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Microscopical Characters, Heavy Metals Level and Histopathological Effects of *Lawsonia inermis* L. Leaves in Female Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Author MRH designed the study. Authors HNM, SAA and SSS did the laboratory work, wrote the protocol and wrote the first draft of the manuscript. Author LAG did the microscopical investigation. Author ERE performed the histopathological analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study is intended to compare the freshly collected henna (*Lawsonia inermis* L.) and the market henna in term of microscopical key elements and heavy metals contamination. Moreover, this study is aimed to investigate the effect of henna and its oily additives on kidney histology in female mice.
Study Design: Department of Pharmacognosy, Faculty of Pharmacy, University of Tripoli and Animals House, Biotechnology Research Center in 2010.
Methodology: The powders of collected and market henna have been subjected to a microscopical study with magnification of 5x then 40 x to investigate the henna key elements, which are calcium oxalate clusters, anomocytic stomata, starch grains and fibers. Three elements: arsenic (As), mercury (Hg) and lead (Pd) were analyzed by atomic absorption spectroscopy (AAs)

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for the collected and market henna. For histopathological study, an aqueous extracts of *L. inermis* leaves and *L. inermis* leaves-oils_were prepared by maceration. Eighteen female Albino Wister mice (3-4-months, 20-25 gm) were injected with the prepared extracts subcutaneously by dose 50 ml/kg/day for 5 days. Kidneys were collected and subjected to histopathological study.

Results: From this study, the microscopical investigation exhibited the presence of some elements which are never mentioned as the henna key elements. Both market and collected henna were contaminated with a high level of heavy metals specially lead (Pd). The histopathological findings implied that there are many histological changes on the kidney tissues such as aggregation of round cells and congestion of blood vessels.

Conclusion: The market henna might be adulterated with other types of plant. The presence of a high concentration of lead (Pd) in the collected henna as well as the market henna might be considered as the cause of some *L. inermis* adverse effects. *L. inermis* leaves and *L. inermis* leaves-oils aqueous extracts implied many abnormalities in the kidney tissues.

Keywords: Lawsonia inermis; henna additives; heavy metal; kidney; adulteration.

1. INTRODUCTION

Henna (*Lawsonia inermis* L.) which belongs to family Lythraceae [1] has been used for more than 6000 years as the old Egyptians have applied henna to their mummies' hair and fingernails, and it is commonly called as (henna, mhendi, shudi, madenrang, mendi, goranti) [2,3]. Henna leaf is rich with constituents including, lawsone (2-hydroxy-1,4-naphthaquinone), that is responsible for henna coloring properties through its affinity to bind with keratin, blood serum protein and albumin [4]. In addition to various phenolic glycosides, coumarins, flavonoids such as luteolin, and 7-O-glycoside, fats, resin and henna-tannin [5]. Besides to cosmetic field, henna has a valuable medicinal use against various diseases. It has been reported to have analgesic, antipyretic and anti-inflammatory effects [6], antioxidant [7], hepatoprotective against carbon tetrachloride [8], immuno-modulatory effects [9], antibacterial activities [10], antifungal activity [11], tuberculostastic effect [12], hypoglycemic agent, hypolipidemic agents [13], anti-ulcer [14] and wound healing effect [15].

Usually natural henna does not have any side effects, and it seems to be safe for most people [16], but the application of henna has resulted in a life-threatening episode of hemolytic anemia, particularly in individuals with a genetic deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity, and the causative agent is thought to be the lawsone. An oxidant stress-associated hemolytic response to lawsone or the hemolytic response to henna may be restricted to individuals with compromised antioxidant defenses [17]. Henna also has been documented to cause dose-dependent nephrotoxicity in laboratory rats [18].

Generally, the incorporation of henna additives is mainly to shorten the time of application and produce a darker shade. Para-phenylenediamine (PPD) is mixed with natural henna to a produce product called black henna, and there have been some reports of allergic contact dermatitis caused by PPD in henna tattoo mixtures [19]. Heavy metals like lead, cadmium, mercury, copper, cobalt, nickel and zinc are mixed with henna to strengthen the color [20,21], and they are reported to have spermatotoxic and gonadotoxic effects [22]. Other additives such as tea leaves, coffee, charcoal, lampblack, lemon oil, vinegar, eucalyptus oil and clove oil [20]. There is some popular oil in Libyan markets that many women have used like Sheih oil.

The study is intended to compare the freshly collected henna (*Lawsonia inermis* L.) and the market henna in term of microscopical key elements and heavy metals contamination. Moreover, this study is aimed to investigate the effect of henna and its oily additives on kidney histology in female mice.

