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Original Article

EXTRACTION, BIOACTIVITIES, PHYTOCHEMICAL INVESTIGATION AND *IN-VIVO* TOXICITY STUDIES OF *MESUA FERREA* L. STAMENS

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ABSTRACT

Objective: To investigate *in-vitro* antibacterial and free radical scavenging potentials of *Mesua ferrea* L. stamens which are used in traditional medicinal preparations along with their phytochemical investigation and *in-vivo* toxicity studies

Methods: Various extracts of *M. ferrea* stamens were prepared by kinetic maceration method using four organic solvents. Potent antibacterial n-hexane extract of stamens was selected in the preliminary screening for antibacterial activity which was performed by an agar diffusion method for further studies. Quantification of antibacterial activity of n-hexane extract was carried out using broth microdilution method as per CLSI guidelines. Phytochemical investigations of the same extract were performed using qualitative tests for the detection of various phytochemical groups and Libermann-Burchard colorimetric assay for determination of total terpenoid content. *In-vivo* safety of the extract was determined by acute oral toxicity studies in mice as per OECD guidelines test no. 420.

Results: n-hexane extract of *M. ferrea* stamens was found most potent amongst other extracts studied for antibacterial activity; moreover it exhibited bactericidal activity against selected bacterial pathogens. The same extract exhibited good free radical scavenging activity in DPPH assay (IC_{50} value = 66.3 µg/ml). Phytochemical investigation of extract revealed presence of sterols, terpenoids and volatile oil components and the total terpenoid content was estimated as 102.8 mg/ml of dried extract (in terms of lupeol equivalents). The extract was found safe in mice in acute oral toxicity studies.

Conclusion: *M. ferrea* stamens exhibited broad spectrum antibacterial activity along with good free radical scavenging potential and *in-vivo* safety which encourage further studies in the area.

Keywords: *Mesua ferrea* L, Antibacterial, Free radical scavenging, Phytochemical investigation, Acute oral toxicity.

INTRODUCTION

Mesua ferrea Linn is a medium to large evergreen tree from the family *Clusiaceae*; widely distributed in countries like India, Burma, Thailand, Indochina and New Guinea [1, 2]. In India, it occurs in the lower Himalayas from Nepal eastwards to Bengal, Assam, Eastern and Western Ghats, North Canara, Konkan region of Maharashtra state, Andhra Pradesh and Andaman & Nicobar Islands ascending up to 1500 meters [3]. The plant is extensively used in traditional medicine for various pharmacological actions and constitutes a part of the number of traditional medicinal preparations such as *Nagkeshara-adi-churna, Nagkeshara Yoga, Eladi churna, Lavangadi churna* and *Dasamoolarishta* etc [2].

M. ferrea seeds, leaves and stem bark have been studied for the array of medicinal properties such as antioxidant and antimicrobial activity, analgesic, antispasmodic and anti-venom activities, immunomodulatory and anti-arthritic potentials etc [2]. Flowers of *M. ferrea* have also been studied for their various medicinal potentials. The dried flowers of the plant are aromatic and used for the treatment of bleeding piles, dysentery, cough and also used as carminative [4]. The flower extract is an ingredient of Ayurvedic preparation *'Arshina'* tablets used for the treatment of piles [5]. *M. ferrea* flowers have also been studied for their anti-convulsant, anti-neoplastic, hepatoprotective and immunomodulatory activities [2]. Methanolic and dichloromethane extracts of *M. ferrea* flowers have been studied for antibacterial activity against various bacterial pathogens [2]. *In-vivo* and *in-vitro* antioxidant activity of methanol and ethanol extracts of *M. ferrea* flowers have also been reported [2]. Volatile oil from *M. ferrea* flowers exhibited antibacterial, antifungal and anthelmintic activities [6]. However, only a limited amount of research work has been reported for the stamens of *M. ferrea* which represent a major constituent of *M. ferrea* flowers as they are abundantly present in the flowers. They also constitute drug preparations such as '*Nagkeshar*' which is used for treatment of fever, dispepsia and renal diseases [2, 6]. But only a smaller amount of scientific data is available regarding their medicinal potentials, phytochemical analysis and *in-vivo* safety.

