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Savita Kulkarni
Department of Pharmacognosy and Phytochemistry, Bombay college of Pharmacy, Kalina, Santacruz (E), Mumbai-400 098, Maharashtra, India

Vanita Kale
Department of Pharmacognosy and Phytochemistry, Bombay college of Pharmacy, Kalina, Santacruz (E), Mumbai-400 098, Maharashtra, India

Ketki Velankar
Department of Pharmaceutics, Bombay college of Pharmacy, Kalina, Santacruz (E), Mumbai-400 098, Maharashtra, India

Correspondence:
Savita Kulkarni
Department of Pharmacognosy and Phytochemistry, Bombay college of Pharmacy, Kalina, Santacruz (E), Mumbai-400 098, Maharashtra, India
Email: Svt_kulkarni[at]yahoo.co.in

To study the Photodynamic antimicrobial activity of Henna extract and preparation of topical Gel formulation

Savita Kulkarni*, Vanita Kale, Ketki Velankar

ABSTRACT

Henna, known to possess antibacterial & antifungal activities is reported to contain naphthoquinone derivatives which have potential as photodynamic agents. Current study comprises of evaluation of Photodynamic antimicrobial activity of alcoholic extract of henna, followed by preparation & evaluation of a topical gel formulation. The alcoholic extract prepared by maceration was analyzed for phytochemicals. Lawsone (2-Hydroxy-1,4 naphthoquinone) was isolated from Henna and was characterized by UV, IR & MASS to confirm its structure. The HPTLC fingerprinting of extract and quantification of lawsone in extract and fraction was carried out. The effect of light was tested on extract and fraction to assess its photosensitizing property. The antimicrobial activity of the extract was tested where one set was exposed to light and other kept in dark and zone of inhibition calculated for both sets.

The yield of fraction was found to be 0.5±0.1% w/w. The quantity of lawsone in alcoholic extract & fraction was found to be 1.3 ± 0.02 % w/w and 59.57 ± 1.01 % w/w respectively. The extract is nearly 75% as active as standard Gentamicin and the activity gets 20% potentiated on exposure to light for all test samples indicating Photodynamic antimicrobial activity. The gel was evaluated for its stability with developed HPTLC method and also for physicochemical parameters. The gel containing henna extract, exhibiting good Photodynamic antifungal & antibacterial properties, was found to be stable. The current study thus has resulted in developing a potentially effective herbal topical formulation for management of skin, scalp & hair infections

Keywords: *Lawsonia inermis* (Henna), Antimicrobial activity, Photodynamic therapy(PDT), HPTLC analysis, Gel formulation.

INTRODUCTION

Natural products, specifically, plant derived products have been used for treatment of various diseases and health conditions since ancient times. Almost 50% of the treatments used today are derived from plant sources [1]. Henna, which is commonly referred to *Lawsonia inermis* Linn. Family: Lythraceae is long known for its coloring ability and used extensively for the same. However, also reported for henna is its antibacterial, antifungal, antidiabetic, antifertility and immunomodulatory activity [2, 3]. In this research, we focused on the antifungal and antibacterial activity of Henna. Henna which is reported to contain lawsone upto 1-1.4%, 2- hydroxy-1:4 naphthoquinone, 2-methoxy-3-methyl-1,4-Naphthoquinone, contains other constituents besides naphthoquinones like gallic acid, glucose, mannitol, fats, resin (2 %), mucilage and traces of an alkaloid. Leaves also contain hennatannic acid and an olive green resin, soluble in ether and alcohol [4].

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for potential antimicrobial activity. One such alternative antimicrobial therapy is Photodynamic antimicrobial chemotherapy (PACT) which is still in infancy but follows similar principles to those of Photo Dynamic Therapy (PDT) [5, 6].

Photodynamic technique involves the administration of a photosensitizer followed by application of light of a specific wavelength. Photodynamic diagnosis and therapeutics are each based on distinct physical and physicochemical concepts. When light interacts with the photosensitizer intracellularly, the photon energy absorbed by the sensitizing molecule generates mainly fluorescence or photo-oxidation. Whereas fluorescence is used for photomedical diagnosis of cancer cells, photo-oxidation leading to the cell-destroying process is used for Photodynamic antimicrobial chemotherapy (PACT) [7, 8].

Some phytochemicals which have been reported to possess photosensitizer properties include psoralen, fagopyrins, Porphyrins, thiophenes, furanocoumarins, naphthoquinones, chlorins & purpurins,

etc. [9-11] So, in this current study, the Henna extract containing 1, 4 naphthoquinone derivatives were explored for photosensitizing antimicrobial activity.

Pharmaceutical gels are often simple-phase, transparent semi-solid systems that are being increasingly used as pharmaceutical topical formulations. The liquid phase of the gel may be retained within a three-dimensional polymer matrix. Drugs can be suspended in the matrix or dissolved in the liquid phase [12]. Gels are stable over longer periods of time and have a good appearance. They are suitable vehicles for applying medicaments to skin and mucous membranes, giving high rates of release of the medicament and rapid absorption. In the current study, Henna Gel was prepared for the management of skin, hair and scalp infections [13].

MATERIALS AND METHODS

Plant material and preparation of extract

The source comprised of dried leaves of *Lawsonia inermis* (Lawsonia alba) Linn. Family: Lythraceae which were powdered for extraction. The leaves of *Lawsonia inermis* (LI) were purchased from local market in Mumbai. The sample was authenticated by Dr. Harshad M. Pandit, Department of Botany, Guru Nanak Khalsa College, Mumbai. The authentication certificate is as attached (Specimen #: vg p 1050170) as shown in fig. no. 1.

HARSHAD M PANDIT, Ph.D.(Botany)
(Formerly Head and Associate Professor of Botany)
302 KAVERI
Bhardawadi Road
Andheri(West), Mumbai 400058
Maharashtra, India
Email: drhmpandit@gmail.com
Mobile: +9197699555574

July 18th, 2016

Vanita Gurale
Ph.D. Student
Department Of Pharmacognosy and Phytochemistry
Bombay College Of Pharmacy
Kalina, Santa Cruz(East)
Mumbai 400098, Maharashtra, India.

Lawsonia inermis Linn.

This is with reference to material in the form of twig with leaves. The material consists of leaves arranged in opposite decussate with small petiole, elliptic, acute, mucronate, entire base narrow. In transverse section leaf shows upper and lower epidermis single layered, mesophyll differentiated into upper palisade and lower spongy with anomocytic stomata, presence of rosettes of calcium oxalate crystals especially in the midrib region, xylem arranged in an arc in the midrib region. The material (**specimen #: vg p 1050170**) is the Twig/leaves of ***Lawsonia inermis*** Linn. synonym ***Lawsonia alba*** Lam. belonging to Lythraceae. It is commonly known as Jamaica mignonette, Henna, Marudaam.

Figure 1 :Opposite leaves Figure 2 : T.S.thru midrib-crystals Figure 3: T.S. thru lamina

Your sincerely
Harshad M. Pandit

Figure 1: Authentication certificate of Leaf of Henna

Chemicals

The standard Lawsone was purchased from Sisco Research Laboratory, Mumbai, India. All the solvents used were of analytical grade.

Method for extraction

Preparation of alcoholic extract

The alcoholic extract from coarse henna leaf powder was prepared by maceration with methanol, using herb: menstruum ratio of 1:10, with occasional stirring for 24 hours. Extract after maceration was collected, filtered through clean muslin cloth & the filtrate and concentrated to obtain a semi solid mass. The retained marc was subjected to extraction by repeating the same procedure for three times.

