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In-vitro Antioxidant, Anti-inflammatory and Anti-Bacterial Studies of Nagkesar: An Uncommon but Potential Indian Spice

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ABSTRACT

The present study evaluates the pharmacological activities of water and alcoholic extracts of Nagkesar enriched with valuable phytochemicals. Nagkesar or Nagchampa (*Mesua ferrea)* is a prominent Indian herbal spice with therapeutic potential in preventing various diseases. There are only a few reports available to date on the antioxidant and anti-inflammatory activities of Nagkesar spice. There is a need to further investigate and elaborate on the pharmaceutical properties of this spice for its role in health and disease. Phytochemical screening and thin-layer chromatography of extracts were performed for the identification of phytoconstituents. The extracts were further quantified for phenolics, and flavonoids, and their antioxidant potential was assessed by Total antioxidant capacity and Ferric reducing activity assay. The anti-inflammatory activity was evaluated through the inhibition of HRBC membrane stabilisation whereas the antibacterial susceptibility was determined by employing the well diffusion assay. Findings revealed that the spice possesses phytoconstituents like phenols, alkaloids, saponins, flavonoids and other related compounds. The Nagkesar spice extracted in ethanol exhibited significant pharmacological and antibacterial efficacies due to the existence of various phytochemicals. Phenolic phytochemical content was found to be 3.5±0.168 *mgGAE/gm* extract and flavonoid presence was 0.589±0.211 *mgQE/gm* extract. The extract exhibited good TAC, (2.71±0.153 *mg AAE/gm* extract) very high hemolytic inhibition (75.86±0.367 %), and effective inhibition against bacterial growth. This research shows that Nagkesar spice might be a potential future candidate for drug development against various challenging and emerging diseases.

Keywords: *Mesua ferrea;* Antimicrobial; Anti-inflammatory; Nagkesar; Hemolytic

NOMENCLATURE

°C	Degree Celsius
min	Minutes
M	Molar
mM	Milimolar
ml	Mililiter
gm	Gram
µg	Microgram
µl	Microliter
Rf	Retention factor
nm	Nanometer
rpm	Rotations per minute
%	Percentage
v/v	volume/volume
WHO	World Health Organisation

1. INTRODUCTION

Spices have been used by humans in food preparation and as herbal medicine for the prevention of various diseases since ancient times[1]. India is a hotbed of spices" and the glory of Indian spices is known throughout the world for their flavor and aroma. The market of spices in India, being the world's top producer, consumer, and exporter, is predicted to grow to INR 298909 crores by 2028. There are many spices available in India such as turmeric, coriander, cumin, mustard, fenugreek, black pepper, clove, etc. These spices play a vital role in the medicinal system of Ayurveda, Unani, Siddha, Chinese, and Egyptian for health benefits[3].

Being rich in antioxidants, spices are reported to inhibit inflammation induced by oxidative stress in a scientific study[4]. Nagkesar is one of India's less-known spices and is found in a few places. Therefore, it is not commonly used in cooking. Nagkesar plant belongs to Guttiferrae family. Its scientific name is *Mesua ferrea* and can be easily seen in several Asian nations like Burma, and Cambodia and places like Nepal, Myanmar, Malaysia, Sri Lanka, Philippines, Thailand, and Sumatra[5]. The world's hardest commercial red timbers come from *Mesua ferrea* which is considered as national tree of Sri Lanka[6].

Nagkesar has many health benefits and studies show that it contains phytochemicals like coumarins, xanthones, flavonoids, terpenoids, and steroids which show various pharmacological activities[7]. The dried flowers of *Mesua ferrea* manifest antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, and liver protective

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activities whereas, the leaves have analgesic and anti-venomous properties, while the seed has potential for anti-spasmodic and anti-arthritic activities[8,9]. Traditionally different varieties of *Nagkesar* spices are utilized by the people of Asian countries for the treatment of multiple ailments[10]. The Ironwood flower buds referred to as Nagkesar are frequently utilized in Ayurveda for relieving bleeding piles and irregular uterine bleeding, as well as to assist digestion and treat cough, fever, and urinary tract issues. Nagkesar can be used to cure pain and inflammation as it has analgesic and anti-inflammatory properties. Its phytochemicals inhibit the activity of chemicals such as histamine, prostaglandin, etc that are responsible for pain and inflammation[11,12]. Nagkesar spice also has antiseptic and disinfecting properties[13]. Antimicrobial activities of *M. ferrea* have been studied against a variety of bacterial and fungal strains responsible for infectious diseases[14].

