

Anti-Leech and Disinfection Activities of Methanolic Extracts of Walnut (*Juglans regida* L.) and Oleander (*Nerium oleander* L.) on *Limnatis nilotica*

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Abstract: This experimental study was designed to evaluate *in vitro* anti-leech effect and disinfectant activity of *Juglans regida* L. and *Nerium oleander* L. on *Limnatis nilotica*. After exposure of leeches with *J. regida* (2×10^5 and 4×10^5 ppm), *N. oleander* (2×10^5 and 4×10^5 ppm), Piperazine (500 and 1000 mg), Praziquantel (50 and 500 mg), Chlorquine (150 and 300 mg), Levamisole (300 mg), Niclosamid (625 mg), Copper sulfate (4×10^2 ppm), Physiological water (10^2 ml) and Chlor (4×10^4 ppm), for 720 min, the mean death time of *L. nilotica* was measured by anti leech assay and disinfectant test. There are statistically significant differences between groups ($P < 0.05$). Data between groups were analyzed using one-way ANOVA statistical method by Sigma State 2.0 software. The results showed the *J. regida* and *N. oleander* have no anti leech effect but Piperazine, Praziquantel, Chlorquine, Levamisole, Niclosamid, Copper sulfate and Chlor killed leeches at 720 ± 0 , 40.22 ± 19.05 , 720 ± 0 , 19.22 ± 8.82 , 17.11 ± 4.13 , 16.25 ± 6.49 and 6.75 ± 2.98 minutes after exposure, respectively. Therefore, it seems the tested compound couldn't be used as a complementary treatment in leech infestation.

Key words: *Limnatis nilotica* • *Juglans regida* • *Nerium oleander* • Anti Leech Assay • Disinfection Activities

INTRODUCTION

Leeches are invertebrates of the Annelida phylum and Hirudinea class. There are approximately 650 species, but almost of these pose a problem to humans [1]. Leeches vary in shape, in color and in length [2]. They are characterized by suckers at both ends of their flexible body and display a unique inch-worm like mode of locomotion. They may be divided into two classes: land leeches and aquatic leeches [3]. Aquatic leeches are good swimmers and have a worldwide distribution [4]. In human, ectoparasitism by this parasite is frequent in rural areas. Habitual drinking of water from springs, which is not rare in the rural areas of Turkey, may give rise to leech infestation [5]. Leeches in water are taken into the body when water is drunk and they localize on the mucosa of pharynx, tonsils, esophagus, nose or nasopharynx [5, 6]. Leeches, which belong to the class Hirudinea and have a world-wide distribution, are well-known for their ability to cause anaemia [7].

Whereas most bloodfeeding leeches feed as ecto-parasites for short periods of time, those that feed on mucous membranes have been known to stay in an orifice for days or weeks on end [8, 9]. Aquatic leeches have a worldwide distribution and it is reported that they can attach to mucosal membranes accidentally from springs and freshwater in endemic areas. They have also been described in sites like nose, tonsils, conjunctiva, pharynx/larynx, trachea/bronchi, esophagus, rectum and vagina [10,11]. The *Limnatis nilotica* species, commonly called the 'horse leech', occurs in lakes and streams in Southern Europe, North Africa and the Middle East including streams in Iran [12]. These leeches can cause haemoptysis and anaemia and may also act as vectors of animal and human pathogens [13]. Morphological characterization of the *L. nilotica* include is a Dark green colour surface, Dorsal surface with rows of green spots and Yellowish-orange and dark green bands on either side [13].

For leech therapy is only supportive of traditional and ethnic medicines in the pharmacopoeia also for the treatment leech biting is not registered according to statistics released by the World Health Organization (WHO) provides a source of drugs for humans is plants. Factors such as lack of access to the majority of people, high cost of production of chemical drugs, the therapeutic effects of chemical drugs because of its resistance to pathogens and the side effects and cause side effects that led to the day on traditional medicine and the treatment with medicinal plants to be more positive in societies [14].

