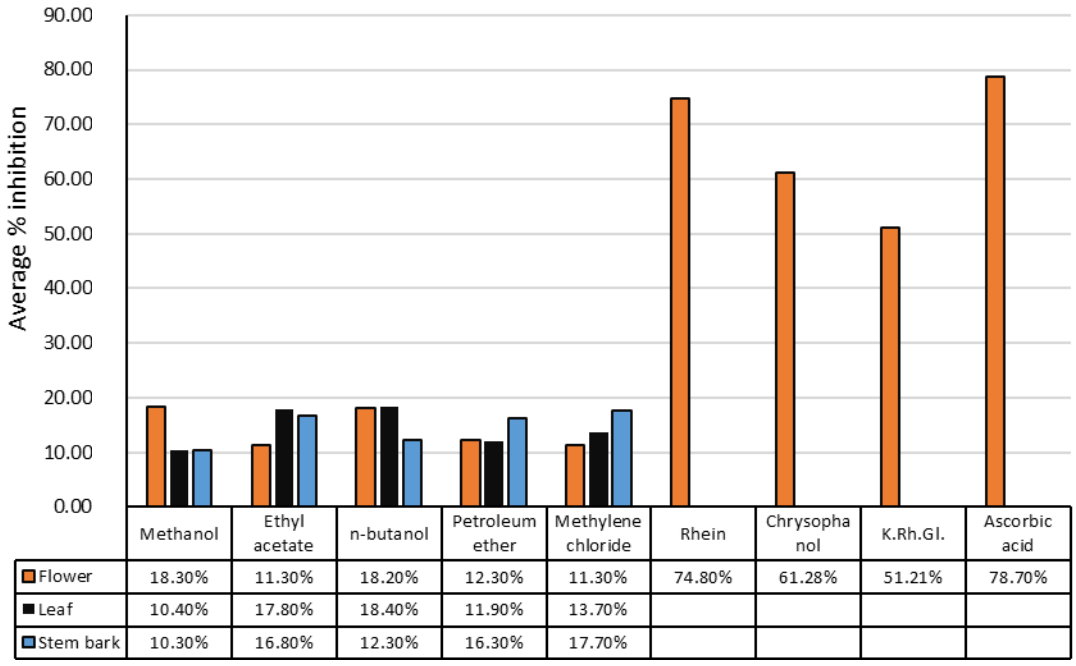
activity (+ve control). Damage to DNA in the presence of a bleomycin-Fe complex has been adopted as a sensitive and specific method to examine potential pro-oxidant agents. If the samples are able to reduce the bleomycin-Fe^{3}+ to bleomycin-Fe^{2}+, DNA degradation in this system would be stimulated, resulting in a positive test for pro-oxidant activity (Gutteridge et al., 1981). Chrysophanol had the highest ability to protect DNA from the induced damage by bleomycin.

Rhein and chrysophanol displayed a significant cytotoxic activity against different cell lines, especially rhein. It is interesting to notice that the high cytotoxic activity of anthraquinones in this study was in agreement with the fact that many herbal formulas-containing anthraquinones were successfully used for treatment of cancer. The most extensively studied anthraquinones, emodin, aloe emodin and rhein have been reported to inhibit proliferation of breast, lung, cervical, colorectal and prostate cancer cells (Cha et al., 2005; Chang et al., 1996; Kuo et al., 1997; Zhang et al., 1995), which is coincident with the results of this study. Several mechanisms have been proposed to explain this anticancer activity, including the intercalation of DNA (Hsiao et al., 2008), inhibition of DNA topoisomerase II (Perchellet et al., 2000), production of free radicals and subsequent cleavage of DNA (Fisher et al., 1992).

Methanolic extract of stem bark, petroleum ether fraction of leaves and flowers methanolic extract and methylene chloride fraction of leaves methanolic extract showed strong to moderate cytotoxic activity, which was in agreement with the fact that the plant is useful traditionally in the indigenous medicine as its leaves and pods had cytotoxic effect (Rizk and Heiba, 1990).

