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GC/MS PROFILING, ANTIBACTERIAL AND ATOMIC FORCE MICROSCOPIC STUDY OF BACTERIAL CELL MEMBRANE AFFECTED BY *FARSETIA HELIOPHILA* BARK EXTRACT ALONG WITH WOUND HEALING ACTIVITY

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ABSTRACT

The aim of the study was to evaluate phytochemically, biologically and pharmacologically *Farsetia heliophila* (*F. heliophila*) using *in vivo* wound healing technique. Phytochemically, *F. heliophila* positive test for tannins, saponins, flavonoides, triterpenoids and alkaloids. The chemical composition of *F. heliophila* evaluated through gas chromatography (GC), gas chromatography/mass spectroscopy (GC/MS) and Fourier transformed infra-red (FTIR) reveled that *F. heliophila* contain important bioactive compounds like barrigenol R1, ethyl iso-allocholate, lupeol acetate, isorhamnetin, α-amyrine, α-tocopherol, l-(+)-ascorbic acid 2,6-dihexadecanoate, ascorbyl palmitate, 2-pyrazolin-5-one, 3,4,4-trimethyl- isoquercetin, pyrimidine, indole and cyclolaudenol. The crude extract was further evaluated for antibacterial activity, (AFM) study of extract treated bacterial cells, acute oral toxicity and *in vivo* wound healing potential. The MIC_{50} values of *F. heliophila* extract against *B. subitils*, *S. typhi E. coli* and *P. aeruginosa* were 25, 50, 75 and 100 µg/mL at ($p<0.01$) and ($p<0.05$). The AFM images showed that the cell membrane of *B. subitils*, *S. typhi* and *E. coli* were significantly damaged with cytoplasm leaked from the bacterial cell. *P. aeruginosa* cell membrane was partially damaged. The extract did not show any acute toxicity at higher dose of 2000, 3000 and 5000 mg/kg. The results of wound healing capabilities showed that the wounds were significantly healed in animals treated with *F. heliophila* extract at 10 and 15 % ointment dose at ($p<0.01$ and $p<0.001$). The epithelialization process was also accelerated at 10 an 15 % dose of *F. heliophila* extract ointment and took 20.4±2.0 and 18.5±1.2 days for complete wound healing. The results of this study provide scientific support for alternative use of *F. heliophila* as a therapeutic agent in the treatment of skin wounds and infections.

Keywords: *Farsetia heliophila*; GC/MS; Antibacterial; AFM; Acute toxicity, Wound healing

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INTRODUCTION

The scarcity of novel antibiotics and increasing resistance to existing antibiotics aid to global prevalence of infections produced by microbes (Zhang *et al*., 2006; Paterson, 2008). Therefore, search for new, safe and economic drug is a need of the day. Since antiquity, plants have been a valuable source of natural products for human health conservation, particularly in the last decade, with additional thorough studies and research for natural therapies. Currently a progressive increase in the demand of phytochemicals for pharmaceutical purposes has observed in many countries. Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine (Sukanya *et al*., 2009). The scientific communities also pay much attention toward antimicrobial compounds. Currently, about 1340 plants has been defined with antimicrobial activities, from these plants around 30,000 antimicrobial compounds have been isolated (Tajkarimi *et al*., 2010). *F. heliophila* belongs to brassicaceae family, found in Balochistan province. The plant is locally known as Shakari, used in the bacterial infections of skin. The other species of genus *Farsetia*, like *F. aegyptia* was previously investigated for their cytotoxicity, antibacterial and antifungal activity. The important compounds identified previously in *F. aegyptia* were betulin, friedelin, β-amyrin, scopoletin and coumarin. The important flavonoids identified in *F. aegyptia* include kaempferol and apigenin (El-Sharkawy

et al., 2013). The leaves were also reported to contain glucosinolates (Marzouk *et al*., 2009; Gil and Macleod, 1980). Flavonoids were reported from *Farsetia hamiltonii* specie in Pakistan (Hayat *et al*., 2015). The main aim of this report is to evaluate the antibacterial, wound healing and screening of phytochemicals content in *F. heliophila*. To the best of our knowledge this is the first report on acute oral toxicity, antibacterial, morphological study of bacterial cells using atomic force microscopic and wound healing potential on hydroethanolic extract of *F. heliophila* extract.