Hence, the present study was undertaken to evaluate broad spectrum antibacterial potential of various extracts of *M. ferrea* stamens, quantification of antibacterial activity of potent extract and evaluation of antioxidant activity and phytochemical investigation for various phytochemical groups present in the potent extract. Quantification of Total Terpenoidal Content and assessment of *in-vivo* safety of the potent extract by acute oral toxicity studies in mice were also part of the study.

MATERIALS AND METHODS

Collection and authentication of plant material

Stamens of *M. ferrea* were procured from local herb trader. They were authenticated from Agharkar Research Institute (ARI), Pune (India), with voucher specimen number I/F-30.

Extraction of *M. ferrea* stamens

Extraction of stamens was carried out using the method of ' kinetic maceration' in which plant material is kept in constant motion with the extraction solvent [7]. Four organic solvents differing in their polarities were used for extraction purpose. The solvents were namely; n-hexane, dichloromethane, ethyl acetate and ethanol. Extraction was carried out at room temperature using rotary test tube shaker at 30 RPM for 6 hours. 1 g of plant material was extracted using 10 ml of organic solvent. The extracts were filtered to remove particulate matter. The organic solvents after extraction was evaporated under vacuum and dried extracts were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 5 % w/v for further microbiology work.

Pathogenic bacteria procured for the study

Six pathogenic bacteria known to cause an array of infections in human, were selected for evaluation of antibacterial activity of various extracts *M. ferrea*. The authentic, pathogenic strains of bacteria were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India). The six pathogenic bacteria were as follows:

The gram positive bacteria selected for the study were

1. *Staphylococcus aureus*, MTCC No. 96, pathogenic strain

2. *Staphylococcus epidermidis*, MTCC No. 435, isolated from skin lesion

3. *Streptococcus mutans*, MTCC No. 497, isolated from carious dentine

The gram negative bacteria selected for the study were

1. *Pseudomonas aeruginosa,* MTCC No.3542, pathogenic strain isolated from human

2. *Proteus mirabilis,* MTCC No. 425, isolated from urine of patient with kidney stones

3. *Serratia marcescens,* MTCC No. 97, pathogenic strain isolated from pond water

All the cultures (except *S. mutans*) were maintained on nutrient agar. *S. mutans* was maintained on Brain Heart Infusion (BHI) agar as per MTCC recommendations.

Preliminary antibacterial screening

Preliminary screening for antibacterial activity of the various extracts of *M. ferrea*, was carried out using zone of inhibition-agar diffusion assay. Mueller Hinton (MH) Broth, Brain-Heart Infusion (BHI) broth and Mueller Hinton (MH) Agar (all manufactured by Himedia) were used for the assay.

All the bacterial cultures were freshly grown overnight in the broth media recommended by MTCC. The turbidity of all the cultures was adjusted to match 0.5 Mcfarland standard (approx. matching to 1.5 x 10^8 CFU/ml) using sterile MH broth. 1 ml of the turbidity adjusted inoculum was mixed with 20 ml of sterile molten MH agar. The mixture was poured in petri plates (BOROSIL) and allowed to cool and solidify. After solidification of the seeded agar in petri plates, wells of 8 mm diameter were punched through the surface. 50 µl of all test extracts of *M. ferrea* were added to the stipulated wells and petri plates were incubated at 37 ^{0}C for 24 hours (except for *S. marcescens,* which was incubated at 30 ^{0}C as per MTCC recommendations). After the incubation period, the plates were observed for clear zones of inhibition of bacterial growth due to antibacterial compounds present in the test extracts. The zones of inhibition around wells were measured in millimetres. 0.02 % v/v of Clindamycin (Cipla Ltd., India) and 0.01 % v/v Gentamicin (Aquafine Injecta Pvt. Ltd., Pune, India) were used as a positive control for antibacterial activity against gram positive and gram negative bacteria respectively as per Clinical Laboratory Standards Institute (CLSI) guidelines [8]. The experiment was repeated three times and results are expressed as a mean of three experiments with standard deviation.

Determination of MIC and MBC values

The most potent antibacterial n-hexane extract of *M. ferrea* which was shortlisted in preliminary screening study; was evaluated for its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values. MIC and MBC were determined by broth microdilution assay using 96-wells microtitre plates as per CLSI guidelines with some modifications [9].

