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In vitro study of antibacterial activity of Kanocha seeds (*Phyllanthus maderaspatensis*) against some gram-positive and gram-negative bacterial strains

Sada Akhtar*[,1,+], Abdur Rauf, Sumbul Rehman & Mohd Zakir Siddiqui[2]

[1]Department of Ilmul Advia, Faculty of Unani Medicine, Aligarh Muslim University (AMU), Aligarh 202 002, Uttar Pradesh, India
[2]Department of Biotechnology, Faculty of Natural Sciences, Jamia Millia Islamia, New Delhi 110 025, India
E-mail: [+]akhtarsada44@gmail.com

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Kanocha is the dried seeds of *Phyllanthus maderaspatensis* of family Phyllanthaceae. It has been long utilising in Unani system of medicine to cure many ailments including genitourinary infectious diseases. In the current investigation, the aqueous, alcoholic and hydroalcoholic extracts of Kanocha seeds were screened for their antibacterial actions against both gram-positive (*Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Corynebacterium xerosis* and *Bacillus cereus*) and gram-negative (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris*) bacterial strains compared with the standard Drug: Ciprofloxacin (SD060) 5 µg/disk for gram-positive bacterial strains and Gentamicin (SD016) 10 µg/disk for gram-negative bacterial strains using Zone of Inhibition (ZOI) with the help of Agar well method and minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC) with the help of Nutrient Broth method. The data was analysed using Gpad INSTAT programming, one-way ANOVA and post-test Bonferroni. Alcoholic and hydroalcoholic extract showed significant antibacterial activity than the aqueous extract but not up to the mark as compared to standard group indicating that the alcoholic and hydroalcoholic extract has the capability of extracting more phytochemicals than aqueous extract which are responsible for their antibacterial activity. It could be concluded that the present drug possesses antibacterial property.

Keywords: Antibacterial, Bioactive compounds, Kanocha, *Phyllanthus maderaspatensis*

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Kanocha (seeds of *P. maderaspatensis*) belongs to family *Phyllanthaceae*. The family Phyllanthaceae has largest genus Phyllanthus, which has remarkable diversity of forms including perennial, annual, herbaceous, arborescent, phyllocladus and Pachycauls. Kanocha (*P. maderaspatensis*) is an annual shrub dispersed in Indonesia, Australia, Africa, China, Sri Lanka and India[1]. It is frequently known as Madras leaf flower hence the name also called Mela Nelli, Nala-Userekee in Tamil, Kanochha, Hazarmani in Hindi, Bazarmani in Gujrati, Marur, Maru, Rehan-ul-Shyookh in Arabic, Marushatu and Marushak in Persian[2,3,4,5]. It is an erect or decumbent herb or sometimes an under shrub, 30-90 cm. high, annual, very variable in habit[6]. The seeds and leaves of *P. maderaspatensis* are used in Unani medicine. The seeds have a bad taste and it is used as carminative, laxative, diuretic, diaphoretic and demulcent. With other demulcent decoctions, they are used in urinary affections such as gonorrhoea and internal inflammations[2,4]. Seeds mainly contain stearic acid, palmitic acid, oleic acid, myristic acid and linolenic acid[7].

The phytochemical analysis of the aqueous extract of the whole plant for the presence of active compounds showed the existence of tannins, proteins, carbohydrates and saponins[1]. Rajasekhar *et al.*, (2014) reported the phytochemical analysis of aqueous and alcoholic extract of leaves and found that it contains the carbohydrates, steroids, phenols, proteins and flavonoids[8]. In another study conducted by Akhtar *et al.*, 2019, preliminary phytochemical examination of the seeds of Kanocha divulged the existence of secondary metabolites such as alkaloids, carbohydrates, glycosides and sterol/terpenes[9].

To validate the claims of Unani physicians being effectiveness of Kanocha in infectious diseases, the current work was aimed to check the antibacterial potential of test drug, which has been long utilised for

*Corresponding author

the treatment of different infectious ailments by using Agar well method.

Materials and methods

Plant material

The seeds of *P. maderaspatensis* were obtained from the local market of Aligarh city, India in the month of April 2017. The identity was confirmed with the help of literatures available and pharmacognosy section, department of Ilmul Advia, Ajmal Khan Tibbiya College, AMU, Aligarh. The sample was further authenticated by CSIR-National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (NISCAIR /RHMD/ Consult/ 2017/3089/38-1). The specimen of the test drug was submitted to Mawalid-e-Salasa Museum of the department for future reference with the voucher No of (SC-0216/17).