MATERIALS AND METHODS

Plant sample collection and extraction: The plant *Farsetia heliophila* was collected from Loralai, Baluchistan, Pakistan during May, 2019. The plant was identified by a botanist at Federal Urdu University of Arts Science and Technology (FUUAST), Department of Botany and a voucher specimen was deposited. The collected plants were rinsed with tap water and dried under shade. The 1.5 kg bark were pulverized to fine powder and macerated in commercial grade methanol for 15 days. After 15 days the extract was filtered with Whatman filter paper and concentrated with rotary evaporator (B-490, Buchi) at 45°C. A greenish extract of about 132 gm was obtained.

Gross phytochemical investigation: The hydromethanolic extract of *F. heliophila* (HMFH) was screened for the presence of tanins, saponins and flavonides (Sofowora, 1996). Triterpenoids and alkaloids were screened as reported by (Nayak and Pereira, 2006; Oyedapo *et al*., 1999).

Gas chromatography (GC) and gas chromatography mass spectroscopy (GC/MS) analysis: The GC analysis of purified HMFH was carried out on (Agilent USB-393752, USA) with capillary HHP-5MS (5%) phenylmethylsiloxane capillary 0.25 µm) assembled with FID detector. The GC/MS of HMEFH sample was performed on (Agilent HP-5973, USA). An HHP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25mm×0.25µm) and FID detector was used. The experimental conditions and sample running for GC and GC/MS was in accordance with previous report (Burki *et al*., 2018). Further, the obtained spectra were matched with Wiley and NIST library (Stein *et al*., 2002; Adams, 2007), while the mass spectra were correlated with the available data in literature.

FTIR analysis: The FTIR of HMFH was analyzed on (Thermo Nicolet FT-IR Nexus) in the mid-IR region i.e., 4000-400 cm^{-1} at resolution 4 cm^{-1} as reported by (Latif *et al*., 2020).

Anti-bacterial assay of HMFH

Microorganisms tested: The 4 common human pathogenic microbial strains were used in this study. The Gram-positive bacterial strain *Bacillus subtilis* (*B. subtilis*), and Gram-negative *Salmonella typhi* (*S. typhi*) *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were selected for antibacterial activity. The bacterial cultures were obtained from Essa Laboratory and were maintained at appropriate agar slant at 4 ℃ prior to experimental use. The strains were further sub-cultured on Muller Hinton agar plate at 4 ℃ and grown on 37 °C when required.

Quantitative antibacterial activity assay: The frequently used and accepted sensitive tetrazolium microplate method (Piaru *et al*., 2012) was adopted for this antibacterial activity. Briefly the overnight bacterial cultures were adjusted to McFarland standard 1, equivalent to 3.08×10^8 cfu/mL (*B. subtilis*), 3.7×10^8 cfu/mL (*S. typhi* and *E. coli*) 3.2×10^8 (*P. aeruginosa*). The serial dilutions 5, 10, 25, 50, 75, 100 and 125 µg/ml of HMFH *were* prepared. About 200 µL of each dilution was added to each well started with lowest concentration. The bacterial strains were also added to each well and incubated further for 18-24 hours at 37± 0.5 ºC. After incubation the 50 µl of MTT was added to the microtiter plate. The absorbance was measure at 570 and 650 nm after incubation period of 30 minutes. Ciprofloxacin was used as a positive control. The IC_{50} was calculated as follows,

$$IC_{50} - \left[\left(\frac{\text{O.D in control} - \text{O.D of test}}{\text{O.D in control}}\right)\right]\times 100$$

Atomic Force Microscopic study of extract treated bacteria: The HMFH treated bacterial cells of *B. subtalis, S. typhi, E. coli* and *P. aeruginosa* were harvested from the microtiter plate. The bacterial culture were diluted with density of 10^5cfu. From this prepared culture about 10 µL of culture was applied on polylysine mica slides. The slides were subject to drying process at ambient temprature. After drying the slides were studied on AFM.

The 3D topography images of the treated bacteria (*B. subtalis, S. typhi, E. coli* and *P. aeruginosa*) were obtained. The PicoView 1.2 imaging analysis software was used for image processing as discussed by (Allison *et al*., 2011).

Animal studies: The swiss albino mice of (20-25 g) were obtained from Dow university of health sciences. The animals were maintained under standard nutritional and environmental conditions as reported by (Burki *et al*., 2018). Animal ethical committee clearance was obtained from institutional review committee (FH-SM-19C).