Determination of free radical scavenging potential

Free radical scavenging activity of n-hexane extract of *M. ferrea* was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay. 3.94 mg of DPPH was dissolved in 100 ml of methanol to give 0.1 mM DPPH reagent. Ascorbic acid was used a standard for the assay at concentration range of 10-34 µg/mL. Dried n-hexane extract of *M. ferrea* dissolved in methanol was used a test solution at a concentration range of 20-120 µg/ml. 3 ml each of the solutions of the test extract was mixed with the 1 ml of the prepared DPPH reagent and allowed to react in dark for 45 minutes at room temperature. Afterwards, the absorbance of all the test mixtures was measured using spectrophotometer (JASCO V-630) at 517 nm. Methanol was used as a blank and methanol (without plant extract) added with DPPH reagent was used a control for the assay. Absorbance values of all the test samples and standards were recorded in triplicates. The free radical scavenging activity was calculated by following formula:

$$\%\ \text{DPPH reduction} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

The IC_{50} values of test extract and standard ascorbic acid were calculated using linear regression analysis and compared.

Phytochemical analysis

Qualitative tests for the detection of various groups of phytochemicals present in the n-hexane extract of *M. ferrea* stamens, were carried out using standard methods of *Jones & Kinghorn*, (2006) [10] and *Sasidharan et al,* (2011) [11].

Determination of total terpenoid contents

From a stock solution (400µg/mL) of lupeol standard in chloroform; 6, 7, 8, 9 and 10 ml of solutions were taken out and evaporated separately to give concentration range of 2400-4000 µg. Similarly, 3 ml of n-hexane extract of *M. ferrea* (10 mg/ml) was evaporated to dryness. To these dried standards and test extract, 0.5 ml of glacial acetic acid and 5 ml Liebermann-Burchard reagent was added. The volumes of mixtures were made to 10 ml using chloroform. The mixtures were allowed to stand for 20 minutes. Absorbance values of all the concentrations of the standard and test extract were read at 618 nm using UV-Visible spectrophotometer (JASCO V-630). Appropriate blank was used for the assay. Total terpenoidal content of the test extract was determined from the equation ($y = 8E^{-05} \times 0.01$) obtained by linear regression analysis of the calibration curve of standard lupeol and expressed as lupeol equivalents (mg) per gram of dried extract. Experiment was performed in triplicates and results were expressed as mean value with standard deviation.

***In-vivo* acute oral toxicity studies**

In-vivo acute oral toxicity study for safety assessment of n-hexane extract of *M. ferrea* was carried out as per Organization for Economic Co-operation and Development (OECD) guidelines test no. 420. Study was performed on Swiss Albino female mice at Animal Testing Centre of Ramnarain Ruia College, Mumbai (India); after an approval (KB-130620-01) from Institutional Animal Ethics Committee. Fixed dose procedure at a dose level of 2000 mg/kg body weight of an animal was followed. Daily food and water intake, body weight changes, mortality, colour and consistency of feces, breathing abnormalities, gait etc. were the parameters observed for the animals during the study.

RESULTS

Extraction of *M. ferrea* stamens at room temperature by kinetic maceration method gave different percent extraction yields for various organic solvents used, as shown in table 1. The yields were in the decreasing order for ethanol (14.2%) > ethyl acetate (12.6%) > n-hexane (7.2%) > dichloromethane (5.1%). It was observed that the yields were more for mid-polar and polar solvents than the non-polar ones.

Results obtained for preliminary screening for antibacterial activity by the zone of inhibition-agar well diffusion method are given in table 1. Zones of inhibition values mentioned in the table are means of three different experiments performed on three different days with the standard deviation values. All the four test extracts of *M. ferrea* stamens exhibited broad spectrum antibacterial activity. It was observed that n-hexane extract of *M. ferrea* stamens demonstrated fairly potent broad spectrum antibacterial activity as it exhibited larger diameters for inhibition zones against all the test pathogenic bacteria. Hence the n-hexane extract of *M. ferrea* stamens was shortlisted for further studies. The inhibition zone diameters for

n-hexane extract of *M. ferrea* stamens ranged between 17.3-11.7 mm. Largest zone diameters were observed against *S. mutans* and *P. mirabilis* whereas the smallest zone diameter was obtained for *S. marcescens*. The zone diameters obtained for n-hexane extract of *M. ferrea* stamens were comparable with the zone diameters exhibited by synthetic positive controls (standard antibiotics) used.