2. MATERIALS AND METHODS

2.1 Plant Material

Lawsonia inermis shrub was collected from area 20 km south of Tripoli, Libya. The plant was authenticated by Dr. Fathi Erteeb a plant taxonomist at Department of Botany, Faculty of Science, University of Tripoli. The leaves were picked and dried in shade for 2-3 weeks, and the

dried leaves were grinded mechanically into fine powder.

2.2 Oil Additives and Henna Products

Oil additives (Sheih oil) of unknown composition and henna products belong to names (Taj, Royal and Al-bashah) were purchased from the Libyan market.

2.3 Microscopical Study

Small amount of (collected henna leaves and commercial products) powders were placed individually on clean dry slide. One drop of diluted glycerin was added on each slide and covered slowly with coverslip then cleaned with filter paper to remove any powder residue. Magnification of 5x then 40 x was used for investigation of key elements, which are calcium oxalate clusters, anomocytic stomata, starch grains, and fibers.

2.4 Determination of Heavy Metals

The collected henna powder and all market henna samples were sent to the Libyan Petroleum Institute to be analyzed by atomic absorption spectroscopy (AAs, Varian, USA). Microwave induced preparation of the 500 mg sample of henna with nitric acid (Sigma-Aldrich, Germany) in autoclave (Heidolph, Laborota 4000, Germany). The resulting solution was filtered and then diluted to 50 ml using demineralized water. Blanks were prepared in the same way. Three elements: arsenic (As), mercury (Hg), and lead (Pd) in all prepared solutions were quantified.

2.5 Biological Study

2.5.1 Preparation of extracts

Two types of aqueous extracts were prepared by cold maceration method. The procedure of extraction simulates the folk preparation of henna paste. 50 grams of the collected *L. inermis* leaves were mixed with 40 ml of oil (Sheih) and 50 ml of distilled water, which is enough to make paste and left to stand for 3 hours in a covered container at room temperature and named as extract B. For preparation, the *L. inermis* free oil extract, the same procedure was applied but the volume of oil was substituted with distilled water and named as extract A. Then the two pastes were pressed through a clean cotton sheet to get crude extracts, which were filtrated and stored at 4°C in clean glass bottles.

2.5.2 Experimental animal

Eighteen female Albino Wister mice (3-4-months), weighing between 20-25 gm. The mice were kept in plastic cages in Biotechnology Research Center, Tripoli, Libya at room temperature of 23± 2°C for one week before injection for acclimatization. They were fed with commercial chick mash and given tap water.

2.5.3 Extract injection

Eighteen Albino Wister mice were randomized and divided into three groups. Group A for extract A, Group B for extract B, and normal control group (normal saline). Each extract and the normal saline were injected subcutaneously in a dose of 50 ml /kg/day for 5 days.

2.5.4 Kidneys collection

After seven days of the first extract injection, the mice were anesthetized, then kidneys were removed and kept in 10% formalin (Research Products International Corp, USA). Samples were sent to the Marine Biology Research Center for histopathological study (the slides were stained with hematoxylin-eosin). Processing and molding were done by using paraffin (Sigma-Aldrich, Germany); thereafter transverse incision thickness of 5-7 µm was prepared. Slices were colored with haematoxylin and eosin staining method [23]. Then, evaluated using pathological parameters including epithelisation, inflammatory cells, and vascularity.

2.6 Statistical Analysis

The data were statistically analyzed by One-sample T test using (IBM SPSS statistics 20). The *P* values less than 0.05 were considered as statistically significant.

3. RESULTS

3.1 Microscopical Study

After microscopical examination, calcium oxalate clusters were relatively larger in market henna compared with the freshly collected henna, which contained a fairly small cluster. The anomocytic stomata were found in collected henna, Royal, and Taj henna but absent in Al-bashah henna. Different types of fibers were found in market henna and freshly collected one. Starch grains in market henna were smaller than the collected henna which showed large ones with centered

slit hilum. Unicellular trichome with rough edges was found in Royal henna, while bicellular glandular trichome was detected in Taj henna and crystal sheet in Al-bashah henna. It is not usual for these elements to be existed in natural henna Table 1.

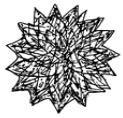
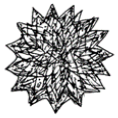
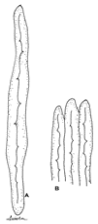
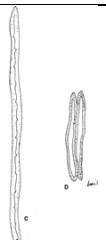
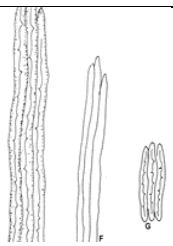
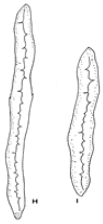
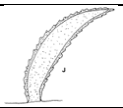
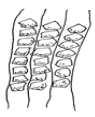
3.2 Heavy Metals Level

In this study, we have tested three henna products in addition to freshly collected one for the presence of three heavy metals using atomic absorption spectroscopy. The distribution of heavy metals in samples studied is shown in Table 2. Metals were detected in most of the samples in varying concentrations. However, we did not observe clear patterns indicating that metal concentrations were related to specific brands, color, or cost. The distribution frequency of metals among the studied henna samples were; Pb>As>Hg.

