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HPLC-ESI-MS Characterization Of Certain Compounds Of Methanolic Extract Of Nerium Oleander And Its Fractions As Well As Evaluation Of Their Potential Against *Schistosomiasis mansoni*

Eman abdallah Morsi[1], El-Sayed Saleh Abdel-Hameed[1], Mortada Mohamed El-Sayed[1], Ibrahim Aly Rabia[2]

1 Medicinal Chemistry Laboratory, Theodor Bilharz Research Institute, Giza, Egypt.

2 Parasitology Laboratory, Theodor Bilharz Research Institute, Giza, Egypt.

Abstract

Due to the expansion of praziquantel (PZQ) schistosome resistant strains, the detection of novel antischistosomal agents is of high seniority. Therefore, in this study, the *in vitro* and *in vivo* effects of the defatted methanolic extract of Nerium *oleander* leaves as well as ethyl acetate and butanol fractions against *Schistosoma mansoni were* evaluated. *In vitro* effect of extracts of *N.oleander* leaves have been carried against *S. mansoni* adult worms. The results appeared that the extracts have activity against adult worms and the mortality was highest with ethyl acetate fraction. On other hand, the vivo antischistosomal activities of *N.oleander* leaves extracts on infected mice with *S. mansoni* were carried out compared with praziquantel. The results showed a high reduction in immature and mature eggs. Also, the number of dead eggs increased by treating infected mice with the three extracts. Besides, the antioxidant activity of infected mice was improved by treating with *N.oleander* leaves extracts. The defatted methanolic extract of *N.oleander* leaf as well as two fractions ethyl acetate and butanol, were submitted to HPLC-ESI-MS analysis. The results produce to the identification of mixture phenolic acid derivatives and flavonoid glycosides in each plant extract which exhibit a respectable effect against *S. mansoni* infections.

Keywords: Flavonoid; *Nerium oleander*; phenolic acid*;* Praziquantel*; Schistosoma mansoni.*

1. Introduction

Schistosomiasis (bilharzias), an illness happened because trematode flatworms of the genus *Schistosoma*, is prevalent tropical diseases in the World[1]**.** It is the second extreme serious parasitic disease worldwide after malaria [2]. Infecting of tissues of the vertebrate host with this parasite raises oxidative stress due to a cumulative reduction in the levels of endogenous antioxidants and excess the generation of free radicals [3, 4]**.** Moreover, the parasite suppresses the steward enzymatic detoxification activities which play a function in the pathogenesis of S. mansoni [3]. The acute phase of *S. mansoni* infection is recognized by the formulation of inflammatory granulomatous around deposited parasite eggs [4, 5]**.**

The therapy and dominance of this disease rely on one drug, praziquantel. (PZQ) is the most active and applicable drug for therapy and dominance of schistosomiasis [6, 7]. But the presence of PZQ-resistant schistosome strains and activity only at the adult phase of the worm [6]. Because of the release of reactive free radicals when infected with disease, therefore any compounds or plant extracts with antioxidant properties can be used as an antischistosomal drug. Plants are regarded as a rich source of antioxidant compounds so many plants are submitted for studies to discover of a new drug for this disease [8, 9]**.**

*Nerium oleand*er (family Apocynaceae) is an evergreen shrub that grows in Mediterranean tropical and subtropical regions. It is commonly known as oleander [10]. Many secondary metabolites such as alkaloids, flavonoids and steroids have been recorded in this plant species. So, several studies reported that this plant species showed many pharmacological properties such as antibacterial, anthelmintic, anti-inflammatory, hepatoprotective, immunopotential, antipyretic, antioxidant, antifungal, anticancer and anti-HIV activity [10, 11]**.**

*Corresponding author e-mail: e.morsi@tbri.gov.eg. (Eman A. Morsi)
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The present work attempted to examine the potential of antischistosomal activity of the defatted methanolic extract, butanolic fraction and ethyl acetate fraction of *Nerium oleander* leaves versus experimentally infected mice. Besides, the identification of the major abundant chemical constituents of the defatted methanolic extract, butanolic fraction and ethyl acetate fraction by using HPLC-ESI-MS technique.

Materials and Methods

Plant material

Nerium oleander leaves were collected from El-Orman garden, Giza, Egypt. The voucher plant sample was characterized by Mrs. Treaze Labib, a high specialist of Plant Taxonomy, Department of Flora and Taxonomy, El-Orman Garden, Giza, Egypt. The voucher sample has been kept in the medicinal chemistry laboratory, Theodor Bilharz Research Institute. The plant severs to little pieces, dehydrated in the shade, finely ground with an electric mill, and the dry powder was protected at room temperature (27 °C) for the extraction process.