The scientific name of walnut is *Juglans regia* L. Of Juglandaceae in traditional medicine and herbal medicine are used around the world [15, 16]. Walnut is tree that reaches a height of 15-12 meters and has a lot of leaves [17]. Walnut (*Juglans*) have three species includes *J. regia*, *J. cinerea* and *Juglans nigra*, Which the only species in Iran is *J. regia* and are going regions of North, South, West of Iran. Arabic name of this plant in traditional medicine nutmeg (Joz) and in French language it named is tree and named it Noyer cimmun and in England called Walnut tree [18, 19].

Walnut is one of the plants that in traditional medicine of leaves for treating rheumatic pains, fever, diabetes, skin disease and its roots for the treatment of diabetes and its flowers for malaria and rheumatic pains are used [20, 21]. Phenolic acids and flavonoids, phenolic compounds, two major groups are found in walnut leaves.

The phenolic acids and acid Kafeoeileo acid are in walnut. The flavonoids quercetin in walnut leaves are such as Galactoside, Pentosid derivative of quercetin, quercetin arabinozid, Glycosid quercetin, quercetin ramnozid and derived of Pentosid Kampfrol [19, 22]. Antimicrobial effects [23], anti-cancer and anti-inflammatory [24] and anti-diabetic walnut [25] has been documented in literature, but is no evidence to the anti-leech effect of walnut.

Some studies on the anti leech activities have been reported. For example, The result of a study [26] showed that the methanolic extract of *Nicotina tabacum* had the anti *Limnatis nilotica*. Previous works showed that garlic tablets have the anti *Limnatis nilotica*. Bahmani *et al.* [27] studied the anti leech effects of five Medicinal plants (*Quercus brantii*, *Achillea* spp., *Scrophularia deserti*, *Artemisia kermanensis*, *Artemisia* spp.) used in Iranian traditional medicine were investigated against *L. nilotica*.

Iranian herbal medicine sources from walnut and oleander plants are named with the antiparasitic plants [14] but so far little scientific studies to prove this therapeutic effect has been made. Since the usefulness of medicinal plants should be established through investigation and research, in this study the effect of methanol extracts of walnut and oleander plants in Iranian traditional medicine as an anti parasitic plants have been mentioned [14] in analogy and comparing to Levamisole, Niclosamid, Piperazin, chloroquine and some chemical disinfectant drugs Praziquantel were investigated with two test methods such as Anti leech assay and disinfection test.

MATERIALS AND METHODS

Preparation of Leeches: In May 2012, a number of mature form of *L. nilotica* leeches (140 in total) were selected from spring waters from the Dehloran city of Ilam province (West part of Iran). The dark green color surface with rows of green spots on the dorsal surface and yellowish-orange and dark green bands on either side were the main signs for detection of *L. nilotica* species [Bahmani *et al.*, 2012]. The length of leeches was 50-110 mm (Fig. 1).

The Plants and Extraction: *Nerium oleander* and *Juglans regia* plants were prepared in May 2012 from flower garden of Isfahan and Urmia city.



Fig. 1: *Limnatis nilotica*

Table 1: Ethnopharmacologic information of studied plants.

Scientific name and family name	Local name	Part used	Extract type	Traditional treatment [14]
<i>Juglans regia</i> L.	Walnut	Leaf	Methanolic extract	Anti-parasite
<i>Nerium oleander</i> L.	Oleander	Leaf	Methanolic extract	Anti-parasite

The methanolic extracts of *J. regia* and *N. oleander* were prepared by adding 1:3 ratio of plants and methanol, respectively and this was subjected to percolation extraction for 72 h according to the prescribed method of Eidi *et al.*, 2006 [28]. After extraction, the solvent was filtered. The ethnopharmacology information for these plants has been presented in Table 1.