Acute oral toxicity assessment: The animal's mice for acute toxicity were randomly selected and divided in to six groups (n=3). The animals were kept fasted only allowed to water for at least 7 hours. The extract solution

in distilled were prepared at a concentration of 100, 500, 1000, 2000, 3000 and 5000 mg/kg. Animals of six group (1-6) received HMFH solution 100, 500, 1000, 2000, 3000 and 5000 mg/kg, while group 7 animals only received normal saline. At the end of 14-day trial animals were euthanized and its organs heart, liver, kidney and stomach were excised, weight and compared its morphology with the control (normal saline) treated animals.

In vivo* wound healing activity of *F. heliophila

Preperation of ointment: The ointment for the assisment of wound healing activity was prepared by fusion technique. For this purpose white soft paraffin, hard paraffin, wool fat and cetosteryl alchol were heated in an increasing melting point order with constant and gentle mixing. The resultent mixture was cooled and packed in wide mouth container as explained by (Krishna *et al*., 2017). The three formulations (5 %, 10 % and 15 %) of HMFH were prepared by incorporation of the extract into prepared ointment.

Excision wound model: The procedure of wound healing activity was carried out as explained by Krishna *et al*., (2017) with slight modifications. Prier wound excision in animals, the animals were divided in to three groups. Animals in group I were (control) received normal saline, while animals in group II (standered) treated with povidone iodine ointment (5%). The animals in group III were further sub-divided in to IIIa, IIIb and IIIc. Animals in group IIIa, IIIb and IIIc were treated with 5%, 10% and 15 % of HMFH ointment twice a day. In each group (n=6) animals were kept.

Prior wound excision the animals dorsal fur was removed by shaving and the shaved skin was sterlized with 70% ethanol. Furthermore, the animals were anasthetized with 1 ml ketamine hydrochloride at a dose of 10 mg/kg. A wound of about 1 cm diameter, and 0.1cm depth was created in all animals by surgical blades and scissors under sterile conditions and wound was left open. The extract ointments, stander drug and normal saline were applied to their respective groups as explained above. The wound area was periodically monitored and measure with the help of transparent graph paper and marker on day 1st, 4th, 7th, 10th,14th and 17th. The time consumed on re-epithelialization was also calculated by recording number of days for complete wound healing. The percentage of area wound contraction was calculated as follows,

$$\% \text{ of wound contraction} = \frac{\textit{initial wound size} - \textit{specific day wound size}}{\textit{initial wound size}} \times 100$$

Statistical Analysis: Data analysis was carried out on GraphPad prism for statistical and graphical analysis 5.01, (La Jolla) Software Inc. The antibacterial analysis was performed in triplicate. Values were calculated as mean ± SEM. For verification of statistical difference one-way analysis of variance (ANOVA) was performed according to experimental protocol. For antibacterial and wound healing activity $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Phytochemical investigation of HEMF: In this study the plant extract was intend to cure skin wounds along with its antibacterial effects. Due to lack phytochemical investigation, *F. heliophila* extract was initially screened for the presence of important bioactive constituents. Since, natural products are considered as rich source of biologically active compounds (Rawat *et al*., 2018). Further, the HMFH extract was examined for antibacterial and wound healing potential. Globally, ethnopharmacologists also deliver the latest information on therapies from natural sources (Cooper, 2008). There is a growing need for finding of medicinal plants especially antibacterial agents that could help to eradicate the pathogenic bacteria and to avoid relapse of skin infections. In the whole extraction process of this study methanol was used as an extraction medium, as it is one of the ideal solvents for the process of extraction of polar and moderately polar antimicrobial phytoconstituents bearing antimicrobial property (Cowan, 1999; Vaghasiya and Chanda, 2007). Previously, *Farsetia hamiltonii* specie from Pakistan was phytochemically investigated (Hayat *et al*., 2015). The qualitative phytochemical investigation revealed that HMFH contain flavonoids, alkaloids, saponins, tannins and tri-terpenoids, which shows important pharmacological activities (Parameswari *et al*., 2019). Initially the qualitative phytochemical screening of HMFH was performed and revealed the presence of flavonoids, alkaloids, saponins, tannins and tri-terpenoids Table 1.