Extraction and fractionation process

Powdered *Nerium oleander* leaves (800 g) were extracted 3 times with 85 % methanol (MeOH) at 25^0C. The methanolic extract was filtered out of a filter paper (Whatman No. 1) and intensified to desiccation under reduced pressure using a rotatory evaporator (BUCHI, Switzerland). The methanolic extract was defatted with petroleum ether. The watery defatted methanolic was split with ethyl acetate (EtOAc) and butanol (BuOH), respectively. The defatted methanolic extract, ethyl acetate, and butanoic fractions were desiccation to dryness and kept at the room temperature in closed vials for biological and chemical studies.

Preliminary phytochemical screening:

The preliminary phytochemical screenings of the defatted methanolic extract and butanoic and ethyl acetate fractions of *Nerium oleander* leaves were carried out to determine qualitative contents of carbohydrates, alkaloids, saponins, flavonoids, tannins, sterols, terpenes and phenolic compounds. The experiments were performed according to methods that reported by [12, 13].

Animals

Healthful male Swiss albino mice (CD-1 strain) with an average weight (24 ± 2 gm) were supplied by the Schistosoma Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (TBRI), Giza, Egypt.

The mice were begotten under environmentally controlled conditions, preserved under the standard laboratory care (21oC, 45-55% humidity), purified drinking water, 24 % protein, 4 % fat and 4.5 % fiber diet. The processing and treatment of the experimental animals were procedure according to the international ethical conditions and valid guidelines adopted by Theodor Bilharz Research Institute.

Acute toxicity assessment

Oral acute toxicity assessment of a defatted methanol extract of *Nerium oleander* leaves as well as butanoic and ethyl acetate fractions derived from the methanol extract of the plant was done as per OECD test guideline 425 [14, 15]. 36 normal mice weighing (24 ± 2gm) were randomly split into 6 groups each group contains 6 mice. All treatment groups were acclimatized to the laboratory environment for one week before this study. The first group (control group) contains tween 80; the other groups received oral different concentrations of each extract from 1000 mg/kg to 5000 mg /kg. An individual mouse was observed hair erection, walking status, diarrhea, death and body weight measurement after 30 minutes from dosing, and one hour as well as every 6 hours during the first 24 hours until 14 days.

Drug preparation

Praziquantel tablets (600 mg) (Distoside) were sold from Chandra Bhagat Pharma Pvt Ltd, India. The tablets were ground into a white powder and pendent in 13 mL of 2 % cremophore-El and other extracts were dissolved in 1 % DMSO. Praziquantel (PZQ) and the extracts were freshly prepared and orally managed to mice using a stainless steel oral cannula.

Schistosoma mansoni cercariae

S. mansoni cercariae were gained from Schistosome biological supply center, TBRI, and infection was performed directly after shedding from *Biomophlaria Alexandrina* snails.

Mice infection

Male CD1 albino mice were infected with (60±10) *S. mansoni cercaria*e via the subcutaneous route suspended in 0.1 mL solution. *Cercariae* were pendent in 0.1 ml distilled water. The ethical obligations to experimental animals were followed. The worms were recovered after 8 weeks to be used in vitro study. The experiments were executed at Parasitology Department, Theodor Bilharz Research Institute

Adult worms were combined by intestinal and hepatic perfusion at 8 weeks after infection according to the method described by Duvall and De Witt [16].

***In vitro* bioassay screening:**

This test was assayed according to the method described by Jiwahinda *et al.* **(2002).** *S. mansoni* worms were rinsed many times in sterile RPMI-1640 media (pH 7.5, with HEPES 20 mM and supplemented with penicillin (100 U/ml), sterptomycin (100 mg/ml) and 10% fetal calf serum (Gibco). In 35 mm diameter (35 X 10 mm) polystyrene petri dish, ten adult *S. mansoni* worms were cultured in 10 ml sterile RPMI-1640 media with concentrations of different plant extracts as (10µg/ml, 50µg/ml, 500µg/ml and 5mg/ml) at 37 °C for 24 hours. In parallel, the adult worms were cultured in RPMI-1640 media containing 400 µg/ml of 10% DMSO (served as a negative control) and 10µg/ml of PZQ (served as a positive control). The efficiency of various concentrations of plant extracts, viability and motility of worms was spotted using a stereomicroscope at different time intervals starting from 1 hour, 3 hours and 24 hours of incubation **[16].**

***In Vivo*-assessment**

60 male mice 8 weeks were allocated into 6 groups with 10 animals of each as follows in table 1: 3 doses of extracts per week were given starting from the 4th week; the animals were sacrificed after the 8 week post infection to gain adult worms. After the end of 8 weeks of the experiment, all mice of all experimental groups were sacrificed to gain adult worms. After aspirate, small pieces of liver and intestine were used to locate the number of ova per gram intestinal or liver tissues after the digestion overnight in 5 % KOH. Perfused liver samples were stored at 8 °C to appreciate the different parameters of oxidative stress.

