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In Vitro Activities of *Lawsonia inermis* L. (Henna) Leaves Extract

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Abstract
Lawsonia inermis (henna) is the medicinal plant. The study has been done to evaluate the preliminary phytochemical, proximate analysis and the bioactive potential of *L. inermis*. Phytochemicals viz., flavonoids of flavonoid, phenol, steroids, resins, tannins and carbohydrates were found to be present. Thin layer chromatography was also done for the phytochemical analysis. Total phenol content by using Folin-Ciocalteau reagent and total flavonoid content were determine by using Woisky and Salatino method. Phenol and flavonoid content found to be 1158mg/g and 322.5mg/g respectively. In fluorescent analysis acetone, ethanol, methanol, glacial acetic acid, chloroform, diethyl ether, petroleum ether and benzene gave characteristics red fluorescence under long UV_{365} nm. Methanol extract of henna leaves found to have antimicrobial activity against *Pseudomonas aeruginosa and Bacillus subtilis* using disc and well diffusion method. Extract was used to evaluate antiarthritic potential. In which soxhlet water extract showed remarkable inhibition of protein denaturation and was found to be 71.41 ± 0.01. In thrombolytic activity aqueous extract was found to be significant which showed maximum about 52.62 ± 0.017 of clot lysis. The free radical scavenging by DPPH method of methanolic extract of henna leaves showed 71.7 ± 0.02 antioxidant activity. The soxhlet methanol extract of henna leaves showed 1101± 0.02 µg/ml antioxidant activity.

Keywords
Anti-arthritic, antidiabetic, antioxidant, Bioautography, *Lawsonia inermis,* phytochemical, proximate analysis, Thin Layer Chromatography.

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1. INTRODUCTION:
Lawsonia inermis L. belongs to Lythraceae family is commonly called as Henna. It is a medicinal plant and also a popular dye plant [24]. *Lawsonia inermis* is a shrub mainly found in Asian origin and also in warm temperature regions [31]. Since a long time, henna has been used as herbal dye to color hands, nail, feet etc. For this coloring purpose the leaves of henna are mainly utilized. It is also used as deodorant, excellent conditioning agent and also used as an ingredient in shampoos, hair dyes and rinses [31]. The dye is also used in staining leather hides in various industry and also important in commercial uses.
L. inermis also shows medicinal application, it is used as an astringent, anti-hemorrhagic, intestinal antineoplastic activity, cardio-inhibitor, hypotensive, sedative and anti-inflammatory [31]. It is also used against amoebiasis, soothe fevers, nervous disorder, hysteria and also leprosy [31]. The lower concentration of plant leaves used for body pain, skin

infections, reduce lesions after bee sting and also to reduce allergy. The leaves in form of paste also shows various application in skin diseases, boils burns, headache, brulies and also rubbed over the burning feet [31].

L. inermis contains a number of compounds which are present in various part of plant. This compound includes Lawsone, phenolic glycosides, gallic acid, Xanthones, quinones, isoplumbasin and also contains triterpenoids [31]. But the principle compound in the *L. inermis* is Lawsone (2-hydroxy-1, 4-naphthoquinone) it also called as hennonaphthanic acid. Lawsone is used as a sensitive cyanide and electrochemical sensor for various anions such as fluoride, acetate and acetonitrile containing dihydrogen phosphate [12]. Latent fingermarks present on the paper surface also detected by lawsone [13]. Lawsone also shows plasmid curing activity [23]. *L. inermis* (henna) contain various bioactive compound, thus present study evaluated the bioactive potential of *L. inermis* using various method.

2. MATERIAL AND METHODS:

2.1 Collection and Processing of Plant Material:

The henna leaves sample were collected and dried under shade. Grind into fine powder by using mortar-pestle and store in plastic container until the use [32].

2.2 Extraction of Henna leaves:

The extraction had been carried out by cold and soxhlet method. In cold extraction, henna leaves powder and solvents (hexane, methanol, ethyl acetate and water) in (1:10) proportion was used. In soxhlet extraction Soxhlet method was done [19].