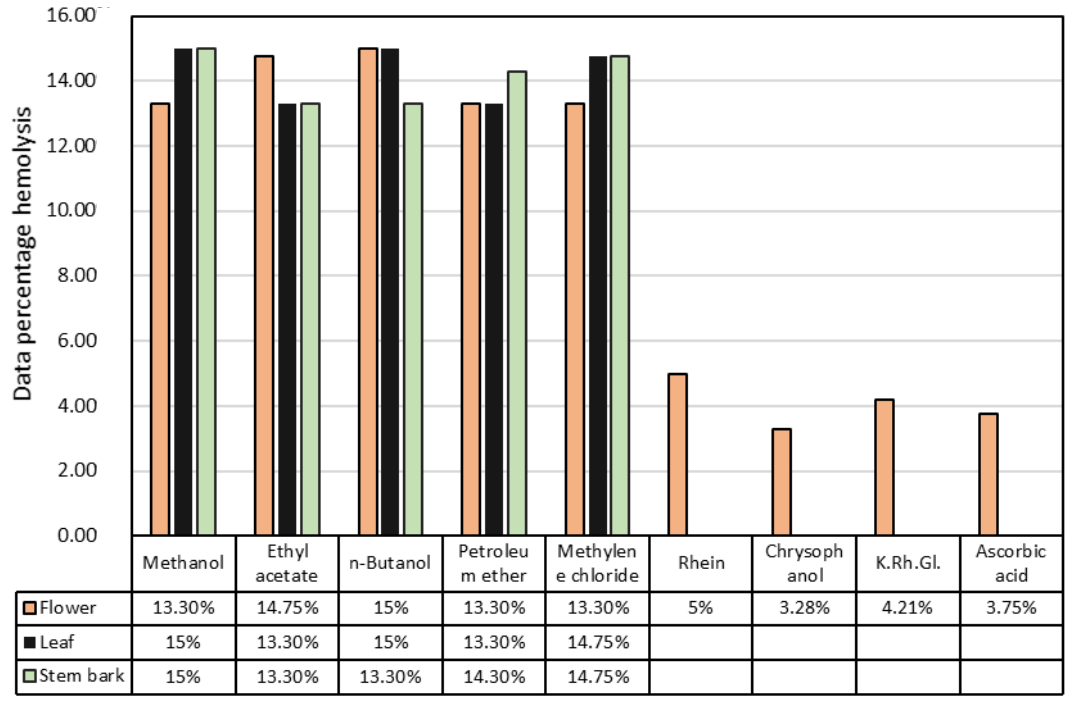
Compound (I)

IR spectrum data revealed that signals at υ_{max} (cm^{-1}) = 3464 is for OH group, 2937 is for aliphatic CH_3 stretching, 1695 is for C=O conjugated, 1608 is for C=C stretching



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	18.30%	11.30%	18.20%	12.30%	11.30%	74.80%	61.28%	51.21%	78.70%
Leaf	10.40%	17.80%	18.40%	11.90%	13.70%				
Stem bark	10.30%	16.80%	12.30%	16.30%	17.70%				

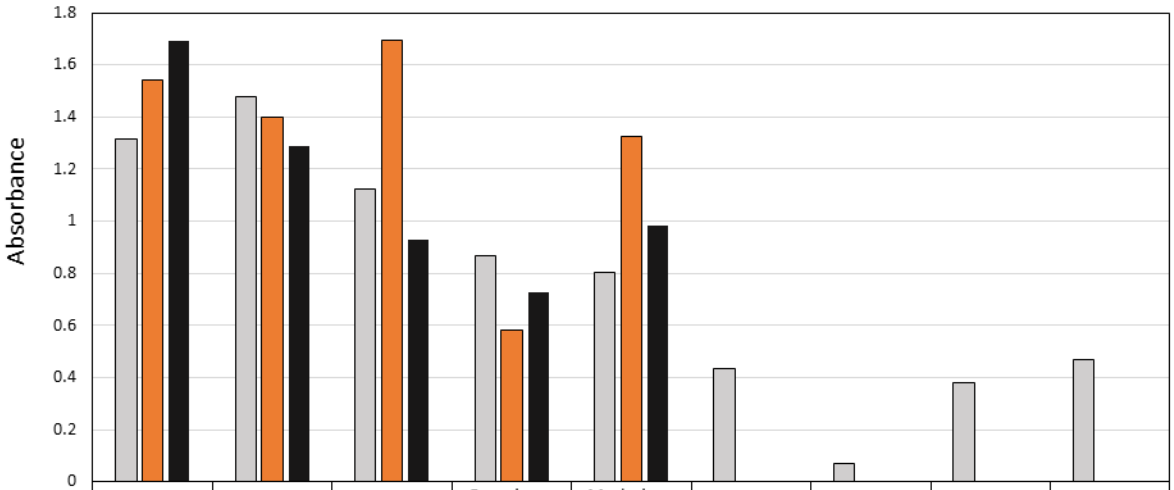
Figure 1. ABTS antioxidant assay.