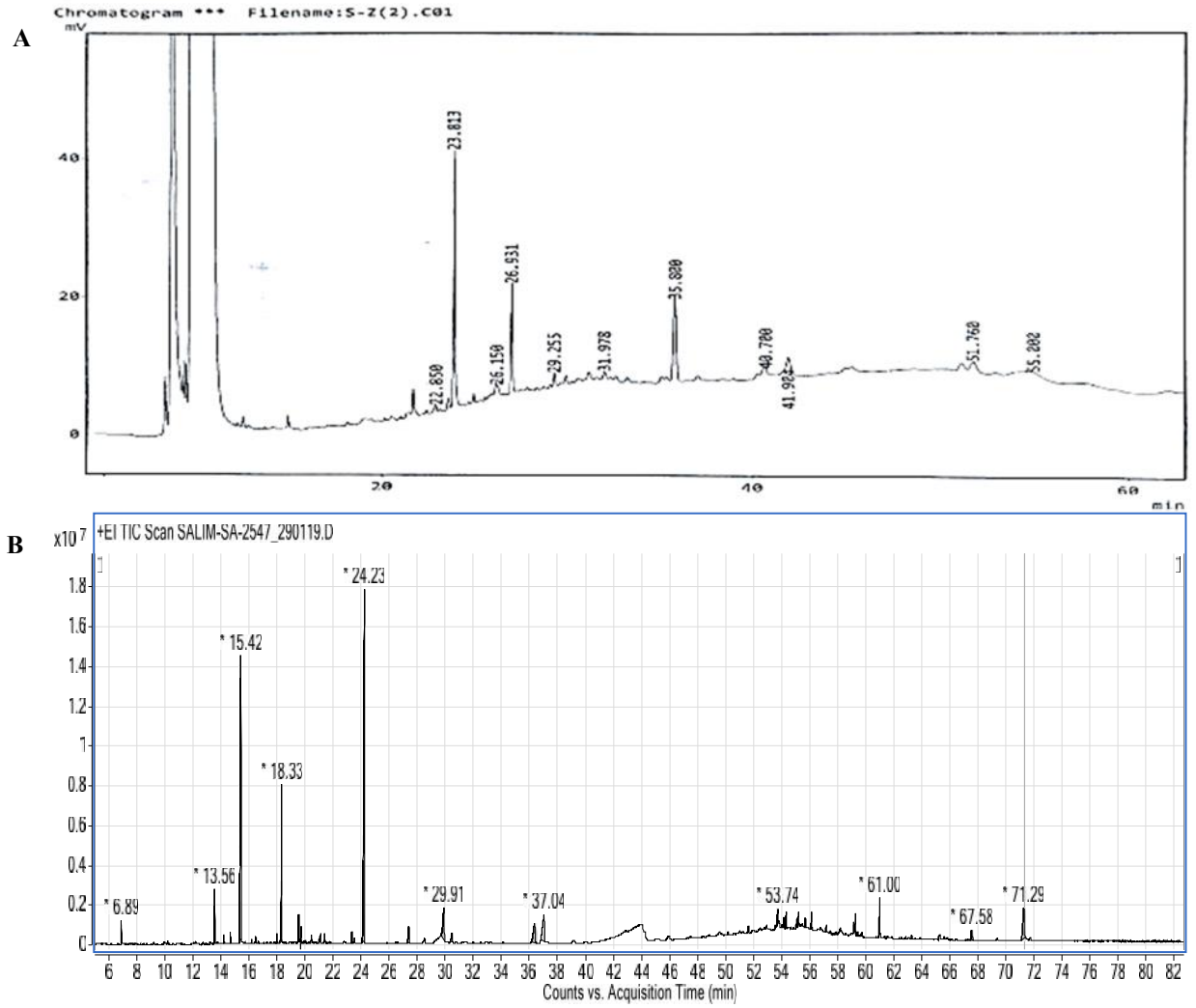
The GC and GC-MS finger printing Figure 1 (A and B) of HMFH conformed and identified more than 50 compounds. Some of the important identified molecule include barrigenol R1, ethyl iso-allocholate, lupeol actate, isorhamnetin, α-amyrine, α-tocopherol, l-(+)-ascorbic acid 2,6-dihexadecanoate, ascorbyl palmitate, 2-pyrazolin-5-one, 3,4,4-trimethyl- isoquercetin, pyrimidine, 4-cyclopropyl-, indole and cyclolaudenol. The FTIR spectra (Figure 1c) conform the functional groups present in the identified compounds. IR peak at (cm^{-1}): 2923 (saturated CH stretching), 2854 (CH_3, CH_2), 1741 (CO, CHO), 1697 (C=N), 1646 (N-H), 1457 (CH_2, CH_3),1375 (C-C double bonds /aromatic) 1234 (carboxylic acid derivatives), 1155 (C-N, single bond), 891 (NH_2), 720(OH). The functional groups were matching the important compounds identified in HMFH. The identified compound like Ethyl iso-allocholate (Malathi and Ramaiah, 2017), barrigenol R1 (Oh *et al*., 2014), lupeol acetate (Gallo and Sarachine, 2009), isorhamnetin (Bhattacharya *et al*., 2016; Ramachandran

