Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Phytochemical analysis and hepatoprotective characterization of AJME

[1]Abdul Wajid Khan, [1]Attaullah Khan, [1]*Saleem Jan, [2]Rahmat Ali Khan, [2]Mushtaq Ahmad, [2]Abdus Saboorshah

1 Department of Chemistry, University of Science and Technology Bannu-28100, Pakistan
2 Department of Biotechnology, University of Science and Technology Bannu-28100, Pakistan

Manuscript Info

Manuscript History:

Received: 25 September 2014
Final Accepted: 29 October 2014
Published Online: November 2014

Key words:
Aervajevanica, CCl_4, Hepatotoxicity, Oxidative stress

***Corresponding Author**

Saleem Jan

Abstract

Objectives:
Aervajevanica (Burm. f.) Juss.ex Schult possess antioxidant activity and hepatoprotective effects, and somay provide a probable therapeutic alternative in hepatic disorders. Hence, the present study was designed to investigate the hepatoprotective efficacy of methanolic extract of *Aervajevanica* (AJME) against carbon tetrachloride (CCl_4) induced toxicity in rats and to check the bioactive constituents responsible for the hepatoprotection.
Methods:
30 chicks were divided into five groups having free access to food and water. Group A (control) and B was given olive oil and DMSO, while group C received only CCl_4 group D received silymarin at a dose of 50 mg/kg however group E was given AJME (200 mg/kg).Various serum marker i.e., alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) were used. Catalase (CAT), peroxidase (POD), glutathione-S transferase (GST), glutathione peroxidase (GSH-Px), glutathione reductase (GSR) activity was measured in liver homogenates. Thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) concentration were also evaluated in liver homogenates. Phytochemical screening was carried out using column chromatography and NMR.
Main results: Hepatotoxicity induced with CCl_4 was evidenced by significant increase in lipid peroxidation products (TBARS) andlevels of serum marker enzymes (ALT, ALP, and LDH). Level of glutathione contents (GSH) determined in liver was significantlyreduced, as were the activities of antioxidant enzymes; CAT, POD, GSH-Px, GSR, GST. Chicks treated with CCl_4.AJME (200 mg/kg bw) and silymarin (50 mg/kg bw) co-treatment prevented all the changes observed with CCl_4-treatedchicks. Phytochemical study revealed the presence of oleanolic acid, stigmasterol and β-sitosterolwhich might be responsible for hepatoprotection.
Conclusion: The results of present work indicate that AJME has a significant protective effect against CCl_4 induced hepatotoxicity in chicks, which may be due to their antioxidant potentialoleanolic acid, stigmasterol and β-sitosterol.

Copy Right, IJAR, 2014,. All rights reserved

Introduction

Background:

Plants are the oldest friends of mankind. They not -only supply foodstuff and shelters but also serve the human race by protecting and curing numerous ailments. Herbs have always been helpful to cure diseases with low side effect. The secondary metabolites of plants such as phenolic compounds, essential oil,saponin and tannins [1]are the natural remedy for different diseases. Medicinal plants offer a countless variety of chemical compounds. That cures many diseases and is used for therapeutic purposes. The medicinal value of plants is due to the presence the secondary metabolites. Secondary metabolites import antioxidant activity and prevent oxidative stress [2]. It is reported that plants posses numerous antioxidants used as a medicine and food additives [3]. In low concentration, antioxidants extensively reduce the oxidation process [4] and protect the cells from injuries. That is why diseases causes by free radicals can be prevented by the use of antioxidants treatment. This focus has got an enormous significance, and modern researchers are directed particularly to-wards the plant origin antioxidants [5].
Liver disease is a great health problem allover the world. Fatty Liver is a metabolic disease in poultry that may occur either due to metabolic disturbances or chemical exposure which is characterized by hyper-cholesterolemia, decreased performance and poor egg production in poultry [6]. It is due to of imbalances in the energy metabolism of energy which leads to change fat metabolism and hence the fatty infiltration in liver [7].$CC1_4$ acts as a direct hepatotoxin resulting necrosis, liver and kidneys injury it also causes destruction of the cells and cellular organelle [8]. A songle dose of CCl_4 (2ml/kg) causes disturbance in the liver enzymes after six hours from treatment [9]. CCl_4 is metabolized in to highly reactive $CC1_3$ radical that initiate lipid peroxidation which ultimately results in cell death [10].*Aervajavanica* (Burm. f.)Juss.exSchult. plants have been analyzed for their biological activity and active constituents. *Aervajavanica* is used in the treatment of hepatitis, kidney stones and as an anti-inflammatory (plant); for cleaning teeth (roots) [11-13]. It is used in Headache, rheumatism. The Woolly seeds stuffed in pillows to relieve headache and protective against rheumatism [14-15]. The present study was designed to investigate the hepatoprotective efficacy of methanolic extract of*Aervajevanica* (AJME) against carbon tetrachloride (CCl_4) induced toxicity in rats and to check the bioactive constituents responsible for the hepatoprotection.