Table 1: It shows the percent extractions yields and inhibition zone diameters for the antibacterial activity of four extracts of *M. ferrea* stamens by agar well diffusion assay

S. No.	Pathogenic bacteria	Diameter of zone of inhibition + Standard deviation (S. D) for *M. ferrea* extracts (5% w/v) with their percent extraction yield				DMSO (Vehicle)	0.02% Clidamycin	0.01 % Gentamicin
		n-hexane (7.2%)	DCM (5.1%)	Ethyl acetate (12.6%)	Ethanol (14.2%)			
1	*S. aureus* (MTCC 96)	15.7 + 0.58	13.3 + 0.58	12.7 + 0.58	12.3 + 0.58	-	11.7 + 0.58	Not Tested
2	*S. epidermidis* (MTCC 435)	16.3 + 1.15	13 + 1	13.7 + 1.52	12.7 + 0.58	-	11.3 + 0.58	Not Tested
3	*S. mutans* (MTCC 497)	17.3 + 0.58	14.3 + 0.58	13.3 + 0.58	14.7 + 0.58	-	15.7 + 0.58	Not Tested
4	*P. aeruginosa* (MTCC 3542)	12 + 1	9.7 + 0.58	9.3 + 0.58	10 + 1	-	Not Tested	12.3 + 0.58
5	*P. mirabilis* (MTCC 425)	17.3 + 1.15	11.3 +1.15	11.3 + 0.58	10.3 + 0.58	-	Not Tested	12.7 + 0.58
6	*S. marcescens* (MTCC 97)	11.7 + 0.58	10.33 + 0.58	9.3 + 0.58	9.7 + 0.58	-	Not Tested	13.7 + 0.58

Abbreviations

DMSO: Dimethyl sulfoxide, DCM: Dichloromethane, -: No zone of inhibition was observed,

Table 2 gives the MIC and MBC values for the quantification of antibacterial activity of n-hexane extract of *M. ferrea* stamens against test pathogenic bacteria. The MIC values ranged between 0.2-0.8 % w/v. Lowest MIC value of 0.2 % w/v was observed against *S. epidermidis*. A sample is said to be bactericidal in nature when the ratio of MBC/MIC ≤ 4 and bacteriostatic when this ratio is > 4 [12]. The n-hexane extract of *M. ferrea* stamens was found bactericidal in nature as it exhibited MBC/MIC ratio 4 or less than 4 against four of the six pathogenic bacteria (for *S. aureus* and *S. marcescens* the MBC value was out of the concentration range selected for the study).

Table 2: It shows MIC and MBC values obtained for the n-hexane extract of *M. ferrea* stamens as per CLSI guidelines

S. No.	Pathogenic Bacteria	n-hexane extract of *M. ferrea* stamens		MBC/MIC ratio*
		MIC	MBC	
1	*S. aureus* (MTCC 96)	0.8%	Above1.6%	-
2	*S. epidermidis* (MTCC 435)	0.2%	0.8%	4
3	*S. mutans* (MTCC 497)	0.8%	0.8%	1
4	*P. aeruginosa* (MTCC 3542)	0.8%	1.6%	2
5	*P. mirabilis* (MTCC 425)	0.4%	0.8%	2
6	*S. marcescens* (MTCC 97)	0.8%	Above1.6%	-

(Concentration range tested for the extract was 1.6% - 0.05% w/v), -: Not calculated, * MBC/ MIC < 4: Bactericidal Activity [12], * MBC/ MIC > 4: Bacteriostatic Activity [12],

Results of the qualitative tests for the investigation of phytochemical groups present in the n-hexane extract of *M. ferrea* stamens are given in table 3. The investigation revealed the presence of steroids, terpenoids and volatile oils in the test extract.

Table 3: It shows the results obtained for the qualitative phytochemical investigation of n-hexane extract of *M. ferrea* stamens

S. No.	Phytochemical groups	Test	Reference	Results*
1	Sterols	Liebermann-Burchard test	*Jones and Kinghorn*, 2006 [10]	+
2	Terpenoids	Salkowski test	*Sasidharan et al*, 2011 [11]	+
3	Volatile oils	NaOH and H_2SO_4 Test	*Sasidharan et al*, 2011 [11]	+
4	Phenolic compounds	Ferric chloride test	*Jones and Kinghorn*, 2006 [10]	-
5	Flavonoids	Shinoda test	*Jones and Kinghorn*, 2006 [10]	-
6	Tannins	Gelatine-Salt test	*Jones and Kinghorn*, 2006 [10]	-
7a.	Alkaloids	Mayer's Reagent Test	*Jones and Kinghorn*, 2006 [10]	-
7b.		Dragendorff's Reagent Test	*Jones and Kinghorn*, 2006 [10]	-
8	Saponins	Foam Test	*Jones and Kinghorn*, 2006 [10]	-