Isolation of Lawsone

The isolation of lawsone from henna sample was carried out using aqueous extraction as per the procedure mentioned by Ashnagar et.al with slight modifications [14]. Powder of dried henna leaves (100 g) was placed in a 1liter Erlenmeyer flask containing a magnetic bar and distilled water (300 mL) and the extraction procedure was carried out as described by Ashnagar et.al. The crude fraction obtained, was subjected to preparative TLC using silica gel plates. The lawsone band was scraped out. The scraped silica was sonicated with methanol to get Lawsone. The solvent was evaporated to dryness to obtain free flowing dried lawsone. The yield of isolated fraction was recorded (Table no. 1).

The isolated fraction of LI was subjected to UV, IR, NMR and MASS analysis for its complete characterization (Fig no. 3,4 &5). The HPTLC method was developed for quantification of lawsone in extract and in fraction as follows:

Protocol for HPTLC method development

Accurately weighed 10mg of standard/extract/fraction was dissolved in methanol in 10 ml volumetric flask and sonicated, followed by making up the volume to get a stock solution of 1000 ppm. Each extract solution was applied on HPTLC plate (silica gel 60 F_{254}, E. Merck) as a band using CAMAG LINOMAT 5 sample applicator equipped with 100 µl syringe. Ascending development to a distance of 85 mm was performed at room temperature (25 ± 2 °C), with Toluene: Ethyl acetate: Glacial acetic acid (6:4:0.1::V/V/V) mobile phase in a CAMAG twin trough chamber previously saturated with mobile phase. After development, the plates were scanned using CAMAG HPTLC SCANNER 3 equipped with WINCAT software at 254 and 366 nm. The procedure was developed with a view to estimate the standard compound in the extract and fraction.

Effect of light exposure on extract and fraction

The study of effect of light on alcoholic extract of LI and isolated fraction containing 1, 4 naphthoquinone derivative was carried out [11, 15]. The study was done in set of two, where the solution of 1000 ppm of LI extract, Lawsone fraction and standard lawsone was prepared in methanol. One set was exposed to the light for red light for 2hr with 320 lux unit and the other set was kept in dark. The change in UV spectra was noted by taking UV scan of both the set of samples [16]. The scan was obtained as shown in fig no. 9,10,11.

Photodynamic antibacterial activity

The naphthoquinone derivatives are reported to have photodynamic activity [11, 15]. So we decided to explore the possibility of enhancing the anti-microbial activity of henna alcoholic extract and isolated fraction. This was carried out in two sets, where one set was exposed to red light (λ_{max} 500 nm) for two hours, and the changes in zone of inhibition were noted. The other set was kept in dark for two hours.

PDT assay Protocol:

Assay Method	:	Agar Well Diffusion Assay
Culture Medium	:	Nutrient Broth
Standard Drug	:	Gentamicin
Bacterial strain used	:	*Bacillus subtilis*
		Staphylococcus aureus
		Klebsiella pneumonia
		Escherichia coli
		Pseudomonas aeruginosa
		Streptococcus mutans
Fungal strain used	:	*Aspergillus niger*
		Candida albicans

Agar well diffusion Method [17]

The preparation of cultures and plates was carried out as per the procedure mentioned by Pelczar, 2011. Briefly, for preparation of cultures,sterile nutrient agar medium was prepared in test tubes as slants. Loopful of culture of desired microorganism was transferred aseptically onto the slants and spread over the surface followed by incubation for 48hours. After 48hours, loopful of colonies on the surface of the slant were transferred aseptically into a test tube containing sterile water. The contents of the test tube were diluted until absorbance of about 0.2000-0.4000 was obtained at λ_{max} 536 nm using a UV-spectrophotometer. This culture was used as the inoculums.

For the preparation of plates, 1ml of the inoculums were added to nutrient agar medium at 50°C and 20ml of this mixture was poured into petri plates and allowed to cool and solidify. Wells were bored in the solidified plates into which test solution was poured aseptically and plates were incubated at 37°C for 24hours.

The samples prepared were 1000 ppm in methanol. Different concentrations were tried for extract and fractions and the dose at which prominent zone of inhibition was observed were noted.

PDT antibacterial activity

The prepared extract, naphthoquinone enriched fraction containing lawsone, standard lawsone, were tested using Gentamicin as a standard. The procedure was followed as mentioned above. The activity was tested in two sets where one set was exposed to red light (λ_{max} 500 nm) of 320 Lux and one set kept in the dark, followed by incubation of plates at 37°C for 24hours. The zone of inhibition was measured and increase in zone of inhibition in exposed set was noted. The potentiation in the photodynamic antimicrobial activity due to light exposure was recorded accordingly. (Fig no. 12-19).

The above procedure has been repeated thrice with three sets of samples each time and the data has been subjected to statistical analysis using two-way ANOVA.

Preparation of Gel formulation

The extract of leaves of LI (2%) was formulated into gel with polymer (Carbopol 980nf) at various concentration levels. The final optimized formula was obtained with Carbopol 980nf at concentration of 1% which resulted in the best gel formulation, which was both-smooth and stable (Table no.2).

Table 2: Optimized formula of Henna gel

Ingredient	Concentration
Extract	2 %
Carbopol 980nf	1%
Glycerine	0.5
Disodium EDTA	0.1
Methyl paraben	0.1
Sodium metabisulphite	0.1
NaOH	q.s.
Distilled water	q.s.

Method for Preparation of Gel Containing Extract

1g of Carbopol 980nf was dispersed in 75 ml of distilled water to swell for half an hour. The other excipients, namely, methyl paraben, disodium EDTA and sodium metabisulfite were dissolved separately in 5 ml of fresh distilled water on water bath. The required quantity of alcoholic extract was dissolved in minimum amount of ethanol and the extract solution was added to the water containing other dissolved excipients. The obtained solution was kept on magnetic stirrer for 2 hours continuous stirring till all ethanol was evaporated. The extract solution added dropwise to polymer solution with continuous stirring on magnetic stirrer. The volume was made up to 100 ml with distilled water. The gel was stirred till it was uniformly mixed. The gel was kept aside overnight for expulsion of air from it. Next day pH of gel was adjusted by using 10% NaOH solution added drop wise to the formulation to attain required skin pH (4-5.5) and to obtain the gel of required consistency. The same method was followed for preparation of control sample without adding extract [18, 19].

Evaluation of gel

The gel was evaluated for a period of three months for its stability and other evaluation parameters as given below:

Physical Evaluation: Physical parameters such as colour and appearance were checked. (Table no.5)

Measurement of pH: pH of the gel was measured by using pH meter for a period of 3 months. The gel was found to be stable.

Spreadibility: It was determined by means of the apparatus which consists of a wooden block, which was provided by a pulley at one end. By this method spreadibility was measured based on slip and drag characteristics of gels. Spreadibility was calculated using the following formula:

$$S = M \times L / T$$

Where,
S = Spreadibility, M = Weight in the pan (tied to the upper slide),
L = Length moved by the glass slide and
T = Time (in sec.) taken to separate the slide completely each other.

Viscosity: Viscosity of gel was measured by using Brookfield viscometer. Spindle no 7 was used for measuring the viscosity so that the dial reading came within the range of 1-100. The apparent viscosity was determined using various speeds (20-100 RPM) of spindle (Table no.5)

Stability Study [20]

The stability study was performed as per ICH guidelines. The formulated gel was filled in the wide mouth container and stored at different temperatures conditions, viz. 0˚ C ± 2˚C and 25˚ C ± 2˚C for a period of three months and studied for its appearance, pH, viscosity and spreadibility. (Observations given in the table no.5)

The gel formulation was assayed by HPTLC method analysis developed earlier. The HPTLC method was developed to assess the stability of gel. The composition of mobile phase was; Toluene**:** Ethyl acetate: Glacial acetic acid (6:4:0.1 v/v/v/v).