	Methanol	Ethyl acetate	n-Butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	13.30%	14.75%	15%	13.30%	13.30%	5%	3.28%	4.21%	3.75%
Leaf	15%	13.30%	15%	13.30%	14.75%				
Stem bark	15%	13.30%	13.30%	14.30%	14.75%				

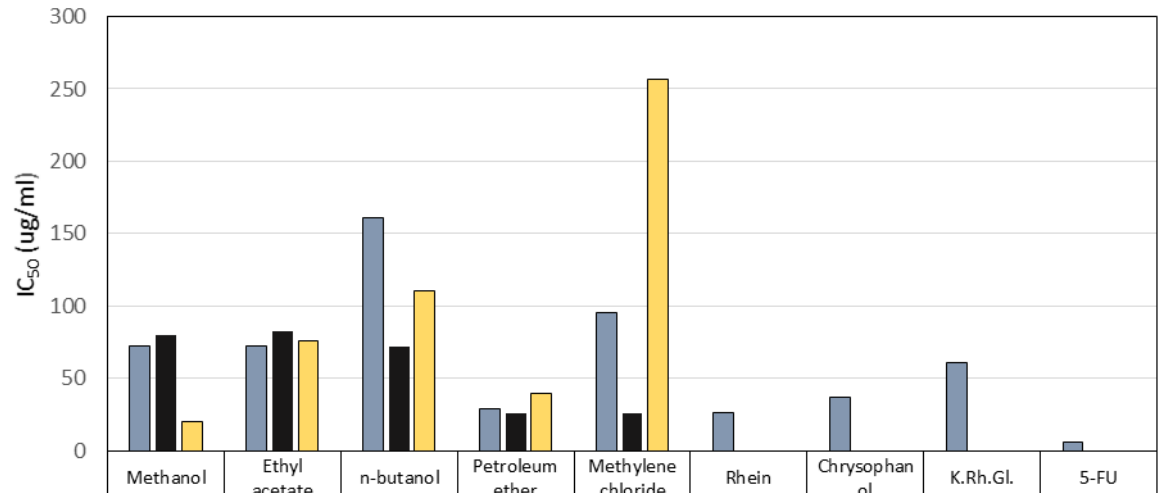
Figure 2. Erythrocyte hemolysis antioxidant assay.

(aromatic), 1431 is for CH_2 bending, 1371 is for CH_3 bending, 1292, 1207 are for C-O. UV spectral data at 348 and 266 nm of compound I was typical of flavonol glycosides substituted at C-3. Shifts obtained with $NaOCH_3$, NaOAC and $AlCl_3$ indicated the presence of free hydroxyl groups at 5, 7, 4` positions. Furthermore,



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	Ascorbic acid
Flower	1.316	1.479	1.124	0.865	0.803	0.433	0.069	0.38	0.47
Leaf	1.54	1.399	1.694	0.584	1.323				
Stem bark	1.692	1.29	0.931	0.727	0.985				

