

Assessment of Vestibulotoxicity of Henna Leaf (*Lawsomnia Inermis*) in A Rat Animal Model

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March 31, 2023

Abstract

BACKGROUND: Otitomycosis is complicated for patients and otologists since it always needs long-term care and follow-up, but the recurrence rate remains high. Management is targeted toward intensive aural toileting with the administration of topical antifungals. Classical antifungal agents, including azoles and polyenes, have shown increased resistance. This has contributed to studies into medicinal plants as an alternative therapy for fungal infections. **OBJECTIVE:** This research aims to examine the potential vestibulotoxic effects of the henna leaf (*Lawsomnia inermis*), an ancient folk medicine, on the inner ear of a rat animal model. **METHODS:** Twenty-four healthy, adult male Wistar Kyoto rats were categorized into three groups (A, B, C) with eight rats each. Baseline vestibular parameters were tested before endoscopic-guided transtympanic instillation of aqueous and ethanol henna extract into the right middle ear of rats in Group A and B, respectively. At the same time, normal saline was instilled in the right middle ear of rats in Group C. Vestibular parameter testing was conducted on rats post-instillation of transtympanic Henna at intervals of 4 hours, 24 hours, 48 hours, 72 hours, one week, two weeks and three weeks. **RESULTS:** All vestibular parameters documented were not affected after the instillation of henna extract into the middle ear. **CONCLUSION:** Henna extract is a safe and potential alternative in treating otomycosis even with tympanic membrane perforation, as it has no vestibulotoxic adverse effects.

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CONCLUSION: Henna extract is a safe and potential alternative in treating otomycosis even with tympanic membrane perforation, as it has no vestibulotoxic adverse effects.

5 succinct key points:

1. Animal phase trials using laboratory rats are widely used for otological studies as the middle and inner ear anatomy is nearly identical to humans.
2. Henna leaf (*Lawsomnia inermis*) is widely available and has anti-fungal properties, but the safety profile has never been studied to be used as an anti-fungal ear drops solution.
3. Henna extract can potentially be used as an alternative antifungal ear drops without any vestibulotoxic effect in an animal study.
4. Henna extract is safe to be used even with tympanic membrane perforation without vestibulotoxic effect in an animal study.
5. Henna extract has the potential to be commercialized as a new drug after thoroughly assessing the cochleovestibulotoxicity effect in human trials.

Keywords :

Animal; Endoscopic; Henna; Ototoxicity; Vestibulotoxicity

Introduction

Otomycosis is an infection of the external auditory canal caused by a fungus commonly observed in the general otology clinic. It has a global prevalence of 9% to 30% among patients with otalgia and otorrhea symptoms [1]. The occurrence of otomycosis differs according to various climatic factors, with higher prevalence in tropical and subtropical regions, where it is warm and humid. The most prevalent pathogenic fungi isolated in otomycosis are *Aspergillus niger* and *Candida albicans*. There is currently no standardized medication regimen for otomycosis that offers novel therapeutic choices, including herbal medication [2].

According to the World Health Organization, herbal plants are one of the ideal sources for obtaining a range of medicines. Hence, these plants should be studied further to discover their benefits, safety, and effectiveness. Henna leaf (*Lawsonia inermis*) has long been valued for its healing and medicinal properties since ancient times. The therapeutic effects of various components of Henna are due to its biologically active compound of 2-hydroxynaphthoquinone (Lawsone) [3]. Studies have shown that henna leaf extracts in Malaysia inhibit the development of pathogenic fungi [4].

For centuries, henna leaves have been used in Africa, the Middle East, and Asian countries due to their healing attributes, such as antifungal, antibacterial, anti-amoebiasis, and antihemorrhagic effects [5]. This is via the action of its phytoconstituents β -sitosterol glucoside, flavonoids, quinoids, naphthalene derivatives, luteolin, betulin, lupeol galic acid, coumarins, xanthenes, and phenolic glycosides [6]. Every part of the henna tree has been studied and found to have antifungal and antitubercular activity [7], while the bark has been found to show anti-inflammatory activity [8]. While its capacity to inhibit the growth of pathogenic fungi causing otomycosis has been well established [4, 5, 7, 9], this medicinal plant's ototoxicity status is still unknown. Ototoxicity is defined as cellular and functional impairment of the inner ear by therapeutic agents and can be classified into cochleotoxic and vestibulotoxic [10].