Materials and Methods:

Plant collection ant formation of Extracts:

One kilogram of plant was collected and identified by ProfessorAbdurRahman,Department of Botany and Government Postgraduate College Bannu. The selected plant was dried under shadow at room temperature for one month and then grinded into finepowdered. The investigated driedpowdered plant materials were extracted with 80% methanol. The methanolic extractwas filtered and concentrated under reduced pressure at 37C^o temperature by using rotary evaporator.

Isolation and purification of bioactive constituents

The hydromethanolic extract (700 g) was initially partitioned into *n*-hexane (84 g). The defatted MeOH extract was dried and then suspended in H_2O (2 L), was successively partitioned with dichloromethane (110 g), and ethyl acetate (27 g). The EtOAc soluble fraction was subjected to *CC* by using normal silica gel. The eluent used were n-hexane, EtOAc, DCM and MeOH in gradient manner to obtain compounds **1**-**3**.

Oleanolic Acid (1):

Physical State Colourless needles; **m.p:** 305-306 ˚C.**[αl $_D^{25}$:** + 78.9 (c = 0.07, $CHC1_3$).

IR v_{max}(KBr)cm^{-1} : 3400 (OH), 1700 (CO), 1660 (C=C) ; **EIMS** *m/z* (rel. int. %):456 $[M]^+$ (2), 248 (99), 208 (8), 203 (65) and 133 (39); **HREIMS** *m/z*: 456.3610 (calcd. for $C_{30}H_{48}O_3$, 456.3603); **^{1}H-NMR** ($CDC1_3$, 500 MHz): See Table-1; **^{13}C-NMR** ($CDCl_3$, 100 MHz): See Table-1.

Stigmasterol (2):

Physical State: Colourless crystalline solid; **m.p :** 170-171 °C; **$[\alpha]_D^{25}$: -**51.5^0 (c = 0.28, $CHCl_3$); **IR** ($CHC1_3$) $v_{max}cm^{-1}$: 3432 (OH), 1648 (C=C); **EIMS** *m/z* (rel. int. %): $[M]^+$412 (8), 396 (12), 394 (20), 379 (27), 369 (35), 351 (71), 327 (60), 301 (18), 300 (67), 273 (30), 270 (24); **HREIMS** *m/z*:412.3920 (calcd. for $C_{29}H_{48}O$, 412.3926).

^{1}H-NMR ($CDCI_3$, 400 MHz): Table-2;**^{13}C-NMR ($CDC1_3$, 100 MHz):** Table-2

β-Sitosterol (3):

Physical State: White needles (45 mg); **m.p**: 135 °C; $[\alpha]_D^{25}$: + 35.5° (c = 0.22, $CHCl_3$)

IR (KBr) v_{max} cm^{-1}:3210 (OH), 3050, 1734 (CO), 1650 (C=C), 1457.3 (CH2); **EIMS** *m/z* (rel. int. %): $[M]^+$ 414 (15), 399 (10), 396 (12), 381 (72), 329 (28), 275 (12), 273 (17), 255 (36); **HREIMS:** *m/z* 414.3845 (calcd. for $C_{29}H_{50}O$, 414.3861).

^{1}**H-NMR** ($CDC1_3$, 400 MHz): Table-3; ^{13}**C-NMR** ($CDC1_3$, 100 MHz): Table-3

***In Vivo* experimental plan**

Six-week-old male chicks weighing were provided with food and water *ad libitum* and kept at 20-22 °C on a 12-h light–dark cycle. All experimental procedures involving animals were conducted in accordance with the guidelines of National Institutes of Health (NIH guidelines). The study protocol was approved by Ethical committee of University of Science and Technology Bannu, KPK, Pakistan. The chicks were acclimatized to laboratory condition for 7 days before commencement of experiment.

EXPERIMENTAL PROTOCOL

Following dosing plan was adapted for the study.
Group A: the normal control received only feed
Group B: Olive oil (0.5 ml/kg b.w., i.p.) + DMSO (0.5 ml/kg b.w. orally)
Group C: CCl_4 twice a week (0.5 ml/kg b.w., i.p., 50% CCl_4/olive oil)
Group D: CCl_4 twice a week (0.5 ml/kg b.w., i.p.) + sylimarin (50 mg/kg b.w., orally)
Group E: CCl_4 twice a week (0.5 ml/kg b.w., i.p.) + AJME (200 mg/kg b.w., orally)
At the end of eight weeks, after 24 h of the last treatment animals were given chloroform anesthesia and dissected from ventral side. All the animals were sacrificed; blood was drawn prior to the excision of organ tissues. The serum was stored at -80 °C after separation until it was assayed as described below. After taking blood the livers were removed and washed in ice cold saline. Subsequently, half of the organs were treated with liquid nitrogen and stored at -80°C for further enzymatic analysis.