Levamisole tablets (an anthelmintic and immunomodulator), Niclosamide and some other antiparasitic drugs such as syrup and tablet of Piperazine, Praziquantel and Chloroquine were studied as a positive control and compared to distilled water. These tablets were powdered and diluted in 10 ml distilled water and added to glass test.

Anti-Leech Assay: For the anti-leech assay, the leeches were located individually in a glass container with 600 ml spring water.

The extracts and chemical drugs were then added and leeches were screened for 720 min and time of paralyze and death of each leech was recorded. The evaluation of death of a leech was based on immobility after stimulation with a needle. The low average paralyzing and death time of these compounds reflects anti-leech properties [27].

The severity of anti leech effect of these compounds/drugs based on time was categorized into five groups: (1) 4+? paralysis and death of each leech within 1-60 min after addition of the drug, (2) 3+? paralysis and death of each leech within 61-120 min after addition of

drug, (3) 2+? paralysis and death of each leech within 121-180 min after addition of drug, (4) 1+? paralysis and death of each leech within 181-240 min after addition of drug, (5) negative ? paralysis and death of each leech within 241-720 min after addition of drug [27].

The efficacy of the drugs which were able to kill leeches within 1-60 min after addition reflects the anti-leech properties of these compounds and therefore, they may be used in the treatment of infestation with *L. nilotica* in the future [27].

For the disinfection test, four leeches were placed in the plastic vessel containing 5 liter of spring water. *J. regia*, *N. oleander*, Chlor and Copper sulfate (as positive control) were used. The experiment was run in three replicates of each treatment. The number of dead and survivor leeches in each plastic vessel was counted after 30 min [27]. The distilled water was used as negative control. The leeches were considered dead if they did not exhibit any internal or external movement.

Statistical Analysis: The differences between the control and treated groups were analyzed using Sigma State 2.0 software program and one-way ANOVA test.

RESULTS

Paralysis and death time after exposure of walnut and oleander extracts with leeches revealed that the methanol extract at different doses have different potential anti leech activity which increasing with the dose.

Table 2: Components/Drugs, mean±SD, dose and severity for anti leech assay

Components/Drugs	Dose (mg)	Mean death time± SD (Min)	Severity Levamisole
	300	19.22±8.82 ^c	4 ⁺
Niclosamide	625	17.11-4.13 ^c	4 ⁺
Piperazine syrup	500	720±0 ^a	-
Piperazine syrup	1000	720±0 ^a	-
Piperazine tablet	500	720±0 ^a	-
Piperazine tablet	1000	720±0 ^a	-
Chloroquine tablet	150	720±0 ^a	-
Chloroquine tablet	300	720±0 ^a	-
Praziquantel tablet	50	720±0 ^a	-
Praziquantel tablet	500	40.22±19.05 ^b	4 ⁺
Physiological water	100	720±0 ^a	-
Methanol extract of the Walnut	300	720±0 ^a	-
Methanol extract of the Walnut	600	720±0 ^a	-
Methanol extract of the Oleander	300	720±0 ^a	2 ⁺
Methanol extract of the Oleander	600	720±0 ^a	2 ⁺

There are statistically significant differences between groups with different superscripts (P<0.05).

Table 3: Components/Drugs, mean±SD, dose and severity for disinfection test.

Components/Drugs	Dose (PPM)	Mean death time± SD (Min)	Severity Copper Sulfate
	4×10 ²	16.25±6.49 ^b	4 ⁺
Chlor	4×10 ⁴	6.75±2.98 ^b	4 ⁺
Physiological water	10 ² ml	30±0 ^a	-
Methanol extract of the Walnut	2×10 ⁵	30±0 ^a	-
Methanol extract of the Walnut	4×10 ⁵	30±0 ^a	-
Methanol extract of the Oleander	2×10 ⁵	30±0 ^a	-
Methanol extract of the Oleander	4×10 ⁵	30±0 ^a	-

There are statistically significant differences between groups with different superscripts (P<0.05).