Preparation of stock solution of formulations

The 2 gm of gel was weighed and transferred into 10 ml volumetric flask. Methanol was added and sonicated for 10 minutes and volume was made up. The contents of flask were filtered through Whatman paper. The filtered solution was used for the HPTLC analysis.

Preparation of stock solution

A stock solution of extract, standard lawsone was prepared by transferring 10 mg of accurately weighed compound into 10 ml volumetric flask, dissolving in 5 ml methanol. It was then sonicated for 5 minutes and volume of solution was made up to 10 ml with methanol to get solution of 1000 ppm.

Preparation of stock solution of excipient

All the excipients used in the formulation except extract were weighed accurately and transferred in to 10 ml volumetric flask, dissolving in 5 ml methanol. It was then sonicated for 5 minutes and contents of flask were filtered through Whatman paper. The filtered content was made up to 10 ml with methanol and used for HPTLC analysis.

Procedure: All the spots were applied in triplicate to the plate as bands 6.00 mm wide and 15 mm from the bottom edge of the HPTLC plate (Silica gel 60F_{254}). After development plates were scanned at 254 nm (Fig no. 20, 21).

RESULTS

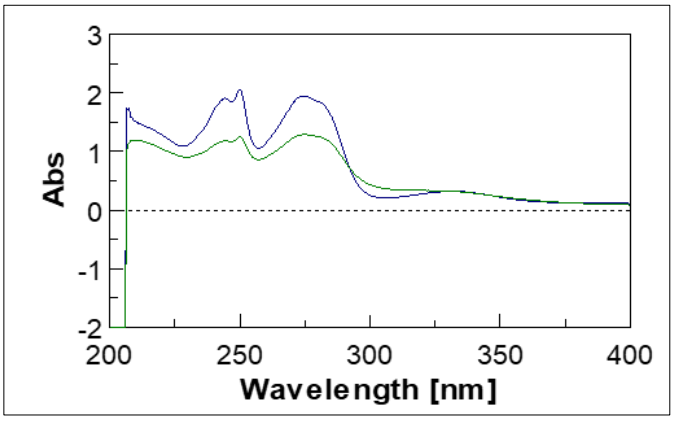
The extraction procedure was repeated thrice and the average yields obtained were as follows:

Table 1: Yield of extract

Name	Yield (% W/W)	
	Aqueous	Alcoholic
Cold extraction	-	10.1±0.12
Lawsone Isolation	0.5±0.1	-

The alcoholic extract of henna was subjected for preliminary qualitative phytochemical analysis which shows presence of carbohydrates, anthraquinones, naphthoquinone derivatives, flavonoid and phenolic compounds present in it. The isolated fraction of LI was subjected to UV, IR, NMR and MASS analysis for its complete characterization.

The UV spectra of isolated lawsone fraction show absorption maxima at 214, 233, 254, 277 and 322nm. The absorption maxima of standard lawsone were at 211.5, 233,254.5, 276 and 333nm.Both absorption maxima for standard lawsone and extract were found to exactly match with each other.



Blue line: STD lawsone, **Green line:** LI Fraction

Figure 2: UV spectra of LI fraction and STD lawsone

The IR values of isolated LI fractions shows –OH stretching at 3332, 3294 cm^{-1}, C=O stretching at 1646 cm^{-1}, C=C aromatic stretching 1578 cm^{-1} and C-O aromatic stretching at 1221 cm^{-1},which match with standard lawsone peaks.

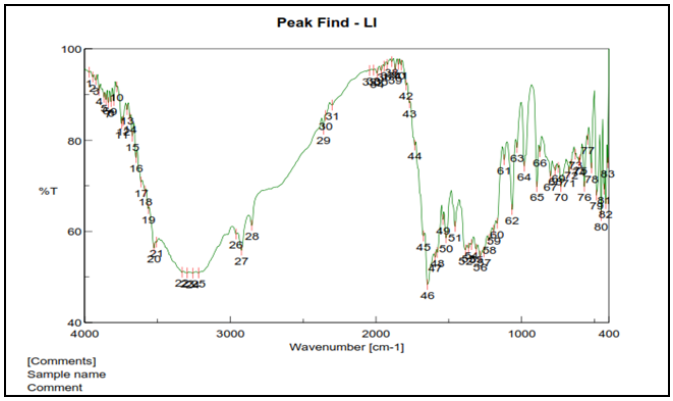
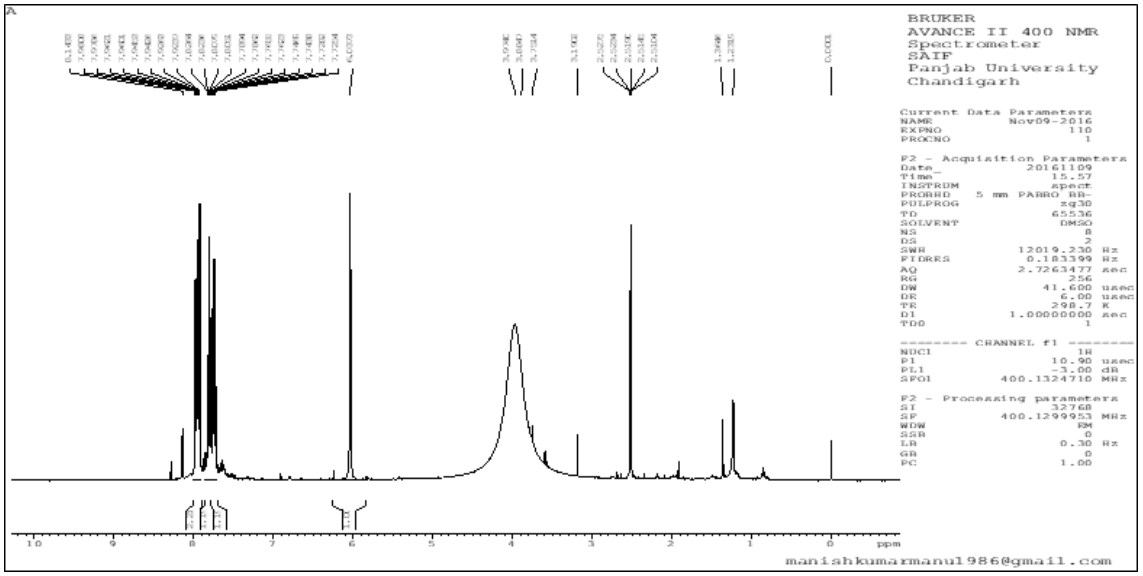


Figure 3: IR pattern of LI fraction

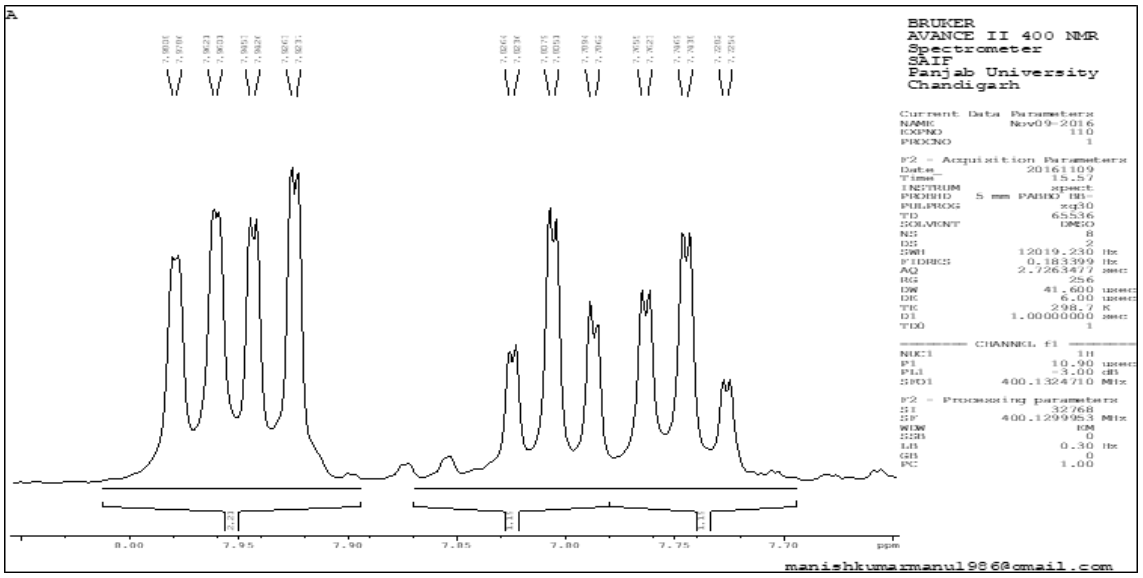
Table 3: IR data of isolated LI fraction