Plant extraction

Coarsely powdered drug Kanocha (25 g in 250 mL solvent) was extracted from double distilled water, ethanol (95%) and hydro-alcoholic mixture (50:50) as a solvent for aqueous, alcoholic and hydro-alcoholic extracts respectively using Soxhlet apparatus for 6 h. The extract obtained was filtered over Whatman No. 1 filter paper and then subjected to dryness at 55ºC on water bath and kept at 4ºC till further use and was reconstituted with Dimethyl Sulphoxide (DMSO) used as a solvent to make stock solution at varying concentration for working with test drug. Aqueous extract was dissolved in double distilled water.

Bacterial strains

Six gram-positive, i.e., *B. cereus, Corynebacterium xerosis, S. pyogenes, S. epidermidis, S. aureus and Streptococcus mutans,* and 4 gram-negative, i.e., *Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *E. coli* bacterial strains were used to assess the antibacterial properties of the test drug. All the bacterial strains were clinical isolates that were obtained from microbiology lab of the department of Ilmul Advia, AMU, Aligarh. Bacterial strains were maintained on 1% semisolid nutrient agar at 4°C and sub cultured every 2 weeks in microbiology lab.

Concentrations of test drug and standard used

The stock solutions of aqueous, alcoholic and hydro-alcoholic extracts were prepared at a concentration of 5 µgm/µL, 10 µgm/µL and 20 µgm/µL. 50 µL/well was used from each stock solution for the study. Standard drugs were ciprofloxacin (SD060) 5 µg/disk for gram-positive bacterial strains and gentamicin (SD016) 10 µg/disk for gram-negative bacterial strains.

Antibacterial screening

The antibacterial testing of aqueous, alcoholic and hydroalcoholic extract of Kanocha was done as per Clinical and Laboratory Standard Institute (CLSI) guidelines against bacterial strains. Results were analyzed on the basis of ZOI, MIC MBC by Kirby Bauer's disk diffusion method and Agar Well Method.

Media preparation

Nutrient Agar No. 2 (HiMedia M1269S-500G) was prepared as per company instructions and autoclaved and then 25-30 mL was poured into sterile disposable (10 cm in diameter) petri dishes on a surface level to give a uniform depth of 4 mm.

Agar Well method

A disinfected cotton bud (PW041, HiMedia Labs, Mumbai, India) was immersed into the inoculums, suspension (10^6 cfu/mL) and pressed on the inner side of the test tube to abolish extra inoculation from it. The dried surface of the nutrient plate (pH 7.2-7.4) was then streaked with the cotton bud containing inoculums many times on the surface and finally the rim of the plate, so as to ensure even distribution over the entire surface of the plate[10]. The bacterial inoculums were allowed to dry for 5-15 min but not more than 15 min, to stop for any excess surface moisture to be absorbed before applying the drugs. The wells of the equal size[11] were then created with the help of a micro tip (6 mm in diameter) in the plate then the wells so prepared were filled by the drug extract (50 µL) in their respective site with the help of a micropipette[12]. The standard antibiotic work disk (HiMedia Labs, Mumbai, India) were placed on the prepared plates with the help of a disinfected forceps and pressed properly to make complete contact with the surface of the medium then the plates were incubated at 37ºC for 24 h placing them at an inverted position[13].

Interpretation

The antibacterial activity was noticed after 24 h, by measuring the diameters of clear halos around the work disk (standard drug) and wells of test drug including the diameter of work disk and wells of test drug, or the inhibition zones using a Hi Antibiotic Zone Scale™ –C (PW297-HiMedia Laboratories Pvt Limited, Mumbai). The larger area indicates that the germ is more sensitive.

Minimum Inhibitory Concentration (MIC)

MIC is the lowest concentration of the drug which causes no visible turbidity in the test tube (bacteriostasis concentration)[1]. MIC of the test drug was tested by Broth Dilution method[14] because the same wells from 96-well microtiter plates can be used for MBC tests also. Among three concentrations (5 µgm/µL, 10 µgm/µL and 20 µgm/µL) of the test drug the intermediate concentration, i.e., 10 µgm/µL was taken for the calculation of MBC and MIC as the highest antibacterial activity was observed at this concentration. The stock dilutions of the drug sample were prepared so that concentrations ranging from 166.6 to 0.02 µg/µL were obtained from the original stock solution (10 µg/µL).