This study aims to determine the vestibulotoxic effect of the henna extract with the possibility of developing it as an antifungal preparation in otomycosis. Balance disruptions as a drug-side effect are frequently overlooked by doctors, whereas patients often see it as part of their condition and affecting their overall health. Most drug-induced ototoxicity findings in patients are only restricted to the cochleotoxic effect. However, studies of the vestibulotoxic effect are scarce. This is a pilot project to evaluate the ototoxic effects of the henna extract by analyzing specifically the vestibulotoxicity in a rat animal model. To date, there is only one study assessing the vestibular side effects of neem leaf extract in an animal study [10].

Methodology

Preparation of Henna Leaf Extract

Six hundred grams of Malaysian henna leaves were collected from a single tree in Hospital Universiti Sains Malaysia (USM) in the district of Kubang Kerian. The henna leaves were collected fresh and healthy from the henna treetop without any fungal or bacterial contamination evidence. The leaves were green in colour; neither brown nor yellow were used to avoid contamination.

The henna preparation was conducted at the Pharmacology Department, School of Medical Sciences, USM. The leaves were cleaned in distilled water and dried at 50 degrees Celsius for two days until brittle. The dried leaves were weighed on a scale balance before being pulverized into a fine powder and stored in a tightly sealed glass jar.

Extraction by using a Soxhlet machine was used to prepare the sample. There were two types of solvents used: aqueous and 70% ethanol. After extensive freeze-drying, 12 grams of ethanol henna extract and 15 grams of aqueous henna extract were produced. These powdered extracts were utilized to determine the exact concentration of Henna required for this research, which is 25% aqueous extract and 25% ethanol extract (Figure 1).

Preparation of the Rat Animal Model

Twenty-four healthy male, mature Wistar Kyoto rats weighing between 300g to 400g were used for this study. They consisted of 24 males aged three months old. They were housed in a typical Macrolon cage (280×520×145mm) with wood shavings as bedding, three to a cage. They were kept in a facility with 12 hours of on/off light cycles, replicating the regular day and night cycle at a constant temperature ($20 \pm 2^\circ\text{C}$) and humidity (55.5%). The rats were classified into three groups: Group A (8 animals), Group B (8 animals), and Group C (8 animals).

The sample size of 24 rats was calculated via the 'resource equation' method, a commonly used method in animal studies. The inclusion criteria were Wistar male rats with normal vestibular parameters and intact tympanic membrane. The exclusion criteria were Wistar rats with the diseased external ear canal, the abnormal external ear canal, abnormal tympanic membrane, abnormal vestibular profile before perforation of the tympanic membrane and female albino rats. The ARRIVE Guidelines 2.0 was the reporting guideline that has been followed in this study.

The four baseline vestibular parameters were tested in all rats in each group. The parameters tested were dyskinetic head movements and circling behaviour, tail-hanging test, air-righting reflex, and contact inhibition of the air-righting reflex. The animals were observed and rated for the presence or absence of the following signs, while the observers were blinded to the treatments given. The scorings were rated 0, 1, and 2 for every parameter tested. Zero indicates normal vestibular behaviour, while one indicates mild abnormalities, and two indicate severe abnormalities observed. The signs of vestibulotoxicity were assessed using published procedures as per the following [11]:

Dyskinetic head movements and circling

Each rat is placed in an observation chamber and monitored for dyskinetic head movements (head weaving) and circling for 2 minutes (Figure 2).

Tail hanging

Each of the rats was raised by the tail, and the response was carefully examined and assessed in the following ways: 0-straight body posture with forelimbs extended toward the earth (normal), 1-slight bending of the body ventrally (intermediate response), and 2-consistently bending the body, occasionally crawling up towards its tail (severe response).

Air righting reflex

The rats were held supine and dropped onto a foam cushion from 30–40 cm height. The response was assessed as follows: 0-successful in righting themselves and landing straight on their feet (normal), 1-inadequate in

righting themselves or landing on the side (intermediate response), and 2-completely unsuccessful in righting themselves and landing on their backs (severe response).