Assessment of antioxidant enzymes

10% homogenate of tissue was prepared in 100 mM KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 x g for 30 min at 4°C. The supernatant was collected and used for the following parameters as described below.

Lipid peroxidation assay:

Lipid per-oxidation was analyzed by the determination of thio-barbituric acid re-active substnces (TBARS) in the liver homogenate by using the method of [16] and modified by [17]. Thio-barbituric acid re-active substnces (TBARS) content was expressed as nM per mg of protein. Protein was determined by the method of [18] using (BSA) bovine serum albumin as a standard.

Catalase (CAT) and Peroxidase assay (POD):

Catalase (CAT) activities were determined as[19] whileperoxidase assay (POD) by the protocol of [20] with some modifications. Changes in absorbance of the reaction solution at 420 and 470 nm respectively were determined after 30 seconds. One unit of CAT and POD activity was defined as an absorbance change of 0.01 units/min.

Glutathione Reductase (GSH) and Glutathione-S-transferase (GST) assay:

Glutathione reductase assay was analyzed by the method [21] and Glutathione-S-transferase[22]. For Glutathione reductase (GSH) 2.0 ml assay mixture contained (100µl ml PMS, 1.65 ml PO_4 buffer, 100 µl EDTA and 50µl oxidized glutathione (1mM), 100µl NADPH 0.1mM) and for GST the reaction mixture consist of (1.475 ml PO4 buffer, 0.200µl reduced glutathione, 1 mM, 25 µl ml CDNB (1mM) and 0.3 ml of liver homogenate in a total volume of 2.0 ml). The enzyme activity was noted by measuring disappearance of NADPH at 340 nm and was calculated as nmol oxidized/min mg protein for GSH and formed conjugate CDNB per mints/mg of protein for glutathione S transferase respectively.

Glutathione per-oxidase assay (GSH-Px):

Glutathione per-oxidase activity was analyzed by [23]. The reaction mixture consisted of 1.49 ml sodium phosphate buffer (0.1mmol with pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml NaN_3 (sodium azide,1mM), 0.05 ml glutathione reductase (1 IU/ml), 0.05ml GSH of 1 mM), 0.1 ml NADPH of 0.2mM), 0.01 ml H_2O_2 of 0.25 mM) and 0.1 ml of liver homogenate in a total volume of 2 ml. The disappearance of NADPH at 340 nm was noted after 30 seconds using spectrophotometer.

Serum Biochemistry:

Before secrifising the chicks, blood was collected from veins and centrifuged at 6000 rpm and separate serum, that was stock at -20°C. For further analysis of serum bilirubin, AST aspartate amino trans-ferase, triglyceride (GT),HDL,LDL, ALT and cholestrol.

Results and Discussion:

Phytochemical Analysis

Oleanolic Acid (1): The small hexane: ethyl acetate (7:3) fraction was further subjected to *CC* by eluting with hexane:ethylacetate (4:6) to obtain colourless needles of compound **1**. It gave positive color reaction, which is a characteristic of a triterpene skeleton. The HR-EI-MS of **1** exhibited the peak for molecular ion at *m/z* 456.3610, which was due to $C_{30}H_{48}O_3$ (calcd. for456.3603).The EI-MS provided other significant peaks at *m/z* 248, 203, 133 which were indicative for the presence of Δ 12-amyrin basic structure [24]. The IR spectrum indicated bands for hydroxyl (3400 cm^{-1}), carboxyl (1700 cm^{-1}) and C-C double bond (1660 and 820 cm^{-1}).The ^{1}H-NMR spectrum of **1** exhibited signals for seven methyls at δ 0.87, 0.93, 0.95, 0.97, 1.11, 1.15 and 1.27. A *dd* signal at δ_H 2.8 and a triplet of a vinyl proton at 5.21 were due to H-18 and H-12, respectively, which suggested an olea-12-ene basic structure. The signal at δ 5.21 (1H, t, J = 3.4 Hz) was due to the olefinic proton. The hydrogen geminal to the OH was resonated at δ_H 3.35 (dd, J = 4.1, 9.9 Hz). This one methine proton showed that **1** had at least one hydroxyl group.The ^{13}C-NMR resonance of different carbons were comprehended by DEPT experiment, which shown seven methyl signals, ten methylene signals, five methines and seven signals for methynes. In ^{13}C-NMR spectrum, the signal of carboxyl C-28 was at δ 183.8. The physical and spectral data of**1** was in covenant to that reported data for oleanolic acid [25-26].