Peak no.	Wavelength (cm^{-1})	Band assignment	Peak intensity
22	3332.39	O-H Stretching	51.00
23	3294.79	O-H Stretching	50.88
25	3217.65	O-H Stretching	50.89
46	1646.91	C=O stretching	48.34
47	1593.88	C=C Stretching	54.31
48	1578.45	C=C Stretching	55.37
56	1284.36	C-O stretching	54.57
58	1221.68	C-O stretching	58.25

The NMR values of isolated LI fraction shows two quartet of ortho and meta coupling, two triplets of ortho and para coupling, and one broad singlet and singlet which predict the possible structure of 2-hydroxy-1, 4 naphthoquinone (Lawsone).



A

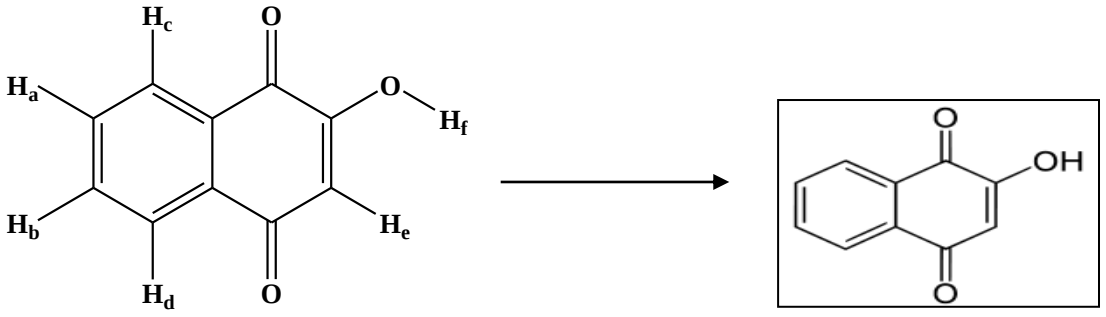


B

Figure 4: A and B NMR spectra of LI isolated fraction

LI isolated fraction interpretation

Possible Structure



2-hydroxy-1, 4 naphthoquinone

Proton	δ value	Splitting	Explanation
H_a	7.92-7.98	Quartet	Ortho coupling with H_b and H_c. Meta coupling with H_d
H_b	7.92-7.98	Quartet	Ortho coupling with H_a and H_d. Meta coupling with H_c
H_c	7.78-7.82	Triplet	Ortho coupling with H_a and para coupling with H_d
H_d	7.72-7.76	Triplet	Ortho coupling with H_b and para coupling with H_c
H_e	6.03	Singlet	
H_f	3.88	Broad singlet	

The fragmentation pattern of isolated LI fraction shows fragments having mass values 174, 173, 146, 145, 119 and 101. The molecular ion peak shows mass value 173, further fragmentation gives molecular ion peak at m/z value 145 due to loss of formaldehyde (HCHO). (Molecular weight of Lawsone is 174).

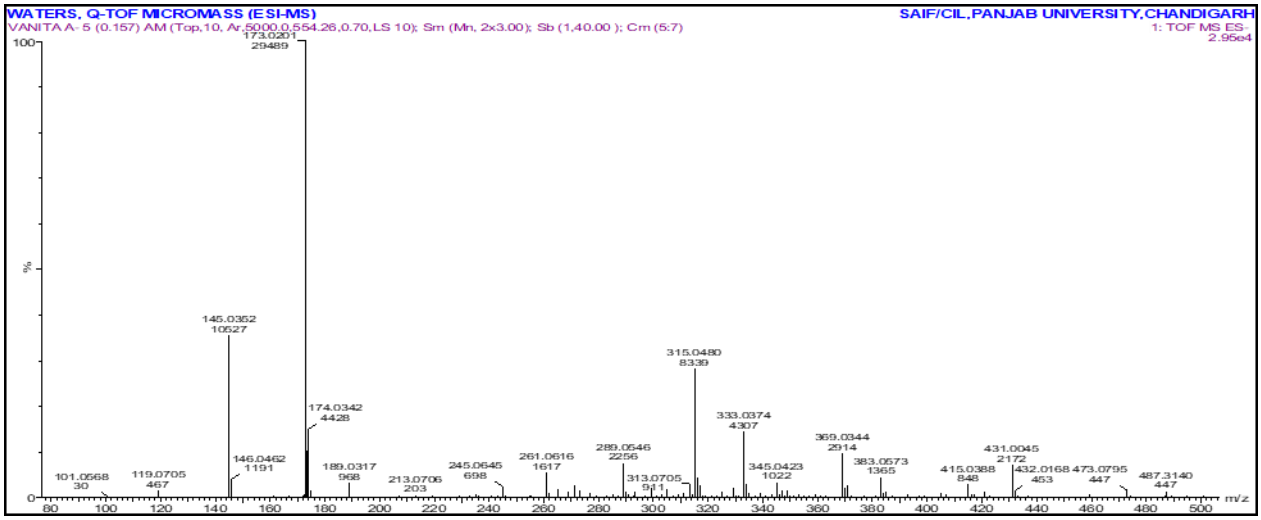
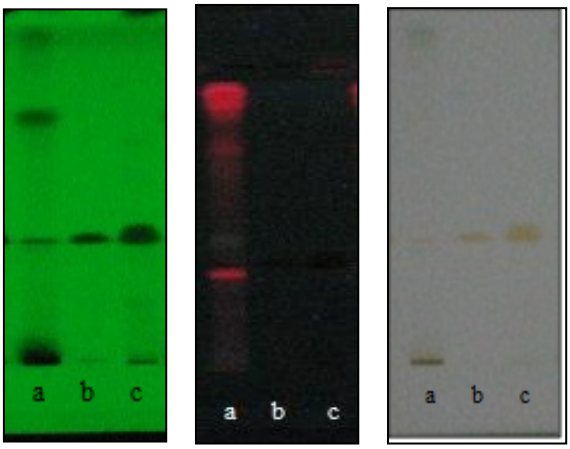


Figure 5: Mass spectra of isolated LI fraction

Hence, from the UV, IR, NMR and MASS data, it was confirmed that the fraction isolated from henna contained lawsone as major constituent.

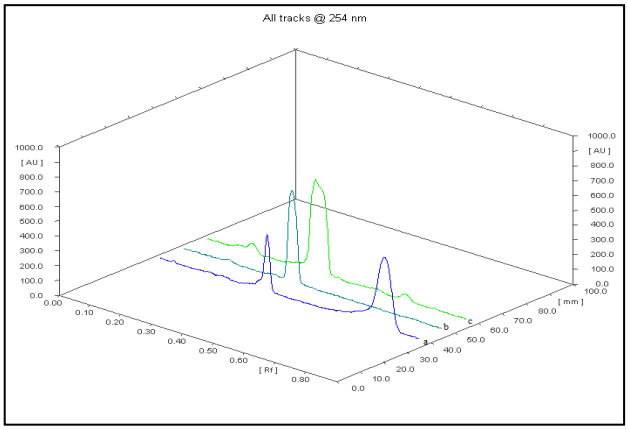
The HPTLC fingerprinting shows presence of Lawsone in extract as well in isolated fraction (fig no. 6,7 & 8). The HPTLC densitometric method was developed for the estimation of Lawsone from the alcoholic extract and the fraction. The amount of lawsone present in the extracts was calculated from the calibration curve of standard lawsone. The quantity of lawsone present in alcoholic extract of LI and the fraction was found to be 1.3 ± 0.02 % w/w and 59.57 ± 1.01 % w/w of Lawsone respectively.