Recent studies are focussed on the use of herbs and spices to reduce the risk of inflammation similar to those seen in the COVID-19 pandemic. WHO is continually encouraging to consume a nutritious and healthy diet enriched with antioxidants, vitamins, and minerals[15]. In light of this, the present research involves an inquiry into the possible health advantages of the more obscure Indian spice Nagkesar by assessing its in vitro pharmacological and antibacterial properties.

2. METHODOLOGY

2.1 Collection of Nagkesar

Indian spice, Nagkesar was purchased from the local market of Indore, Madhya Pradesh, India. The sample was taxonomically identified and authenticated.

2.2 Nagkesar Extract Formation

A 2 *gm* Nagkesar sample as shown in Figure 1 was ground in a mortar with a pestle and extracted with 20 *ml* of ethanol and water separately. The homogenized samples were kept overnight for 12 hours to allow the phytochemicals to seep out into the solvent. The Whatman filter paper was employed for the filtration of extracts and their color, and yields were noted. The extracts were labeled as crude ethanolic and aqueous Nagkesar extracts respectively.

Figure 1. ***Messua ferrea:*** **An Indian spice, Nagkesar.**

2.3 Chromatographic Separation by TLC

The chromatography was performed on silica gel G-coated microscopic glass slides for the separation of the extracted sample in the form of a thin layer (TLC). Samples (7 drops) were spotted using a micropipette and after that, the slides were placed in the developing chamber containing mobile phase as chloroform and methanol in 15:1 ratio[16].

2.4 Phytochemical Analysis of Nagkesar Extract

The phytochemical analysis of Nagkesar ethanolic and aqueous extracts was performed by using standard qualitative methods for the identification of alkaloids, tannins, polyphenols, saponins, glycosides, flavonoids and terpenoids based on color development[17, 18].

2.4.1 Analysis of Phenols

Ferric chloride test: 1 *ml* 5 % $FeCl_3$ was added to 1ml Nagkesar extract. The emergence of a deep blue-black color confirmed phenol.

2.4.2 Analysis of Flavonoids

Alkaline reagent test: 1ml of Nagkesar extract was made alkaline with 1 *ml* of 10 % NaOH solution. The development of a deep yellow color indicated the presence of flavonoids.

2.4.3 Tannins Analysis

Braymer's test: 1 *ml* 10 % $FeCl_3$ was added to 1ml Nagkesar extract. The emergence of a deep blue-black color confirmed the tannins.

2.4.4 Analysis for Alkaloids

Wagner's test: 1 *ml* of Nagkeasr extract was treated with a few drops of Wagner's reagent (2 *g* I_2 and 6 *g* KI in 100 *ml* water). The formation of a brown precipitate indicates the presence of alkaloids.

2.4.5 Analysis of Saponins

Foam test: To 1 *ml* of Nagkesar extract, 5 *ml* of water was added. Shaken vigorously and kept for 3 minutes. The presence of persistent foam confirmed the presence of saponins.

2.4.6 Steroids and Terpenoids Analysis

Salkowski test: 1 *ml* of Nagkesar extract was mixed with 0.5 *ml* of chloroform and 1 ml of concentrated sulfuric acid was added carefully along the sides of the test tube. The result is positive when a reddish-brown color is obtained at the interface of the liquid.

2.4.7 Glycosides Analysis

Keller-Kelliani's test: To 2.5 *ml* extract, glacial acetic acid, and few drops of 5 % $FeCl_3$ solution were added. Heated gently and cooled. This was transferred to a test tube containing sulfuric acid. Brown ring formation at the junction showed positive indication for glycosides.