et al., 2012), α-amyrine (Melo *et al*., 2011), α-tocopherol (Hobson, 2016; Bidossi *et al*., 2017), l-(+)-ascorbic acid 2,6-dihexadecanoate (Karthikeyan *et al*., 2014), ascorbyl palmitate, 2-pyrazolin-5-one, 3,4,4-trimethyl-isoquercetin, pyrimidine 4-cyclopropyl-, indole (Kaushik *et al*., 2013) and cyclolaudenol (Djemgou *et al*., 2010) were reported for significant antibacterial activities.

Table 1. Phytochemical prospecting.

S. No	Test	Observation	Results	(+) (-)
1.	500 mg MBHE + 5 mL dilute NH_3 solution → 2 mL H_2SO_4 (conc.) added	Appearance of Yellowish color	Flavonoids	+ve
2.	1 drop of MBHE solution on TLC plate+ Dragen-dorff's reagent	Appearance of orange /red color	Alkaloids	+ve
3.	200 mg MBHE → boil + 5 mL distilled H_2O→ shudder vigorously→ froth formation + olive oil → shudder vigorously	Emulsion formation	Saponins	+ve
4.	Aqueous aliquot of MBHE + $FeCl_3$ reagent	Appearance of greenish black color	Tannins	+ve
5.	300 mg MBHE + 3 mL $CHCl_3$→ warmed for 0.5 hour → 2 mL H_2SO_4 (conc.) added	Red color appearance in lower layer	Tri-terpenoids	+ve

(+) Presence, (-) Absence



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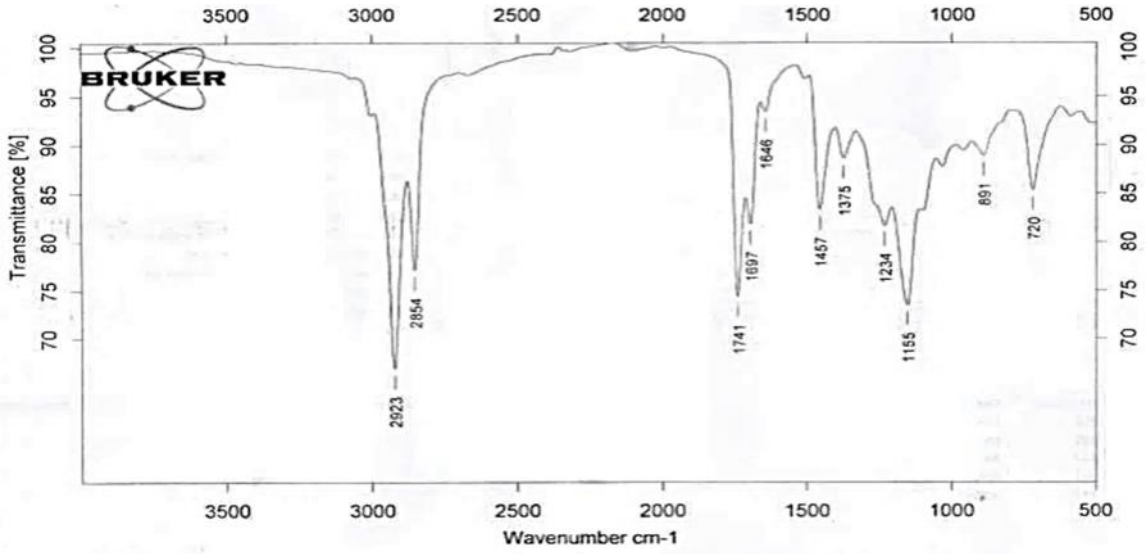

Figure 1. (A) GC (B) GC/MS and (C) FTIR chromatogram of *F. heliophila.*

Anti-bacterial activity of HMFH: The antibacterial effect of HMFH was conformed using tetrazolium microplate assay technique. In this study one-gram positive bacteria *B. subtilis* while three-gram negative bacteria *S. typhi*, *E. coli* and *P. aeruginosa* were tested against HMFH. The HMFH showed maximum *in-vitro* antibacterial activity against *B. subitils*, *S. typhi, E. coli* and *P. aeruginosa* specially at 125 µg/ml concentration. The results also indicate that there is a significant difference in the sensitivity of tested microorganisms against HMFH ($p<0.001$). The Gram-positive *B. subtilis* was more sensitive to HMFH and achieved 100% inhibition at 125 µg/mL, while *S. typhi* achieved 93±1.1% inhibition at 125 µg/mL ($p<0.001$). At 125 µg/mL *E. coli* and *P. aeruginosa* achieved 82±0.6 and 71±0.7 inhibition ($p<0.001$ and $p<0.01$) (Figure 2). *P. aeruginosa* was comparatively less sensitive at HMFH 125 µg/mL. The results were comparable with standard drug ciprofloxacin 30 µg/mL.