Broth Dilution Method

In a disinfected microtiter plates (96-u-shaped wells) 50 µL of the aseptic nutrient broth was filled upto 9th well in the first three rows, then from a fresh inoculums (10^6 cfu/mL diluted with 100 µL Nutrient broth to have 10^6 cfu/mL), 50 µL of the suspension was filled in the first and third row upto 9th well, second row was again filled with 50 µL of aseptic nutrient broth, then the drug solution of 50 µL was added in the first well of first row diluting uniformly from 166.6 to 0.02 µg/µL till the 9th well. The first row was positive control (nutrient Broth+bacterial culture+test drug), the second row was plain control (nutrient broth) and the third row was used as negative control (nutrient broth+bacterial culture).

Minimum Bactericidal Concentration (MBC)

The MBC is the lowest concentration which causes no bacterial growth after 24 h of incubation (Bacteriocidal concentration)[1]. After that MBC was calculated from the broth dilution which was obtained from the MIC (96-u-shaped wells) tubes by sub culturing to aseptic agar plates. In this process, the solution from the MIC tubes was streaked on the surface of the aseptic agar plates with the help of a disinfected cotton buds and then the plates were placed in an incubator at 37° C for 18 h.

Interpretation of MIC and MBC

The MICs of the different extracts were interpreted as the minimum concentration of the drug sample (in other words highest dilution of the sample) which showed transparent fluids without turbidity (turbidity indicates bacterial expansion). Different dilutions from the MIC tubes were taken for the calculation of the MBC and the smallest concentration which showed no bacterial growth was perceived as MBC of the extract.

Statistical Analysis

Statistical analysis was carried out by utilising Gpad (INSTAT) programming, one-way ANOVA and post-test named Bonferroni. Chosen pairs of columns with various comparison were performed considered $p<0.05$ as significant difference.

Results and discussion

The aqueous, alcoholic and hydroalcoholic extracts of the seeds of *P. maderaspatensis* were tested against gram-positive *B. cereus, C. xerosis, S. pyogenes, S. epidermidis, S. aureus and S. mutans* and gram-negative *P. vulgaris*, *P. aeruginosa* , *K. pneumoniae* and *E. coli* bacterial strains by using three different concentrations of 5 µgm, 10 µgm and 20 µgm. However, it was the observed that among three concentrations (5 µgm/µL, 10 µgm/µL and 20 µgm/µL) of the drug extracts, inhibitory effects were exhibited more at the intermediate concentration, i.e., at 10 µgm/µL. All the extracts, i.e., aqueous, alcoholic and hydroalcoholic extracts of the seeds hindered the development of majority of the isolates but the maximum degree of activity was observed by alcoholic and hydroalcoholic extracts. The antibacterial activity of aqueous extract is shown in Table 1. Aqueous extract showed mild to moderate activity against all bacterial strains except for *K. pneumonia* (6.33±0.33; $p<0.05$) which was found resistant. The antibacterial activity of alcoholic extracts is shown in Table 2. Alcoholic extract showed moderate to significant activity against all pathogens. The antibacterial activity of hydroalcoholic extracts of Kanocha is shown in Table 3. Hydroalcoholic extract showed significant antibacterial activity against all human pathogens. Zone of inhibition showed by all the extracts are shown in Fig. 1. MICs and MBCs of the Kanocha are shown in Table 4. Nowadays antibiotic resistance is a big issue in front of the world, According to the WHO. In 2016, 4,90,000 people have created resistance from antibiotics worldwide. Indiscriminate use of commercial antibiotics, negative effects on the host including allergic reactions, immune suppression, hypersensitivity and inadequacy of the present antibiotics impel the revelation of new therapeutic agents from the medicinal plants. Approximately 20% of the plants found on the planet have been submitted to pharmacological or biological tests[15].