2.3 Phytochemical analysis of plant extract:

Phytochemical analysis of plant extract was done to detect secondary metabolites which includes Alkaloids, Flavonoids, Steroids, phenols, Tanins, Resins, Terpenoids and Carbohydrates [20].

2.4 Thin Layer Chromatography:

Qualitative analysis of phytochemical present in henna leaves extract was done by using TLC silica gel 60. The phytoconstituents were qualitatively determined [7, 30]. Retention time (R_f) were calculated.

2.5 Fluorescence study:

Fluorescence study is important for the determination of crude drug. The henna powder with different solvent was analyzed under long UV_{365} and short UV_{254} light as well as visible light. Different type of emitted color was observed [20].

2.6 Total phenol content determination:

Folin-Ciocalteu method [29] was used to determine total phenol content in henna plant. The determination of total phenol content from the sample was expressed in terms of Gallic acid equivalent (GAE) in mg/gm of dry weight.

2.7 Total Flavonoid content determination:

The total flavonoid content of henna plant was done by Woisky and Salatino (1998) method [21]. The determination of total flavonoid content from the sample was expressed in term of quercetin equivalents in mg/gm of dry weight.

2.8 Determination of proximate content:

2.8.1 Ash value: Quality and purity of crude extract is depending on ash values. As per standard protocol [5] total content of ash, water soluble and acid insoluble ash was determined.

2.8.2 Extractive values: The water soluble and alcohol soluble extractive value was determined which shows the presence of active constituents in henna [5].

2.8.3 Moisture content: Moisture is important for activation of enzyme and also provide favorable condition for growth of living microorganism. As per standard protocol of moisture content was determined [5].

2.9 Antimicrobial activity: The antimicrobial potential of henna leaves extract was carried out by using disc diffusion method [14] and well diffusion method (Bauer *et.al.1966*) against pathogenic microorganisms (*Shigella, Pseudomonas, Bacillus, S.paratyphi.* etc.).

2.10 Bioautography: The antimicrobial compound detection was studied by agar overlay method (Immersion bioautography). The Thin Layer Chromatography sheet (silica gel 60) was used to detect antimicrobial activity of henna leaves extract against pathogenic microorganism. Antimicrobial compounds were detecting by using bioautography [22].

2.11 *In vitro* anti-arthritic activity: The inhibition of protein denaturation method was used for *in vitro* anti arthritic activity. Diclofenac sodium solution was used as standard. Bovine serum albumin was used as protein source for protein denaturation. The absorbance was recorded spectrophotometrically at 450 nm. The control solution showed 100% denaturation, the recorded result was compared with the diclofenac sodium as a standard [16].

2.12 *In vitro* thrombolytic test: The *in vitro* thrombolytic test was done for thrombolytic activity [25]. The blood was collected from healthy person and distributed into sterile pre weighted eppendorf tube (1ml in each tube) and incubated at 37^0C for

45min. After the incubation serum was separated from clot and clot weight measured.100µl of test sample and 100µl distilled water in test tube and control tube respectively added. Tubes were incubated at 37^0C for 90min. Release fluid material was removed after incubation and percent clot lysis was calculated.

% of clot lysis = (weight of released clot / clot weight) × 100

2.13 Antioxidant activity: The *in vitro* antioxidant activity of the henna leaves extract was carried out by two methods: Ferric Reducing Antioxidant Power (FRAP) and DPPH Free radical scavenging method.

2.13.1 Ferric Reducing Antioxidant Power (FRAP): Benzie and strain method [4] was used to estimate antioxidant capacity of henna plant. In this method 3.995 ml of freshly prepared FRAP reagent and 5µl diluted plant extract was mixed and instant blue color complex was observed. The blue color complex was due to ferric tripyridyl triazine (Fe^{3+} TPTZ) complex were reduce into ferrous tripyridyl triazine (Fe^{2+}TPTZ) form. The reduce Fe^{3+} was observed at 593nm after incubation of 30min. at 37^0C. Concentration of Fe^{2+} expressed as mg of trolox equivalent per gram of sample.

