Hepatoprotective and *in-vitro* anti-HIV-1 effect of *Alectryon tomentosus* F. Muell leaf extract and its phytochemical profile

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Objectives

This work was designed to evaluate the therapeutic efficacy of *Alectryon tomentosus* F. Muell leaf extract against CCl_4-induced hepatic injury in albino rats and anti-HIV effect *in vitro*. Proximate analysis, phytochemical screening and assay of total flavonoid (TFC) and phenolic contents (TPC), were carried out according to standard methods.

Materials and methods

The TPC and TFC were estimated spectrophotometrically. The median lethal dose (LD_{50}) of the total ethanol extract of *A. tomentosus* F. Muell leaves was evaluated. The effect on acute HIV-1 infectivity was measured with the syncytium formation assay using AZT (3′-azido-3′-deoxythymidine) as a positive control. Moreover, carbon tetrachloride-induced acute hepatic damage was used to evaluate the hepatoprotective effect of the leaves.

Results and conclusion

Proximate analysis of air-dried leaves of *A. tomentosus* F. Muell revealed 8.3% moisture content, 9.2% total ash, 0.48% water-soluble ash and 2.56% acid-insoluble ash. Preliminary phytochemical screening revealed that the dried powdered leaves of *A. tomentosus* F. Muell are rich in carbohydrates and/or glycosides, volatile constituents, sterols and/or triterpenes and tannins; however, flavonoids (free and combined) and coumarins are present in a lesser concentration. Alkaloids, saponins, cardiac glycosides and anthraquinones are absent. The evaluated TPC was 71.59 mg gallic acid equivalent/g dry weight, whereas TFC was 63.64 mg quercetin equivalent/g plant dry weight. The median lethal dose (LD_{50}) of the total ethanol extract was found to be 9.2 g/kg body weight, indicating the safety of the leaves of the plant. In carbon tetrachloride-induced acute hepatic damage model, the methylene chloride extract showed the highest protection percentage as shown by reduction in the level of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase levels, followed by aqueous extract. These may be attributed to their phenolic and flavonoidal content. The anti-HIV-1 activity was assessed by evaluating syncytium formation. The results showed that the extract was minimally toxic and showed a weak anti-HIV-1 activity in comparison with AZT.

Keywords:
Alectryon tomentosus F. Muell, anti-HIV-1, flavonoids, hepatoprotective, phytochemical profile

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Introduction

Phenolic compounds are plant substances that possess in common an aromatic ring bearing one or more hydroxyl groups. There are about 8000 naturally occurring plant phenolic compounds and about half of which are flavonoids [1]. Previous studies support the use of phenolic compounds in the treatment of hepatitis and other medical benefits, attributed to their anti-inflammatory effect [2–4]. Some phenolic compounds showed a potential anti-HIV-1 activity [5]. Furthermore, phenolic compounds possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic, as well as the ability to modify the gene expression [6]. *Alectryon* is a genus of trees in the family Sapindaceae and is found in the rainforests of Australia, Hawaii, Indonesia, Malaysia, New Zealand, Papua, New Guinea, Philippines and Samoa [7]. There are no previous phytochemical or biological studies reported on *Alectryon tomentosus* F. Muell leaves. Thus, the objective of this study was to determine the phytochemical constituents in general, and the total flavonoid (TFC) and the total phenolic content (TPC) in particular.

Materials and methods

Plant material

Fresh leaves of *A. tomentosus* F. Muell were obtained from Orman garden, Giza, Egypt, in April 2012.

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The plant was identified by Mrs Therese Labib, a plant taxonomist at Orman Garden, Giza, Egypt, and confirmed by the taxonomist, Dr M. El-Gebaly, NRC. A voucher specimen was kept in the Pharmacognosy Department, NRC. The leaves of the plant were air-dried, powdered and kept in tightly closed containers.

Preparation of the aqueous methanolic extract

The air-dried powdered leaves of the plant (200 g) were extracted by maceration with 70% aqueous methanol until exhaustion. The solvent was evaporated to dryness under reduced pressure to yield the crude extract of the plant leaves.

Preparation of the ethanolic extract

Extraction was carried out with 95% ethanol in a continuous extraction apparatus (Soxhlet) and evaporation of the solvent under reduced pressure.