Stigmasterol (2): Compound **2** was isolated as colourless crystalline solid from the ethylacetate soluble fraction. The IR spectroscopic analysis, gave band at 3373 cm^{-1} that was the characteristic of O-H stretching, which showed that **2** has the hydroxyl functionalities. Absorption at 2940 cm^{-1} and 2867 cm^{-1} were due to aliphatic C-H stretching. Other absorption frequencies include 1641 cm^{-1} (C=C stretching), 1457 cm^{-1} (cyclic CH_2). The absorption frequency at 1030 cm^{-1} showed cycloalkane. The out of plane C-H vibration of un-saturated part was observed at 881 cm^{-1}. These absorption frequencies resemble the absorption frequencies observed for Stigmasterol [27].The molecular formula ($C_{29}H_{48}O$) was established through HR-MS showing molecular ion peak at *m/z* 412.3920 (calcd. for 412.3926). The loss of H_2O from the molecular ion was indicated by the presence of a fragment ion at *m/z* 394. The subsequent fragment at *m/z* 379 was due to the loss of a methyl group. The characteristic feature of this fragmentation was the presence of a fragment ion peak at *m/z* 271 due to loss of the side chain of stigmasterol, followed by the loss of two hydrogen atoms. The mass spectrum showed characteristic fragmentation pattern of $\Delta^{5.27}$ sterol [24]. The ^{1}H-NMR spectrum of **2** was identical to that of the Stigmasterol [28]. It displayed two tertiary methyls (δ_H 0.84, 0.65), multiplets for three olefinic protons (δ_H 5.33, 5.15, and 3.28).The ^{13}C-NMR showed other signals at 145.2 and 121.7, which were assigned to C-5 and C-6 double bond, respectively, as in other Δ^5spirostene. The ^{13}C-NMR spectrum of **2** indicated twenty nine carbons for six methyls, nine methylenes, eleven methines and three quaternary carbons. The alkene carbon appeared at δ145.2, 139.8, 121.7 and 118.8. The above data was compared with the literature of identified compound **2** and revealed as stigmasterol.

***β*-Sitosterol (3):**The compound **3** was isolated as needles from the ethylacetate soluble fraction. The molecular formula ($C_{29}H_{50}O$) was recognized through HREIMS showing $[M]^+$ at *m/z* 414.3845 (calcd. for $C_{29}H_{50}O$; 414.3861), corresponding to five degrees of unsaturation. The IR spectrum exhibited the bands at 3050, 1650 and 815 cm^{-1}, which indicated trisubstituted double bond along with hydroxyl band at 3446 cm^{-1}.EI-MS spectrum of **3** indicated characteristic fragment ions at *m/z* 399, 396, 381, 329 and 303. The ions at *m/z* 329 and 303 were characteristic for sterols having Δ^5. Other prominent peaks were at *m/z* 273 and 255 for $[M\text{-side chain}]^+$ and $[M\text{-side chain-}H_2O]^+$, respectively.The ^{1}H-NMR spectrum of **3** revealed six methyls, out of which two were tertiary (δ 0.68 and 1.01), three secondary (δ 0.92, 0.83 and 0.81) and one primary (δ 0.84). The signals at δ 3.36 (1 H, m) and 5.11 (1 H, m) were due to carbinylic and olefinic protons, respectively.The ^{13}C-NMR spectrum of **3** indicated resonance for six methyls, eleven methylenes, nine methines and three quaternary carbons. The comparative study of NMR data with the reported data revealed compound **3** to be β-sitosterol.

In Vivo Studies

CCl_4 induced hepatic damages are usually used as a models for the screening of hepatoprotective drugs. It is reported that CCl_4 is converted to reactive CCl_3 through cyto-chrome P450. These free radicals attach to un-saturated fatty acid, producing of lipidperoxide and elevated levels of serum marker enzymes, reduction of GSH, decreased protein synthesis, triglyceride accumulation, increased lipid peroxidation, and thus damage liver cells [29]. The present study revealed that the activity of CAT, POD, GSH-Px and GST was significantly *($P<0.05$)* decreased in CCl_4 group (Table 4& Table 5). Chicks treated with AJME revealed significantly *($P<0.05$)* increased

the activity of antioxidant activities as compared to CCl_4 group. The same outcome is reported in broiler intoxicated with lead [30].GSH metabolite free radicals and reduce triberbitoric acid reactive substances (TBARS) contents. Intoxication of CCl_4 GSH contents and increased TBARS formation in liver as shown in Table 6.Supplementation of AJME reversed the contents and protects cells from lipidperoxidation.200 mg/kg of the plants extracts showed excellent effectiveness as that of silymarin. The result of this present study has closely resemblance with [31-32].Due to CCl_4 toxicity resulted into fatty liver and the damage of liver cells there by the fat metabolism in the liver is affected leading to hyper-cholesterol-emia and increased levels of serum triglycerides, HDL and LDL in birds [33]. ALT, ALP and serum bilirubin level are significantly increased in CCl_4 group shown in Table 7 treatment of CCl_4 significantly elevated ALT and ALP in serum relative to group control group. The groups exposed to CCl_4 along of AJME significantly restore the toxicity induced by CCl_4 and the activity of ALT and ALP reversed closely towards the silymarin group. The levels of serum cholesterol, triglycerides, HDL and in ALT, ALP were found to be significantly increased in CCl_4 intoxicated group as compared to control group. The elevated level is reduced by AJME towards silymarin group. The results found, has resemblance to [34].Treatment of CCl_4 significantly increased the lipid profile, total cholesterol, LDL and triglycerides compared with the control group as shown in Table 8. HDL contents were significantly reduced because of CCl_4 administration comparetively to the control group. The groups treated with AJME efficiently reduced the levels of total cholesterol, LDL and triglycerides whereas increased the levels of HDL in the serum of chicks to the control group.This finding has closely resemblance with the results of [35].