2.13.2 Free radical scavenging DPPH: The free radical DPPH is a stable compound which was used to measure free radical scavenging activity. Methanol was used for solubilization of DPPH (0.3mM). 1ml of methanolic henna extract and 2ml DPPH was mixed well, and tubes were incubated in dark at room temperature for 30min. After incubation absorbance was taken at 517 nm. Ascorbic acid was used as standard, as per standard protocol [3].

% Antioxidant activity= (Abs control - Abs sample / Abs control) × 100

2.14 *In vitro* Antidiabetic Activity: *In vitro* antidiabetic activity of henna leaves extract was carried out by two methods: Inhibition assay for α-amylase activity and Glucose diffusion inhibitory study.

2.14.1 Inhibition assay for α-amylase activity: Four different concentration of henna plant extract (25mg/ml, 50mg/ml, 75mg/ml and 100mg/ml) was prepared in double distilled water. These four concentrations were used as per standard protocol for inhibition assay for α-amylase activity [1].

2.14.2 Glucose diffusion inhibitory study: A dialysis membrane (Dialysis membrane -70 approximately capacity 2.41ml/cm) containing prepared henna leaves extract and glucose solution (0.2mM in 0.15M sodium chloride) was kept in beaker which contain 40ml of 0.15M sodium chloride and 10 ml distilled water. The beakers were placed at room temperature on orbital shaker, and glucose movement into external solution was observed. The protocol was carried out in triplicates [1].

2.15 Antihelminthic Activity: An adult Indian earthworm *Asonia putida* was used for antihelminthic activity of soxhlet methanol extract, soxhlet water and aqueous extract of henna leaves [8].

3. RESULT & DISCUSSION:

The primary phytochemical analysis of henna leaves extract for the four solvents such as aqueous, ethyl-acetate, hexane and methanol extract are given in table-1. In this extract the phytochemical analysis showed the presence of flavonoids, phenols, steroids, resins, tannins and carbohydrates in the different concentration. Alkaloids, terpenoids, quinines, carboxylic acid and proteins were completely absent in all four extract.

Different types of phytochemicals phytochemicals were known to have significant in medicinal and industrial science. Some of these compounds are recorded to show the important roles of henna leaves extract such as: Steroid are known to significant for cardiotonic and antimicrobial activity. Steroids are widely used in herbal medicine and cosmetics [6]. Tannin is also recorded for the antibacterial, antitumor and antiviral activity. Some tannin is recorded for the inhibition of HIV [11]. The secondary metabolites in different concentration are present or absent in the plant leaves extract. Solubility of metabolites content of sample is different in various solvent [15]

Table 1: Phytochemical analysis henna leaf extract:

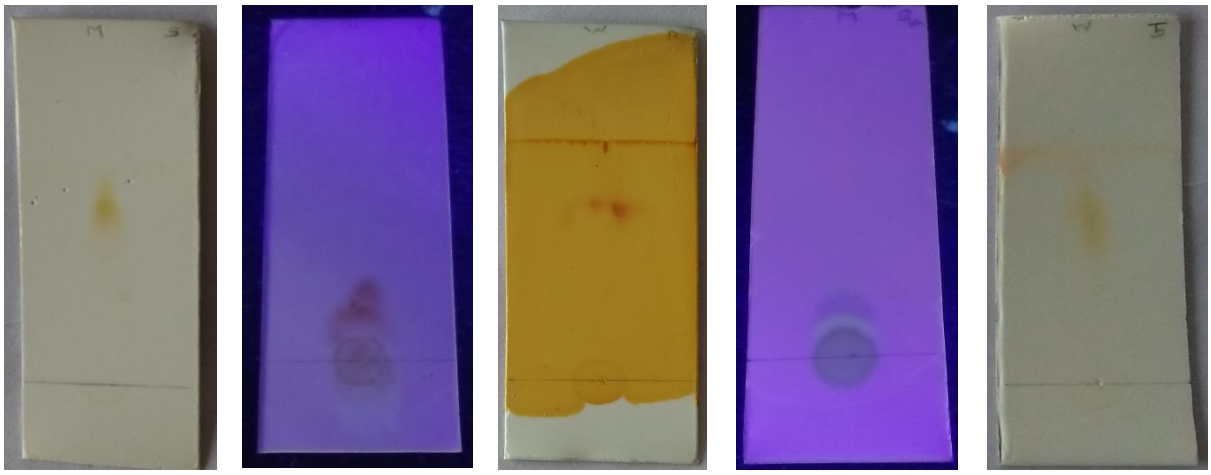
	Henna leaf Extract using			
	Aqueous	**Methanol**	**Ethyl- acetate**	**Hexane**
1. Alkaloids				
a) Mayer's test	–	–	–	–
b) Wagner's test	–	–	–	–
2. Flavonoids	+	+	–	–
3. Phenols	+	–	+	+
4. Terpenoids	–	–	–	–
5. Steroid	+	+	–	–
6. Resins	–	+	–	–
7. Tannins	–	–	+	+
8. Quinones	–	–	–	–
9. Carboxylic acid	–	–	–	–
10. Proteins	–	–	–	–
11.Carbohydrate	–	+	–	–

