IJPSR (2024), Volume 15, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 May 2023; received in revised form, 11 August 2023; accepted, 20 September 2023; published 01 January 2024

IN-VITRO ANTIOXIDANT, ANTIBIOTIC COMPLEMENTARY OR SUPPLEMENTARY EFFECTS AND BACTERICIDAL ACTIVITIES OF THE SEEDS OF A WEED: *HYGROPHILA AURICULATA* (K. SCHUM) HEINE AGAINST UROPATHOGENS

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Keywords:

Antibacterial, Antioxidant, *Hygrophila auriculata* (K. Schum) Heine, Phytochemical analysis, Uropathogens

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ABSTRACT: Upgradation of conventional medications with additional therapeutic phytocompounds to combat emerging resistant pathogenic strains, is uptrend now-a-days. The current work investigates the precise *in-vitro* bactericidal efficacy of the methanolic extract of the unexplored seeds of a swampy weed, *Hygrophila auriculata* (K. Schum) Heine (MHA), Family – Acanthaceae against different uropathogens. It was observed that, 200µg of MHA has 20% of supplementary inhibitory effect on the antibiotic disc of Amoxicillin-clavulanic acid 30 against *Enterococcus faecalis* and *Escherichia coli*. Similarly, MHA treated Nitrofurantoin 300 disc shows 25%, 4.5% and 5% of supplementary inhibitory effect against *E. faecalis*, *E. coli* and *Staphylococcus aureus*, respectively. Ciprofloxacin 5 is supplemented by 2.8% and Streptomycin 10 by 4.5% against *S. aureus.* 39.28%, 42.39%, 53.36% and 90.4% of increased inhibition was recorded against *E. faecalis, E. coli, Pseudomonas aeruginosa* and *S. aureus* respectively in terms of cfu/mL, on treating with a higher concentration of MHA. Present study has noted a minimum inhibitory concentration of 2.5mg/mL of MHA each against *E. coli*, *P. aeruginosa* and 1.25mg/mL against *E. faecalis*, *Proteus vulgaris* and *S. aureus*, respectively. Alkaloids, carbohydrates, coumarin, flavonoids, phenols, proteins, steroids, and tannins were tested as the likely bioactive components for the antibacterial response. The phenol, flavonoid content and IC_{50} value of MHA is quantified as 20.42 ±1.2 mg/g of Gallic acid equivalent, 348 ± 8.6 mg/g of Rutin equivalent and 58.37µg/mL, respectively to potentiate its antioxidant capacity. Our findings imply warranted development of potent antibacterial agent from the MHA in pharmaceutical sector.

INTRODUCTION: Since, decades, the local community and traditional healers in India have skillfully investigated the potential of the country's diverse medicinal flora for treating a wide range of human disorders [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.15(1).153-65

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.15(1).153-65

The latest scientific attention on oriental medicines has increased the context of developing novel medications supplemented by phytoextracts, for upgrading the present therapeutic sources against various ailments.

One-third of the total drugs (35%) in USA and 80% of drugs used in fast developing countries such as China and India are derived from the phytoextracts [2]. That is why, Ayurveda, Siddha and Unani type of traditional medicinal systems, hinge on herbal products or their derivatives. The phenolic compounds and tannins like antimicrobial

constituents of the plant products with definite physiological effects, can be used to potentiate the modern system of medicines [3].

After the rice harvest, in the month of November, bare paddy fields are seen to be overgrown by prickly weeds known as *Hygrophila auriculata* (K. Schum) Heine, which are also known as *Asteracantha longifolia* (Family - Acanthaceae). The plant has tetragonal, hairy, and nodally stiffened stem. The leaves are hispid and elliptic-lanceolate, while the bark is dark brown. The blossoms are purple-blue with violet undertones. The fruit has a four-sided shape, is linear and glabrous, and has roughly one-cm-long seeds that are orbicularly hairy, brown in colour, and resemble rice grains in shape [4].

According to the literature gathered between 1995 and 2010 using the search engines Google Chrome and Sci-Hub, the plant is said to have potential medical benefits with aphrodisiac, antinociceptive, antibacterial, antioxidant, antidysentery, hematopoietic, and anticancer effects [5]. It also attributes curative properties against liver disorders, jaundice, hepatic obstruction, posing zero side effects. Aqueous extract of the entire plant was discovered to have hepatoprotective characteristics, in contrast to the ethanolic extract of aerial portions, which exhibits diuretic [6], aphrodisiac [7], hypoglycemic [8], spasmolytic, and hypotensive qualities [9]. Petroleum ether: methanolic extract of leaves has promising antioxidant action due to the presence of phenolic components and flavonoid, which boosts WBC count considerably and has hematopoietic activity [7].