A B C

Track details: a) LI extract, b) Standard Lawsone, c) LI fraction

Figure 6: Photo of HPTLC plate containing LI extract, standard lawsone and Isolated lawsone (A=at 254nm, B=366nm, C=visible light)



etails: a) LI extract, b) Standard Lawsone, c) LI fraction

Figure 7: 3D graph of plate showing LI extract, standard lawsone and isolated lawsone fraction peak

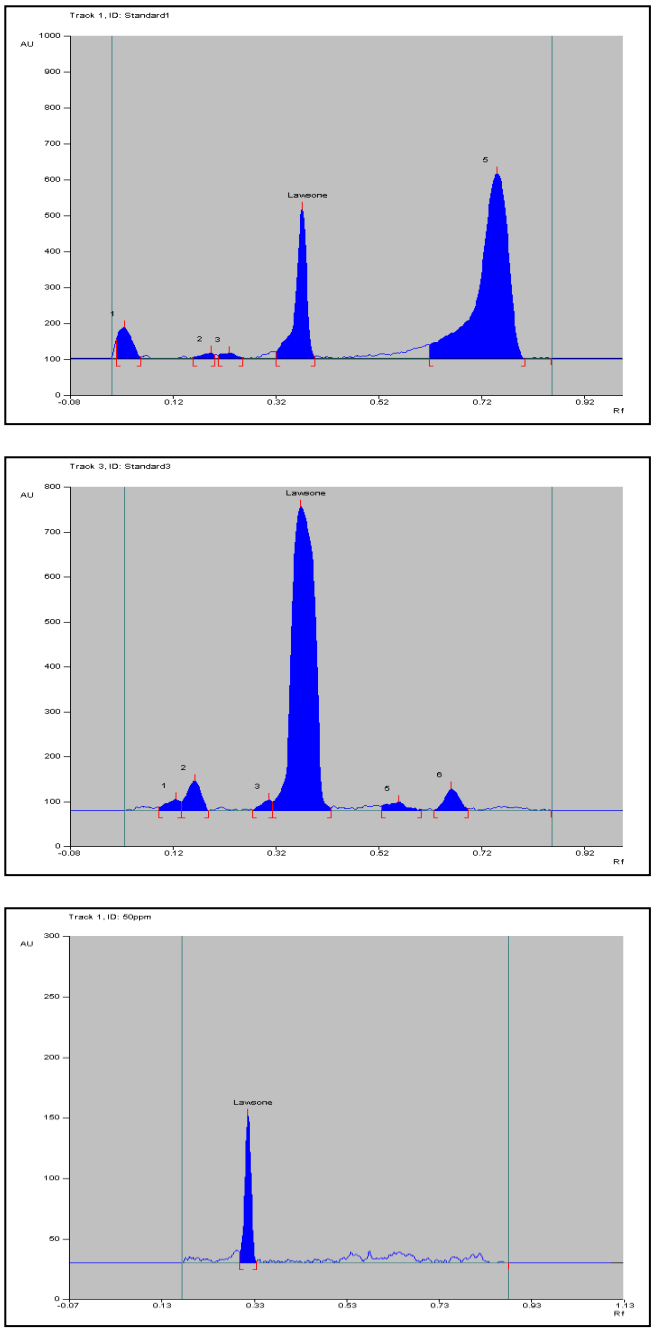
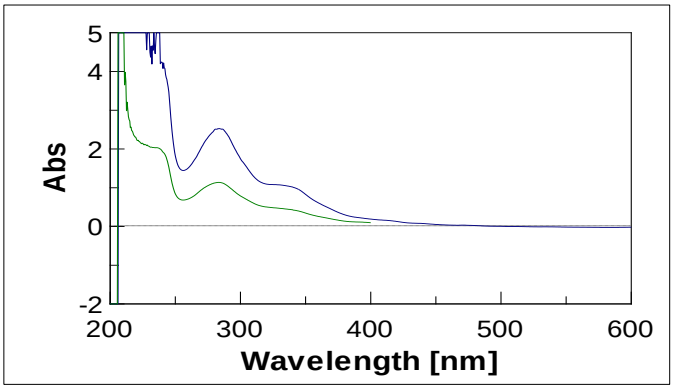


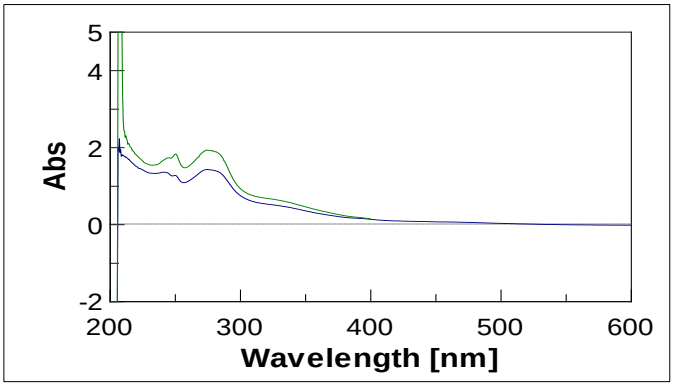
Figure 8: Standard Lawsone peak in (a) Extract of LI, (b) Fraction isolated of LI, and (c) Standard lawsone

Effect of light on extract: The UV spectra of extract, fraction and standard exhibited a change in UV spectra when exposed to red light and the changes in spectra have been recorded. Hence, it can be concluded that 1,4 naphthoquinones which are present in extract, fraction and standard, get activated due to light i.e. naphthoquinones may get excited from ground state to triplet state and shows change in UV absorbance. (Fig no. 9, 10, 11) Hence, 1,4 naphthoquinone derivative present in extract and fraction are responsible for Photodynamic activity which can be further explored as photodynamic therapy (PDT).



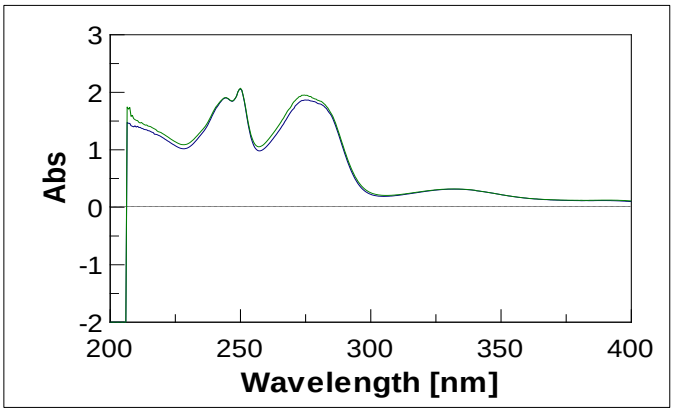
Green line: when kept in dark, **Blue line:** when exposed to light

Figure 9: UV absorption maxima of LI extract



Blue line: when kept in dark, **Green line:** when exposed to light

Figure 10: UV absorption maxima of LI fraction



Blue line: when kept in dark, **Green line:** when exposed to light

Figure 11: UV absorption maxima of standard Lawsone

Different doses were tried for extract, isolated lawsone and standard lawsone to assess antimicrobial activity. Dose of 400, 200 and 100µg of extract, fraction and standard lawsone respectively showed maximum zone of inhibition when tested against different bacteria

and fungi. The zone of inhibition got increased by 20% when the set was exposed to the red light of specific wavelength. Hence, the extract, fraction and standard has shown to exhibit photodynamic antimicrobial activity. The activity got potentiated by 20% when exposed to light.