Preparation of successive extracts

The air-dried powdered leaves of the plant (100 g) were successively extracted with petroleum ether, methylene chloride, ethyl acetate, butanol and water. Each extract was evaporated to dryness under reduced pressure. The solvent-free residue in each case was weighed.

Proximate analysis

The percentages of moisture content, total ash, water-soluble ash and acid-insoluble ash values were determined according to the Egyptian Pharmacopeia [8].

Preliminary phytochemical screening

All extracts were qualitatively screened for the presence of various groups of phytoconstituents using different chemical tests [9,10].

Total phenolic assay

The TPC was determined applying the Folin–Ciocalteu colourimetric method using gallic acid as a standard and was expressed as milligrams of gallic acid equivalents/g of the dry plant material [11].

Total flavonoid assay

The TFC was measured using aluminium chloride colourimetric assay. Calibration curve was established using quercetin as a standard. TFC was expressed as mg quercetin equivalent/g of the dry plant material [12].

Bioactivity studies

Experimental animals

Adult albino rats of Sprague–Dawley strain weighing 130–150 g and albino mice weighing 25–30 g were obtained from the animal house colony of the National Research Centre, Dokki, Egypt. They were kept under the same hygienic conditions and well-balanced diet and water. Medical research ethical committee (MREC) in NRC has approved the work.

Normal diet

It consisted of vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), 95% pure casein (10.5%) and starch (54.3%).

Drug dosage

Doses of the standard drugs were calculated [13] and administered orally by means of a gastric tube.

Standard drugs, chemicals and biochemical kits

Carbon tetrachloride (Analar, Sigma, St. Louis, USA) was used as a hepatotoxic agent. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen and blood glutathione kits were used (QCA, Tarragona, Spain). AZT (3´-azido-3´-deoxythymidine), used as a standard anti-HIV drug, was purchased from Sigma. AZT was dissolved in RPMI-1640 and stored at –20°C.

HEPES [*N*-2 (2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid)], MTT [3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], DMF (*N*, *N*'-dimethyl formamide), penicillin, streptomycin sulphate and glutamine were purchased from Sigma (Sigma, St. Louis, USA). 2-ME (2-mercaptoethanol) was purchased from Bio-Rad (Bio-Rad., California, USA). RPMI-1640 and fetal bovine serum were purchased from Gibco (for anti-HIV test).

Cell lines

HIV cell line: C8166 cells (a fusion of primary umbilical cord blood cells with an HTLV-1-producing line from an adult T-cell leukaemia lymphoma patient containing a defective HTLV-1 genome) and HIV-1IIIB were kindly donated by Medical Research Council, AIDS Regent Project. The cells were maintained at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivating fetal bovine serum (Gibco). The HIV-1IIIB was prepared from the supernatants of H9/HIV-1IIIB cells. The 50% HIV-1 tissue culture infectious dose (TCID50) in C8166 cells was determined and calculated [14]. Virus stocks were stored in small aliquots at –70°C.

Determination of median lethal dose (LD_{50})

The median lethal dose (LD_{50}) of total ethanol extract of *A. tomentosus* F. Muell was determined [15]. Male albino rats were divided into groups, each of six animals. Preliminary experiments were undertaken to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen between these two doses, and each dose was injected into a group of six animals subcutaneously. The mice were observed for 24 h; symptoms of toxicity and mortality rates in each group were recorded and LD_{50} was calculated.

Evaluation of anti-HIV activity

Cytotoxicity assay

All experimental procedures were performed at the BSL-3 laboratory according to the safety guidelines of Kunming Institute of Zoology, Chinese Academy of Sciences. The cellular toxicity of the extracts on C8166 cells was assessed with the MTT colourimetric assay. A measure of 100 µl of 4×10^5 cells was plated into 96-well plates. This was followed by the addition of100 µl of various concentrations of compounds and incubation at 37˚C in a humidified atmosphere of 5% CO_2 for 72 h; 100 µl of supernatant was discarded. MTT reagent was added and incubated for 4 h; and 100 µl 50% DMF-20% SDS was added. The formazan formed was dissolved completely; the plates were read by means of a Bio-Tek EL ´800 ELISA reader at 570 nm/630 nm. The concentration that produces 50% cytotoxicity (CC_{50}) was calculated [16].