Contact inhibition of righting reflex

The rats were positioned supine on a horizontal surface, and another horizontal surface was placed slightly in touch with the supine animal's feet soles. The rating was done as follows: 0-the animal successfully righted itself (normal), 1-partial righting, in which the animal exerts some effort (intermediate response), and 2-complete loss of righting, in which the animal faces up its feet and walks on the upper surface (severe response).

Ketamine (75-90 mg/kg, Ketalar, Pfizer, Istanbul, Turkey) and xylazine (5-8 mg/kg, Rompun, Bayer, Leverkusen, Germany) were used to anesthetize the rats intraperitoneally. The pedal reflex was used to determine the depth of anaesthesia, and further anaesthesia was provided in half-dose increments as needed. Each rat's external ear canal and tympanic membrane were examined following general anaesthesia using a zero-degree rigid Karl Storz surgical scope. Rats with abnormal external ear canals or a perforated tympanic membrane were excluded from this study.

After the baseline vestibular parameters testing, an endoscopic-guided transtympanic instillation of aqueous and ethanol henna extract was conducted into the right middle ear of the rats in Group A and B, respectively (Figure 3). At the same time, normal saline was instilled in the right middle ear of rats in Group C. A total of 0.1ml instillation of aqueous, ethanol extract, and saline for each rat was sufficient to fill the whole middle ear cavity of the rat.

The rats were given time to recover from the anaesthesia. The four vestibular parameters testing were assessed on the rat's post-instillation of transtympanic Henna at intervals of 4 hours, 24 hours, 48 hours, 72 hours, one week, two weeks, and three weeks.

The rats would then be euthanized intraperitoneally with thiopental sodium (200 mg/kg Pentothal; Abboth, Campoverde di Aprilla, Italy).

Results

In Group A (n=8), where the rats were subjected to transtympanic instillation of 25% ethanol henna extract, no vestibular dysfunction was observed at 4 hours, 24 hours, 48 hours, 72 hours, one week, two weeks, and three weeks post-instillation. Similar results were observed in Group B (n=8), where the rats were subjected to transtympanic instillation of 25% aqueous henna extract, and Group C (n=8), where the control group received transtympanic instillation of normal saline.

Provided the scores were 0 for the baseline and all post-test parameters (Table 1), statistical analysis could not be performed for this research. In other words, there was no impairment of all vestibular parameters documented post-instillation of both aqueous and ethanol henna extract in the middle ear, indicating that Henna has no vestibulotoxic effects after it is administered into the middle ear mucosa.

This study used 24 Wistar Kyoto rats consisting of 8 male rats for Groups A, B, and C, with the latter serving as a control group. The rats were three months old when this study was performed and weighed between 300 to 400g each.

Discussion

Otomycosis refers to fungal infections of the external ear, including the auricle, auditory canal, eardrum, and middle ear. The symptoms include otorrhea, otalgia, itchiness, and it is usually a recurrent disease [12, 13]. Fortunately, this entity is not life-threatening, and complications are uncommon except in immunocompromised patients as it is an opportunistic infection. However, this disease presents difficulties for the otolaryngologist and the patient, as it has a high recurrence rate and residual disease. Additionally, it necessitates long-term treatment and monitoring [13]. The mainstay of treatment for otomycosis is thorough

removal and cleaning of the infected area with topical antifungal ear drops [14]. Topical medications are preferable to systemic medications because they have a low risk of systemic side effects and a high concentration in the targeted infected area.

Aspergillus and *Candida* are the most prevalent fungal species isolated in otomycosis [13]. There has been a report of growing resistance to classical antifungal agents that contain azoles, polyenes, and echinocandin, which are usually effective against *Aspergillus* and *Candida* [15]. This is described as clinical resistance, which is the inability to eliminate a fungal infection despite the antifungal agents having in vitro action against the pathogen [15]. Currently, there is no FDA-approved antifungal otic solution for otomycosis treatment [16]. Physicians have struggled to discover the most efficient antimycotic medication to treat this ailment since there are numerous medicines available with varying antimycotic properties. This opens up the research of using herbal plants as an alternative drug to treat otomycosis.