* +: Presence of Phytochemical Group, -: Absence of Phytochemical Group

Total Terpenoid Content of n-hexane extract of *M. ferrea* stamens was determined using Libermann-Burchard colorimetric assay. Lupeol was used as a standard and the Total Terpenoid Content was found to be 102.8 mg lupeol equivanents per gram of dried extract (table 4). Fig. 1 illustrates the linear regression analysis for the calibration curve of the standard lupeol.

Table 4: It shows the Total Terpenoid Content of n-hexane extract of *M. ferrea* stamens

Test extract	Total terpenoid content
n-hexane extract of *M. ferrea* stamens	102.8 + 33.5 mg/g of dried extract (Lupeol Equivalents)

Table 5: It shows the Percent DPPH inhibition recorded for ascorbic acid standard and n-hexane extract of *M. ferrea* stamens

S. No.	% DPPH inhibition for ascorbic acid		% DPPH inhibition for n-hexane extract of *M. ferrea* stamens	
	Concentration (µg/ml)	Mean	Concentration (µg/ml)	Mean
1	10	26.48	20	1.35
2	14	38.85	40	12.39
3	18	43.13	60	56.96
4	22	66.12	80	67.19
5	26	79.26	100	88.98
6	30	86.48	120	95.64
7	34	98.18	-	-
	IC_{50} Value = 17.9 µg/ml		IC_{50} Value = 66.3 µg/ml	

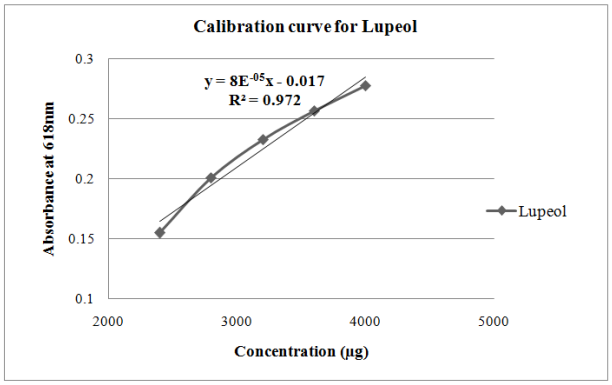

Fig. 1: It shows the calibration curve for lupeol standard from which Total Terpenoid Content of n-hexane extract of *M. ferrea* stamens was calculated

DPPH assay was used for the determination of free radical scavenging potential of n-hexane extract of *M. ferrea* stamens for which ascorbic acid was used as a standard. The IC_{50} value of 66.3 µg/ml was obtained for the test extract as compared to the IC_{50} value of 17.9 µg/ml for the ascorbic acid standard. table 5 shows the % DPPH inhibition values as per concentration for test extract as well as the ascorbic acid standard. fig. **2** illustrates the combined graph for the concentration versus % DPPH inhibition values for the test extract and the ascorbic acid standard.

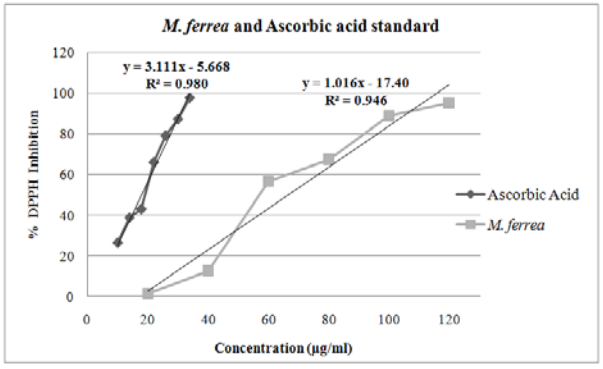

Fig. 2: It shows the combined graph for the concentration versus %DPPH inhibition values of the test extract and the ascorbic acid standard

DISCUSSION

Extraction of *M. ferrea* stamens in various organic solvents gave different extraction yields. Percent extraction yields for polar and midpolar solvents such as ethanol and ethyl acetate were comparatively more than non-polar solvents such as n-hexane and dichloromethane, which represent that the stamens of *M. ferrea* contains more amount of mid-polar and polar phytoconstituents than the non-polar ones.