Key: - = Absent, + = Present

The henna leaves extract was summarized by Thin Layer Chromatography Technique. The different solvent systems were used for different phytochemical analysis. Every phytochemical showed different retention factor value with their corresponding solvent system. The R_f value of different phytochemical are showed in table no: 2. The retention factor (R_f) value of flavonoid was found to be maximum which is 0.73 and the minimum R_f value was found in phenol which is 0.26(fig.1).

Table2: R_f values of the chemical in the henna leaves extract:

Sr no.	Phytochemicals	R_f
1)	Steroids	0.7
2)	Alkaloids	0.28
3)	Flavonoid	0.73
4)	Tannin	0.63
5)	Phenols	0.26



a b c d e

Fig.1 Thin Layer Chromatography (Silica gel 60) of phytochemicals
a. Steroids b. Alkaloids. c. Flavonoids. d. Tannin. e. Phenols

Seventeen different chemical reagents were used for fluorescence analysis of henna leaves powder was shown in (Table 3). The result of this analysis showed green, dark green color under visible light whereas green and brown shades under short UV_{254} light. Out of these seventeen different solvents, henna leaves powder with acetone, ethanol, methanol, chloroform, diethyl ether, petroleum ether, benzene showed characteristics Red fluorescent under long UV_{365} light (fig.2) [8]

Table 3: Fluorescence analysis of henna leaf powder:

Sr.no	Solvents	Visible light	Short UV_{254nm}	Long UV_{365nm}
1	Distilled water	Faint brown	Brown	Greenish red
2	Acetone	Dark brown	Dark green	Red Fluorescent
3	Ethanol	Faint green	Faint green	Red Fluorescent
4	Methanol	Faint green	Dark green	Red Fluorescent
5	Glacial acetic acid	Dark green	Dark brown	Red Fluorescent
6	Sulphuric acid	Blackish green	Brownish black	Black
7	Nitric acid	Greenish yellow	Orange	Yellowish red
8	Hydrochloric acid	Blackish green	Greenish black	Black
9	5%Fecl3	Greenish black	Dark brown	Black
10	5%I2	Brown	Brown	Green
11	Picric acid	Yellowish green	Dark yellow	Greenish red
12	1N NaOH	Dark green	Dark brown	Dark green
13	1NNaOH+Methanol	Dark green	Dark brown	Greenish red
14	Chloroform	Greenish	Brown	Red Fluorescent
15	Diethyl ether	Faint bottle green	Green	Red Fluorescent
16	Petroleum ether	Faint bottle green	Yellowish green	Red Fluorescent
17	Benzene	Dark green	Brown	Red Fluorescent