2.5 Phytochemical Estimations

2.5.1 Polyphenol Estimation

The colorimetric method was employed for the estimation of total polyphenols in the ethanolic and aqueous extracts of Nagkesar[19]. 20 *µl* of Nagkesar extracts were treated with 2.5 *ml* of Folin-Ciocalteu's reagent (10 %) and 2 ml of sodium carbonate (7.5 %). The standard solution of gallic acid ranging from 25 *µg/ml* to 200 *µg/ml* was used for the generation of its calibration curve. After 15 min incubation, the reading in absorbance was determined at 750 nanometers. The concentration of polyphenols in the sample was calculated as *mg* gallic acid equivalents (GAE) per gram of the extract. Every measurement was carried out in triplicates.

2.5.2 Flavonoid Estimation

The standard aluminum chloride assay was adopted for the estimation of flavonoids in Nagkesar extracts[20]. 20 *µl* Nagkesar sample or standard solutions of quercetin (100 *µg/ml* to 500 *µg/ml*) were treated with one volume of water. This was followed by the addition of $AlCl_3$ (10 %) and 1M potassium acetate. After dilution with required volume of water the mixture was incubated for 30 minutes. The absorbance reading was taken against a blank at 415 nanometers in a colorimeter. The standard graph was used to determine the amount of flavonoid present in the test samples as mg of quercetin equivalents (QE)/gram.

2.6 Antioxidant Capacity Determination

2.6.1 Phosphomolybdate Method

The sample antioxidant capacity was estimated by the phosphomolybdate method using standard antioxidant[21]. To 20 *µl* of sample solution, 1.2 *ml* distilled water and 2.2 *ml* phoshphomolybdate reagent were added. The mixture was kept in boiling water bath for 90 minutes. After cooling, optical density (OD) was measured taken at 765 nanometers. Ascorbic acid was used as a standard antioxidant (25-125 *ug/ml*) for plotting of calibration curve. The antioxidant capacity of the Nagkesar sample was measured as mg ascorbic acid equivalents (AAE) per gram of the extract.

2.6.2 Ferric Reducing Ability Assay (FRAA)

The ferric-reducing ability of the Nagkesar sample was established by colorimetric method with slight modification[22]. Briefly, the sample (20 *µl*) along with sodium phosphate buffer (0.2 *M*, pH 6.6) and potassium ferricyanide (1 %) was incubated for 20 minutes at 50 °*C*. TCA (10 %) was added to the mixture for the reaction termination. The supernatant was collected after centrifugation at 3000 *rpm* for 10 minutes and then treated with 0.1 *ml* ferric chloride (0.1 %). After 10 minutes incubation, the OD was determined at 770 nanometers. Standardisation of assay was done by preparing a standard curve using 25–125 *ug/ml* solutions of ascorbic acid.

2.7 Anti-inflammatory Activity Assessment Using Human Blood Cells

It employs the principle that the erythrocyte membrane is similar to the lysosomal membrane for anti-inflammatory activity determination by hemolytic assay. RBC membrane stabilisation by phytoconstituents is indicative of its anti-inflammatory response. The method was performed for in vitro anti-inflammatory activity of Nagkesar extracts using membrane stabilisation with slight modifications[23-25].

Whole blood collected from healthy person was reconstituted as 10 % suspension with isosaline. 20 *ul* Nagkesar extracts, standard drug, and control (saline) were separately mixed with 100 *ul* of 10 % red blood cell suspension and 3 *ml* of 10 *mM* phosphate-buffered saline (pH 7.4). The standard drug was taken as Aspirin (15 *mg/ml*). Heat-induced hemolysis was initiated at 54 *ºC*. The absorbance of the resulting supernatant after centrifugation was taken at 560 nanometers. As per the methodology, we determined the percentage inhibition of hemolysis of the sample and standard solution.