3.3 Histopathological Study of Kidney

The histopathological examination of the kidney in Albino Wister mice female of the control group showed the glomeruli with normal size and normal renal tubules Fig. 1(I). The kidney histological examination of the group A has showed a perivascular interstitial aggregation of round cell, hydropic degeneration, and damage of tubular epithelium Fig. 1(II). Group B exhibited a congestion of blood vessels and capillaries Fig. 1(III).

Table 1. Comparison of market henna with *L. inermis* powder under the microscope

Key element	Collected henna (*L. inermis*)	Royal henna	Taj henna	Al-bashah henna
Calcium-oxalate cluster				
Anomocytic stomata				--
Fibers				
Starch grains				
Trichomes	--			--
Crystal sheet	--	--	--	

A: Single fiber. B: Group of fibers. C: Few single fibers with lumens. D: Group of fibers with large lumens. E: Group of large lignified fibers. F: Group of transparent fibers. G: Group of small fibers. H: Long single fiber. I: short single fiber. J: Unicellular trichome with a rough edge. K: Bicellular glandular trichome. (--): Not detected

Table 2. Heavy metals levels of the market and the collected henna

Elements (ppb)	Collected henna	Royal henna	Taj henna	Al-Bashah henna	USP oral limit µg/kg
Arsenic (As)	<200[a]	<200[a]	<200[a]	<200[a]	1500[a]
Mercury (Hg)	22.41[b]	8.29[b]	12.13[b]	36.72[b]	1500[a]
Lead (Pd)	**3130[b]**	**1140[b]**	**3030[b]**	**3910[b]**	1000[a]

- *Values with different superscript letters are significantly different from the specified USP limit (P <0.05).*
- *<: less than the detected limit.*
- *USP: United State Pharmacopeia*
- *ppb: part per billion*