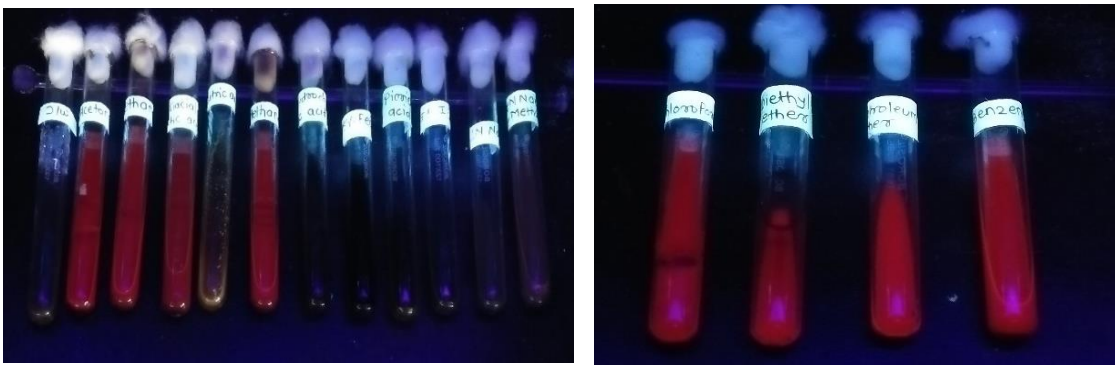


Fig 2. Fluorescence analysis under long UV_{365nm}

Ash value is important factor in determining the authenticity as well as purity of henna leaves powder. It is considered as major qualitative standard. The total cash value of henna leaves powder was found to be 41%. The extractive value containing acid soluble ash and water-soluble ash was found to be 73% and 12% respectively. It is found that the acid soluble ash is maximum than the water-soluble ash which was 73% [5].

Moisture is the important factor which plays crucial role in deterioration of plant powder and also an inevitable compound. The moisture content of henna leaves powder was found to be 5%. Low moisture content gives the higher stability to the compound [5].

Total solid content is the amount of residue remains after the release of moisture is found to be 95% for henna leaves powder. The carbohydrate fraction that remains after the acid and alkali treatment is the crude fibre content which is found to be 15.35% for henna leaves powder. (Table 4). The extract was found to contain 1158 mg GAE/g DW phenolic Content. Various biological effect found due to flavonoid and phenol compound of plant material have been reported. A Coloured complex from *L. inermis* 2 % $AlCl_3.6H_2O$ measured at 440nm. From standard quercetin curve (Y=0.0031x) the total

flavonoid content 322.5 mg/gm was found in *L. inermis* leaves.

Table 4: Physicochemical constants analysis and phenolic and flavonoids content of henna leaf:

Sr.No.	Experimental studies	Observations for powdered leaf of *L. inermis*
1)	Total ash	41%
2)	Acid-insoluble ash	73%
3)	Water-soluble ash	12%
4)	Moisture Content	5%
5)	Total solid content	95%
6)	Crude fibre content	15.35%
7)	Total phenolic content	1158mg/g
8)	Total flavonoid content	322.5mg/g

To develop a new chemotherapeutic agent, plants are main source. In present study, Methanolic henna extract was used against *Shigella, S. paratyphi, Pseudomonas* and *Bacillus.* The methanol extract showed significant activity in both disc diffusion as well as in well diffusion method against *Bacillus* species, which is found to be 18±2.65 mm, 24.3± 2.08 mm respectively (Table 5).

Table 5: Disc diffusion and well diffusion method:

Name of Pathogen	Mean ± SD [Zone of inhibition using Disc diffusion method (mm)]	Mean ± SD [Zone of inhibition using Well diffusion method (mm)]
	Methanol Extract	Methanol extract
Shigella spp.	4.66± 1.52	33±2.64
S. paratyphi	1.6± 0.5	7.3±1.15
Pseudomonas aeruginosa	9.3± 1.15	14.6±2.51
Bacillus subtilis	18± 2.65	24.3± 2.08

In bioautography, Rf value of bioactive compound found to be 0.9. The zone of inhibition found to be 19 mm for *Bacillus subtilis* and 17 mm for *Shigella*. Bioautography is one of the techniques for testing antimicrobial activity of plant extracts. This technique not just separates the components but also tests its antimicrobial activity (fig.3).