Table 1: ^{1}H and ^{13}CNMR spectral data for Oleanolic acid (**1**)

Position	^{13}C-NMR (δ_C)	^{1}H-NMR (δ_H)	J_{HH} (Hz)
1	CH_2	38.4	-
2	CH_2	27.2	
3	CH	79.0	3.35
4	C	38.7	-
5	CH	55.2	-
6	CH_2	18.3	-
7	CH_2	32.6	-
8	C	39.1	-
9	CH	47.6	-
10	C	37.1	-
11	CH_2	23.4	-
12	CH	122.7	5.21
13	C	143.6	-
14	C	41.6	-
15	CH_2	27.7	-
16	CH_2	23.4	-

17	C	46.5	-
18	CH	41.0	-
19	CH_2	45.9	-
20	C	30.6	-
21	CH_2	33.8	-
22	CH_2	32.4	-
23	CH_3	28.1	1.27
24	CH_3	15.6	1.15
25	CH_3	15.3	1.11
26	CH_3	17.1	0.97
27	CH_3	25.9	0.95
28	C	183.4	-
29	CH_3	33.0	0.93
30	CH_3	23.5	0.87

Table 2: ^{1}H and ^{13}CNMR spectral data of Stigmasterol (**2**)

Position	^{13}C-NMR (δ_C)	^{1}H-NMR (δ_H)	J_{HH} (Hz)	
1	CH_2	37.5	-	-
2	CH_2	31.8		
3	CH	71.9	3.28	m
4	CH_2	42.2	-	-
5	C	140.9	-	-
6	CH	121.7	5.33	m
7	CH_2	31.9	-	-
8	CH	32.2	-	-
9	CH	50.3	-	-
10	C	36.6	-	-
11	CH_2	21.0	-	-

12	CH_2	39.7	5.20	m
13	C	42.5	-	-
14	CH	57.0	-	-
15	CH_2	24.4	-	-
16	CH_2	28.9	-	-
17	CH	56.0	-	-
18	CH_3	12.4	0.65	s
19	CH_3	19.4	0.80	s
20	CH	40.5	-	-
21	CH_3	21.1	0.90	d, J = 6.5
22	CH	138.4	5.15	dd, J = 6.5, 8.4
23	CH	129.4	5.02	dd, J = 15.2, 8.6
24	CH	51.3	-	-
25	CH	32.0	-	-
26	CH_3	19.0	0.83	d, J = 6.6
27	CH_3	21.2	0.81	d, J = 6.5
28	CH_2	25.4	-	-
29	CH_3	12.0	0.84	t, J = 7.0

Table 3: ^{1}H and ^{13}CNMR spectral data of ***β***-Sitosterol (**3**)

Carbon No.	Multiplicity (DEPT)	^{13}C-NMR (δ_C)	^{1}H-NMR (δ_H)	J_{HH} (Hz)
1	CH_2	37.3	-	-
2	CH_2	31.8		
3	CH	71.9	3.36	m
4	CH_2	42.4	-	-
5	C	140.9	-	-
6	CH	121.9	5.11	m
7	CH_2	32.1	-	-

8	CH	32.0	-	-
9	CH	50.8	-	-
10	C	36.6	-	-
11	CH_2	21.1	-	-
12	CH_2	40.3	-	-
13	C	42.6	-	-
14	CH	56.8	-	-
15	CH_2	24.3	-	-
16	CH_2	28.2	-	-
17	CH	56.2	-	-
18	CH_3	11.9	0.68	s
19	CH_3	19.4	1.01	s
20	CH	36.3	-	-
21	CH_3	19.1	0.92	d, *J* = 6.2
22	CH_2	34.0	-	-
23	CH_2	29.3	-	-
24	CH	50.4	-	-
25	CH	26.2	-	-
26	CH_3	18.8	0.83	d, *J* = 6.5
27	CH_3	19.8	0.81	d, *J* = 6.5
28	CH_2	23.1	-	-
29	CH_3	11.9	0.84	t, *J* = 7.0