Very few *in-vitro* antibacterial works are been performed for the validation of the stem and leaves of *H. auriculata*, whereas the seeds are yet to be explored. This piece of research work, is a step towards the exploration of these seeds towards their pharmacological efficacies. The present study is designed to investigate the seeds of this plant scientifically for their constituents and possible anti-urobacterial potentials.

If these unexplored seeds can be tested for their therapeutic constituents, they may serve as a great help to the pharmaceuticals to enhance their potential antibacterial components against Urinary Tract Infections (UTI). These can be cultivated on the paddy fields during the off-season without hampering the harvest and agricultural output if they are deemed therapeutically effective enough. At the same time, the bioactive components of these seeds can also be used to create high-quality medications.

MATERIALS AND METHODS:

Bacterial Strains: The uropathogens like *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris,* and *Staphylococcus aureus* are examined for their *in vitro* inhibitory activity by methanolic extract of seeds from *Hygrophila auriculata* (K. Schum) Heine. Subcultures of the aforementioned strains are preserved in aseptic conditions at a low temperature in Nutrient agar (NA), Luria Bertani (LB), or Muller Hinton (MH) agar. One-hour activations in LB or MH broth of the sub-cultured bacterial strains are used for the antibacterial experiments on *H. auriculata* seed extract.

Plant Material Collection: The healthy seeds are removed from the dried plants of *H. auriculata*, which are procured in November 2021 from the swampy region of Digapahandi, Ganjam, Odisha state, India, with coordinates 19.3777^0 N and 84.5668^0 E **Fig. 1**.

Hygrophila auriculata plant with flowers Dried plant Separated Seeds

FIG. 1: IMAGE OF THE PLANT, COLLECTED DRIED PLANT AND THE SEEDS

Preparation of Crude Extract: The seeds of dried plants of *H. auriculata* were separated and ground into a fine powder, and weighed as W_2. Using the Soxhlet equipment, methanol extract is produced from the *H. auriculata* seed powder (MHA). The aforementioned is further evaporated, and the crude extract is then collected, weighed as W_1, and maintained at a temperature of 4^0C. Each antibacterial trial began with the preparation of a new working concentration of the extract. Around 0.02 g of the crude extract is dissolved in 1 mL of lukewarm water to create an aqueous solution of the crude sample, which is then kept in a labeled Eppendorf tube for future use.

Antibacterial Sensitivity Test (AST): With regard to *E. faecalis*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *S. aureus*, the inquiry was conducted to determine the minimum inhibitory concentration (MIC) of the MHA and a positive control drug, Ciprofloxacin. The susceptibility of bacterial strains is tested through all other possible methods through the disc diffusion, swabbing and pour plate method of agar well diffusion, modified agar well diffusion, supplementing or complementing effects of antibiotic discs treated with MHA and cfu/mL determination in different concentrations of MHA through spread plate method following the standard lab protocol.

Determination of Minimum Inhibitory Concentration (MIC): The MIC analysis was performed *via* broth microdilution techniques according to CLSI guidelines (National Committee for Clinical Laboratory Standards) procedures for aerobic testing in 96-well microtiter plate with some modifications [10]. Stock extract solution is prepared by dissolving 250 mg of drug in 1mL of Luria Bertani Broth. Around 5 mL of sterile broths are inoculated with 10µL of bacterial strains at log phase after 6–7-hour activation at 37^0C, to get 500 times dilution, to ensure the concentration of bacterial suspensions approximately up to 10^6 cfu/mL. Ciprofloxacin is taken as the positive control. In the first column wells of a microtiter plate, media was taken, in second 200µL bacterial culture, then positive control 0.5mg of CIP and 5mg of MHA in 200µL of broth was taken in the first row in duplicates. These are subjected to serial half fold dilution and then 100µL of bacterial suspension was added to each well. MHA without bacteria served as blank. Each plate was wrapped loosely with para-film to prevent dehydration, and finally incubated at 37^0C for 20-24 hours. Following the incubation, 40µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added at a concentration of 0.2mg/mL to each of the well and incubated at room temperature for 30 minutes and the optical densities of microplates were taken in Elisa reader at 595nm. Bacterial growth was observed as purple coloration of the wells. The well of lowest coloration was observed and the corresponding concentration was referred to as the MIC value.