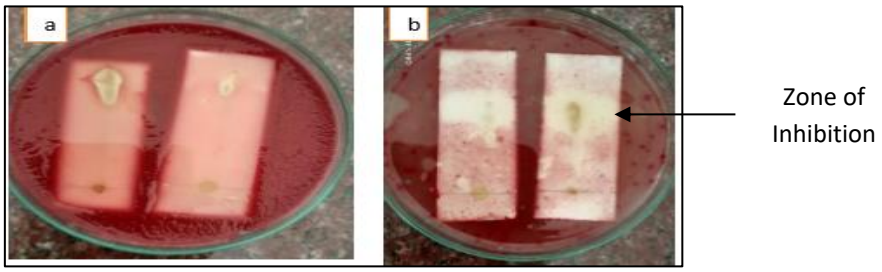


Fig3. Bioautography of henna leaves extract against a) *Bacillus* b) *Shigella*

In vitro anti-arthritic activity of henna leaves soxhlet aqueous extract exhibit maximum activity about 74.41±0.01 than the aqueous and soxhlet methanol extracts. The result compared with Diclofenac sodium (standard) showed 100% *in vitro* anti arthritic activity (Table 7).

Table 6: Thrombolytic test of henna leaf extract

Sr.No	Test Sample	Weight of the empty tube(A)gm	Weight of the tube with clot(B)gm	Weight of clot. (C) C=B-A	Weight of the tube with clot after lysis(D)gm	Weight of lysis(E) (B-D)	Mean ± SD (% of clot lysis)
1)	Aqueous extract of *L. inermis* leaf powder	1.03	1.980	0.95	1.48	0.5	52.62±0.017
2)	Soxhlet water extract of *L. inermis* leaf powder	1.02	2.030	1.01	1.65	0.38	37.62±0.015
3)	Soxhlet Methanol extract of *L. inermis* leaf powder	1.04	2.140	1.136	1.63	0.51	44.88±0.015
4)	Distilled Water	1.04	2.030	0.99	2.029	0	0

Clot lysis measured through various method [28]. Aqueous extract of henna leaves showed maximum clot lysis up to 52.62%. In the tube with distilled water (negative control) there was no release of clot (Table 6). The problem associated with blood circulation is mainly due to blood clot formation. Blood clot blocks the blood flow [28].

Table 7: *In vitro* anti-arthritic and thrombolytic activity of henna leaf extract

Sr no.	Test sample	Antiarthritic (% activity)	Thrombolytic (% activity)
1	Aqueous extract of *L. inermis* leaf powder	45.44±0.015	52.62±0.017
2	Soxhlet water extract of *L. inermis* leaf powder	74.41±0.01	37.62±0.015
3	Soxhlet Methanol extract of *L. inermis* leaf powder	29.83±0.76	44.88±0.015
4	Standard	100 ±0.02	100± 0.02

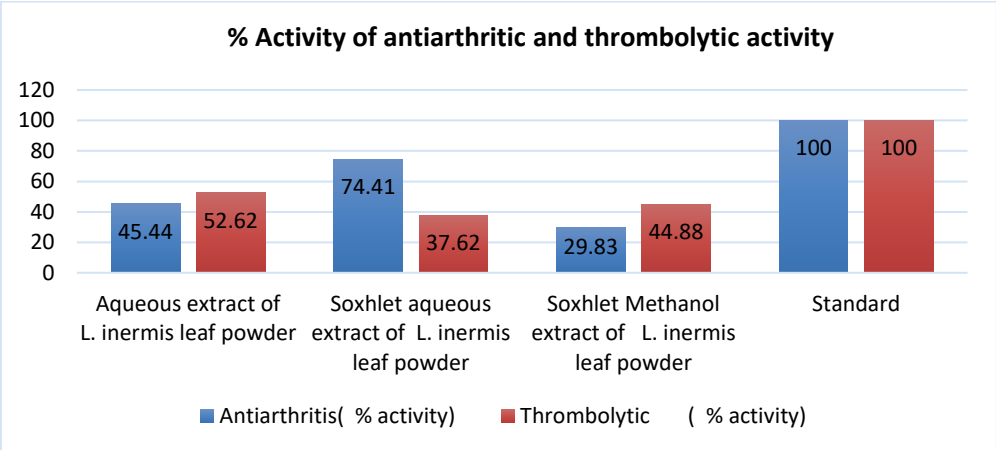


Fig 4. Antiarthritic and thrombolytic activity of *L. inermis*

The soxhlet aqueous extract of henna leaves showed 71.41 ±0.01 % activity for antiarthritic and 37.62 ±0.015 % activity for thrombolytic. The soxhlet aqueous extract was potent for antiarthritic activity than the thrombolytic activity (Fig.4.)