According to the World Health Organization, herbal plants are one of the ideal sources for obtaining a range of medicines [17]. Hence, these plants should be studied further to evaluate their benefits, safety, and effectiveness. Henna leaf (*Lawsonia inermis*) has long been valued for its healing and medicinal properties since ancient times. The therapeutic effects of various components of Henna are due to its biologically active compound of 2-hydroxynaphthoquinone (Lawsone) [3]. Studies have shown that henna leaf extracts in Malaysia inhibit the development of pathogenic fungi [4]. Although commonly used as a natural remedy to treat skin, outer ear, and hair lesions, the safety status and possible ototoxic effects of henna leaves are still to be reported. Ototoxicity is identified as functional disability and cellular degradation of the inner hair cells by therapeutic drugs and chemical compounds and is categorized into cochleotoxicity and vestibulotoxicity [18].

Two Malaysian henna extracts were used, which are aqueous extract and ethanol extract, which, their fungitoxic activity has been established [4]. Yaroko et al. tested the antifungal activity of Malaysian henna leaf extract against two prevalent otomycotic pathogenic fungi, which were *Aspergillus niger* and *Candida albicans*. He discovered that the aqueous extract of Malaysian Henna is superior in inhibiting the growth of *A. niger*. In contrast, ethanol extract is more effective in suppressing the growth of *C. Albicans* [4]. Three concentrations of aqueous and ethanol henna extracts were used in this study; 25mg/ml, 50mg/ml, and 75mg/ml. Statistical analysis revealed significant inhibition of *A. niger* with aqueous extract concentrations of 75mg/ml and 50mg/ml, while ethanol extract was significant with a concentration of 75mg/ml only. Meanwhile, henna ethanol extract demonstrated near-significant inhibition against *C. Albicans* with a 75mg/ml concentration. The least inhibition was recorded with a 25mg/ml concentration for both extracts [4]. However, to ensure that the substance is administered into the ear canal and absorbed by the inner ear and middle ear mucosa, aqueous and ethanol henna at a concentration of 25% were used and instilled transtympanically [4]. Nadjib et al. also studied using the same concentration of 25% by mixing 25g of powdered henna plant with 100 ml of different solvents to test against *C.albicans* and various microbes [9]. Meanwhile, Zakaria et al. recommended 20mg/ml as the best concentration to inhibit many pathogenic fungi in aqueous or ethanol extract [19]. This concentration has been shown to have a sufficient antifungal effect in a thin diluted solution. It is suitable for physicians and patients to administer in the form of an ear drop.

Wistar rats are widely used in otological studies and were chosen in our study [20, 21]. The middle and inner ear anatomy is nearly identical to humans. The rat cochlear comprises two and a half turns, making it a good model for cochleotoxicity and vestibulotoxicity research [22]. The rats were all three months old when used in the experiment, equivalent to a 20-26-year-old human [23]. This is due to the widespread recognition of the age connection between mice and humans, particularly regarding neurological development.

The four established vestibular parameters observed in rats after transtympanic instillation of ototoxic agents were dyskinetic head movements and circling, tail hanging, air righting reflex, and contact inhibition of righting reflex [11]. A normal baseline vestibular parameters were ensured in all rats following the inclusion criteria. The henna extract was injected into the right middle ear of each rat for constant observational behaviour. Vestibular parameters were recorded at four hours, twenty-four hours, seventy-two hours, one week, two weeks, and three weeks after transtympanic instillation. Acute vestibular changes can be observed

in rats as early as 4 hours and as late as three weeks after transtympanic instillation. This is because rats can reestablish normal postural locomotor function following a temporary vestibular insult on one side [23]. The findings recorded by blinded observers were zero or normal responses in all rats at every vestibular interval tested. Hence, statistical analysis could not be implemented in this study.

To conclude, henna extract in aqueous and ethanol extract can safely be used in otomycosis without vestibulotoxic effect in an animal model, even with a tympanic membrane perforation. Malaysian Henna showed potential to be used as an alternative topical antifungal eardrop in treating otomycosis. We recommend conducting a histological study of the cochlear in an animal model to assess the effect on inner hair cells to fully establish the safety efficacy of Henna before human clinical trials.