Disc Diffusion Method: Discs of approximately 6mm diameter were loaded with 100µg, 200µg, 300µg and 400µg concentrations of MHA separately. These discs were placed on sterilized nutrient agar plates swabbed with the one-hour activated urobacteria. The plates were incubated overnight at 37^0 C and checked for zone of inhibitions (ZOIs) in terms of millimeter.

Agar Well Diffusion by Swabbing: In sterilized nutrient broth, a single bacterial colony was suspended and incubated at 45^0C for 15 minutes for activation after proper shaking. Sterilized nutrient agar plates were swabbed by these activated bacterial strains and incubated for 15 minutes. Three wells made in the plate were loaded with 400µg, 800µg, 1200µg of MHA and the proper diffusion was allowed at room temperature. The ZOIs were noted down after overnight incubation at 37^0 C.

Agar Well Diffusion by Pour Plate Method: Around 100µL of activated culture was plated with nutrient agar media aseptically. Three wells were made and MHA is loaded into them at different concentrations (400µg, 800µg, 1200µg) to verify the drug dependent ZOIs.

Antibiotic Supplementing or Complementing Potential of MHA: Aseptically placed standard antibiotic discs (AMC 30- Amoxycillin and Clavulanic Acid 30, NIT 300- Nitrofurantoin 300, CIP 5-Ciprofloxacin 5, CFM 5- Cefixime, S10-Streptomycin 10) on uropathogens swabbed nutrient agar plates, were subjected to overnight incubation followed by measurement of inhibitory zones. The resultant AST is considered as standards

for comparison with another set of plates of MHA supplemented antibiotics. The differences in the inhibition zones present the level up to which the MHA supplements or complements the inhibitory potential of the antibiotic discs.

Spread Plate Method: Three test tubes with 3mL of nutrient broth each are inoculated with 100µL of activated culture. Of these, first one without drug served as control, second one is added with low dose of MHA (100µg) and third one was treated with a high dose of MHA (600µg). All three were incubated for 4 hours at 37^0 C. Aliquots of these cultures were subjected to two rounds of serial dilution (10µL culture in 990µL of sterile distilled water). Then 30µL of this diluent is spread on sterilized agar nutrient plates and the cfu/mL is calculated by counting the colonies after overnight incubation.

Thin Layer Chromatography (TLC): The TLC profiling was performed to find out the possible number of major phytocompounds as per description of Biradar et al. with required modifications, for finding the probable number of therapeutic constituents like alkaloids, flavonoid, tannin and phenols in the seed sample [11, 12]. Methanolic sample of the seed extract was prepared and added at the origin point of TLC plates by capillary tubes. After drying, the plates were placed in variable glass chambers containing saturated solvents such as Hexane: Ethanol or Diethyl ether: Ethyl acetate at a ratio of 8:2, 7:3 and 6:4, respectively. Mobile phase was allowed to move through absorbent phase till 1cm away from the tip of the plate. Then the TLC plates were air dried and developed in iodine chamber to observe the phytocompounds.

Qualitative Phytochemical Screening: Standard methods were used to perform a primary qualitative analysis on the methanolic crude extract of seeds to determine the presence of bioactive components [13, 14].

Quantitative Analysis of Phytochemicals: The standard protocols of Folin-Ciocalteu method [15] and the $AlCl_3$ assay [16] respectively, were used to plot the calibration curves to quantify the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of the seed extract.

Determination of Antioxidant Activity: The ability of the methanolic extracts to scavenge free radicals was assessed with little modification using the DPPH assay [17]. Variable concentrations of solution of plant extracts such as 20, 40, 60, 80, 100, 200, 500µL are taken in a series of test tubes and the volume was made up to 3mL by addition of methanol, which were been combined with 1mL of a methanolic solution containing 1, 1 Diphenyl-2-picrylhydrazyl(DPPH) radicals with ultimate 0.4mM concentration. After 30 minutes of dynamic shaking and standing, the mixture was tested for absorbance at 517 nm. The standard utilized was Ascorbic acid. The following formula was used to determine the sample's percentage of DPPH decolorization.

$$\% \text{ decolorization} = (\text{Abs. of control} - \text{Abs. of sample} / \text{Abs. of control}) \times 100$$

RESULTS:

Plant Extract: The crude extract of MHA, is dark brown coloured, with mucilaginous texture, soluble in Luke-warm water and presents 5.7% of final yield, by using standard formula for yield calculation with minor modifications [18].

$$\% \text{ Yield} = W_1/W_2 \text{ X } 100$$

Where, W_1 = The weight of the methanolic extract in grams (1.75g), and W_2=The weight of the initial dried sample in grams (30.4g).