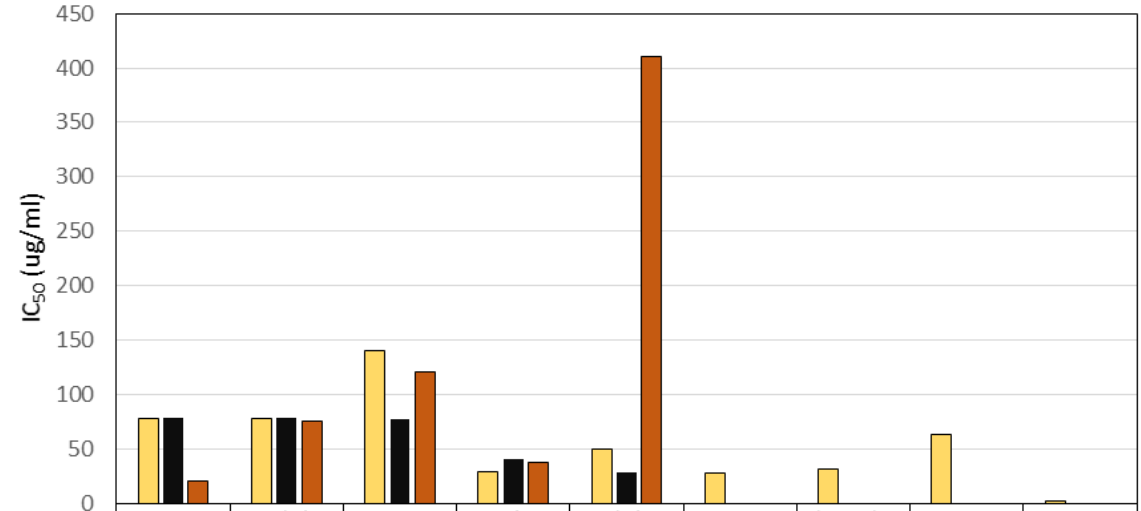
Figure 3. Bleomycin dependent DNA damage assay.



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	72.4	72.4	160.6	28.8	95.6	26.3	36.9	60.7	6.5
Leaf	80.3	82.3	72.4	25.8	26.3				
Stem bark	20.5	76.4	110.4	39.6	256.6				

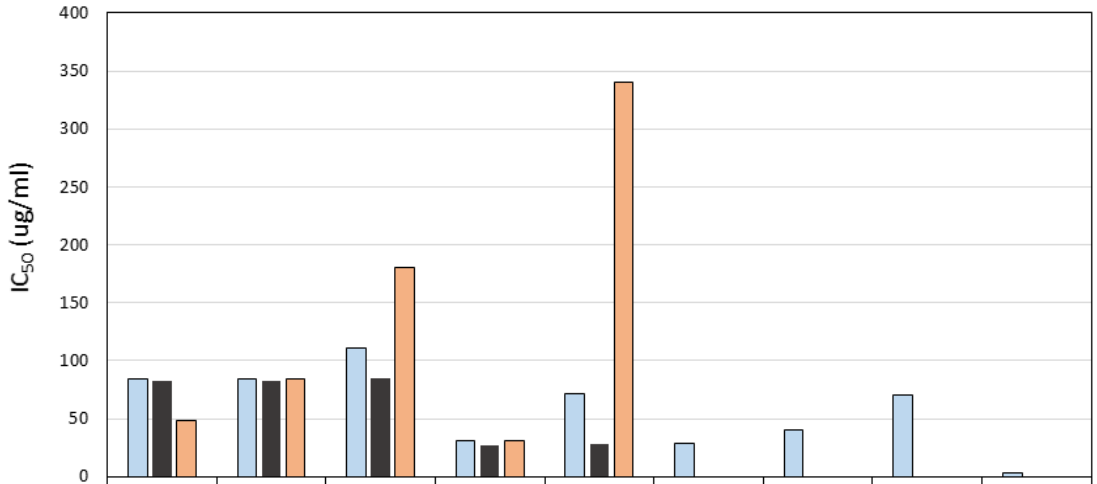
Figure 4. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on VERO cell line.