2.8 Antibacterial Activity

The anti-bacterial susceptibility of ethanolic and aqueous extracts of Nagkesar was detected on the nutrient agar plate by agar well diffusion with slight modification[26]. One Gram-positive and one Gram-negative bacterial culture was used. Four wells were made by gel puncture into nutrient agar surface. Bacteria were seeded into a sterile nutrient agar plate by direct streaking. Into the 4 wells made on each plate, 20 and 40 microliters of ethanolic and aqueous extracts of the Nagkesar sample were pipetted out separately. The plates were allowed to stand for 1 hour for diffusion of solution and incubated overnight at 37 °*C*. The clear zone around wells was observed after incubation for anti-bacterial activity.

2.9 Analysis of Statistical Data

Analyses were performed on triplicate samples to ensure accuracy. Statistics were presented in mean and standard deviation form. Microsoft Excel program was used for the preparation of standard graphs and statistical calculations.

3. RESULTS AND DISCUSSION

The goal of the research study is to evaluate the phytochemicals and antioxidant activities of the ethanolic and aqueous extracts (Figure 2) of Nagkesar. The extractive yield of different solvents along with colour is given in Table 1. Maximum yield was found in ethanolic extract (82.5 %) than in aqueous extract (65 %) of Nagkesar.

3.1 TLC Analysis

TLC of the ethanolic extract of Nagkesar revealed the presence of only one phytochemical compound with an R_f value of 0.813 when chloroform and methanol (15:1) were used as a solvent phase. However, with water extract, no bands were observed with the same

Figure 2. Crude aqueous and ethanolic extracts of Nagkesar.

Table 1. Extraction yield of extracts of Nagkesar extracts of Nagkesar

Extract	Final volume (ml)	Color of extract	Yield
Ethanolic	16.58	Golden yellow	82.5%
Aqueous	13	Dark brown	65%

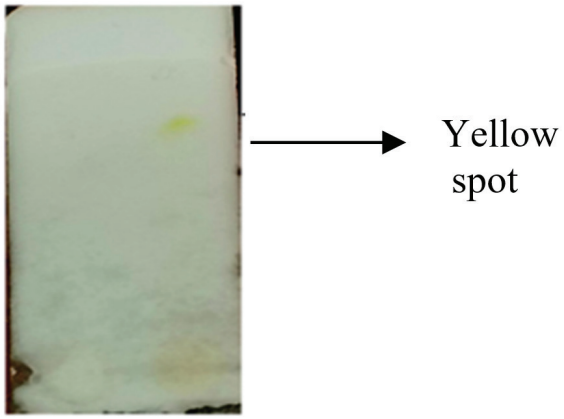

Figure 3. TLC profile of ethanolic extract of Nagkesar.

solvent phase. The TLC profile of the ethanolic extract is shown in Figure 3 and the results of chromatographic separation of the ethanolic extract of Nagkesar are shown in Table 2.

3.2 Evaluation of Phytoconstituents

The phytoconstituent evaluation of ethanolic and aqueous extracts of Nagkesar demonstrated the presence of most of the phytoconstituents such as polyphenols, flavonoids, alkaloids, saponins, tannins, terpenoids, and glycosides. The results of the qualitative determination of phytochemicals are presented in Table 3. Figure 4 depicts color development in phytochemical tests. Among these phytochemicals, only alkaloids were found to be present in lesser amounts in aqueous extracts as compared to the ethanolic extract. All these phytochemicals are reported to manifest multiple physiological effects like anti-inflammatory and anticancer properties due to their potent antioxidant potential.

Table 2. TLC separation of extracts of Nagkesar

Extract	Spot color	Distance travelled by sample (cm)	Distance travelled by mobile phase (cm)	Rf value
Ethanolic	Yellow	3.9	4.8	0.813
Aqueous	-	-	-	-

3.3 Polyphenol Estimation

The result for polyphenol content (TPC) of Nagkesar in ethanolic and aqueous solvents is shown in Table 4. This assay is performed in aqueous medium and basic condition so that polar polyphenols majorly contribute towards the antioxidant capacities of the extracts. The polyphenol concentration in the extracts was obtained from the straight line equation presented in Figure 5. The results are reported in *mg GAE/gram* of extract The polyphenolic concentration of aqueous extract of *M. ferrea* was found to be significantly higher than its ethanolic extract.