Antibacterial Activities of MHA: The minimum inhibitory concentration of the MHA is recorded against each of aforementioned pathogenic strains. A concentration of 2.5mg/mL was found effective against *E. coli*, *P. aeruginosa* and 1.25mg/mL against *E. faecalis*, *P. vulgaris* and *S. aureus*, respectively. At the same time, 0.125mg/mL of CIP was found effective against *E. coli*, *P. aeruginosa* and 0.062mg/mL of CIP was found effective against *E. faecalis*, *P. vulgaris* and *S. aureus*, respectively.

The bactericidal efficacy of MHA against the aforesaid bacterial strains was measured in terms of ZOI in mm *via* disc, agar well and modified agar well diffusion methods along with MHA treated antibiotic sensitivity test followed by cfu/mL count through spread plate method.

Disc Diffusion Method: The bactericidal effectiveness of MHA against the aforementioned bacterial strains was assessed by using different discs loaded with different amounts of MHA and the zone of inhibitions were measured in millimeter. **Table 1** provides an overview of the disc diffusion of MHA findings, and **Fig. 2** shows a graphical comparison.

TABLE 1: *IN-VITRO* ANTIBACTERIAL POTENTIAL SCREENING OF MHA THROUGH DISC DIFFUSION METHOD, EXPRESSED IN MM

Name of the bacterial strain	ZOI measured in mm at different conc. of MHA in µg /disc			
	100 µg	200 µg	300 µg	400 µg
E. faecalis	16.33 ± 0.27	17.66 ± 0.27	-	19 ± 0.47
E. coli	13 ± 0.47	15.33 ± 0.27	16 ± 0.27	16.66± 0.47
P. vulgaris	11 ± 0.94	13 ± 0.94	13 ± 0.94	15 ± 0.47
P. aeruginosa	-	12.33 ± 1.18	14.66 ± 0.27	19 ± 0.94
S. aureus	7.33 ± 0.72	16.33 ± 0.72	17.66 ± 0.27	19.33 ± 0.27

Note: Values represent the average ± SEM of triplicate sets of experiments and "- "presents no zone of inhibition.

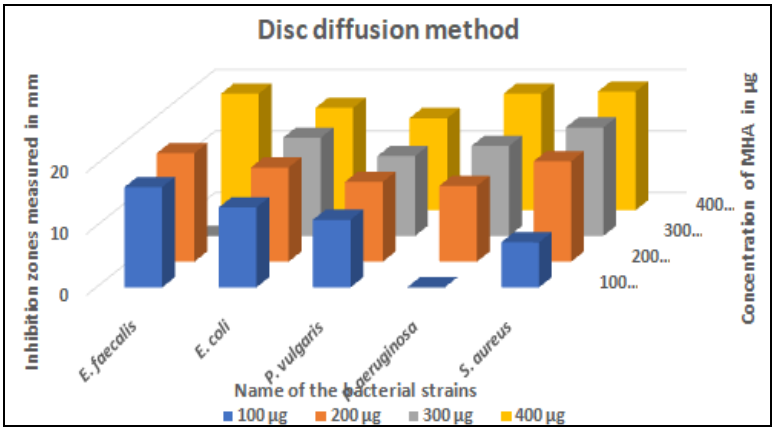


FIG. 2: GRAPHICAL PRESENTATION OF COMPARATIVE INHIBITORY POTENTIALS OF MHA AGAINST BACTERIAL STRAINS IN DISC DIFFUSION

According to **Table 1** and **Fig. 2**, at 400µg/disc concentration of MHA, a maximum zone of inhibition of 19±0.47, 19±0.94, 19.33± 0.27mm was noted against *E. faecalis*, *P. aeruginosa* and *S. aureus*, respectively.

MHA is effective against *E. coli* and *S. aureus* at a concentration of 300µg/disc with ZOIs 16 ± 0.27 and 17.66 ± 0.27 mm. A maximum ZOI of 15.33 ± 0.27, 16.33 ± 0.72, 17.66 ± 0.27mm was noted at a concentration of 200 µg/disc against *E. coli*, *S. aureus* and *E. faecalis*. Similarly, 100µg of MHA is better effective against *E. faecalis* (16.33 ± 0.27mm) followed by *E. coli* (13 ± 0.47mm). It is found that the increasing concentration of drug facilitates greater resistance to the pathogenic strains.

Agar Well Diffusion by Swabbing and Pour Plate Method: The results of agar well diffusion through swabbing and pour plate method are presented in **Table 2** and analyzed graphically in **Fig. 3**.