The recent study shows DPPH, FRAP assays are commonly used for antioxidant activity

determination [26]. Reducing power serves as a significant factor for antioxidant activity. Coloured produced through ferrous tripyridyltriazine (Fe $^{2+}$ TPTZ)) from reduction of ferric tripyridyltriazine (Fe3+ TPTZ) complex measures the antioxidant reacting capability of henna leaves extract. The absorbance of blue colored complex was taken at 593nm result from the study (table 8.1) shows the Soxhlet Methanol extract has higher antioxidant capacity than other extracts. 1101 ± 0.02 µg per trolox equivalent recorded for Soxhlet methanol extract of henna whereas 400.66 ± 0.04 µg per trolox recorded for henna aqueous extract, showed lowest reducing power. Total phenolic content and antioxidant activity shows linear correlation [26] which agreemental statement with reported [34] the concentration of Fe $^{2+}$ for different henna leaves extract. The oxygen capacity of many plant product is due to the phenols [29].

Table 8.1: Antioxidant activity by FRAP assay

Sr.no	Test Sample	Mean ± SD (Trolox per µg equivalent)
1)	Aqueous extract of *L. inermis* leaf powder	400.66 ± 0.04
2)	Soxhlet water extract of *L. inermis* leaf powder	795 ± 0.02
3)	Soxhlet Methanol extract of *L. inermis* leaf powder	1101 ± 0.02

DPPH method is a rapid and easy method to evaluate the antioxidant activity [33]. Methanolic extract studied for free radical scavenging capacity (DPPH). Structural confirmation gives the relation between potential antioxidant and DPPH [33].

Table 8.2: Antioxidant activity by free radical scavenging DPPH assay

Sr no.	Test sample	Mean ± SD (% Activity)
1)	Methanolic extract of *L. inermis* leaf powder	71.7 ± 0.02

Over worldwide, more than 100 million people (6% of population) suffer from Diabetes mellitus (DM). The result obtained from DNSA study was given in (table 9.1). All concentration showed significant inhibitory activity, 50 mg/ml concentration of henna leaves extract showed maximum inhibition of α-amylase enzyme whereas 100% mg/ml plant extract exhibits lowest enzyme activity .*Allium sativum*, *Aloe vera*, *Murraya koeingii* reported plants with antidiabetic activity [9]

Table 9.1: Inhibition assay for α-amylase activity:

Sr. no	Concentration of henna leaves extract (mg/ml)	Mean ± SD (% Activity)
1)	25	75.3± 1.15
2)	50	76.3 ±1.52
3)	75	71.6 ± 1.52
4)	100	66 ± 1

Aqueous extract of *L. inermis* showed effective antidiabetic activity at different time intervals. After 120 minute 30 ± 0.015 maximum inhibitory activity of glucose diffusion was observed (Table 9.2).

Table 9.2: Effect of aqueous extract of *L.inermis* on glucose diffusion

Sr no.	Time (min.)	Control	*L. inermis*	Mean ± SD (% Relative movement)
1	30	25	12.5	50 ± 0.01
2	60	25	10	60 ± 0.015
3	90	25	15	40 ±0.012
4	120	25	17.5	30 ±0.015
5	150	25	15	40 ± 0.01
6	180	25	15	40 ± 0.02

Concentration of glucose (µg/ml)