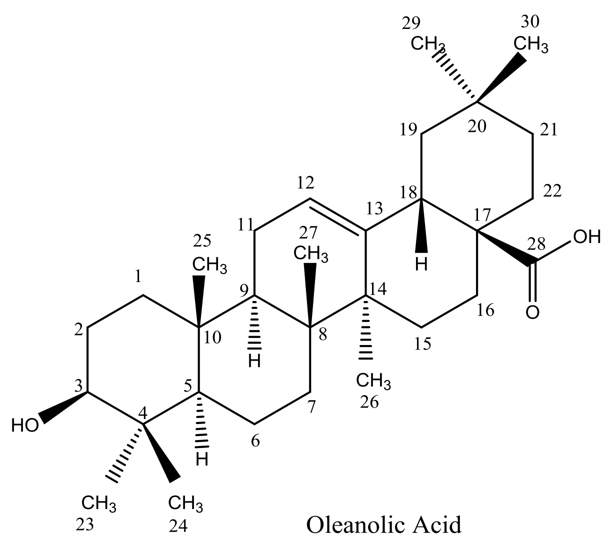

Figure 1: Oleanic acid

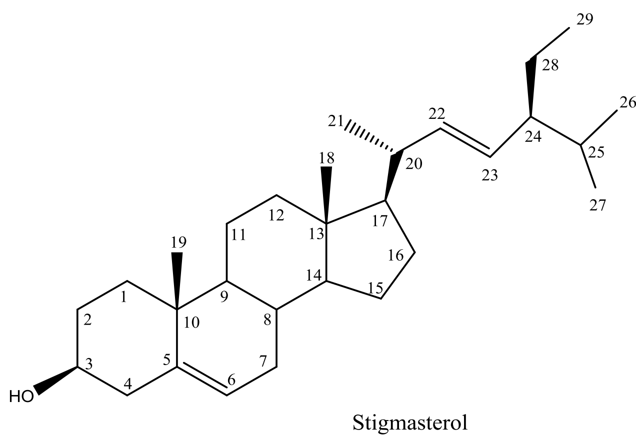

Figure 2: Stimasterol

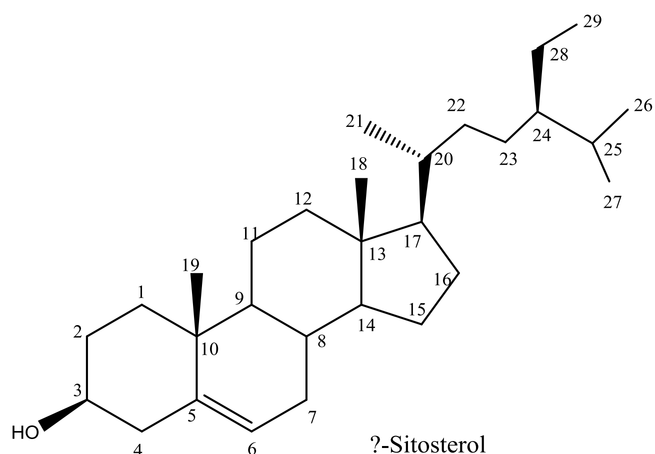

Figure 3: *β*-Sitosterol

Table 4 Effect of AJME on the protein and antioxidant enzymes in liver of chicks

Group treatment	Protein(µg/mg tissue)	CAT(U/min)	POD(U/min)
Group A	183±6.22++	0.82±0.05++	0.80±0.03++
Group B	175±3.5++	0.81±0.06++	0.73±0.09++
Group C	115±6.9**	0.35±0.02**	0.32±0.02**
Group D	170±7.5++	0.51±0.07++	0.56±0.01++
Group E	156±8.0++	0.42±0.1++	0.40±0.06++

Mean ±SE (n=6 number)
** indicate significance from the control group at P<0.05 probability level
++ indicate significance from the CCl_4 group at P<0.05 probability level

Table.2. Effect of AJME on GSH-Px and GST in chick's liver

Group treatment	GST(nmol/min/mgprotein)	GSH -Pox (mol/g tissue)
Group A	52.54±7.03++	307±14.3++
Group B	52.23±11.0++	295±19.6++
Group C	10.94±2.90**	163.6±11**
Group D	47.50±9.21++	292.0±17++
Group E	34.44±5.83++	226.0±9.0++

Mean ±SE (n=6 number)
** indicate significance from the control group at P<0.05 probability level
++ indicate significance from the CCl_4 group at P<0.05 probability level

Table 3Effects of AJME on TBARS and GSH in chick's liver

Group treatment	GSH(mol/g tissue)	TBARS(nmol/ min/ mg protein)
Group A	17.07±4.0++	3.12±1.2++
Group B	16.02±3.2++	4.68±0.9++
Group C	4.21±7.1**	6.00±2.4**
GroupD	14.01±3.8++	3.63±1.0++
Group E	12.61±6.4++	4.16±1.7++