TABLE 2: *IN-VITRO* ANTIBACTERIAL POTENTIAL SCREENING OF MHA THROUGH AGAR WELL DIFFUSION IN SWABBING AND POUR PLATE METHOD, EXPRESSED IN MM

Names of Bacteria	ZOI measured in mm at different concentrations of MHA in µg /well					
	Swabbing method			Pour plate method		
	400µg	800µg	1200µg	400µg	800µg	1200µg
E. faecalis	15.33±0.27	20.33±0.27	22.66±0.54	7.33±0.72	10.33±0.27	11.66±0.54
E. coli	18.66±0.27	19±0.47	19.66±0.54	16.33±1.18	16±0.94	17.33±0.27
P. vulgaris	22.33±0.27	23.66±0.54	26.66±0.54	12.33±0.72	19.66±0.98	26.33±0.27
P. aeruginosa	18.66±0.54	25.33±0.27	26.33±0.27	7.66±0.27	20.66±0.27	22.66±0.98
S. aureus	12.33±0.27	22.66±0.98	25±0.94	13.66±0.54	17±0.94	18.33±0.27

Note: Values represent the average ± SEM of triplicate sets of experiments.

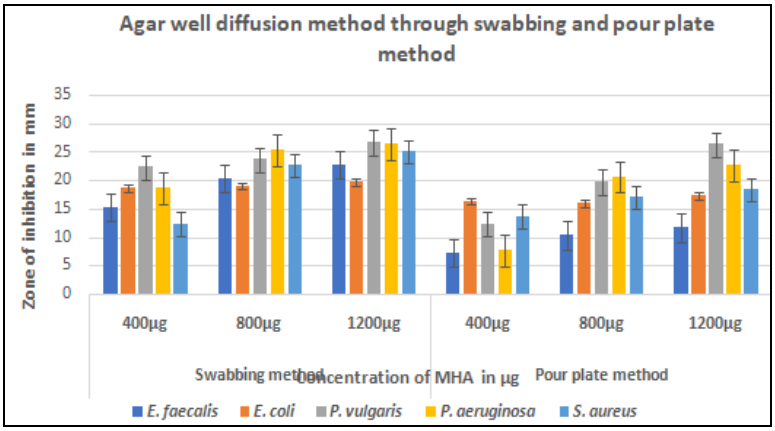


FIG. 3: GRAPHICAL PRESENTATION OF COMPARATIVE INHIBITORY POTENTIALS OF MHA AGAINST BACTERIAL STRAINS IN AGAR WELL DIFFUSION BY SWABBING AND POUR PLATE METHOD

From the agar well diffusion through the swabbing and pour plate method, presented in **Table 2** and **Fig. 3**, it was clear that the *P. aeruginosa*, *P. vulgaris* are the most vulnerable bacterial strains followed by *S. aureus*, when treated with different doses of MHA. The maximum ZOI of 26.66±0.54mm, 26.33±0.27mm is obtained against *P. vulgaris* through swabbing and pour plate method of agar well diffusion, respectively at a concentration of 1200 µg/well. But at a lower concentration i.e., 400µg of MHA, 22.33±0.27mm and 12.33±0.72mm are found against *P. vulgaris.* At the same time, a minimum ZOI of 19.66±0.54mm and 17.33±0.27 was noted against *E. coli* at the same concentration of 1200µg/well. Better antibacterial effects are been noticed in swabbing method than the pour plate method at same concentrations against *E. faecalis*. This probably happened due to improper or indifferent diffusion rate of MHA.

Modified Agar Well Diffusion Method: In modified agar well, maximum ZOI of 14mm was found against *S. aureus* followed by 11 mm each of *P. vulgaris* and *E. coli*. Whereas *E. faecalis* and *P. aeruginosa* are found to be more resistant to MHA with a minimum ZOI of 6mm each. This presents a comparative inhibitory result of MHA against the bacterial strains. The result of modified agar well is represented graphically in **Fig. 4**.

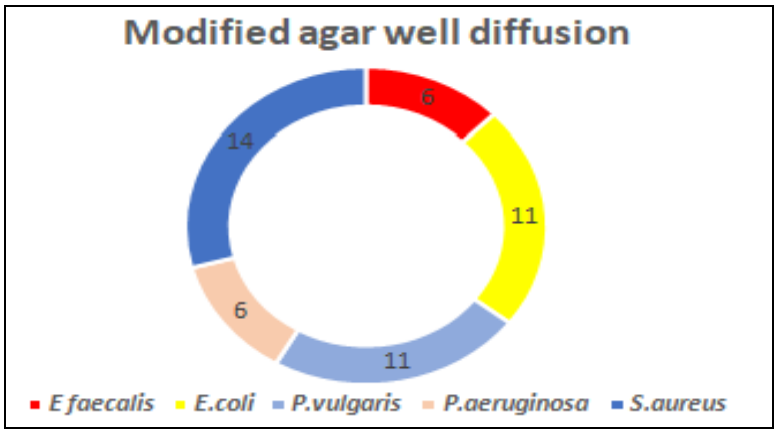


FIG. 4: GRAPHICAL PRESENTATION OF COMPARATIVE INHIBITORY POTENTIALS OF MHA AGAINST BACTERIAL STRAINS IN MODIFIED AGAR WELL DIFFUSION METHOD