Table 3. Result of phytochemical screening of different extract of Nagkesar

S. no	Colored reaction for phytochemicals	Ethanolic	Aqueous
1.	Yellow for flavonoid	++	++
2.	Blue-black for polyphenols	++	++
3.	Blue-black for tannins	++	++
4.	Reddish-brown ring for glycosides	++	++
5.	Reddish-brown for terpenoids	++	++
6.	Reddish-brown for steroids	++	++
7.	Brown for alkaloids	++	+
8.	Foam for saponins	++	++

++, indicates high presence; +, indicates faint presence

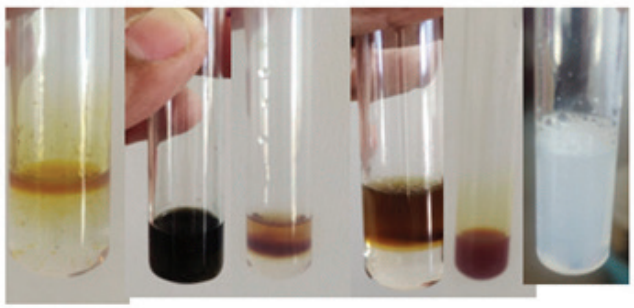

Figure 4. Color development in phytochemical tests.

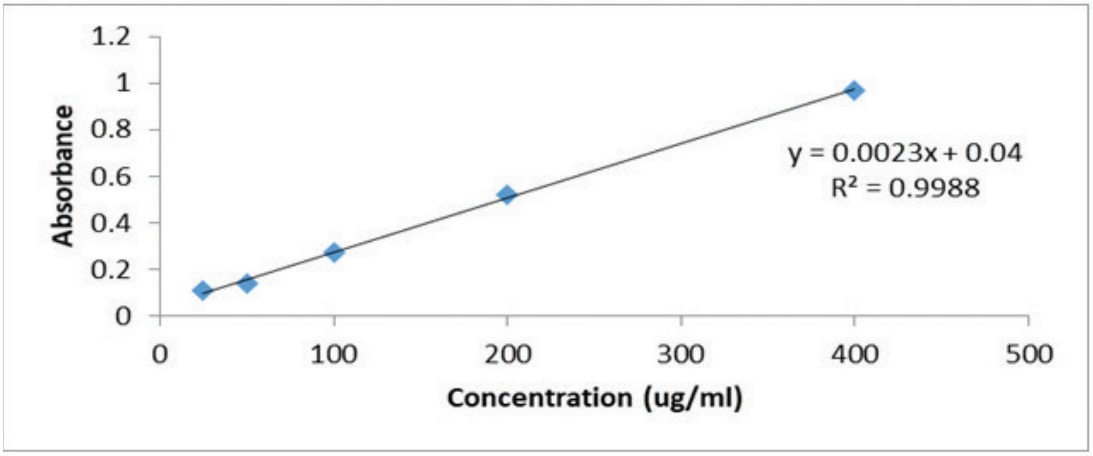

Figure 5. Standard graph of Gallic acid.