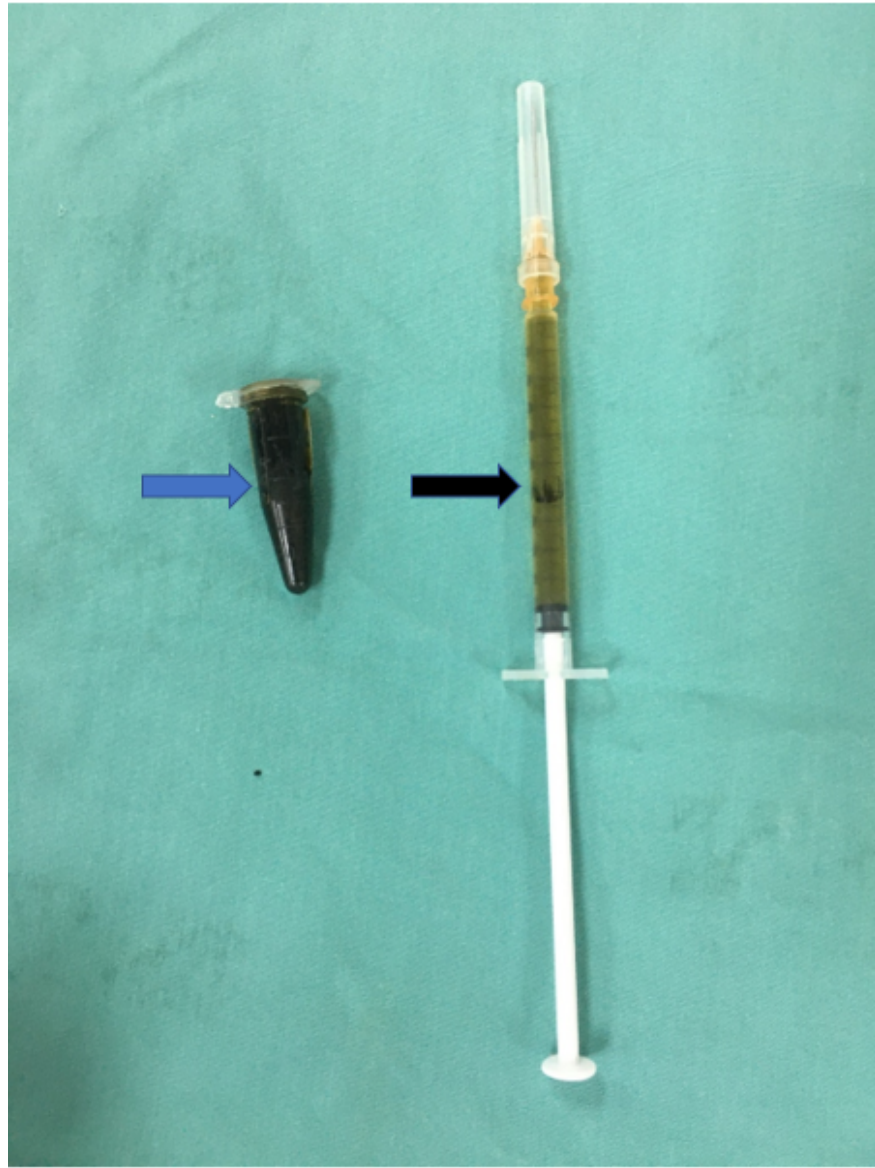
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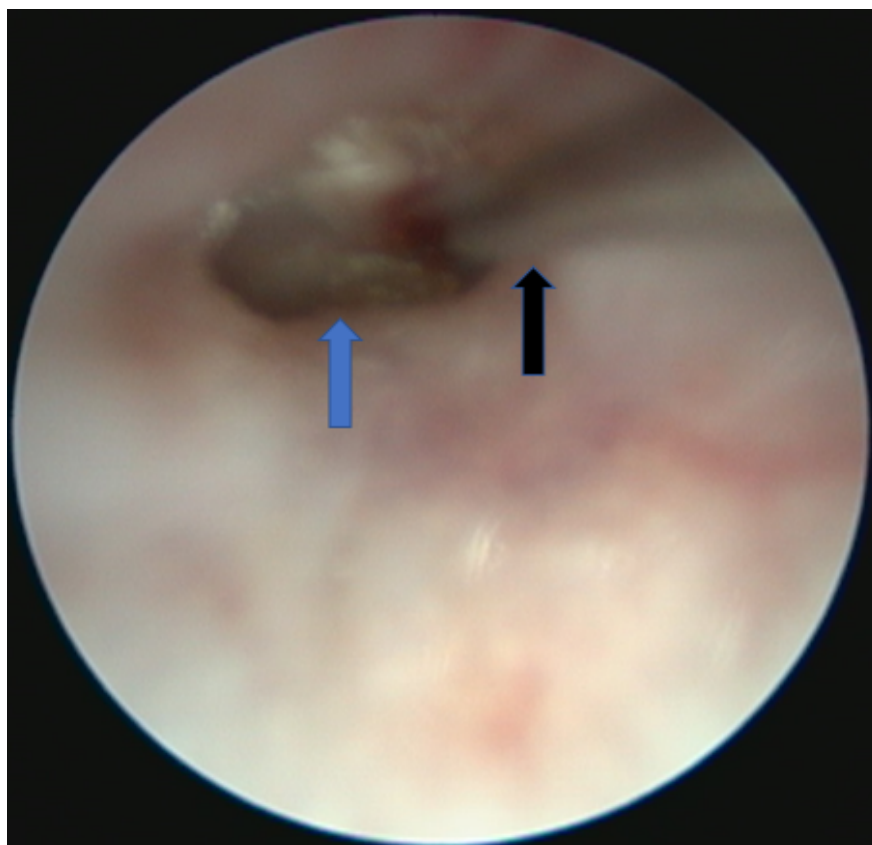
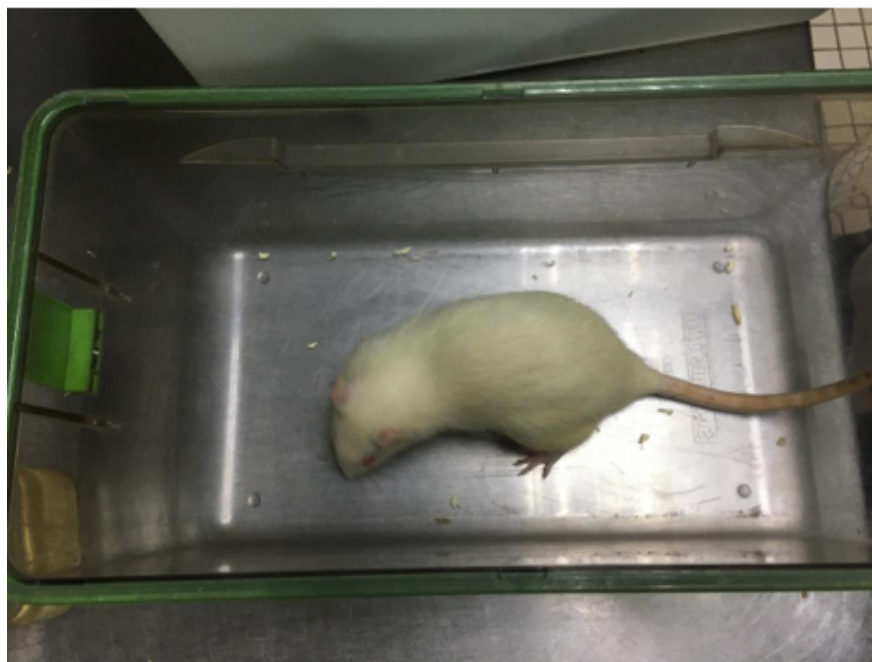
- [1] Agarwal P, Devi LS. Otomycosis in a rural community attending a tertiary care hospital: Assessment of risk factors and identification of fungal and bacterial agents. *J Clin Diagn Res* 2017; 11:Dc14.
- [2] Saniasiaya J, Salim R, Mohamad I, Harun A. Antifungal effect of Malaysian aloe vera leaf extract on selected fungal species of pathogenic otomycosis species in in vitro culture medium. *Oman Med J* 2017; 32:41.
- [3] Tripathi RD, Srivastava HS, Dixit SN. A fungitoxic principle from the leaves of *Lawsonia inermis* Lam. *Experientia* 1978; 34:51-2.
- [4] Yaroko AA, Salim R, Mohamed Z, Mohamad I. Antifungal activity of Malaysian Henna leaves extracts on pathogenic fungi of otomycosis. *Int Med J* 2015; 22:389-91.
- [5] Khattak SG, Gilani SN, Ikram M. Antipyretic studies on some indigenous Pakistani medicinal plants. *J Ethnopharmacol* 1985; 14:45-51.
- [6] Lal JB, Dutta SB. Constitution of the colouring matter of *Lawsonia alba* or Indian Mehndi. *J Indian Chem Soc* 1933; 10:577-9.
- [7] Bhatnagar SS, Santapau H, Desa JD, Maniar AC, Ghadially NC, Solomon MJ et al. Biological activity of Indian medicinal plants. Part I. Antibacterial, antitubercular and antifungal action. *Indian J Med Res* 1961; 49:799-813.