Inhibition of syncytium formation

Syncytium is a viral protein fusion at the host cell membrane during viral replication or induction of virus by host-cell–cell fusion resulting in a multinucleated giant cell. The effect of extract on acute HIV-1 infectivity was measured using the syncytium formation assay [17]. In the presence or absence of various concentrations of samples, 4×10^4 C8166 cells were infected with HIV-1 at a multiplicity of infection of 0.015 and cultured in 96-well plates at 37˚C in 5% CO_2 for 3 days. The AZT was used as a positive control. At 3 days after infection, the cytopathic effect was measured by counting the number of syncytia in each well of 96-well plates under an inverted microscope (×100). The inhibitory percentage of syncytium formation was calculated by the percentage of syncytium number in a sample-treated culture compared with that in infected control culture. The 50% effective concentration (EC_{50}) was calculated according to the method described by Reed and Muench [14]. Fifty percent cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) were determined from dose–response curve. The therapeutic index of anti-HIV activity is CC_{50}/EC_{50}.

$$\text{Cell viability (\% of control)} = \frac{\left(OD_{test} - OD_{blk}\right)}{\left(OD_{ctrl} - OD_{blk}\right)} \times 100,$$

$$\text{CPE inhibition } (\%) = \left(\frac{1 - CPE_{test}}{CPE_{ctrl}}\right) \times 100,$$

where OD is optical density.

Evaluation of hepatoprotective activity

Induction of liver damage

Liver damage in rats was induced by means of intraperitoneal injection of 5 ml/kg of 25% carbon tetrachloride in liquid paraffin [18].

Experimental design

Forty-two male albino rats were randomly divided into seven groups of six rats each:

The first group, the control group, received a daily oral dose of 1 ml saline for 1 week before and after liver damage (negative control).

The second, third, fourth, fifth and sixth groups included liver-damaged rats pretreated with a daily oral dose of 100 mg/kg body weight of petroleum ether, methylene chloride, ethyl acetate and butanol extracts, respectively, for a week.

The seventh group included liver-damaged rats pretreated with a daily oral dose of 25 mg/kg body weight silymarin as a standard. Administration of the extracts and drug was continued after liver-damage for a week.

After an overnight fast, whole blood sample was withdrawn from the retro-orbital venous plexus through the eye canthus of anaesthetized rats. Blood samples were collected at zero time point, 1 week after extract adminstration, 72 h and 1 week after CCl_4 injection. Serum was isolated by centrifugation. Serum AST, ALT [19] and ALP [20] were measured.

Results and discussion

Proximate analysis

The estimated moisture content, total ash, water-soluble ash and acid-insoluble ash of the leaves were 8.31, 9.29, 0.48 and 2.56, respectively. These constants could be used as criteria for the identity and purity of *A. tomentosus* F. Muell leaves.

Phytochemical screening

Preliminary phytochemical screening revealed that the dried powdered leaves of *A. tomentosus* F. Muell are rich in carbohydrates and/or glycosides, volatile constituents, sterols and/or triterpenes and tannins, whereas flavonoids (free and combined) and coumarins are present in a lesser concentration. Alkaloids, saponins, cardenolides and anthraquinones are absent. Successive extracts were shown (Table 1) as percentage of yield, physical characters and phytoconstituents. The extracts prepared with polar solvents were rich in carbohydrates, flavonoidal contents and tannins. Sterols and/or triterpenes were present in the extracts prepared with nonpolar solvents.

Total phenolic and flavonoid contents

The TPC in *A. tomentosus* F. Muell leaves was found to be 71.59 mg gallic acid equivalent/g plant dry weight, whereas TFC in *A. tomentosus* F. Muell leaves was found to be 63.64 mg quercetin equivalent/g plant dry weight.

Median lethal dose (LD_{50})

LD_{50} of the total ethanol extract was found to be 9.2 g/kg body weight, indicating the safety of the plant.