The following tables and graphs gives the comparison between two sets with and without exposure. The increased zone of inhibition was recorded and plotted into graphs for each microorganism handled as follows:

Table 4

Micro organism	Test Sample	Zone of inhibition in cm (Mean± SD)	
		WE	E
B.Subtilis Fig. No 12	LI Ext	2.13±0.05	2.57±0.02
	LI fraction	2.66±0.11	2.97±0.02
	Standard lawsone	2.1±0.1	2.56±0.06
	Gentamicin	3.1±0.1	3.14±0.02
S. aureus Fig. No 13	LI Ext	2.25±0.05	2.63±0.03
	LI fraction	2.52±0.02	2.89±0.15
	Standard lawsone	2.15±0.05	2.73±0.08
	Gentamicin	3.14±0.01	3.12±0.02
K. pneumoniae Fig. No 14	LI Ext	2.17±0.01	2.54±0.03
	LI fraction	2.56±0.02	2.87±0.02
	Standard lawsone	2.03±0.05	2.55±0.026
	Gentamicin	3.05±0.057	3.04±0.017
E. coli Fig. No 15	LI Ext	2.3±0.1	2.79±0.19
	LI fraction	2.69±0.03	2.92±0.06
	Standard lawsone	2.43±0.06	2.78±0.16
	Gentamicin	3.16±0.017	3.15±0.017
P. Aeruginosa Fig. No 16	LI Ext	2.3±0.1	2.68±0.08
	LI fraction	2.54±0.02	2.89±0.03
	Standard lawsone	2.21±0.02	2.66±0.06
	Gentamicin	3.16±0.011	3.15±0.01
S. mutans Fig. No 17	LI Ext	2.32±0.02	2.62±0.02
	LI fraction	2.74±0.04	2.93±0.03
	Standard lawsone	2.24±0.04	2.58±0.06
	Gentamicin	3.20±0.011	3.2±0.01
A. niger Fig. No 18	LI Ext	2.18±0.02	2.57±0.04
	LI fraction	2.67±0.01	2.54±0.37
	Standard lawsone	2.18±0.02	2.58±0.03
	Gentamicin	3.10±0.011	3.11±0.01
C. Albicans Fig. No 19	LI Ext	2.31±0.02	2.64±0.04
	LI fraction	2.72±0.03	2.95±0.04
	Standard lawsone	2.4±0.02	2.55±0.03
	Gentamicin	3.06±0.05	3.07±0.06

WE: Without light exposure; E: Light Exposure
Dose of test samples(µg): LI Ext= 400; LI fraction=200; Standard lawsone=100; Gentamicin=100

All Values are means ± S.E.M. (n=3). Significance was determined by Two-Way ANOVA with $p < 0.0001$ when compared with exposed group.

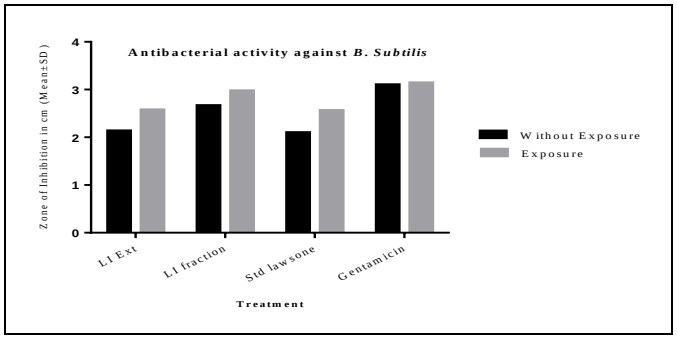


Figure 12: Graph of zone of inhibition versus extract and fraction treatment against *B. subtilis*

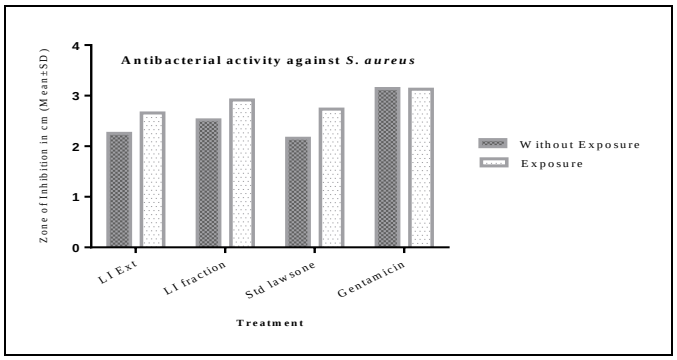


Figure 13: Graph of zone of inhibition versus extract and fraction treatment against *S. Aureus*

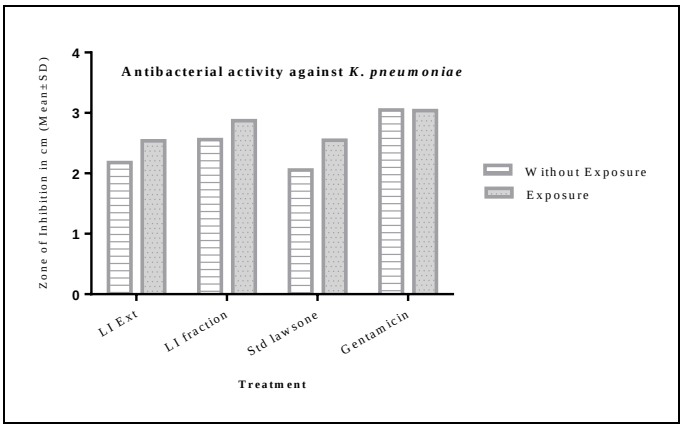


Figure 14: Graph of zone of inhibition versus extract and fraction treatment against *K. Pneumonia*

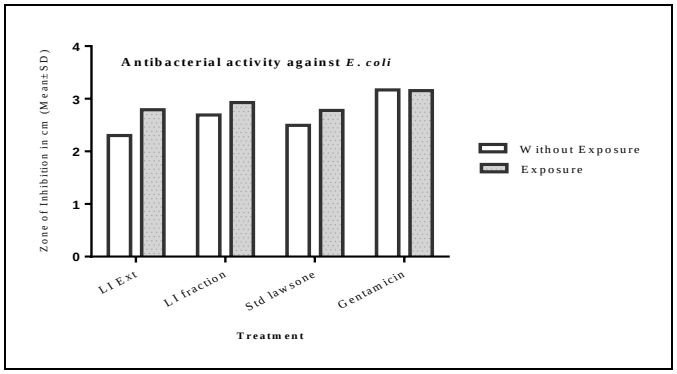


Figure 15: Graph of zone of inhibition versus extract and fraction treatment against *E. Coli*

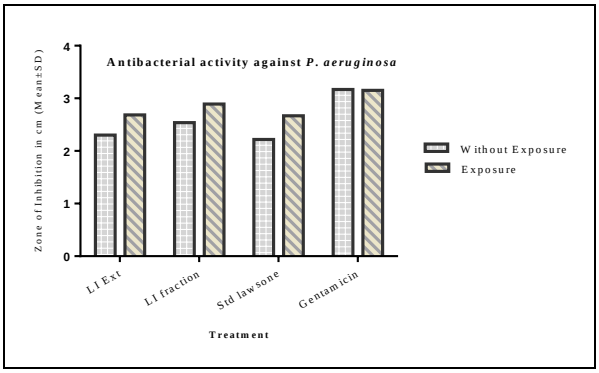


Figure 16: Graph of zone of inhibition versus extract and fraction treatment against *P. Aeruginosa*