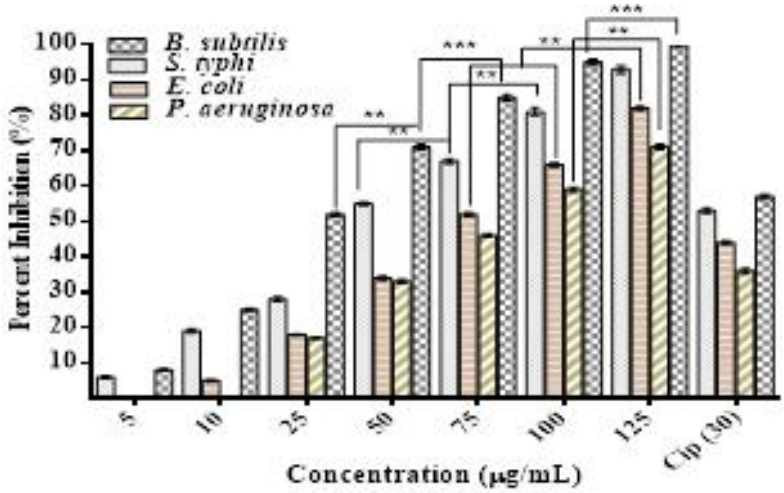

Figure 2. Antibacterial activity of *F. heliophila* extract against resistant Gram-negative and Gram-positive bacteria strains. Each value is represented as mean±SEM values are represented as (n=3), (*p < 0.05; **p < 0.01; ***p < 0.001), Cip= ciprofloxacin

The MIC_{50} values of HMFH were also significant against tested microorganisms. The MIC_{50} of HMFH ranged between 25-100 µg/mL. The MIC_{50} against *B. subitils* and *S. typhi* was 25 and 50 µg/mL at ($p<0.01$). *E. coli* and *P. aeruginosa* showed MIC_{50} at 75 and 100 µg/mL at ($p<0.05$) Table 2. and the results were comparable with standard drug ciprofloxacin. The significant antibacterial activity may be due to important biologically active compounds in the extract of *F. heliophila.*

Table 2. Minimum inhibitory concentration (MIC_{50}) of crude methanolic extract of *F. heliophila* against strains of Gram-positive and negative bacteria.

Bacteria	MIC_{50} (µg/mL)	p-value
B. subtilis	25	p<0.01
S. typhi	50	p<0.01
E. coli	75	p<0.05
P. aeruginosa	100	p<0.05

Atomic force microscope study of HMFH treated bacteria: The antibacterial activity of HMFH was further confirmed via atomic force microscopic 3D images of the extract treated bacteria. The images of tested bacterial strains against HMFH are captured and have been presented in (Figure 3). The AFM images reveled that *B. subitils* and *S. typhi* were extensively damaged. The appearance of cell membrane of microbes in these images looked fragile and their cytoplasm appears to be irreversibly leaked from the cell. The effect of HMFH extract on *E. coli* and *P. aeruginosa* cell membrane was also appreciable but surprisingly *E. coli* and *P. aeruginosa* maintained their cellular integrity (Figure 3). Nonetheless, these results could be linked and justify the significant antibacterial activity.