Table(1): **Experimental design**.

Groups	Treatment	Description
Normal control	Normal control	Non-infected, non-treated group
Negative control	Infected control	Infected non-treated group
positive control	PZQ	Infected and treated at 6 weeks post-infection (p.i.) with 500 mg/kg PZQ[19]
Group A	MeOH extract	Infected and treated 3 times weekly with defatted methanolic extract of N. oleander (400 mg/kg). Start from 21th day p.i. to the end of experiment[20]
Group B	EtOAc fraction	Infected and treated 3 times weekly with EtOAc fraction derived from the methanolic extract of N. oleander (400 mg/kg). Start from 21th day p.i. to the end of experiment[20]
Group C	BuOH fraction	Infected and treated 3 times weekly BuOH fraction derived from the methanolic extract of N .oleander (400 mg/kg). Start from 21th day p.i. to the end of experiment[20]
Mice were infected by the subcutaneous (s.c.) injection of 100 S. mansoni cercariae/mouse. For all treatments, each concentration was administrated at a different doses (50 µl extract + 150 µl distilled water)		

Study of parasitological criteria

Worm burden:

Worms (males, females and coubles) were collected from infected mice by perfusion according to the technique of Duvall and De Witt **(1967)**. The collected worms were visualized under a stereo-microscope. Percentage reduction in worm burden (due to treatment) was studied by the following formula:

Reduction% = Mean worms (control group) - Mean worms (test group) / Mean worms (control group)* 100

(MDA) and Reduced glutathione (GSH) parameters were measured using Biodiagnostic assay kits (Biodiagnostic).

Lipid peroxidation

Lipid peroxidation was examined by measurement thiobarbituric acid-reactive substances (TBARS) and expressed in terms of malondialdehyde (MDA) content according to the method Draper and Hadley **[18].** The MDA values were studied using standard (1, 1, 3, 3-tetraethoxypropane) and expressed as nmol of MDA/g liver **[19].**

Glutathione (GSH)

200 µL of supernatant was added to 1.1 mL sodium phosphate buffer (0.25 M, pH 7.4) followed by the addition 130 µL of 0.04 % DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Sigma-Aldrich, Germany). Then, the mixture was diluted to 1.5 mL with distilled water and the absorbance was measured using a spectrophotometer at 412 nm.The concentration of glutathione was expressed as µg GSH/µg protein **[20].**

HPLC-ESI-MS Analysis

The defatted methanolic extract, ethyl acetate and butanoic fractions of *Nerium oleander* leaves (5 mg/mL) solution were ready using HPLC analytical grade solvent mixture of CH_3CN / MeOH / H_2O (1: 1: 2; v/v/v), filtered using a membrane disc filter (0.45 µm) then analyzed by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) according to the method described by Abdel-Lateef et al **[21].** Analysis was performed using HPLC system (Waters Alliance 2695, Waters, USA) and mass analyzer (Waters 3100). 20 μL of the sample was injected into the HPLC instrument equipped with reverse phase C-18 column (Phenomenex 250 mm, 5 μm particle sizes). Sample mobile phase was prepared by filtering using 0.45 μm filter membrane disc and degassed by sonication before injection. Mobile phase elution was made with the flow rate of 0.4 mL/min using gradient mobile phase comprising two solvents: Solvent A contains water acidified with 0.1 % HCOOH (FA) and solvent B is CH_3CN/ MeOH (1: 1; v/v) acidified

with 0.1% FA. Elution was performed using the following gradient: 5 % B; 0-5 min, 5-10 % B; 5-10 min, 10-50 % B; 10-55 min, 50-95 % B; 55-65 min, and 5 % B; 65-70 min. The parameters for analysis were carried out using negative ion mode as follows; source temperature 150 °C, cone voltage 50 eV, capillary voltage 3 kV, desolvation temperature 350 °C, cone gas flow 50 L/h, and desolvation gas flow 600 L/h. Mass spectra were detected in the ESI negative ion mode between *m/z* 50-1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively fixed by comparing its retention time (RT) and mass spectrum with the mention data.

Statistical analysis

The statistical analysis was performed using SPSS (16) software. The results were given as means ± standard deviation (SD) for all experimental. The significant variances were assessed using one-way analysis of variance (ANOVA) using SPSS software package. P values less than 0.05 were considered significant.