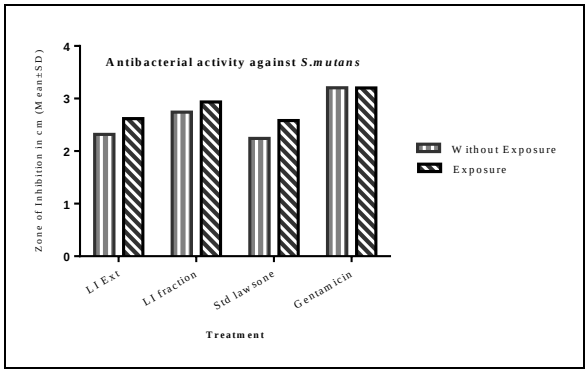


Figure 17: Graph of zone of inhibition versus extract and fraction treatment against *S.mutans*

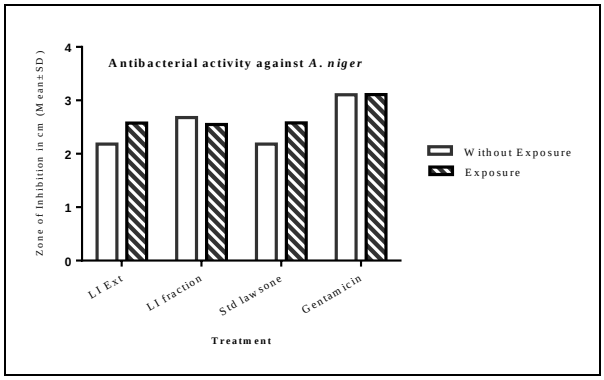


Figure 18: Graph of zone of inhibition versus extract and fraction treatment against *A.niger*

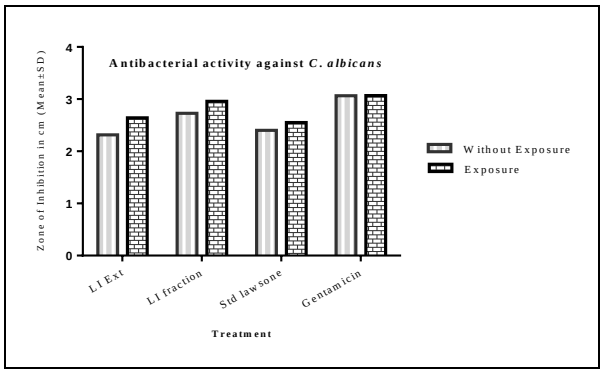


Figure 19: Graph of zone of inhibition versus extract and fraction treatment against *C. Albicans*

The PDT antibacterial and antifungal activity for LI extract, LI fraction shows potentiation in activity when exposed to light. Hence, the LI extract was subjected for gel preparation, the gel evaluated with respect to different parameters where it shows stability. (Table no. 5)

Table 5: Evaluation of topical gel of Henna leaf extract

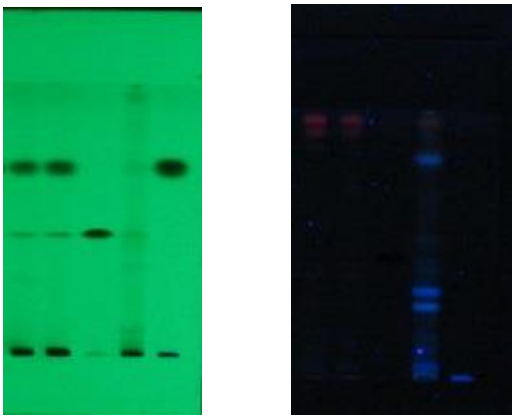
Sr. No.	Parameter	at 0˚C ± 2˚C	at 25˚C ± 2˚C
1	Colour	Greenish	Greenish
2	Appearance	Homogeneous	Homogeneous
3	pH	5.99	6.01
4	Spreadibility	15 (gm.s/cm)	18 (gm.s/cm)
5	Viscosity		
	(at 20 RPM, spindle#7)	20000 cps	21000 cps
	(at 50 RPM, spindle#7)	10800 cps	10000 cps
	(at 100 RPM, spindle#7)	6600 cps	6000

Analysis of Henna gel by HPTLC method

2 g of 2% Gel was dispersed in 10 ml methanol. The final extract concentration was 40mg extract in 10 ml methanol (i.e. 4mg/ml). The resultant solution was used for HPTLC study.

Table 6: Tract details of Henna gel HPTLC plate

Sr. no.	Name	Volume applied	Concentration
1	Henna gel (at 0˚C ± 2˚C)	20µl	4mg/ml (4000 ppm)
2	Henna gel (at 25˚C ± 2˚C)	20µl	4mg/ml (4000 ppm)
3	Standard Lawsone	5µl	1000ppm
4	Henna extract	20µl	1000ppm
5	Excipient solution	20µl	1000ppm



(a) **(b)**

Figure 20: HPTLC plate photo of Henna gel at 254 and 366nm

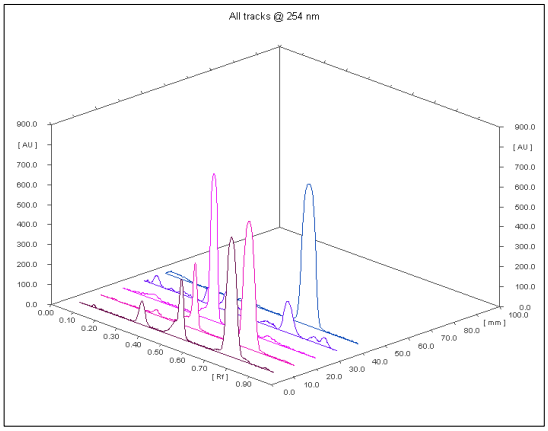


Figure 21: 3D graph of Henna gel HPTLC study

Although, the gel was tested for stability for a period of three months, the formulation has been found to be stable for more than one year.

DISCUSSION

The presence of naphthoquinone derivatives in henna leaves is widely known. The leaves of henna which were extracted by cold maceration technique gave a yield of 10.1±0.12 %W/W. The alcoholic extract of henna was subjected for preliminary qualitative phytochemical analysis which shows presence of carbohydrates, anthraquinones, naphthoquinone derivatives, flavonoid and phenolic compounds present in it. The isolation of lawsone from leaf powder of henna was also attempted. The fraction rich with 1,4-Naphthoquinone derivative (lawsone) was then subjected for structure elucidation by UV, IR, NMR and MASS technique. The UV spectra of isolated lawsone fraction and absorption maxima of standard lawsone were both exactly matching with each other. The IR values of isolated LI fractions shows –OH stretching, C=O stretching, C=C aromatic stretching, and C-O aromatic stretching which match with standard lawsone peaks.The NMR values of isolated LI fraction shows two quartets of ortho and meta coupling, two triplets of ortho and para coupling, and one broad singlet and singlet which predict the possible structure of 2-hydroxy-1, 4 naphthoquinone (Lawsone).The fragmentation pattern of isolated LI fraction shows fragments having mass values 174, 173, 146, 145, 119 and 101. The molecular ion peak shows mass value 173, further fragmentation gives molecular ion peak at m/z value 145 due to loss of formaldehyde (HCHO). (Molecular weight of Lawsone is 174). Hence, it can be confirmed that the fraction isolated from henna contained lawsone as major constituent.

The HPTLC densitometric method was developed for the quantification of Lawsone in alcoholic extract and isolated fraction. The HPTLC fingerprinting for extract as well as isolated fraction shows presence of Lawsone in extract as well in isolated fraction (fig no. 6,7 & 8). By estimation of lawsone in extracts, done by calibration curve, the quantity of lawsone was found to be present in alcoholic extract and the fraction 1.3 ± 0.02 % w/w and 59.57 ± 1.01 % w/w of Lawsone respectively.