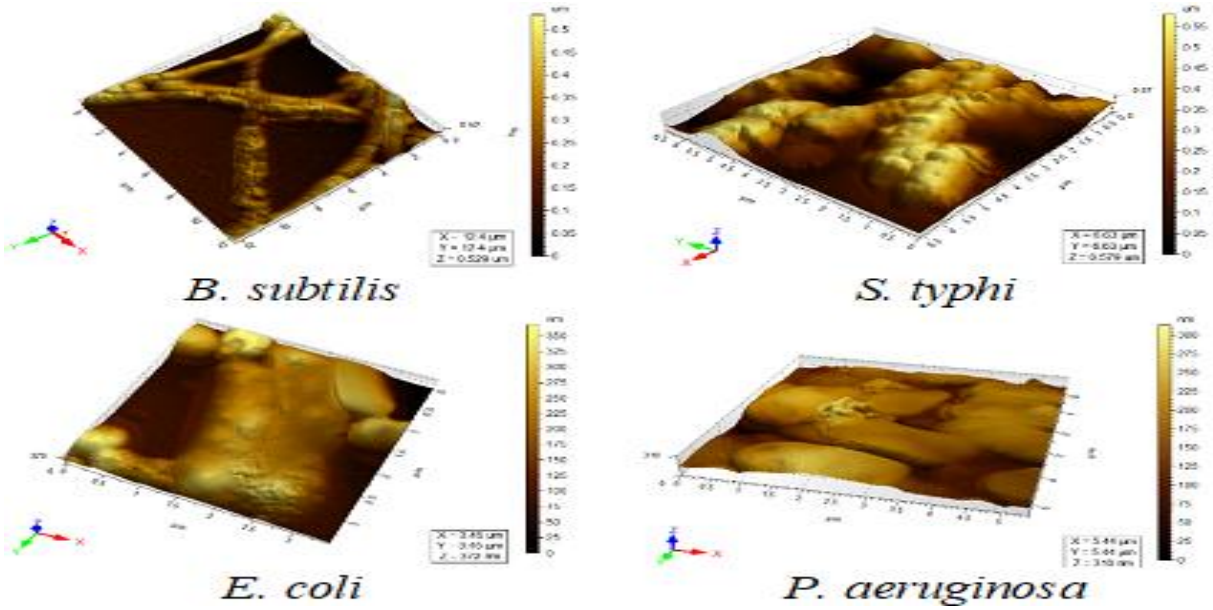

Figure 3. Atomic force microscopic 3D images of *B. subtilis*, *S. typhi*, *E. coli* and *P. aeruginosa* treated with *F. heliophila* extract

Acute oral toxicity assessment of HMFH: In the acute oral toxicity test, HMFH presented no toxicity and mortality to the experimental animals on the oral administration of HMFM. In the (Figure 4) are the excised organs (liver, heart, kidney and stomach) of animals treated with extract 2000, 3000 and 5000 mg/kg. The organs of extract treated animals were compared to that of the control animals. There was no change in the color and the weight of organs of mice treated with extract 2000 and 3000 mg/kg, upon comparison with that of control animals. The organs of animals treated with 5000 mg/kg were slightly darkish on comparing with the organs of animals treated with 2000 and 3000 mg/kg dose and control. It is revealed that the LD_{50} of HMFH is > 5000 mg/kg dose. The results revealed that HMFH has a relative margin of safety as a therapeutic agent.

Wound healing activity of HMEFH: Wound healing is complex biological process in order to maintain and restore tissue integrity. Inflammation, inflammatory cells and markers are responsible for the delayed wound healing process (Hunt, 1988; Koh and DiPietro, 2011). The bacterial colonization and resistance also aid in the delayed wound healing process (Edwards and Harding, 2004). Therefore, HMFH was evaluated against skin infection using wound healing method by creating full thickness skin wound on the back of mice. Each wound was studied for a period of 17 days.

The data indicates that the HMFH (10 and 15 %) ointment was considerably effective in reducing the wound size as compared to positive control Povidone iodine ointment 5 %. A dose of 10 % ointment was capable of healing the wound 63.4±1.0 ($p<0.01$), 78.1±2.4, and 87.6±3.0 % ($p<0.001$) on day 10, 14 and 17^{th}, while 15 % dose HMFH ointment was capable to reduce wound size by 63.4±1.0 ($p<0.01$), 78.1±2.4 and 87.6±3.0 ($p<0.001$) on 10^{th}, 14^{th} and 17^{th} day. Povidone iodine 5 % ointment exhibited 57.7±2.0, 70±1.6 and maximum 83.5±2.3% ($p<0.001$) wound healing

capability on 10th, 14th and 17th day. The re-epithelialisation process was also dose dependent. At a dose of 10 % the re-epithelialisation took 20.4±2.0 days, while at 15 % dose this process complete in 18.5±1.2 days. The wound healing results HMFH were comparable with standard Povidone iodine ointment 5.0% Table 3. The significant wound healing property of HMFH could be linked with important antibacterial and anti-inflammatory agents like quercetin. Quercetin has been previously reported as strong anti-inflammatory agent (Li *et al*., 2016). The HMFH also showed positive result to qualitative flavonoids test. Flavonoids have astringent and antimicrobial property, which appear to be responsible for wound healing and accelerate epithelialization process (Tsuchiya *et al*., 1996). The tannins present in HMFH also have astringent activity (Cowan, 1999). Therefore, the wound healing potential of HMFH could also be linked with tannins present in the HMFH.

a **B** **c**

Figure 4. Excised organs of mice treated with *F. heliophila* extract (a) 2000 mg/kg (b) 3000 mg/kg and (c) 5000 mg/kg.