Results and discussions

Preliminary phytochemical screening

Results of the phytochemical constituents of the defatted methanolic extracts, ethyl acetate and butanoic fractions of Nerium oleander leaves reported in Table 2. The phytochemical screening showed the presence of terpenoids, alkaloids, flavonoid, saponins, tannin and carbohydrates by different amounts. These results are in agreement with previous studies on the plant [22, 23]

Table (2): Results of Phytochemical analysis of plant extracts

Chemical Category	*Nerium oleander*		
	MeOH	EtOAc	BuOH
Carbohydrates	++	-	+
Alkaloids	+	-	+
Flavonoid	++	++	+
Saponin	++	+	++
Phenolic	++	++	+
Tannins	+	+	+
sterols and or/ triterpenes	++	+	++

(++): high amount, (+): moderate amount, (-): Absent.

The spread of schistosomiasis in endemic areas and the increasing infection rate need for new drug discovery. Research on medicinal plants has provided new drugs for the treatment of parasite diseases [24, 25]. So in this study, the effect of *N. oleander* against *S. mansoni* has been evaluated.

Acute toxicity assessment

Before the *in vitro* and *in vivo* studies, it is very important to take knowledge about the range of the safety of the extracts used in the present study. The results of the acute toxicity of the plant extracts (defatted methanol of *Nerium oleander* leaves as well as two fractions derived the methanol extract) appeared that no effect of these extracts in mice by the absence of death of mice and no diarrhea was observed across the study groups during the study period. These results indicated that LD_{50} is greater than 5000mL /kg. So the used extracts are safe up to a dose of 5000 mg/kg. These results are agreement the results of Abdou *et al* [26].

***In- vitro* study**

The current study showed that culturing of adult *S. mansoni* worms in different concentrations (10μg/ml, 50μg/ml, 500μg/ml and 5mg/ml) of each (defatted methanol of *N. oleander* leaves as well as the two fractions e derived from the methanol extract) for one hour led to the instant shrinking of worms, continued muscle contraction and death ranged from 75% to 100% of worms. After exposure time 3 and 24 hours of incubation, the percentage of worm death increased to 100%. Also, the results appeared that the ethyl acetate fraction has a high rate of mortality than other extracts. These results indicated that *N. oleander* leaves extracts possess *in-vitro* schistosomicidal activity against *S. mansoni* adult worms. A similar finding has been reported by several authors who estimated the *In-vitro* effects of the many plant extract. [27]. Although in vitro assays have not been considered appropriate to cover all the aspects of the anthelmintic activities of drugs, mostly for pharmacological and immunological host interactions but, it provides the first guide of anthelmintic effects and insight into the style of action. Thus, they may lead to the expansion of new therapeutic approximations like drug targeting [28].

***In-vivo* study**

Many studies showed that the relation between *Schistosoma* and its host is extremely complex. The infected animals with *S. mansoni* led to liver injury connected with the product of excessive reactive oxygen species (ROS) which induce hepatic stress [25]. Also, it has been reported that after an infection (8 weeks) of the experimental mice with *S. mansoni* causes a rather quiet infection in the host until the parasite has accomplished ova position. So some eggs lay in mesenteric vessels by the blood flow and turn into trapped inside the liver followed by deposition of collagen around these eggs. So these eggs raise a granulomatous reaction and demonstrate the positive correlation between liver tissue, egg counts and septal

as well as total fibrosis degrees [25, 17]. In the present research *in-vivo* Antischistosomial effects of the defatted MeOH extract of *N. oleander* leaves beside two fractions ethyl acetate and butanoic were evaluated. And the results have been listed in tables 3 and 4

Percentage egg developmental stages (Oogram pattern)

Table 3 has exhibited the administration of each extract (MeOH extract, ethyl acetate fraction and butanol fraction) on Oogram pattern led to a depression in immature, mature eggs and an excess in dead eggs as compared with negative control. (Group B) ethyl acetate fraction a highly significant relief in the percentage of immature, mature eggs (32±2.2 % and 26±0.6%) beside an increase in the percentage of dead eggs (42±0.7 %) was recorded. This study is full agreement with the previous study, which showed a high reduction the immature and mature eggs in the infected animal group with *S. mansoni* when treated with *Echinacea purpurea* and mefloquine extract. Also, there was a rise in the percentage of dead eggs [17].

Table (3): Effect of different groups of *N. oleander* and PZQ on Oogram pattern of S. mansoni.