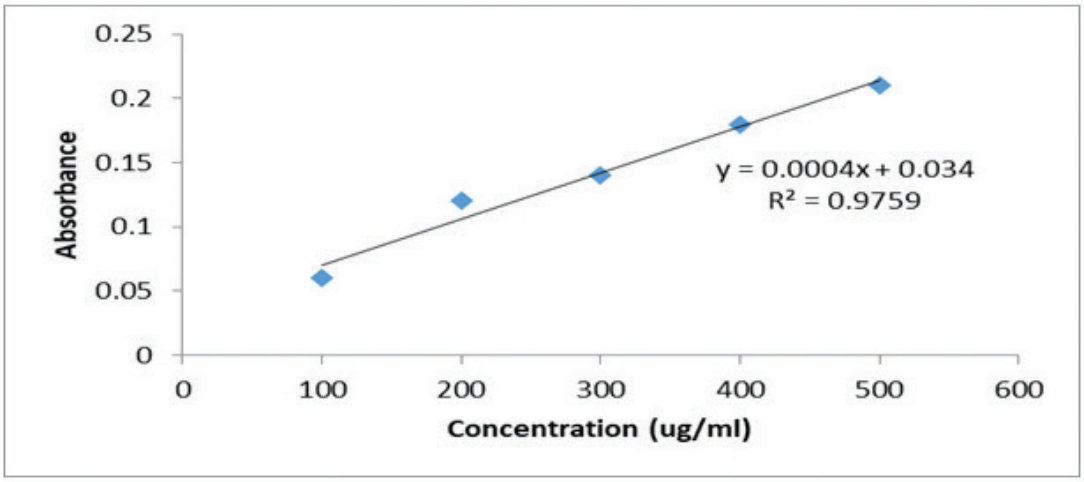

Figure 6. Standard graph of Quercetin.

3.4 Flavonoid Estimation

The concentration of flavonoids (TFC) in Nagkesar extracts was obtained by extrapolation from the calibration curve using a range of quercetin concentrations against absorbance (Figure 6) and indicated in *ug* of quercetin equivalence (QE) per gram. The TFC of ethanolic and aqueous extract of Nagkesar is shown in Table 4. The amount of flavonoid in ethanolic extract was almost double (589 *ugQE /gram* of extract) than aqueous extracts (254 *ugQE /gram* of extract).

3.5 TAC

Phoshphomolybdate assay for determination of total antioxidant capacity is quantitative since it is expressed as ascorbic acid equivalents. Mo(VI) is converted to green-colored phospho Mo (V) complex by reduction in the presence of Nagkesar extracts when measured at 695 *nm*. The TAC of different extracts of Nagkesar was evaluated by plotting a calibration curve of absorbance of standard ascorbic acid for its range of concentrations (25-125 *ug/ml*) as depicted in Figure 7. The results were calculated from the straight-line equation formed by the plot. The TAC of both the extracts of Nagkesar is found to be similar and thus more oxidative stress can be handled by both of them. The values were shown in terms of *mgAAEq/gram* of extract (Table 4).

3.6 FRAA

The reducing potential of different extracts of Nagkesar was evaluated by their abilities to transfer electrons to FRAP reagent in this assay. Ascorbic acid (1 *mg/ml*) was taken as standard for this assay. From Table 4 we can conclude that the aqueous extract displayed significantly higher antioxidant activity than ethanolic extract as measured by the FRPA method. The strong reducing power of the aqueous extract of *M. ferrea* is also a testament to its higher polyphenol content in comparison to its ethanolic extract.

3.7 Anti-inflammatory Effect on Red Blood Cells

Oxidative stress can cause inflammation and the first of the processes that takes place at the site of inflammation is cell lysis through membrane destabilisation. It has been studied that certain phytochemicals can protect the cell from lysis and this part of the study dealt with the same principle. Both the extracts of *M. ferrea* at 20 *ul* volume showed significant stabilisation towards the HRBC membrane as their absorbance was lower than that of the control. Drug aspirin (standard) acts weaker than ethanolic but stronger than the aqueous extract of Nagkesar in protecting the RBC membrane. It is assessed that the ethanolic extract showed higher hemolysis inhibition so can be effectively used for treating inflammation. The