Table 3. Wound surface area (cm^2) in mice treated with *F. heliophila.*

Group	Days	Group I	Group II	Group III dose (w/w %)		
				5 %	10 %	15 %
Percent wound healing (%)	1	0.0	0.0	0.0	0.0	0.0
	4	13.6±1.4	22.5±2.4	20.7±2.4	24.6±2.5	27.7±2.3
	7	27.4±1.5	41.8±2.3	40.5±2.5	42.5±1.5	45.5±1.2
	10	41.2±1.5	57.7±2.0	56.8±2.6	63.4±1.0*	66.8±2.4*
	14	55.2±2.5	70±1.6*	69.5±1.8*	78.1±2.4‡	80.2±2.2‡
	17	64.2±2.0	83.5±2.3‡	81.8±2.2‡	87.6±3.0‡	92.6±3.1‡
Period of epithelialization (days)	N/A	24.8±1.3	21.4±2.9	23.8±1.6	20.4±2.0	18.5±1.2

Values represented as mean ± SEM of all groups on different days, Statistically; * p<0.05, *p<0.01 and ‡p<0.001

The images in (Figure 5) showed the wound size reduction treated with different formulation of HMFH ointment. The wound size reduction pattern was dose dependent, nevertheless the results were comparable with standard povidone iodine ointment 5 %.

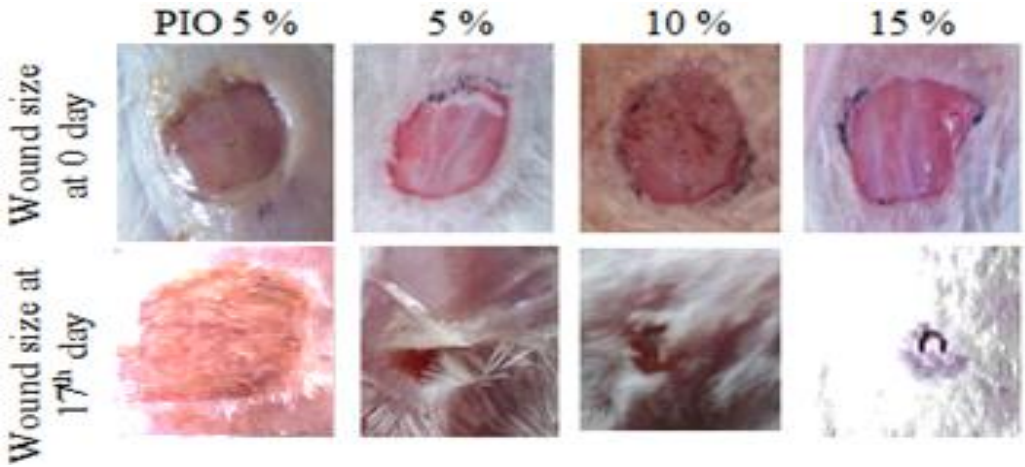

Figure 5. Excision wound model in mice, Group III (each sub group) treated with *F. heliophila* extract at a dose of 5, 10 and 15 % w/w body weight (topically). Povidone iodine ointment (PIO) 5 %, (topically), treated with in paste form.

The antibacterial, AFM, oral acute toxicity and wound healing results suggested that HMFH safe and effective medicinal plant. However, further studies on skin infections and its histopathology are required.

Conclusion: It is concluded that *F. heliophila* bark extract is enriched with important antimicrobial and anti-inflammatory compounds. The results showed that HMFH is safe and has broad therapeutic index. The significant antibacterial and wound healing activities of HMFH suggest that it could be a part of complementary medicine and alternative therapy.

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Conflict of interest: The authors declared no conflict of interest.

Contributions: SB conducted the whole study with ZGB and Mehjabeen collaboration. SB and ZGB wrote the paper and help during the practical work. MK and IA were responsible for acute toxicity study and histology. SA help in phytochemical characterization, drafting and writing the final version of the manuscript. All authors performed data analysis in addition they read and approved the final manuscript.

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