Despite anti parasitic effects of Walnut and Oleander which mentioned in traditional medicine literatures in Iran, methanol extracts of this plant had no effect on *L. nilotica*. The highest affectivity was found for levamisole and niclosamide. The exact timing of drug treatments for paralysis and death are shown in Table 2 and 3.

Statistical analysis shows the different drug treatments for paralysis and death factors, there is no significant difference between the leeches.

DISCUSSION

In this study the effect of walnut methanol extract and oleander on *L. nilotica* was investigated. The results of this research shown that methanolic extracts of walnut and oleander despite recommendations from the Iranian traditional medicine literature based on their anti-parasitic effect of the plants, showed no such inference can be made ??anti leech activity, Which perhaps methanol not ability to draw out the active ingredients, So recommended the aqueous, alcoholic and ethanolic extracts of this plants on *L. nilotica* can be used and tested.

Praziquantel is group of anthelmintic drug that increase dopamine cream Ca⁺² exiting the exhaust pipe, causing muscle paralysis and consequently their detachment from the vessel wall. In this study, Praziquantel with dose of 500 mg likely by listed mechanism can kill the leeches but with low dosage (50 mg) wasn't to kill the leeches.

Nazemi Rafiei [29] showed that ethanol extract of *Nerium oleander* L., *Lavandula officinalis* L. and *Ferula assafoetida* L. the interdiction of the *Tribolium castaneum* (Herbst) but the extracts in high concentration hadn't significant effect on flour lice. Nazemi rafi and Moharram [29] in another study showed that acetone extract of the gum comparing to red and white extracts of oleander leaves and lavender flowers is repellency power with a significant very high. Nutritional effects of oleander leaf extract has been done on the fly Stables [26].

Khalili dehkordi *et al.* [30] showed that the walnut leaf extract had antiparasitic effects on *Trichomonas vaginalis*. Previous study showed that important alkaloids of oleander is indole. The result of recent researches shown that oleander has cardiac glycosides, which effective in cardian muscle activity. Secondary compound of oleander is such as phytosterol, stroides, limnoides, á-amirim, steroidal saponins, cardiac glycosides, triterpenoid saponins, cucurbitancins and quassinoids [14].

A speices of walnut is *Juglans nigra* L. that includes Juglen [14]. Oleander is qualified cardiac glycosides (Odoroside and Oleandrin) which are stimulating and exciting compound that in ancient times has been used for fishing. Also Oleander (*Nerium oleander* L.) with have Cardenolide compound is Iranian toxic plant [14]. In disinfection test, oleander methanol extract could not kill the leeches. The results of our study weren't consistent with fatality effect in fishing.

Bahmani *et al.*, 2011 [33] reported that *Allium sativum* L. methanol extract exhibited the anti immature *limnatis nilotica* activities. Also Eftekhari *et al.*, 2011 [34], evaluated the anti leech effect of *A. sativum* L. methanol extract and Levamisole on *L. nilotica* (Mature form). The result of this study showed that garlic methanol extract had average dead time 144.55±57.217 min. In other study by Gholami-Ahangaral *et al.*, 2012 [35] reported which grape methanolic extract (*Vitis vinifera* L.), niclosamide and ivermectin on *L. nilotica* were evaluated and found that grape had the anti *L. nilotica* effect. Bahmani *et al.* [36] studied the disinfection effect of *Nicotiana tabacum* extract on *L. nilotica* with LC₅₀ for tobacco 13×10⁴ (ppm) and for copper sulfate and ammonium chloride 8×10⁴ and 370×10⁴ (ppm), respectively.

Therefore, treatments such as Levamisole, Niclosamid, Praziquantekl, Chlor, Copper sulfate are probably involved potentially the anti leech but *J. regida* L. and *N. oleander* L. plants can not anti parasite activity. These results suggest that walnut and oleander couldn't be used potentially for the prevention and controlling

leech pollution. However, research on the elucidation of the exact mechanism of chemical drugs for the anti-*L. nilotica* beneficial effects are needed.

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