However, remarkably higher antibacterial activity with larger zones of inhibition was exhibited by non-polar n-hexane extract of *M. ferrea* stamens as compared to other extracts in the preliminary screening for antibacterial activity by an agar diffusion method. It indicates that the higher extraction yields do not necessarily correspond to higher antibacterial activity. In the case of *M. ferrea* stamens, the phytoconstituents responsible for potent antibacterial activity must be non-polar in nature and hence they were extracted in non-polar solvent such as n-hexane, following the principle of 'like-dissolves-like'. Various extracts of *M. ferrea* stamens exhibited broad spectrum antibacterial activity. The n-hexane extract was found bactericidal in nature as it gave MBC/MIC ratio < 4 [12] for most of test bacterial pathogens; which are known causative agents of various infections in human such as skin and soft tissue infections, urinary and respiratory tract intections, nosocomial infections, tooth decay and dental caries, eye infections etc.

Qualitative phytochemical tests for the potent antibacterial n-hexane extract of *M. ferrea* stamens revealed presence of classes of phytoconstituents such as terpenoids, steroids and volatile oil components. This was found in agreement with the fact that generally; lipophilic components in plants such as alkanes, fatty acids, sterols, and terpenoids are extracted in nonpolar solvents [13]. The monoterpenes and sesquiterpenes are chief constituents of essential oils and steroids are biologically the triterpene derivatives [14, 13]. Hence following the presence of sterols, terpenoids and volatile oil components in the test extract as per qualitative tests, the total terpenoid content of n-hexane extract of *M. ferrea* stamens was estimated using Liebermann-Burchard colorimetric assay. Lupeol, a medicinally active triterpene, was used as a standard for the assay which demonstrated significantly higher values of total terpenoid content i. e. 102.8 mg/g in the dried test extract in terms of lupeol equivalents.

The n-hexane extract also exhibited significant free radical scavenging activity in the present study with IC_{50} value of 66.3 µg/ml in comparison with the IC_{50} value of 17.9 µg/ml obtained for ascorbic acid standard. The terpenoidal classes present in the test extract may be responsible for the free radical scavenging activity as monoterpenes and sesquiterpenes (which are main constituents of volatile/ essential oils) as well as diterpenes, phenolic terpenes show antioxidant activity in plants [14, 15]. *In-vivo* and *in-vitro* antioxidant activity of *M. ferrea* flowers have already been reported. In an *in-vivo* study dried methanolic extract of *M. ferrea* flowers had exhibited antioxidant activity in female wistar mice at 100 and 200mg/kg concentration [2]. Ethanol extract of *M. ferrea* flowers had also been studied for nitric oxide assay for free radical scavenging activity [2]. Present study demonstrates that the stamens which are abundantly present in *M. ferrea* flowers, exhibit the same free radical scavenging activity. Hence it can be assumed that the antioxidant activity of *M. ferrea* flowers could mainly be due to the antioxidant components present in the *M. ferrea* stamens.

Furthermore, the n-hexane extract of M. ferrea stamens was found completely safe in mice at a dose level of 2000 mg/kg body weight of an animal. There were no significant changes in food and water intake, no significant decrease in body weights and no mortality was recorded for

the test group in comparison with the control group. The results are in agreement with the toxicological data reported for acetone extract of stamens which was found safe upto 1600 mg/kg p. o.[1].

CONCLUSION

In the present study, the n-hexane extract of *M. ferrea* stamens was found to possess significant bactericidal and antioxidant activities. In addition, the *in-vivo* safety data obtained for the plant validates its use in traditional medicinal preparations. The medicinal properties of *M. ferrea* stamens evaluated in present study can be attributed to the presence of steroids, terpenoids and volatile oil components and the higher total terpenoid content as demonstrated. Hence the present research establishes the medicinal worth of *M. ferrea* stamens. The bioactivity-guided isolation and identification of pharmacologically active components and further extensive studies on the therapeutically potential and safe extract may provide some important leads in the area.

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CONFLICTS OF INTERESTS

Authors have no conflicts of interest to declare.

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