Animal group	Immature (%)	Mature (%)	Dead (%)
Negative control	50±3.1	40±0.2	10±1.5
Group A	40±1.3*	32±1.02*	28±2.3*
Group B	32±2.2*	26±0.6*	42±0.7*
Group C	36±1.5*	30±1.7*	34±1.03*
Positive control	4±2.1*	2±2.6*	94±2.3*

***=Significant differences decrease as compared with negative control p<0.05**

2- Tissue egg load

The results in table 4 showed that the administration of mice with defatted methanol extract of *N. oleander* leaves, ethyl acetate and butanol fractions led to a significant depression in the total number of tissue eggs compared with non- treated mice. Also, the results appeared that mice treated ethyl acetate led to high decreasing in the number of intestine tissue eggs (46.2%) and liver eggs (38.3%) than that treated with defatted methanol extract and butanoic fraction. The reduction of tissue eggs in the infected mice is in full agreement with the previous study when mice treated with alcoholic or aqueous stem extracts of *Calotropis perocera* [29].

Antioxidant activities

Schistosomiasis is connected with the release of free radicals and disorders in the cellular antioxidant system. As the contagion becomes decided, the parasite comes under oxidative stress produced by the host immune system which is counteracted by the parasite antioxidant protection mechanism [29]. Reduced glutathione (GSH) is an intracellular reductant and plays a master role in catalysis, metabolism and transfer. It protects cells versus free radicals, peroxides and other toxic compounds [30]. Also, it has been reported that schistosomiasis caused a failure of the liver GSH content of mice, thus serving to decrease the antioxidant ability of the liver and leading to the obstetrics of lipid peroxides that may in switch play a central role in the pathology associated with schistosomiasis [29].

In the present work, there is decreasing in the hepatic tissue of the infected mice group compared to the normal group. Therapy of infected mice with plant extract led to improvement of GSH and high results were recorded with ethyl acetate comparing with the positive control. These results are in agreement with the reports of Jatsa *et al* [8]. On the other hand, the decreasing of MDA in treated groups with methanol extract, ethyl acetate and butanol fractions compared with the infected group prevent the formation of free radicals and improvement of antioxidant of infected mice. These results agree with many previous results [29, 2]

LC-ESI-MS Analysis

The MeOH extract beside the two fractions (Ethyl acetate and butanol) were submitted to the negative ion mode of the HPLC ESI-MS analysis. Ionization situations in the mass spectrometer were optimized in order to detect the m/z corresponding to the precursor ion $[M - H]^-$. Identification of the major components of these extracts based on its RT(min), the identify the deprotonated molecular ions [M-H]-, the major ESI-MS/MS fragment ions and the bibliographic references. The Neutral losses of 132 (pentose), 162 (hexose), 146 (deoxyhexose), 176 (hexuronic acid), 86 (malonyl residue) and 100 (succinyl residue) were identified according to Guarnerio *et al.,* [31]. The fragmentation of each compound in the defatted MeOH extract as well as BuOH and EtOAc fractions in tables 6-8 showed a mix of phenolic acid derivatives and flavonoid glycosides

Table (4): Effect of different groups of N. oleander and PZQ on worm burden and Tissue egg load of *S. mansoni*

Animal group	Mean no.of worms ±SEM	Reduction %	Mean no. of Ova count ±SEM/g tissus			
			liver	Reduction %	Int.	Reduction %
-ve control	30±3.2		12000±353		7800±347	
Group A	26±1.2*	13.3%	10200±203*	15%	6100±365*	22%
Group B	12±2.3*	60%	7400±135*	38.3%	4200±202*	46.2%
Group C	22±2.01*	26.7%	9400±238*	22%	5800±156*	25.6%
Positive control PZQ	1.3±2.5*	1.3%	892±215*	92.7 %	454±101*	95 .6%

*=Significant differences decrease as compared with negative control p<0.05

Table (5): Effect of of different groups of N. oleander and PZQ on GSH and MDA enzymes of liver tissue.

GROUPS	GSH mg/g tissue	MDA nmol/g tissue
Normal control	10.7±1.45	29.8±2.13
Negative control	5.2±0.63*	52.3±6.32*
Group A	7.5±1.18*	41.5±4.33*
Group B	5.9±0.38*	46.9±4.05*
Group C	8.9 ±1.15*	37.3±3.72*
Positive control	9.1±1.09*	34.2±3.24*

***=Significant differences decrease as compared with normal control p<0.05**

Table (6): Retention and HPLC-ESI –MS data of compounds in defatted methanolic extract of *Nerium*