The naphthoquinones are reported to have photodynamic activity [10, 11].

The effect of light on extract and fraction was tested and the change in UV spectra was recorded. It was observed that the extract, fraction and standard were showing change in UV spectra when exposed to red light. This change in spectra may be due to 1,4 naphthoquinones present in extract, fraction and standard compound which get affected due to light i.e. naphthoquinones may get excited from ground state to triplet state and shows change in UV absorbance. (Fig no. 9, 10, 11) Hence, 1,4 naphthoquinone derivative present in extract and fraction was responsible for Photodynamic activity which was further explored as photodynamic therapy (PDT).

To further study this activity and thereby potentiation in the antimicrobial activity of lawsone, the extract, fraction and standard were subjected to antimicrobial activity in a set of two where one set was exposed to light and the other unexposed. The result was found to be that the isolated lawsone fraction and standard lawsone at the dose of 200 and 100µg shows zone of inhibition in the range of 2.66±0.1 and 2.23±0.1 respectively, when tested against different bacteria and fungi. The activity gets potentiated by 20% when the set was exposed to the red light of specific wavelength. The standard lawsone was found out to be most active in a low dose of 100 µg followed by isolated fraction at dose of 200 µg and lastly the alcoholic at dose of 400 µg. But, the potentiation in the antimicrobial activity was observed in all tested groups which were exposed to light. This may be attributed to the synergistic effect of 1,4 naphthoquinone derivatives and related compounds which are present in extract and fraction which may be responsible for potentiation in the activity of extract and fraction. Hence, it can be concluded that the activity was potentiated due to presence of 1,4 naphthoquinone derivatives in extract, fraction and standard compound due to their photodynamic activity.

After successful attempt of PDT, the LI extract was subjected for preparation of topical gel formulation. The physicochemical properties of lawsone, make it feasible for formulating a gel. The gel was formulated by optimized formula and evaluated with respect to different parameters like color, appearance, pH, spreadability, viscosity etc. It was found that the gel was stable for the stated period. The HPTLC method was developed to analyze the stability of extract in gel formulation. The result shows the gel was stable for period of three months when tested by developed HPTLC method of analysis. (Table no. 5)

The gel was tested for stability for the period of three months, nonetheless, the formulation has been found to be stable more than one year.

CONCLUSION

The current study was aimed at assessing the photosensitizer properties for Henna and studying its application in the Photodynamic antimicrobial chemotherapy (PACT).The study was also extended to preparation of a suitable formulation to deliver the active constituent. The extraction method for henna leaves was standardized, and the yield of extract was found to be 10.1±0.12 %W/W. The isolation of 1,4 naphthoquinone rich fraction was carried out which contained 60% of Lawsone present. The yield of 1,4 naphthoquinone rich fraction was found to be 0.5±0.1 %W/W. The HPTLC fingerprinting confirmed the presence of Lawsone in extract as well as in isolated fraction. The characterization of isolated fraction using UV, IR, NMR, MASS was carried out which confirms that the major chemical constituent of fraction is lawsone. The quantification of lawsone in alcoholic extract as well as in isolated fraction was carried out by HPTLC analysis. Hence, the detail standardization method will be available for henna extract and fraction.

The photodynamic antimicrobial activity is possibly reported for the first time for Henna through this study. The photodynamic antimicrobial activity of alcoholic extract was tested against different types of microorganisms. The henna extract, fraction and standard gentamicin at the dose of 400, 200 and 100 µg showed good antimicrobial activity. The antimicrobial activity gets potentiated up to 20% in the set which was exposed to the light. The zone of inhibition of exposed set of plates were almost equivalent to that of standard gentamicin. The activities of standard lawsone and the extract containing only about 10% lawsone have similar zone of inhibition.This probably may be attributed to the synergistic effect of structurally similar compounds present in the extract. It may be concluded that the henna extract and fraction containing 1,4 naphthoquinone derivatives is responsible for photodynamic antimicrobial activity due to which the potentiation in the activity was observed for the set which was exposed to light.

The topical gel formulation was successfully prepared and evaluated for parameters like appearance, color, odour and pH and was found to be stable. The stability of gel was evaluated up to three months by using HPTLC method of analysis, but it was observed that the gel was stable for more than one year. The topical gel formulation which has been prepared from Henna extract can be used for skin, hair and scalp treatment because of its promising antibacterial and antifungal activity. Hence, this topical formulation would possibly emerge as a novel therapeutic option for lawsone therapy and benefit the end users.

REFERENCES

1. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Pharmacognosy reviews. 2014; 8(16):73.
2. Habbal O, Hasson S, El-Hag A, Al-Mahrooqi Z, Al-Hashmi N, Al-Bimani Z, *et al*. Antibacterial activity of Lawsonia inermis Linn (Henna) against Pseudomonas aeruginosa. Asian Pacific journal of tropical biomedicine. 2011; 1(3):173-6.
3. Yadav S, Kumar A, Dora J, Kumar A. Essential perspectives of Lawsonia inermis. Int J Pharm Chemical Sci. 2013; 2:888-96.
4. Sahu L, Roy A, Satapathy T. A Phytopharmacological Review on Lawsonia Inermis L. Research Journal of Science and Technology. 2012; 4(3):II.
5. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). The Journal of antimicrobial chemotherapy. 1998; 42(1):13-28.
6. Maisch T, Szeimies R-M, Jori G, Abels C. Antibacterial photodynamic therapy in dermatology. Photochemical & Photobiological Sciences. 2004; 3(10):907-17.
7. Prasad PN. Introduction to biophotonics. John Wiley & Sons, 2004.
8. Wainwright M. Photosensitisers in biomedicine. John Wiley & Sons, 2009.
9. Wyss P. History of photomedicine. Photomedicine in Gynecology and Reproduction: Karger Publishers; 2000, p. 4-11.
10. Staab HA, Weiser J, Baumann E. Photoinduced electron transfer in porphyrin-quinone cyclophanes, 5. Quinone-porphyrin-quinone and quinone-porphyrin-donor cyclophanes: Syntheses, structures and electron-transfer-related properties. European Journal of Inorganic Chemistry. 1992; 125(10):2275-83.
11. Mukherjee T. Photo and radiation chemistry of quinones. Proceedings-Indian National Science Academy part A. 2000; 66(2):239-66.
12. Chittodiya P. Topical Gel A Review. International Journal of Pharmaceutical & Biological Archive. 2013; 4(4).
13. Bangarwa S, Garg S, Aseri A. A review on antifungal gels: as a topical drug Delivery system. IJPTB. 2014; 1(1):48-55.
14. Ashnagar A, Shiri A. Isolation and characterization of 2-hydroxy-1, 4-naphthoquinone (lawsone) from the powdered leaves of henna plant marketed in Ahwaz city of Iran. IJ Chemtech Res. 2011; 3:1941-4.
15. Brahmia O, Richard C. Phototransformation of 1, 4-naphthoquinone in aqueous solution. Photochemical & Photobiological Sciences. 2003;2(10):1038-43.
16. Kulkarni SR, Khatwani PF, Gurale V. International Journal of Pharma and Bio Sciences ISSN.
17. Michael J. Pelczar. Microbiology, An application based approach. 3rd reprint, ed. ed. New Delhi: Tata Mcgraw Hill education, 2011.
18. Dwivedi S, Gupta S. Formulation and evaluation of herbal gel containing *Sesbania Grandiflora* (L.) Poir. Leaf extract. Acta Chim Pharm Indica. 2012; 2(1):54-9.
19. Bankar AM, Dole MN. Formulation and evaluation of herbal antimicrobial gel containing musa acuminata leaves extract. JPP. 2016; 5(1):01-3.
20. Guidelines I. Stability Testing of New Drug Substances and Products Q1A, 2003.

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