Hepatoprotective activity

Tables 2–4 revealed that pretreatment of rats with the successive extracts of the leaves for a week

Table 1 Percentages and physical and chemical characters of the successive extracts of *Alectryon tomentosus* F. Muell leaves

Character	Petroleum ether extract	Methylene chloride extract	Ethyl acetate extract	Butanol extract	Water extract
Weight (g/1000 g powder)	5	20	8.8	15.46	6.6
Percentage dry w/w	0.5	2	0.88	1.54	0.66
Colour	Greenish brown	Greenish brown	Brown	Dark brown	Brown
Condition	Waxy	Waxy	Semisolid	Semisolid	Solid
Carbohydrates and/or glycosides	–	–	+	+++	+++
Sterols and/or triterpenes	++	++	–	–	–
Tannins	–	–	+	+	+
Flavonoids	–	+	++	++	+
Anthraquinones	–	–	–	–	–
Coumarins	–	–	–	–	–
Saponins	–	–	–	–	–

++, moderately positive response; +, weakly positive response; –, not detected.

Table 2 Effect of successive extracts of *Alectryon tomentosus* F. Muell leaves and silymarin drug on serum aspartate aminotransferase (m/l) enzyme level on liver-damaged rats

Group	Time			
	AST (μ/l)			
	0	7 days	72 h	7 days
Control 1 ml saline	47.2 ± 2.4	46.4 ± 1.2	152 ± 4.9*	163.4 ± 6.2•*
Petroleum ether extract (100 mg/kg)	43.9 ± 1.4	42.4 ± 1.6	126.9 ± 3.2*	105.9 ± 2.3•*
Methylene chloride extract (100 mg/kg)	48.2 ± 2.1	43.7 ± 1.6	72.4 ± 2.1*	56.3 ± 2.1•*
Ethyl acetate extract (100 mg/kg)	44.7 ± 2.3	41.9 ± 1.8	87.8 ± 2.6*	61.2 ± 2.6•*
Butanol extract (100 mg/kg)	46.3 ± 1.9	45.8 ± 1.4	93.1 ± 4.1*	79.4 ± 2.4•*
Aqueous extract (100 mg/kg)	41.8 ± 1.7	39.2 ± 1.3	82.2 ± 2.5*	69.3 ± 2.2•*
Silymarin (25 mg)	42.6 ± 1.4	39.7 ± 1.3	51.3 ± 2.1*	35.7 ± 1.2•*

AST, aspartate aminotransferase; *Significantly different from zero time at $P < 0.01$; •Significantly different from 72 h after CCl_4 at $P < 0.01$.

Table 3 Effect of successive extracts of *Alectryon tomentosus* F. Muell leaves and silymarin drug on serum alanine aminotransferase (m/l) enzymes level on liver-damaged rat

Group	Time			
	ALT (m/l)			
	0	7 days	72 h	7 days
Control 1 ml saline	36.7 ± 2.8	37.2 ± 1.3	144.7 ± 5.3*	149.6 ± 5.7•*
Petroleum ether extract (100 mg/kg)	39.3 ± 1.6	39.41 ± 0.1	124.3 ± 4.9*	97.3 ± 3.8•*
Methylene chloride extract (100 mg/kg)	33.9 ± 1.1	31.2 ± 1.1	79.3 ± 2.1*	48.6 ± 2.1•*
Ethyl acetate extract (100 mg/kg)	382 ± 1.4	36.4 ± 1.2	81.2 ± 2.3*	51.3 ± 1.7•*
Butanol extract (100 mg/kg)	34.1 ± 1.2	35.2 ± 0.9	102.6 ± 4.8*	75.9 ± 3.6•*
Aqueous extract (100 mg/kg)	38.4 ± 1.5	37.6 ± 1.3	79.6 ± 2.7*	62.3 ± 2.1•*
Silymarin (25 mg)	36.2 ± 1.4	34.7 ± 1.3	56.8 ± 1.7*	33.9 ± 0.8•

ALT, alanine aminotransferase; •Statistically significantly different from 72 h after CCl_4 at $P < 0.01$; *Statistically significantly different from zero time at $P < 0.01$.

before liver damage did not result in a significant change in AST, ALT and ALP as compared with silymarin, which is considered to be safe on the liver. After a week, liver damage was induced by means of intraperitoneal injection of CCl_4. The methylene chloride leaf extract showed the least damage effect as shown by AST levels after 72 h of liver damage induction and thus has the highest level of protection followed by aqueous leaf extract. The methylene chloride extract showed the best treatment results as well, followed by ethyl acetate, after 7 days of treatment.