Table 1 — Antibacterial activity of aquous extracts of Kanocha (*P. maderaspatensis*) against gram-positive and gram-negative bacterial strains

S. NO.	Test Strains (Clinical Isolates)	Zone of Inhibition (in mm) expressed as Mean±SEM[Probability of error]			Standard Ciprofloxacin/ Gentamicin 5 µgm/disc	Plain Control DMSO (50 µL)
		Aquous extract (50 µL/well)				
		5 µgm/mL	10 µgm/mL	15 µgm/mL		
1	Streptococcus mutans (SM)	11.33±0.33***	6.66±0.33***	6.66±0.33***	42.66±0.33	6.33±0.33
2	Staphylococcus aureus (SA)	10.33±0.33***	15.66±0.33***	6.66±0.33***	43.33±0.33	6.33±0.33
3	Staphylococcus epidermidis (SE)	6.66±0.33***	7.33±0.33***	6.66±0.33***	26.66±0.33	6.33±0.33
4	Streptococcus pyogenes (SP)	8.66±0.33***	6.66±0.33***	6.66±0.33***	35.66±0.33	6.33±0.33
5	Corynebacterium xerosis (CX)	10.66±0.33***	6.66±0.33***	6.33±0.33***	40.33±0.33	6.33±0.33
6	Bacillus cereus (BC)	7.66±0.33***	6.66±0.33***	16.33±0.33***	30.66±0.66	6.33±0.33
7	Escherichia coli (EC)	10.66±0.33***	6.33±0.33***	12.33±0.33***	30.66±0.33	6.33±0.33
8	Klebsiella pneumoniae (KP)	6.33±0.33***	6.33±0.33***	12.66±0.33***	30.33±0.33	6.33±0.33
9	Pseudomonas aeruginosa (PA)	16.66±0.33***	6.33±0.33***	12.33±0.33***	32.33±0.33	6.33±0.33
10	Proteus vulgaris (PV)	12.66±0.33***	6.33±0.33***	10.66±0.33***	30.33±0.33	6.33±0.33

Significance: ***=p<0.001, **=p<0.01, *=p<0.05; NS= Not Significant

Table 2 — Antibacterial activity of hydroalcoholic extracts of Kanocha (*P. maderaspatensis*) against gram-positive and gram-negative bacterial strains

S. NO.	Test Strains (Clinical Isolates)	Zone of Inhibition (in mm) expressed as Mean±SEM[Probability of error]			Standard Ciprofloxacin/ Gentamicin 5 µgm/work disk	Plain Control DMSO (50 µL)
		Aquous extract (50 µL/well)				
		5 µgm/mL	10 µgm/mL	15 µgm/mL		
1	Streptococcus mutans (SM)	15.66±0.33***	14.66±0.33***	11.33±0.33***	42.66±0.33	6.33±0.33
2	Staphylococcus aureus (SA)	11.66±0.33***	11.33±0.33***	11.66±0.33***	43.33±0.33	6.33±0.33
3	Staphylococcus epidermidis (SE)	13.66±0.33***	10.66±0.33***	10.66±0.33***	26.66±0.33	6.33±0.33
4	Streptococcus pyogenes (SP)	15.33±0.33***	11.66±0.33***	8.66±0.33***	35.66±0.33	6.33±0.33
5	Corynebacterium xerosis (CX)	14.66±0.33***	14.66±0.33***	13.33±0.33***	40.33±0.33	6.33±0.33
6	Bacillus cereus (BC)	11.66±0.66***	12.66±0.33***	12.66±0.33***	30.66±0.66	6.33±0.33
7	Escherichia coli (EC)	15.33±0.33***	12.33±0.33***	2.66±0.33***	30.66±0.33	6.33±0.33
8	Klebsiella pneumoniae (KP)	15.66±0.33***	10.66±0.33***	2.33±0.33***	30.33±0.33	6.33±0.33
9	Pseudomonas aeruginosa (PA)	10.66±0.33***	10.33±0.33***	12.33±0.33***	32.33±0.33	6.33±0.33
10	Proteus vulgaris (PV)	18.33±0.33***	16.66±0.33***	13.33±0.33***	30.33±0.33	6.33±0.33

Significance: ***=p<0.001, **=p<0.01, *=p<0.05; NS= Not Significant

Phytochemical constituents present in the drugs may vary, not only from plant to plant but also among different samples of same species. Fundamental phytochemical trial of the seeds of *P. maderaspatensis* uncovered the existence of secondary metabolites such as alkaloids, carbohydrates, glycosides and sterol/terpenes. Eloff (1998) revealed that methanol was the best solvent for plant extraction than water and hexane[16]. In the current work the solvents utilised were ethanol, ethanol-water (50-50) and distilled water for extraction of the plant material and the present study showed minimum activity by aqueous extract and these findings confirmed the Eloff observations. There are some previous antibacterial studies which have been carried out on *P. maderaspatensis* like Leelaprakash and Dass (2011) they performed antibacterial activity on aqueous extract of the whole plant and the results showed that aqueous extract possess antibacterial activity but the inhibitory impacts were more as the concentration of the extracts increase[1]. In an Another study carried out by Rani and Raju (2014), ethyl acetate, ethyl alcohol and distilled water extracts of roots and shoots of Kanocha were screened for