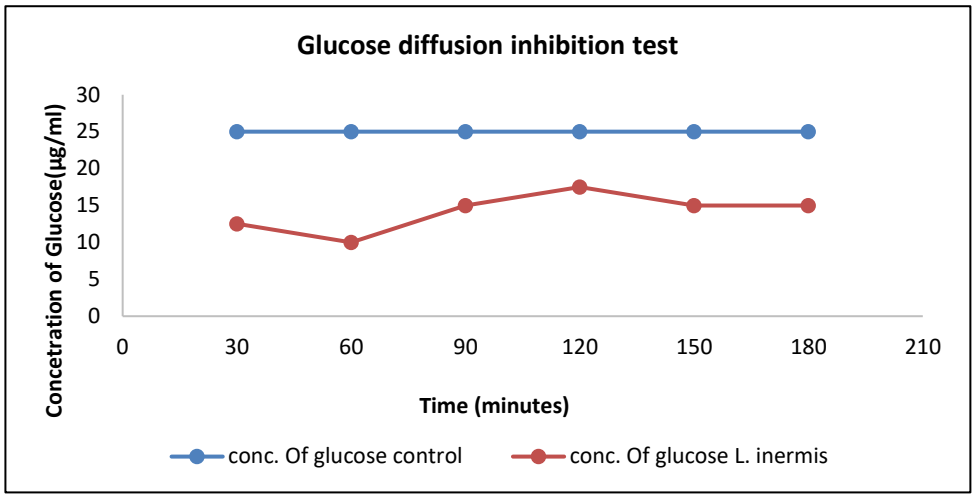
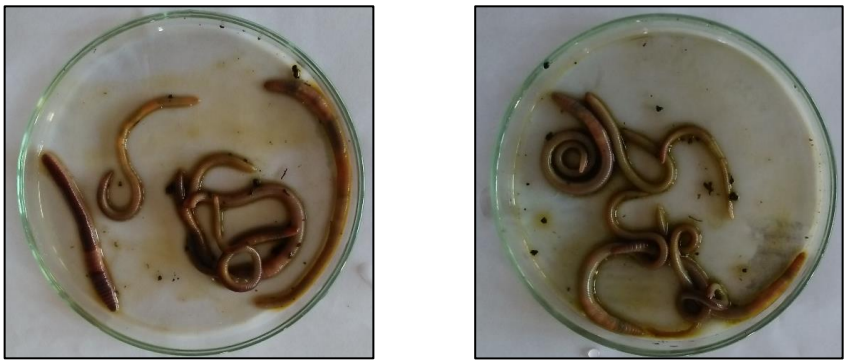


Fig 5. Effect of aqueous extract on glucose diffusion

An Indian earthworm *Asonia putida* was used to carry out antihelminthic activity. Both extract of solvent gives the activity against *A. putida*, but the soxhlet methanol extract gives the efficient result as compare to the soxhlet water extract (Table 10). In the presence soxhlet methanol extract, earthworm got readily paralyzed within 10min. and after 5min. earthworms were died whereas the earthworm in the presence of water does not showed paralysis or death (fig.6).

Table 10: Analysis of Antihelminthic activity:

Sr. no.	Treatment	Paralysis time(min.)	Death time (min.)
1)	Soxhlet water extract of *L. inermis* leaf powder	15 ± 0.01	17 ± 0.02
2)	Soxhlet Methanol extract of *L. inermis* leaf powder	10 ±00	15 ± 0.01
3)	Water (control)	-	-



a. Effect of Soxhlet methanol extract of *L.inermis* on *Asonia putida*
b. Effect of Soxhlet aqueous extract of *L.inermis* on *Asonia putida*

4. CONCLUSION:

L. inermis is one of the traditional medicinal plant in India. Its active component is lawsone (2-hydroxy-1, 4-naphthoquinone) which is used as coloring dye for skin and hair and also for treatment of epilepsy and jaundice. In the present study *L. inermis* found to contain flavonoid, phenol, steroids, resins, tannins and carbohydrate. Soxhlet extraction of henna leaves was done using methanol solvent. It possesses antimicrobial activity against various pathogenic microorganisms. Its methanolic extract found to have antioxidant, thrombolytic, antidiabetic and antihelminthic activity.

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