Spread Plate Method: Bactericidal activity is assessed in a liquid broth culture media by growing different strains in absence and presence of MHA and then the colony forming units per mL is determined in wild *i.e.*, without MHA, and taken as the control. The cfu/mL of the same bacterial strains are determined for 100µg and 600µg MHA treated strains through the spreading method. The comparative study of number of colonies facilitates the dose-dependent inhibition of MHA. 39.28% increase of inhibition is observed against *E. faecalis* at 600µg as compared to 100µg. Similarly a higher dose of MHA (600µg) shows an increase of 42.39% of inhibition for *E. coli.* Around 53.36%

of increase in inhibition is observed against *P. aeruginosa*, whereas a maximum increase of 90.4% is observed for *S. aureus* at a higher conc. of 600µg than the lower conc. of 100 µg **Table 3**. This proves that, the MHA at a higher concentration is more inhibitive against the pathogenic bacterial strains. These results are graphically presented in **Fig. 5**.

TABLE 3: CFU/ML IN 10^6 IS DETERMINED IN WILD AND MHA TREATED BACTERIA AT LOG PHASE BY SPREAD PLATE METHOD

Name of the bacterial strain	Control (No MHA)	Lower dose of MHA (100µg)	Higher dose of MHA (600µg)	Increased % of inhibition
E. faecalis	3.26 x 10^6	2.8 x 10^6	1.7 x 10^6	39.28
E. coli	13.8 x 10^6	9.2 x 10^6	5.3 x 10^6	42.39
P. aeruginosa	6.03 x 10^6	1.93 x 10^6	0.9 x 10^6	53.36
S. aureus	11.3 x 10^6	8.96 x 10^6	0.86 x 10^6	90.4

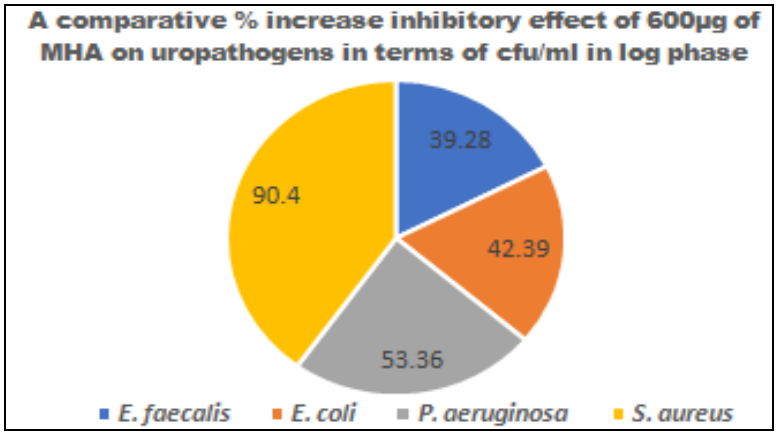


FIG. 5: GRAPHICAL PRESENTATION OF THE COMPARATIVE ANALYSIS OF THE INCREASED PERCENTAGE OF INHIBITION AT HIGHER DOSE OF MHA AS COMPARED TO LOWER DOSE

Antibiotic Complementary or Supplementary Activities of MHA: The sensitivity of traditional antibiotic discs like Amoxycillin and Clavulanic Acid 30 (AMC 30), Nitrofurantoin 300 (NIT 300), Ciprofloxacin 5 (CIP5), Cefixime 5 (CFM 5), Streptomycin 10 (S10) is tested against the aforesaid bacterial strains.