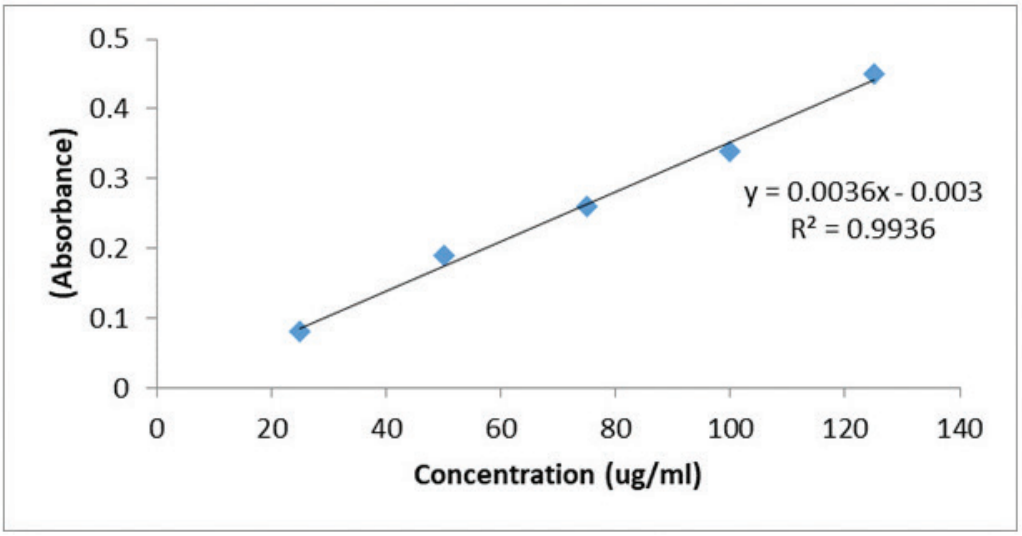

Figure 7. Calibration curve of ascorbic acid for TAC of Nagkesar extract.

Table 4. Qunatification of polyphenols, flavonoids and antioxidant capacity by TAC and FRAA in Nagkesar extracts

Sample	Total phenolics	Total flavonoids	TAC	FRAA
Ethanolic	3.5±0.168	0.589±0.211	2.71±0.153	1.65±0.341
extract	mgGAE/g	mgQE/g	mgAAE/g	mgAAE/g
Aqueous	7.5±0.19	0.254±0.321	2.71±0.198	2.85±0.276
extract	mgGAE/g	mgQE/g	mgAAE/g	mgAAE/g

Table 5. Results for percentage inhibition of hemolysis by Nagkesar extracts

Sample	Volume (µl)	Absorbance	% hemolysis inhibition
Control	20	0.29	Maximum hemolysis
Ethanolic	20	0.07	75.86±0.367
Aqueous	20	0.18	37.93±0.263
Aspirin (15 *mg/ml*)	20	0.16	44.87±0.511

Values are represented as mean ± SD

inhibition percentage of hemolysis from two different extracts is depicted in Table 5.

3.8 Antibacterial Potential Determination

The ethanolic extract of Nagkesar flower bud showed strong antibacterial activity against both test bacteria as evidenced by a clear zone on an agar plate. The ethanolic extract (Et) of Nagkesar at 20 and 40 *ul* concentrations showed the highest inhibitory activity against bacteria. The aqueous extract (Aq) at the same volume was devoid of this inhibitory action. The clear zone around wells as a result of the inhibitory action of the ethanolic extract of Nagkesar is seen in Figure 8. The presence of many

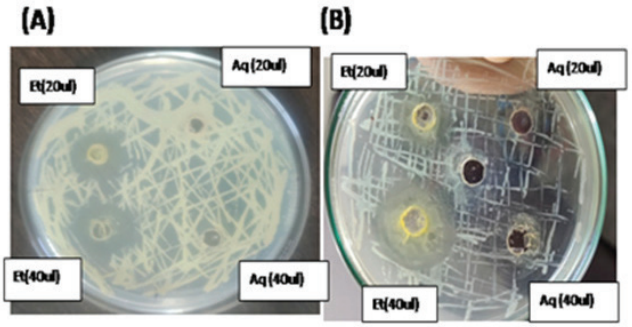

Figure 8. Antibacterial activity of ethanolic (Et) and aqueous (Aq) extracts of Nagkesar against (A) Gram-positive and (B) Gram-negative bacteria.

bioactive antioxidant components may be the cause of the ethanolic extract of Nagkesar's antibacterial ability.

4. DISCUSSION

M. ferrea, commonly known as Nagchampa or Nagkesar is a well-known herbal Indian spice with potential therapeutic applications. In this study, dried stamens of *M. ferrea* which is used as spice were extracted with ethanol and water to evaluate its phytochemical, antioxidant, anti-inflammatory, and antibacterial activities. Water as well as alcohol both are considered to be universal solvents for phytochemical analysis[27].