No.	compound	fragments	M-H	MW	Rt
.1	Estradiol derivative	273(100), 159, 387, 503	503	504	1.86
.2	Behenic acid	71,129(100),297,341	339	340	2.13
.3	Caffeic acid –O- hexoside dimer	341(100),179,161,135	683	684	2.67
.4	quinic acid hexoside derivative	533, 191(100),85	695	696	2.8
.5	quinic acid	191, 79, 62(100)	191	192	4.8
.6	Hydroxybenzoic acid	137, 93	137	138	7.74
.7	3-O-caffeoyl quinic acid dimer	375, 353,191(100), 179, 135, 85	707	708	18.69
.8	5-o-caffeoyl quinic acid hexoside	375, 353, 191(100), 85	537	538	21.63
.9	1-o-caffeoyl quinic acid dimer	375, 353, 191(100), 85	707	708	22.16
.10	5-o-caffeoyl quinic acid dimer	729,353, 191(100), 85	707	708	23.49
.11	4-o-caffeoyl quinic acid dimer	375, 353, 191, 179 (100)173, 135	707	708	25.36
.12	-Caffeoyl glucaric acid dimer2	743, 371 (100), 121,191	765	766	31.37
.13	Quercetin-3-O-[2''-o- galloyl-glucoside]	637, 615(100), 463, 313, 301	615	616	35.38
.14	Quercetin-O-rutinoside (Rutin)	609(100), 301, 179	609	611	36.71
.15	Quercetin-3-O-glucoside	(100), 181, 179130	463	464	537.6
.16	1,3 di –O-caffeoyl quinic acid	537, 353, 191(100),179, 135	515	516	38.98
.17	3,5 di-O- caffeoyl quinic acid	537, 353, 191(100), 179,135	515	516	39.12
.18	Luteolin-7-O-rutinoside	593(100),285, 161	593	594	39.25
.19	Di galloyl-hexose-malic acid	599(100), 447, 313, 169	599	600	39.92
.20	Kaempferol -3-rutinoside	3(100), 285, 15159	593	594	40.46
.21	luteolin 7-O-glucoside	447(100), 285	447	448	41.25
.22	4,5- di caffeoyl quinic acid	537, 515(100),353, 191,179,173,137	515	516	41.92
.23	Apigenin-6,8-C-diarabinoside-pentoside	533, 515, 353, 191	667	668	45.52
.24	Gentibiosyloleandrin	821 (100),659,497, 179, 161	899	900	49.4
.25	luteolin-O-deoxyhexosylhexoside di pentoside	857(100), 695,593, 533,285, 161	857	858	51.27
.26	Neoglucoerysimoside	857(100), 695, 533, 323	857	858	51.93
.27	Apigenin-6,8-C-diarabinoside hexoside	741, 695(100),533, 515,443, 353, 161	695	696	55.54
.28	Unknown	841(100),679,517, 161	841	842	55.94

Table (7): Retention and HPLC-ESI –MS data of compounds in EtOAc extract of Nerium

No.	compound	fragments	M-H	MW	Rt
1.	Disaccharide dimer derivative	683(100), 341, 17	683	684	2.67
2.	quinic acid	191, 79, 62(100)	191	192	5.07
3.	Hydroxybenzoic acid	137, 93, 80 (100)	137	138	7.87
4.	3,4 di-Hydroxybenzoic acid-O - glucoside	233,153, 109(100)	315	316	16.02
5.	3-O-caffeoyl quinic acid dimer	375, 353,191(100), 179, 135, 85	707	708	18.83
6.	5-o-caffeoyl quinic acid	375, 353, 191(100), 85	353	354	22.03
7.	5-o-caffeoyl quinic acid dimer	729,353, 191(100), 85	707	708	23.77
8.	4-o-caffeoyl quinic acid dimer	375, 353, 191, 179 (100)173, 135	707	708	25.37
9.	4-feruloyl-5-caffeoylquinic acid	375, 353, 193,191(100), 173	529	530	27.5
10.	Galloyl-O-Vanilloyl-O-hexoside	459, 437(100), 313, 169, 125	437	438	29.9
11.	Feruloylquinic acid	191, 179(100), 161, 135	367	368	32.18
12.	Quercetin-3-O-[2’’-o-galloyl-glucoside]	637, 615(100), 463, 313, 301	615	616	35.25
13.	Quercetin-O-rutinoside (Rutin)	609(100), 301, 179	609	611	36.71
14.	Quercetin-3-O-glucoside	301(100), 181, 179	463	464	37.52
15.	3,5-o- di caffeoyl quinic acid dimer	537, 515, 353(100),191	1031	1032	38.85
16.	Di galloyl-hexose-malic acid	599(100), 447, 313, 169	599	600	39.79
17.	Kaempferol -3-rutinoside	593(100), 285, 151	593	594	40.32
18.	luteolin 7-O-glucoside	447(100), 285	447	448	41.25
19.	4,5- di caffeoyl quinic acid dimer	515(100), 353, 191, 173, 137	1031	1032	41.92
20.	Rutin dimer	609 (100),463, 300	1219	1220	44.13
21.	3-Feruloyl-5-caffeoylquinic acid	529(100), 367, 193,191, 179, 161	529	530	44.59
22.	Quercetin –O-sinapoylglucoside	691, 669(100),463, 301	669	670	46.59
23.	4-feruloyl-5-caffeoylquinic acid	529(100), 367, 193, 173, 161	529	530	47.93