The methylene chloride extract showed the least damage percentage as shown by ALT percentage, followed by aqueous extract, after 72 h. The methylene chloride extract showed the best treatment results as well, followed by ethyl acetate, after 7 days. The methylene chloride extract showed the least liver damage as shown by ALP percentage, followed by aqueous extract, after 72 h and 7 days.

These may be attributed to their terpenoidal, flavonoidal and phenolic content.

Table 4 Effect of successive extracts of *Alectryon tomentosus* F. Muell leaves and silymarin drug on serum alkaline phosphatase enzymes level on liver-damaged rats

Group	Time			
	ALP (KAU)			
	0	7 days	72 h	7 days
Control 1 ml saline	7.3 ± 0.1	7.2 ± 0.1	48.6 ± 1.4*	52.1 ± 1.9*•
Petroleum ether extract (100 mg/kg)	7.1 ± 0.1	7.2 ± 0.1	39.2 ± 1.6*	33.6 ± 1.2*•
Methylene chloride extract (100 mg/kg)	7.5 ± 0.1	7.4 ± 0.1	22.1 ± 0.8*	14.3 ± 0.4*•
Ethyl acetate extract (100 mg/kg)	7.1 ± 0.1	7.2 ± 0.1	29.4 ± 0.7*	21.0 ± 0.9*•
Butanol extract (100 mg/kg)	7.2 ± 0.1	7.3 ± 0.1	36.9 ± 1.4*	25.4 ± 1.4*•
Aqueous extract (100 mg/kg)	7.6 ± 0.1	7.4 ± 0.1	24.9 ± 1.3*	17.3 ± 0.8*•
Silymarin 25 mg	7.4 ± 0.1	7.2 ± 0.1	18.3 ± 0.8*	7.6 ± 0.1•

ALP, alkaline phosphatase; •Statistically significant different from 72 h after CCl_4 at $P < 0.01$; *Statistically significant different from zero time at $P < 0.01$.

Table 5 Cytotoxicity of the extracts in C8166 cell

Extract	Concentration (μg/ml)	Cell viability ± SD (%)	CC_{50} (μg/ml)
Methanol 80%	1000	11.04 ± 1.38	375.809
	200	75.11 ± 5.58	
	40	110.75 ± 4.51	
	8	111.20 ± 6.07	
	1.6	99.96 ± 0.69	
	0.32	96.70 ± 2.38	
Drug			
AZT	4000	38.28 ± 0.86	1354.782
	800	86.71 ± 11.06	
	160	87.39 ± 1.77	
	32	88.60 ± 3.24	
	6.4	78.81 ± 2.57	
	1.28	80.42 ± 13.95	

Table 6 Anti-HIV activity of the extract in C8166 cell

Extract	Concentration (μg/ml)	Inhibition ± SD (%)	EC_{50} (μg/ml)
Methanol 80%	1000	100.00 ± 0.00	50.785
	200	100.00 ± 0.00	
	40	41.29 ± 3.04	
Drug			
AZT	4000	98.13 ± 0.87	5.439
	800	93.58 ± 2.13	
	160	56.74 ± 3.56	
	32	28.62 ± 4.34	

In-vitro evaluation of anti-HIV-1 activity of the total methanol extract of *Alectryon tomentosus* F. Muell leaves

The effect of extracts on acute HIV-1 infectivity was measured with the syncytia formation assay. AZT was used as a positive control. The cytopathic effect was measured by counting the number of syncytia (multinucleated giant cells) in each well of 96-well plates under microscope. The results (Tables 5 and 6) showed that the extract was minimally toxic and showed a weak anti-HIV-1 activity in comparison with AZT.

Conclusion

Phytochemical study of the leaves of *A. tomentosus* F. Muell revealed the presence of a variety of constituents in the plant, which contribute to the demonstrated bioactivities of the extracts prepared. Meanwhile, the plant has high LD_{50}, indicating its low toxicity. This study deduced the potential hepatoprotective effect of *A. tomentosus* F. Muell leaves.

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Conflicts of interest

There are no conflicts of interest.

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