Table 3 — Antibacterial activity of hydroalcoholic extracts of Kanocha (*P. maderaspatensis*) against gram-positive and gram-negative bacterial strains

S. NO.	Test Strains (Clinical Isolates)	Zone of Inhibition (in mm) expressed as Mean±SEM[Probability of error]			Standard Ciprofloxacin/ Gentamicin 5 µgm/ work disk	Plain Control DMSO (50 µL)
		Hydroalcoholic extract (50 µL/well)				
		5 µgm/mL	10 µgm/mL	15 µgm/mL		
1	Streptococcus mutans (SM)	16.33±0.33***	14.33±0.33***	11.33±0.33***	42.66±0.33	6.33±0.33
2	Staphylococcus aureus (SA)	10.66±0.33***	30.33±0.33***	15.66±0.33***	43.33±0.33	6.33±0.33
3	Staphylococcus epidermidis (SE)	20.33±0.33***	16.66±0.33***	11.66±0.33***	26.66±0.33	6.33±0.33
4	Streptococcus pyogenes (SP)	20.66±0.33***	15.33±0.33***	13.33±0.33***	35.66±0.33	6.33±0.33
5	Corynebacterium xerosis (CX)	18.33±0.33***	18.66±0.33***	12.66±0.33***	40.33±0.33	6.33±0.33
6	Bacillus cereus (BC)	11.33±0.33***	12.33±0.33***	10.66±0.33***	30.66±0.66	6.33±0.33
7	Escherichia coli (EC)	19.33±0.33***	11.66±0.33***	12.33±0.33***	30.66±0.33	6.33±0.33
8	Klebsiella pneumoniae (KP)	16.66±0.33***	13.33±0.33***	12.66±0.33***	30.33±0.33	6.33±0.33
9	Pseudomonas aeruginosa (PA)	16.66±0.33***	8.66±0.33***	12.33±0.33***	32.33±0.33	6.33±0.33
10	Proteus vulgaris (PV)	24.66±0.33***	13.33±0.33***	10.66±0.33***	30.33±0.33	6.33±0.33

Significance: ***=p<0.001, **=p<0.01, *=p<0.05; NS= Not Significant

Table 4 — Antibacterial activity of hydroalcoholic extracts of Kanocha (*P. maderaspatensis*) against gram-positive and gram-negative bacterial strains

S. NO.	Test Strains	Alcoholic extract				Aquous extract	
		MIC	MBC	MIIC	MBC	MIC	MBC
1	Streptococcus mutans (SM)	18.5	>166.6	55.5	>166.6	55.5	166.6
2	Staphylococcus aureus (SA)	18.5	>166.6	55.5	>166.6	55.5	>166.6
3	Staphylococcus epidermidis (SE)	18.5	>166.6	55.5	>166.6	55.5	>166.6
4	Streptococcus pyogenes (SP)	18.5	>166.6	55.5	>166.6	55.5	>166.6
5	Corynebacterium xerosis (CX)	55.5	>166.6	18.5	>166.6	55.5	>166.6
6	Bacillus cereus (BC)	18.5	>166.6	55.5	>166.6	55.5	>166.6
7	Escherichia coli (EC)	18.5	>166.6	55.5	>166.6	55.5	>166.6
8	Klebsiella pneumoniae (KP)	18.5	>166.6	55.5	>166.6	55.5	>166.6
9	Pseudomonas aeruginosa (PA)	6.17	166.6	6.17	166.6	55.5	>166.6
10	Proteus vulgaris (PV)	6.17	166.6	6.17	166.6	55.5	>166.6