Table (8): Retention and HPLC-ESI –MS data of compounds in BuOH extract of Nerium

No.	compound	fragments	M-H	MW	Rt
1.	quinic acid hexoside derivative	533, 191(100),85	695	696	2.8
2.	quinic acid	191, 79, 62(100)	191	192	4.8
3.	Hydroxybenzoic acid	137, 93, 80 (100)	137	138	7.88
4.	3-o-caffeoyl quinic acid hexoside	375, 353, 191(100), 179, 85	537	538	17.09
5.	3-o-caffeoyl quinic acid dimer	729,353, 191(100), 179,135, 85	707	708	18.83
6.	5-o-caffeoyl quinic acid dimer	729,353, 191(100),135, 85	707	708	22.29
7.	1-O-caffeoyl quinic acid dimer	375, 353, 191(100)	707	708	23.63
8.	4-o-caffeoyl quinic acid dimer	375, 353(100), 191, 179, 173,135	707	708	25.37
9	Feruloyl -3-caffeoyl quinic acid	375, 353, 193, 191(100), 175	530	531	27.5
10.	5-O-caffeoyl quinic acid	375, 191(100), 161	353	354	27.64
11.	Caffic acid -O-(sinapoyl -O-hexoside)	519(100), 387,223, 191, 179	565	566	29.24
12.	Saccharide	371(100), 249,231, 121	371	372	31.24
13	Feruloyl quinic acid dimer	389, 367, 191, 179(100)161, 135	735	736	32.18
14.	Caffic acid -O-(sinapoyl -O-hexoside)	519(100), 387,223, 191, 179	565	566	32.85
15.	Quercetin-3-O-[2’’-o- galloyl-glucoside]	637, 615(100), 463,313, 301	615	616	35.52
16.	Quercetin-O-rutinoside (Rutin)	609(100), 301, 179	609	611	36.85
17.	Quercetin-3-O-glucoside	301(100), 181, 179	463	464	37.78
18.	3,5-o- di caffeoyl quinic acid	537, 353, 191(100),179, 135	515	516	38.12
19.	Di galloyl-hexose-malic acid	599(100), 447, 313, 169	599	600	39.92
20.	Kaempferol -3-rutinoside	593(100), 285, 151	593	594	40.46
21.	4,5- di caffeoyl quinic acid	1032, 537, 515(100),353, 191,179,173,	515	516	41.92

	dimer	137			
22.	Apigenin-6,8-C-diarabinoside-pentoside	533, 515, 353, 191	667	668	44.32
23	3(4-O-glucosyl Feruloyl) - quinic acid	551, 529(100), 367, 191, 179, 161	529	530	44.59
24.	Feruloyl- caffeoyl quinic acid	529(100), 367, 179, 161	529	530	47.93
25.	unknown	741, 695(100),533, 513, 353, 161	695	696	55.54

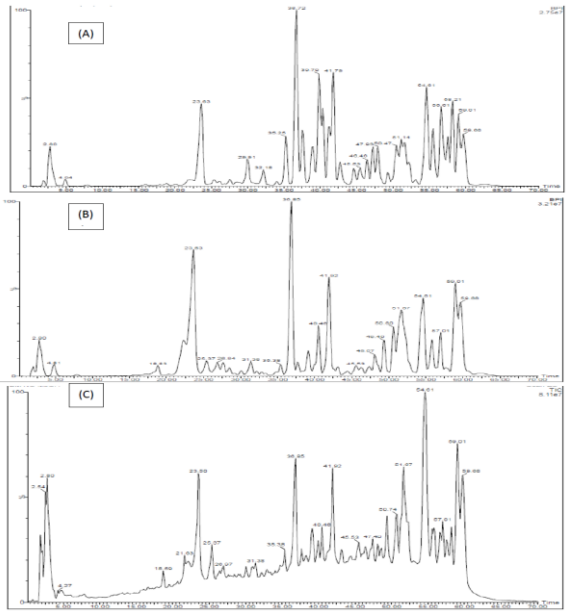

Fig. (1): Total ion current (TIC) chromatogram of MeOH extract (A), EtOAc fraction (B) and BuOH fraction (C) of Nerium