shifts with $AlCl_3$, $AlCl_3$/HCl and NaOAC/H_3BO_3 did not support the presence of dihydroxyl system (Mabry et al., 1970). Glucose and rhamnose were obtained by acid hydrolysis of compound (I), this confirmed with paper chromatography alongside authentic sugar. ^{1}H-NMR displayed the presence of six aromatic protons ascribed to H6, 8, 2`, 3`, 5`, 6` positions beside two anomeric protons as two doublet signals resonating at δ 5.71, 4.43 ppm with *J* value 7.65, 1.1 Hz, respectively. These two later proton signals can be assigned to anomeric protons of glucose and rhamnose moieties, respectively. The ^{13}C-NMR spectral data showed the presence of anomeric carbons at δ 101.2, 98.8 ppm for glucose and rhamnose moieties, respectively. Furthermore examination of ^{13}C-



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	77.5	77.5	140.5	29.1	49.9	28.3	31.7	63.7	2.3
Leaf	79.2	78.2	77.5	40.8	28.3				
Stem bark	20.2	75.5	120.5	37.4	410.6				

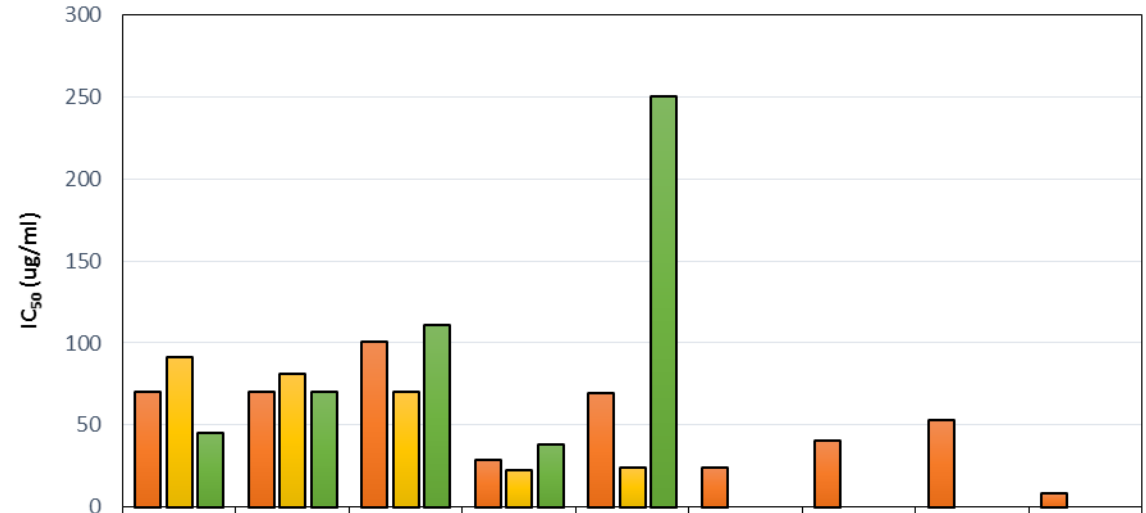
Figure 5. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on MCF- 7 cell line.



	Methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.Rh.Gl.	5-FU
Flower	84.6	84.8	110.6	30.5	71.2	28.4	39.8	70.4	3.2
Leaf	82.4	82.6	84.6	27.3	28.4				
Stem bark	48.2	84.6	180.2	31.1	340.5				

Figure 6. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on WI- 38 cell line.

NMR data indicated rhamno (1→2) glucoside linkage (neohesperidoside). This was evidenced by the lack of shift of C-6`` of glucose, whereas C-2`` signal was de-shielded by 4.5 ppm. Compound (I) was identified as Kaempferol-3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucoside (Kaempferol neohesperidoside) by comparison of spectral data with the published data (Al-Musayeib et al., 2011; Markham and Terani, 1976; Markham et al., 1978;



	methanol	Ethyl acetate	n-butanol	Petroleum ether	Methylene chloride	Rhein	Chrysophanol	K.RH.Gl.	5-FU
Flower	70.2	70.5	100.7	28.7	69.5	23.7	40.7	52.8	8.6
Leaf	91.2	81.2	70.2	22.4	23.7				
Stem bark	45.1	70.2	111	38.1	250.2				

Figure 7. Cytotoxic activity (IC_{50}) of different extracts and pure compounds on HepG2 cell line.

Takemura et al., 2005).