their antibacterial activity and the results showed maximum activity by ethyl acetate extracts of shoot[17]. Karthikeyan M. *et al.*, 2012 performed antibacterial activity of water, methanol, acetone, ethyl acetate and benzene extracts of fresh leaves of Kanocha plant against some human pathogenic bacteria, all the extracts showed potential antibacterial activity but benzene extract was found inactive[15]. In the present investigation, hydroalcoholic and alcoholic extracts showed better antibacterial activity than aqueous extract and it indicates that hydroalcoholic and alcoholic extracts have capabilities of extracting more phytochemicals in comparison to aqueous extract. On the basis of the previous and present studies it may be concluded that the various parts like roots, shoots, leaves and seeds of the plant possess varying degree of antibacterial potential depending upon the presence of phytochemicals and also among all the studies it was observed that the aqueous extracts showed minimum antibacterial activity. The antibacterial activities appeared by the concentrates might be due to the existence of these secondary metabolites through different mechanisms. Some phytochemicals like alkaloids, quinones, terpenoids, flavonoids and tannins have property of precipitating proteins[18]. The proposed antibacterial mechanism of alkaloids may be by inhibiting nucleic acid production, as they repress the catalyst dihydrofolate reductase in cell free measures, by repressing cell division and perturbing the Z-ring, by trading off external layer and cytoplasmic integrity[19]. Similarly, it has been observed that sterols either decrease the activity of *E. coli, S. aureus, P. vulgaris* and *P. pyocyanea* or have no effect in case of *Klebsiella* and *Shigella dysenteriae*[20]. The possible antibacterial mechanism of glycosides may be by inhibiting the RNA nucleic

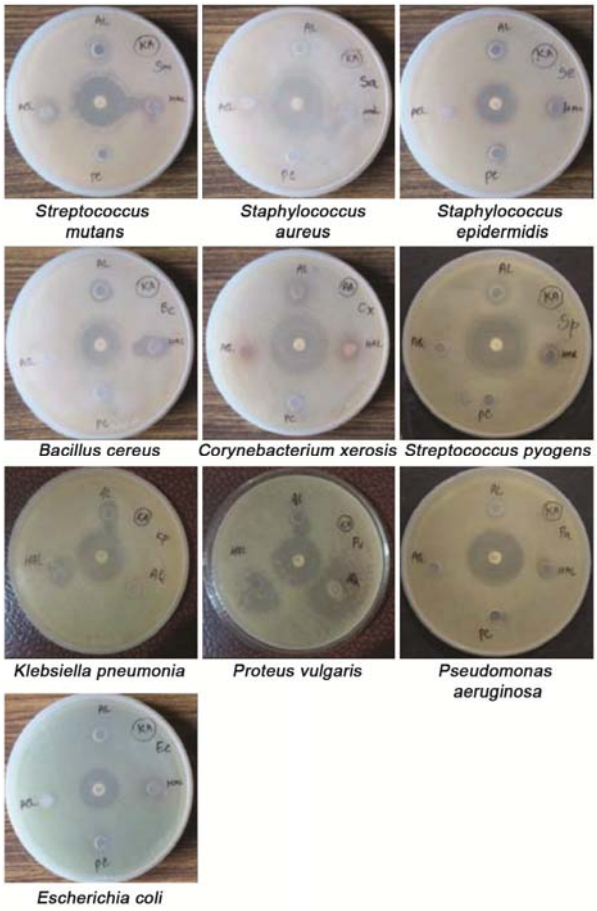

Fig. 1 — Antibacterial activity of aqueous, alcoholic and hydroalcoholic extract of Kanocha, plain control (DMSO) and standard (Ciprofloxacin & Gentamicin) against Gram positive and Gram negative bacterial strains. AQ = Aqueous; AL = Alcoholic; HAL = Hydroalcoholic; PC = Plain control (DMSO); KA = Kanocha

acid synthesis[21]. In the current investigation, alcoholic and hydroalcoholic extracts of the seeds shown much inhibitory effect against *E. coli, K. Pneumoniae* and *P. vulgaris* which are responsible for many infectious conditions thus the study validates the use of medicine in traditional system. Based on the present results the drug may be used to treat wounds, nosocomial contaminations, urinary tract infections, food borne diseases, gastroenteritis, septicemia, mild superficial skin infections and pneumonia. The present study is an indication of usefulness of Kanocha as a potent antibacterial drug which contains many known and unknown phytochemicals. By extracting and recognizing these compounds new medications can be prepared. To make the study more comprehensive it is suggested that further screening may be done to clarify the accurate component and mechanism of activity of medication.

Conclusion

The present study confirms that the alcoholic and hydroalcoholic extracts of the Kanocha (seeds of *P. maderaspatensis*) possess significant antibacterial action against both gram-positive and gram-negative pathogens when contrasted with aqueous extract. These actions might be because of existence of bioactive compounds in the drug or some unknown reasons. It was concluded that the test drug has potent antibacterial activity and may be utilized as an antibacterial agent to cure different infectious ailments.

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