Mean ±SE (n=6 number)
** indicate significance from the control group at P<0.05 probability level
++ indicate significance from the CCl_4 group at P<0.05 probability level

Table 4 Effect of AJME on ALT, ALP and Serum bilirubin

Group treatment	ALT(U/L)	ALP(U/L)	Serum bilirubin(mg/dl)
Group A	93±6.9++	146±17.5++	2.15±1.06++
Group B	92±8.0++	145±21.3++	2.12±1.01++
Group C	185±16**	278±24.7**	8.20±3.19**
Group D	112±21++	140±18.4++	3.65±0.92++
Group E	133±9.8++	177±20.0++	6.60±2.40++

Mean ±SE (n=6 number)
** indicate significance from the control group at P<0.05 probability level
++ indicate significance from the CCl_4 group at P<0.05 probability level

Table 5 Effect of AJME on total cholesterol, HDL, LDL and TG

Group treatment	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	TG (mg/dl)
Group A	76.5±14.0++	30.5±3.9++	35.01±2.8++	80±2.6++
Group B	74.7±17.8++	29.2±5.6++	36.30±4.3++	82±5.8++
Group C	122±31.5**	13.2±4.9**	87.12±9.0**	124±6.3**

Group D	108±10.3++	25.5±7.1++	65.28±7.2++	92±3.4++
Group E	112±19.0++	21.2±1.9++	71.7±3.1++	99±7.0++

Mean ±SE (n=6 number)
** indicate significance from the control group at $P<0.05$ probability level
++ indicate significance from the CCl_4 group at $P<0.05$ probability level

Conclusion:

The results of present work indicate that AJME has a significant protective effect against CCl_4 induced hepatotoxicity in chicks, which may be due to their antioxidant potential oleanolic acid, stigmasterol and *β*-sitosterol.

Competing interest
The authors declare that they have no competing interests.
Authors' contributions
AWK, ASS made significant contribution to acquisition of data, analysis, conception, design of the manuscript. SJ, RAK, MA, AK, BM, and MIC made significant contribution to acquisition of data, analysis, drafting and conception. All the authors read and approved the final manuscript.

References:

1.Kamel C T: Modes of action and roles of plant extracts in non ruminants. Recent advances in Animal Nutrition: P. C. Garnssworthy and J.Wiseman, Nottingham Univ. press, Nottingham, UK, 2001.
2.Kalim MD, Bhattacharyya D, BanerjeeA, Chattopadhyay S: **Oxidative DNA damage preventive activity and antioxidantpotentialofplantsusedinUnani systemofmedicine.***BMCComplem and Altern Med,* 2010, **10**:77.
3. Hazra B, Biswas S, Mandal N: **Antioxidant and free radical scavenging activity of Spondiaspinnoto.***BMC Complement Altern Med* 2008, **8**:63-72.
4. Halliwell B: **The antioxidant paradox**, *The Lancet* 2000, **355**: 1179–1180.
5.GanieSA, Zaragar MA, Masood A, Haq E: ***In vitro* and *in vivo* evaluation of free radical scavenging potential of ethanolic extract of *Podophyllumhexandrum.****AfricJ Biochem Re*2010, **4**:196–203.
6.Simpson CF, and Harms RH:**Aortic atherosclerosis in non laying hens with fatty liver syndrome.***Avian Dis*. 1983, **27:** 652-659.
7. Whitehead CC, Bannister DW, and Cleland ME: **Metabolic changes associated with the occurrence of fatty liver and kidney syndrome in chicks**. *Br J Nutr* 1978, **40:** 221-234.
8. De-Groot H, and Noll, T: **The crucial role of low steady oxygen partial pressure in haloalkanes free radical induced lipid peroxidation**. **Possible implications inhaloalkane liver injury.***BiochemPharmacol* 1986*,* **35:** 15-19.
9.Vanitha A, Murthy KN, Kumar V,Sakthivelu G,Veigas JM,Saibaba P,Ravishankar GA: **Effect of the carotenoid-producing alga, Dunaliellabardawil, on CCl_4-induced toxicity in rats.***Int J Toxicol*2007, **26:** 159-167.
10. Pandit S, Sur T K, Jana Udebnath P K, Sen S,& Bhattacharya D:**Prevention of carbon tetra chloride -induced hepatotoxicity in rats by *Adhatodavasica*leaves**.*Indian J* Pharmcol 2004,**36**:313.
11. Chandel KPS, Shukla, G and Sharma N: **Biodiversity in Medicinal and Aromatic Plants in India**. *National Bureau of Plant Genetic Resources, New Delhi , India*. 1996, Pp 242- 245.
12. Saleh NA, Mansour RM, Markham KR: **An acylatedisorhamnetin glycoside from Aervajavanica**. *Phytochemistry* 1990, **29**:1344-5.
13. Dassanayake MD:, gen. ed. 1981. A revised handbook to the flora of Ceylon. Vol. I. Amerind Pub. Co., New Delhi. pp. 31-32.
14. Parveen; Upadhyay, B.; Roy, S. and Kumar, A. (2007). **Traditional uses of medicinal plants among the rural communities of Churu district in the Thar Desert, India.** J Ethnopharmacol**113:** 387-399.
15. Jain S.C., Jain R. and Singh, R. **Ethnobotanical Survey of Sariska and Siliserh Regions from Alwar District of Rajasthan, India**, *Ethnobotanical Leaflets* 13: 171-88. 2009.