A significant number of the phytochemicals, including polyphenols, flavonoids, alkaloids, tannins, terpenoids, glycosides, and steroids having therapeutic potential, have been identified in the aqueous and ethanolic extracts of Nagkesar. Alkaloids were dominant in ethanolic extract in comparison to aqueous extract. Alkaloids are nitrogenous bases that are widely used as anti-cancer and anti-microbial agents, and CNS stimulants, and play metabolic roles in a cell. Polphenols and flavonoids are known to prevent oxidative cell damage and are thus used in the prevention of diseases induced by oxidative stress like diabetes, and cancer[28]. The antimicrobial, antifungal, antiviral, antiparasitic, anti-hyperglycemic, and anti-inflammatory

activities of terpenoids are well known[29]. Tannins contribute to antimicrobial, and antiseptic activity for the prevention of various diseases. Steroids can contribute to drug formulation for pharmaceutical applications.

It can be seen from Table 4 that, the aqueous extract (7.5±0.192 *mgGAE/gm* extract) of Nagkesar showed maximum phenolic content, almost double that of ethanolic extract (3.5±0.168 *mgGAE/gm* extract). The considerable difference could be due to the greater extractability of polyphenols in water. The amount of flavonoids was found to be lesser than the polyphenols in both the extracts of Nagkesar as indicated in Table 4. Ethanol extract exhibited higher flavonoid content (0.589±0.211 *mgQE/gm* extract). Plekratoke, *et al*. reported the presence of a significant amount of flavonoids in ethanolic Nagkesar extracts[30]. This may be due to the presence of a greater amount of less polar flavonoids in the stamens of Nagkesar. The high concentration of polar phenolic compounds in the aqueous extract of M. ferrea might contribute to its significant antioxidant property (2.85±0.276 *mgAAE/gm* extract) as indicated by FRPA assay (Table 4). It could be inferred that the electron transfer mechanism is predominant in the antioxidant activity of aqueous extract of Nagkesar[31].

The ethanolic extract of Nagkesar showed more significant anti-inflammatory and antimicrobial potential than the aqueous extract which might be due to its strong correlation with total flavonoid content (0.589±0.211 *mgQE/gm* extract) and total antioxidant capacity (2.71 mgAAE±0.153/gm extract). Prasad, et al. also revealed promising invitro antioxidant activity of Nagkesar ethanolic extract[32]. Hemolytic inhibition of ethanolic extract (Table 5) was found to be 75.86±0.367 % as compared to aqueous extract (37.93±0.263) thus allowing it to be used as a potent anti-inflammatory agent. Ragnathan, *et al*. mentioned similar findings on ethanolic extract of *M ferrea*[33]. The agar well diffusion method revealed that promising antibacterial activity was shown only by ethanolic Nagkesar extract against Gram-positive and negative bacteria (Figure 7). This might be due to greater solubility of antimicrobial phytochemicals like flavonoids, and alkaloids in ethanol which can be useful in fighting emerging bacterial infections. Wang, *et al.* showed the antimicrobial action of the Nagkesar flower against bacteria due to the presence of coumarins and xanthones[34]. The antibacterial effect of *M. ferrea* against human pathogens such as S aureus, Proteus, and Bacillus has been reported by Ali[35], *et al*.

5. CONCLUSIONS

It may be inferred from this study that ethanolic Nagkesar extract has potential pharmacological activities due to the abundance of valuable phytoconstituents. The findings demonstrated that *M. ferrea* (Nagkesar) dried flower buds can be used as natural antioxidants and antimicrobials in the pharmaceutical and food industries. Therefore, future studies are required to prove the potential of this Indian spice and to isolate active phytocompounds for therapeutic applications in diseases associated with oxidative stress. Consuming a healthy diet abundant in antioxidants is the need of the hour to combat the next pandemic in the future. More studies are to be performed on the inclusion of Nagkesar spice in animal and human trials for its application in modern medicines.

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