Compound (II)

IR spectrum data showed that signals at υ_{max} (cm^{-1}) = 3433 is for OH group, 2926 is for aliphatic CH_3 stretching, 1655 is for C=O conjugated, 1609 is for C=C stretching (aromatic), 1454 is for CH_2 bending, 1367 is for CH_3 bending, 1269, 1209 are for C-O. UV spectral properties of Compound (II) were those of typical flavonol glycosides with substituted (OH) group at C-3; the presence of absorption maxima at 341 and 265 nm. Bathochromic shift with NaOMe, NaOAC and $AlCl_3$ indicated for the presence of free hydroxyl groups at 5, 7 and 4` positions. Spectra obtained with $AlCl_3$, $AlCl_3$/HCl and NaOAC/$H_3BO_3$ revealed absence of orthodihydroxyl system at 3` and 4` positions of ring B (Mabry et al., 1970). Rhamnose was obtained by acid hydrolysis, this confirmed with paper chromatography with authentic sugar. $^1$H-NMR spectrum exhibited the presence of six aromatic (H-6, 8, 2`, 3`, 5` and 6`) and one anomeric proton at (δ 5.35 ppm). Protons of methyl group of rhamnose was exhibited at (δ 0.9 ppm, J = 5.53 Hz). ^{13}C-NMR spectrum showed signals at δ 102.1 ppm for anomeric carbon of rhamnose, a significant signal at δ 16.3 ppm for CH_3 of rhamnose. Compound (II) was identified as Kaempferol-3-*O*-α-L-rhamnoside by comparison of spectral data with the published data (Diantini et al., 2012; Matthes et al., 1980).

Compound (III)

EI-Mass spectrum displayed molecular ion peak at 283 $[M]^+$. UV spectrum displayed a bathochromic shift after addition of NaOH. ^{1}H-NMR spectrum showed signals for five aromatic protons (H1, 3, 6, 7 and 8). Phenolic proton signal at (δ 11.84 ppm) was also noticed. ^{13}C-NMR spectrum showed the carbonyl carbons at (δ 165.9 ppm), (δ 191.7 ppm) C-10, (δ 181.4 ppm) C-9, the higher δ value for C-10 is due to strong intramolecular hydrogen bonding with hydroxyl groups at C-4 and C-5 (Agarwal et al., 1999). Compound (III) was identified as 4, 5-dihydroxyanthraqiunone-2-carboxylic acid (rhein), by comparison of spectral data with the published data (Singh et al., 2005).

Compound (IV)

EI-Mass spectrum displayed the parent ion peak at 254 $[M]^+$ which was at the same time the base peak. Strong peaks at 226 and 197 were due to successive elimination of (CO) group indicating the anthraquinoidal nature of the compound (Agarwal et al., 1999). UV spectrum displayed a bathochromic shift after addition of NaOH. ^{1}H-NMR spectrum showed signals for five aromatic protons (H2, 4,

5, 6 and 7). A three proton singlet at (δ 2.46 ppm) indicated the presence of aromatic methyl in the compound. Two phenolic proton signals at (δ 12.02 and 12.13 ppm) were also noticed.^{13}C-NMR spectrum showed the carbonyl carbons at (δ 192.6 ppm) C-9 and (δ 182.0 ppm) C-10, the higher δ value for C-9 is due to strong intramolecular hydrogen bonding with hydroxyl groups at C-4 and C-5 (Agarwal et al., 1999). Compound (IV) was identified as 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol) by comparison of spectral data with the published data (Amatya and Tuladhar, 2005).

Conclusion

In our study findings, it was clear that different crude extracts had no antioxidant, anti-hemolytic and DNA protective activities, on the other hand chrysophanol had potent anti-hemolytic and DNA protective activity which exceeded L-ascorbic acid (positive control). Rhein had the best $ABTS^{+}$ scavenging activity. Cytotoxic assessment revealed that stem bark methanolic extract, methylene chloride, petroleum ether fractions of leaves and petroleum ether fraction of flower displayed a significant cytotoxic activity against different cell lines. Rhein as a hydroxy anthraquinone showed a significant anti-tumor activity against all cancer cell lines, when compared to 5-Florouracil (positive control).

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