16. Wright JR, Colby HD, Miles PR:**Cytosolic factors that affect microsomallipid peroxidation in lung and liver.***Arch Biochem Biophysics*1981, **206:** 296–304.
17. Iqbal M, Sharma SD, Zadeh HR, Hasan N, Abdulla M, Athar M:**Glutathione metabolizing enzymes and oxidative stress in ferric nitrilotriacetate (Fe-NTA) mediated hepatic injury.***Redox Report*.1996,**2:**385–391.
18. Lowry OH, Rosenberg NJ, Farr AL, Randall RJ:**Protein measurement withFolin Phenol reagent.** *J BiologChem*1951,**193**:265–275.
19. Manna P, Sinha M, Sil PC:**Aqueous extract of *Terminaliaarjuna* preventscarbon tetrachloride induced hepatic and renal disorders.***BMC Compl and Alter Medic*.2006,**6:** 33.
20. Chance B, Maehly AC:**Assay of catalase and peroxidases. Methods in Enzymol.**1955,**11**: 764–775.
21. Carlberg I, Mannervik EB: **Glutathione level in rat brain**. *J BioloChem* 1975, **250**: 4475–4480.
22. Habig WH, Pabst MJ, Jakoby WB:**Glutathione-S-transferases: the firstenzymatic step in mercapturic acid formation.***J BioloChemy*1974, **249:**7130–7139.
23. Mohandas J, Marshal JJ, Duggin GG, Horvath JS, Tiller DJ, (). **Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy.***BiochemPharmacol*1984, **33**: 1801–1807.
24. Budzikiewicz H, Wilson JM, Djerassi C: **Mass spectrometry in structural and stereo chemical problems**. PartXXXII.Penta cyclic triterpenes.*J Am ChemSoc* 1963, **85**: 3688.
25. Seo S, TomitaY, Tori K: **Determination of the Absolute Configuration of Alcohols by Low Temperature ^{1}H NMR of Aryl(methoxy)acetates.***Tetrahedron Lett*., 1995, **1**:7. 26. Ageta H, Ageta T: **Ericaceous constituents: seventeen triterpenoids isolated from the buds of Rhododendron macrocepalum**, *Chem Pharm Bull*,1984, **32**: 369-372.
27. Wyllie, S. G.; Amos, B. A.; and Tokes, L., J. *Org. Chem*., 1977, **42**: 725-731.
28. Rubinstein I, Goad LJ, Clague ADH, Mulheim JL: **The 220 MHz NMR spectra of phytosterols***Phytochemistry,* 1976, **15**:195-200.
29.Hegde K, &Arun BJ:**Hepatoprotective effect of Carissa carandas Linn root extract against CC14and paracetamol induced hepatic oxidative stress**.*Ind J Exp Biol.* 2009, **47:** 660-667.
30. Lakshmi UD, Adilaxmamma K, Gopala Reddy A, and VykuntaRao V: **Evaluation of Herbal Methionine and *MangiferaIndica* against Lead-induced Organ Toxicity in Broilers**. *Toxicol Intl* 2011, **18:** 58–61
31. KarmiaMoawad M: **Possible prophylactic effects of vitamin E or Lycopene treatment on renal toxicity induced by CCl_4 administration in albino rats**. *World J Zool* 2007, **2**: 19-28.
32. Sonkusale P, Bhandarker AG, Kurkare NV, Ravikanth K,. MainiS, and Sood D: ().**Hepatoprotective Activity of Superliv Liquid and Repchol in CCl4 Induced FLKS Syndrome in Broilers.***Int J of PoultSci* 2011, **10**: 49-55.
33. Ajuwon OR, IdowuOM:**Vitamin C attenuates copper-induced oxidative damage in broiler chickens.***Afri J of Biotechnol* 2010, **9**: 7525-7530.
34. Lakshmi UD, Adilaxmamma K, Gopala Reddy A, and VykuntaRao V: **Evaluation of Herbal Methionine and *MangiferaIndica* against Lead-induced Organ Toxicity in Broilers**. *Toxicol Int.* 2011, 18: 58–61
35. Kumar MR, GopalaAR,Anjaneyulu Y, and. Reddy GD: **Oxidative Stress Induced by Lead and Antioxidant Potential of Certain Adaptogens in Poultry.***Toxicol Intl* 2010,**17:** 45-48.