Phenolic acid derivatives

Results in defatted methanolic extract (table 6) exhibited the identification the eight caffeoyl quinic acid derivative compounds Quinic acid and quinic acid hexoside derivative as well as hydroxybenzoic acid derivative. These compounds were numbered by their elution order as:

Peak 3 with Rt =2.67min, and molecular ion [M-H]- , appeared as a peak at m/z 683. Also the four major peaks at m/z 341, m/z 179 [caffeic acid-H]- , m/z 161 [caffeic acid-H-H2O]-, and 135 [M-H- hexose-CO2]-. It indicated that this compound is caffeic acid-O-hexoside dimer [28]. Peak 4 with Rt= 2.8 min (table 5) showed its molecular ion at m/z 695 [M-H]-, and three major fragments at m/z 533 [M-H-glucose]-, m/z 342 [M-H -quinic acid]-, m/z 191quinic acid, so this compound is quinic acid hexoside derivative [32]. Peak 7 with (Rt = 18. 69 min) appeared molecular ion at m/z 707 [M-H] - and the important two fragments at m/z 353 and 191 .This represented that the compound is 3-O-caffeoylquinic acid dimer [33].

Table 6 showed the main fragmentation pattern of phenolic acid derivatives in the ethyl acetate fraction were identified in ethyl acetate fraction as two Hydroxybenzoic acid derivatives (Peaks 3 and 4) , nine caffeoylquinic acid derivatives (peaks 5-9,15,19-21, 23), one Di galloyl-hexose-malic acid (peak 16). According to the fragmentation pattern of phenolic acid derivatives in butanolic fraction (table 7), these compounds were identified as caffeoylquinic acid derivatives (peaks 4-10, 18, 21, 23) and Caffic acid -O-(sinapoyl -O-hexoside) (Peak 11, 14).

2-Flavonoid glycosides

Three Quercetin derivatives, two apigenin derivatives, three luteolin and Kaempferol -3-rutinoside were appeared in defatted methanolic extract (Table 6) as follows: Peak 13 with Rt = 35.38 in the methanolic extract (table 6) showed a molecular ion peak at m/z 615 [M-H] - and fragments at m/z 463, 313, 301 indicated that this compound is Quercetin-3-O-[2''-o- galloyl-glucoside] [34]. In other hand, peak 14 showed a molecular ion peak at m/z 609 [M-H] - and fragments at m/z 301 and 179 indicated this compound is Quercetin-O-rutinoside (Rutin) [35]. Also peak 15 with Rt =37.65 and fragments at m/z 301,181 and 179 represented the presence of Quercetin-3-O-glucoside [36]. Peak18 with Rt = 39.25 had fragments at 503, 285, and 161 indicated the presence of Apigenin- 6, 8 di-glucoside while peak 27 with Rt = 55.54 and fragments at m/z 533, 513, 353, 161 showed apigenin-6, 8-*C*-diarabinoside hexoside. Peak 25 with Rt = 51.27 and fragments at m/z 695,593, 533, 285,161 showed luteolin-O-deoxyhexosyl hexoside di pentoside [37].

Results in table 8 showed the fragmentation pattern of seven flavonoid glycosides in ethyl acetate fraction. These compounds were Quercetin-3-O-[2''-o- galloyl-glucoside], Quercetin-O-rutinoside, Quercetin-3-O-glucoside, Kaempferol -3-rutinoside, Luteolin -7-rutinoside, Rutin dimer and Quercetin – O- sinapoylglucoside. On the other hand, the flavonoid compounds in butanoic fraction (Table 8) were three Quercetin glycosides (peaks 15 to 17), Kaempferol -7-rutinoside (peak 20) and Apigenin-6, 8-C-diarabinoside-pentoside (peak 22). The present study is in full agreement with a previous study where Siham *et al* [38] identified 19 compounds from *N. oleander* leaves by using HPLC-ESI-MS/MS analysis; the main compounds were chlorogenic acid, rutin and quinic acid esters, such as caffeoylquinic acids and dicaffeoylquinic acids

Conclusion

The defatted methanolic extract of *Nerium oleander* and certain fractions derived from it showed antischistosomal activity through the reduction of worm load and egg count in vivo. HPLC-ESI-MS analysis of the defatted methanolic extract of this plant identified polyphenolic compounds as the major constituents of this extract. Future studies will be needed to further isolate and characterize the chemical compounds from the most active fractions to search for new bioactive antischistosomal secondary metabolites in fruits and vegetables.

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