The ZOIs are measured in millimeters and taken as the control values. The same antibiotics are loaded with 200µg of MHA and their inhibiting potential is tested against the same bacterial strains. The difference in values of ZOIs presented in **Table 4**, are regarded as the extent of complementary and supplementary effects of MHA on the antibiotic discs against each of the bacterial strain. Here complementary effect means, the antibiotic disc itself is ineffective in preventing growth of a particular bacterial strain, whereas a clear ZOI is noticed on addition of MHA. This indicates that, probably the unknown phytocompounds of crude extract of seeds of MHA becomes effective only in presence of the compounds of the antibiotic discs. Similarly, supplementary effect means the inhibitory potential of a particular antibiotic is enhanced by addition of certain unknown components of MHA. It was observed that, the antibacterial effect of AMC 30 is complemented by 20mm, 9mm against *E. faecalis, E. coli* and supplemented by 2mm for *S. aureus* which is around 20% increase of total inhibition by AMC 30 disc. The inhibition of NIT 300 is supplemented by 4mm, 1mm, 1mm for *E. faecalis, E. coli* and *S. aureus* respectively, presenting 25%, 4.5% and 5% increase in inhibition. The MHA complements the inhibition of *E. faecalis, E. coli* by 28mm, 15mm and supplemented by 1mm for CIP5 that is around 2.8% extra of the inhibition zone. CFM5 is complemented 13mm for *E. faecalis*. The MHA complements S10 by 15mm against *E. faecalis* and supplements *S. aureus* by 1mm, which is an increase of 4.5% of the regular ZOI. These complementary and supplementary effects of MHA support the therapeutic potentials of seeds of *H. auriculata*. All the pictures of the plates of in vitro antibacterial susceptibility test are compiled and presented in **Fig. 6**.

TABLE 4: *IN-VITRO* SUPPLEMENTARY AND COMPLEMENTARY ANTIBACTERIAL EFFECTS OF MHA, EXPRESSED IN MM

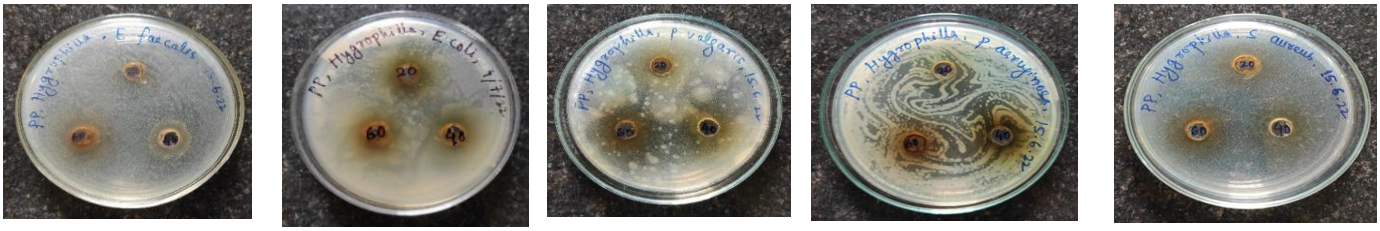
Name of the strain	AMC 30	AMC 30 + MHA	Diff in ZOI	NIT 300	NIT 300 + MHA	Diff in ZOI	CIP 5	CIP 5 + MHA	Diff in ZOI	CFM 5	CFM 5 + MHA	Diff in ZOI	S 10	S10 + MHA	Diff in ZOI
E.f	-	20	20	16	20	4	-	28	28	-	13	13	-	15	15
E.c	-	9	9	22	23	1	-	15	15	-	-	-	23	23	-
P.v	-	-	-	21	21	-	39	39	-	15	15	-	29	29	-
P.a	-	-	-	20	20	-	36	36	-	26	-	-	24	24	-
S.a	10	12	2	20	21	1	35	36	1	23	-	-	22	23	1

Note: AMC 30- Amoxycillin and Clavulanic Acid 30, NIT 300- Nitrofurantoin 300, CIP 5-Ciprofloxacin 5, CFM 5- Cefixime, S10- Streptomycin 10 and *E. f - Enterococcus faecalis, E. c- Escherichia coli, P. a - Pseudomonas aeruginosa, P. v- Proteus vulgaris, S. a- Staphylococcus aureus*.