- [8] Singh VK, Pandey DK. Fungitoxic studies on bark extract of *Lawsonia inermis* against ringworm fungi. *Hindustan Antibiot Bull* 1989; 31:32-5.
- [9] Rahmoun N, Boucherit-Otmani Z, Boucherit K, Benabdallah M, Choukchou-Braham N. Antifungal activity of the Algerian *Lawsonia inermis* (Henna). *Pharm Biol* 2013; 51:131-5.
- [10] Salim R, Daud Mk, Narayanan Ms, Ab Rani A. Assessment of vestibulotoxicity of Neem leaf (*Azadirachta indica*) in a rat animal model. *Indian J Otol* 2020; 26:159.
- [11] Al Deeb S, Al Moutaery K, Khan HA, Tariq M. Exacerbation of iminodipropionitrile-induced behavioral toxicity, oxidative stress, and vestibular hair cell degeneration by gentamicin in rats. *Neurotoxicol Teratol* 2000; 22:213-20.
- [12] Jadhav VJ, Pal M, Mishra GS. Etiological significance of *Candida albicans* in otitis externa. *Mycopathologia* 2003; 156:313-15.
- [13] Ho T, Vrabec JT, Yoo D, Coker NJ. Otomycosis: clinical features and treatment implications. *Otolaryngol Head Neck Surg* 2006; 135:787-91.
- [14] Vennewald I, Klemm E. Otomycosis: diagnosis and treatment. *Clin Dermatol* 2010; 28:202-11.
- [15] Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanisms and clinical impact. *Clin Infect Dis* 2008; 46:120-8.