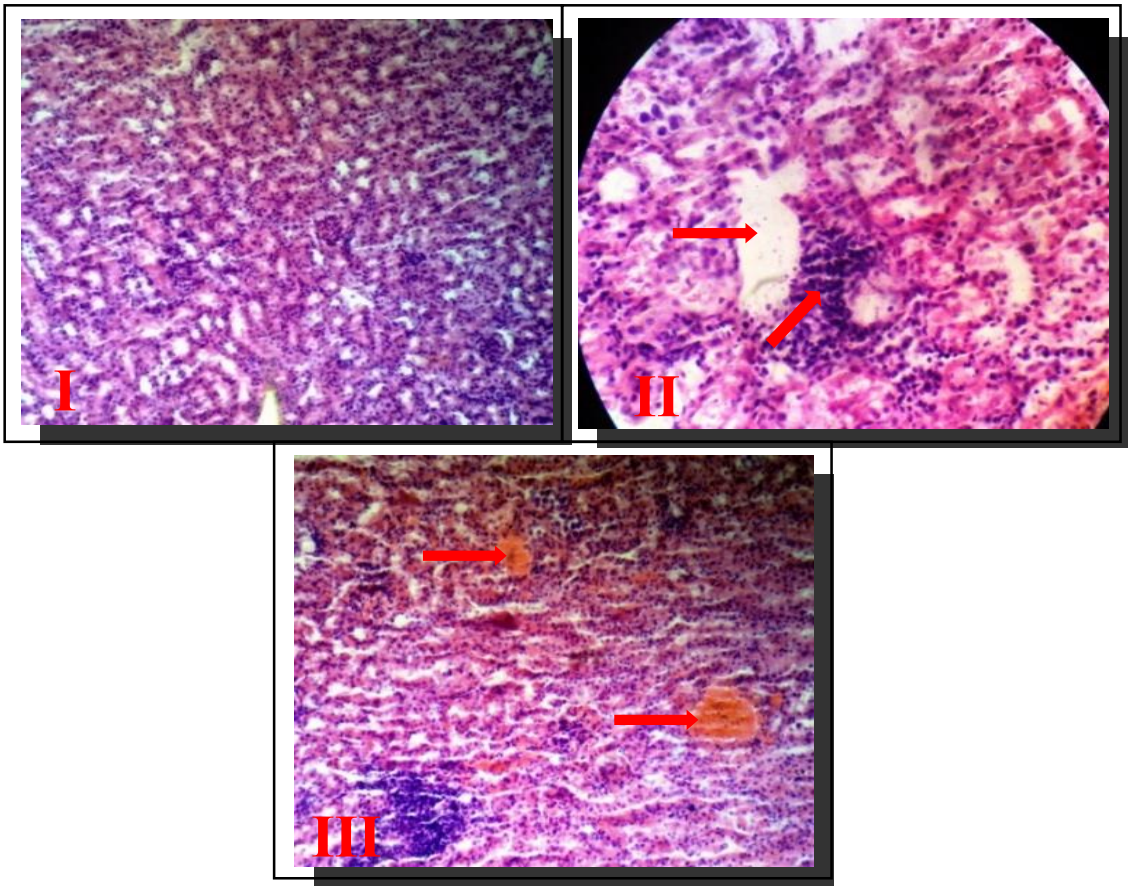


Fig. 1. I: Kidney histopathological features of normal control group. [Kidney tissue was normal (normal renal tubules and glomerulus)]. II: Kidney histopathological features of *L. inermis* extract group A. [Kidney presented many abnormal features such as perivascular, interstitial aggregation of round cells, hydropic degeneration, and damage of tubular epithelium as indicated in red arrows]. III: Kidney histopathological features of *L. inermis* and sheih oil extract group B. [Kidney showed a congestion of blood vessels and capillaries as indicated in red arrows]

4. DISCUSSION

The main characteristics of henna powder leave under the microscope are anomocytic stomata, cluster crystals of calcium oxalate and small groups of lignified fibers [24]. In comparison of market henna with the collected (*L. inermis*) powder under the microscope, some of the basic henna key element was found in the market henna while other important features were absent. Additionally, the microscopical investigation exhibited the presence of some

details which is never mentioned as the henna key features. These findings may indicate that the market henna was adulterated or contaminated with other types of plant such as senna leaves that have a stimulant effect on smooth muscles [25]. Although, the Libyan women complain about the application of henna paste and the appearance of some health issues, which is related to the change in urine color and abortion. Esteki and Miraj have confirmed the abortifacient effect of henna on pregnant mice. They have inferred from the subsequent embryonic absorption as a parameter for the abortion [26]. Bello et al. have found that the aqueous extract of *L. inermis* evoked significant dose-dependent myometrial contraction of myometrial strips from non-pregnant rat's uterus [27]. However, further study could apply the dose-dependent uterus contraction for the henna-oil mixture.

Heavy metals occur naturally or as a result of human activities such as industry and agriculture. Subsequently, plants roots absorb the heavy metals which are crucial for plant growth such as Fe, Zn, Cu, Mg, and Ni. Whereas, some metals as As, Cd, Pb, Se and Hg can be accumulated in plant parts [28]. The consuming of the heavy metals contaminated plants by the human either orally or topically lead to accumulation and disruption the function of vital organs by binding to proteins and nucleic acids of body cells [29]. By comparing the results in Table 2 to USP oral limit, the levels of arsenic and mercury are statistically significant (P >0.05) below the specified limit in all the tested samples, whereas the levels of lead are exhibited to be statistically significant (P <0.05) higher than the specified limit. The side effects of heavy metals exposure are numerous. Muntner et al. [30] have observed that the exposure to small amounts of lead is related to kidney dysfunctions. In further, long exposure to heavy metals is harmful and it might be abortifacient [22].

Concerning the histopathological study, subcutaneous injection of henna and henna-oil aqueous extracts with dose 50 ml/kg/day have resulted in many abnormal histological changes, which consequently affect the kidney functions. In the *L. inermis* group, the presence of inflammation, which is indicated by aggregation and infiltration of round cells and destruction in tubular epithelium showed in Fig. 1 (II). The congestion of blood vessels is the main observed abnormal feature in *L. inermis*-oil group as illustrated in Fig. 1 (III). This work agrees with the previous studies [31,32] that have been conducted on male rats by oral administration of the aqueous extract [31] and ethyl acetate extract [32] of *L. inermis* leaves. Both studies have approved that the oral administration of *L. inermis* extracts may be responsible for many kidney histological deformations such as interstitial hemorrhage peritubular, death of tubules epithelial cells, and congestion and dilatation of vessels. From this study, we can conclude that the administration of *L. inermis* leaves extracts by different routes (orally or subcutaneously) may lead to almost the similar histological changes on kidney. In this study, the abnormal histological changes might be attributed to the main active constituents (lawson) of the collected henna specially to patients with compromised antioxidant defense [17].

5. CONCLUSION

From the microscopical view, the market henna might be adulterated with other types of plant i. e. senna leaves (*Cassia acutifolia*), which could have a serious impact on pregnancy. *L. inermis* and *L. inermis*-oils extracts implied many abnormalities in the kidney tissues. These abnormalities might be due to the presence of a high concentration of heavy metals in the collected henna as well as the market henna.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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