- [16] Nemati S, Hassanzadeh R, Jahromi SK, Abadi AD. Otomycosis in the north of Iran: common pathogens and resistance to antifungal agents. *Eur Arch Otorhinolaryngol* 2014; 271:953-7.
- [17] Rates SM. Plants as source of drugs. *Toxicon* 2001; 39:603-13.
- [18] Hawkins JE, Keidel WD, Neff WD. Handbook of sensory physiology. Drug ototoxicity 1976; 5:707.
- [19] Almola Z. The inhibitory effect of Henna *Lawsonia inermis* leaves on some fungi. *Iraq Acad Sci J* 2010; 10:501-10.
- [20] Fetoni AR, Quaranta N, Marchese R, Cadoni G, Paludetti G, Sergi B. The protective role of tiopronin in cisplatin ototoxicity in Wistar rats. *Int J Audiol* 2004; 43:465-70.
- [21] Rybak LP, Somani S. Ototoxicity. Amelioration by protective agents. *Ann N Y Acad Sci* 1999; 884:143-51.
- [22] Albuquerque AA, Rossato M, Oliveira JA, Hyppolito MA. Understanding the anatomy of ears from guinea pigs and rats and its use in basic otologic research. *Braz J Otorhinolaryngol* 2009; 75:43-49.
- [23] Dutta S, Sengupta P. Men and mice: relating their ages. *Life sci* 2016; 152:244-8.

Figures legend:

1. Figure 1: The final extract in paste form (blue arrow) with 100% concentration. One cc syringe was used for intratympanic instillation after serial dilution into the exact concentration of 25% aqueous and ethanol Henna extract each (black arrow).
2. Figure 2: Observation and scoring of the rat vestibular parameter (dyskinetic head movements and circling) in a glass cube.
3. Figure 3: Endoscopic-guided intra-tympanic instillation of henna extract. The blue arrow shows the rat's right tympanic membrane and the black arrow shows the 25-gauge needle attached to the 1cc syringe for intratympanic instillation.





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