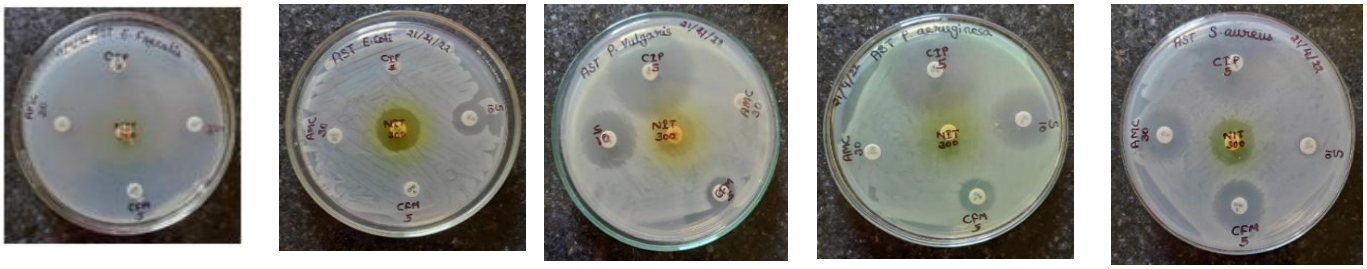
DISC DIFFUSION

AGAR WELL DIFFUSION THROUGH SWABBING METHOD

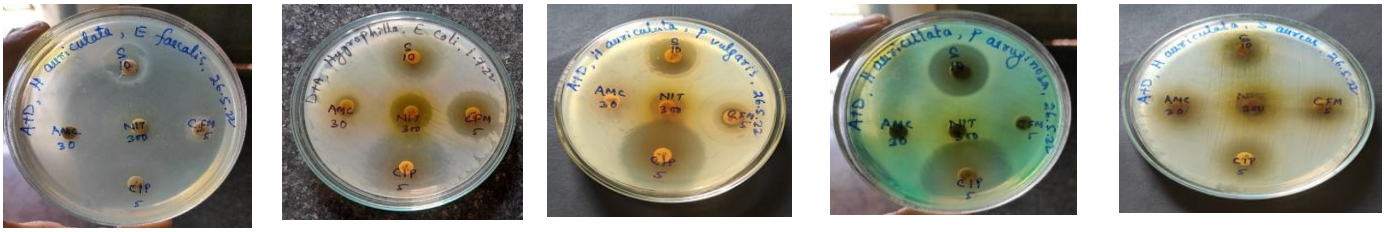
AGAR WELL DIFFUSION THROUGH POUR PLATE METHOD



ANTIBIOTIC SENSITIVITY TEST



ANTIBIOTIC DISCS SUPPLEMENTED WITH MHA



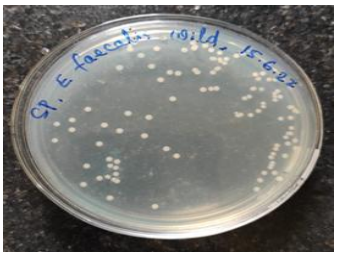
E. faecalis *E. coli (E.c)* *P. vulgaris(P.v)* *P. aeruginosa (P.a)* *S. aureus (S.a)*

(E.f)

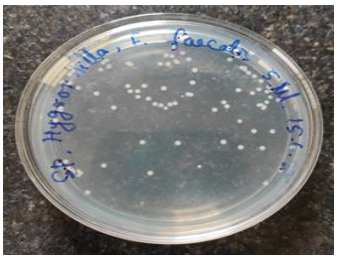
MODIFIED AGAR WELL DIFFUSION

Abbreviations on plate: *E. faecalis (E.f); E. coli (E.c); P. vulgaris (P.v); P. aeruginosa (P.a); S. aureus (S.a)*

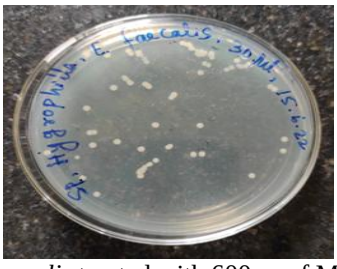
CFU/ML COUNT THROUGH SPREAD PLATE METHOD



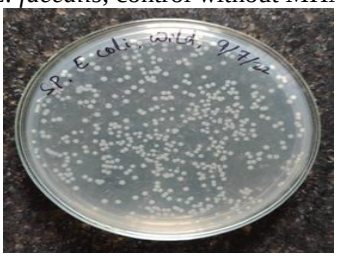
E. faecalis, control without MHA



E. faecalis treated with 100µg of MHA



E.f aecalis treated with 600µg of MHA



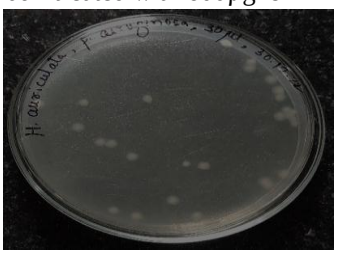
E. coli, control without MHA

E. coli treated with100µg of MHA

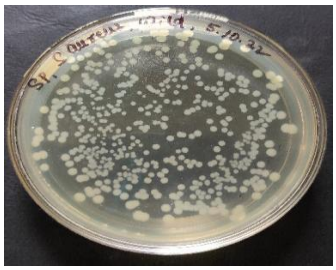
E. coli treated with 600µg of MHA

P. aeruginosa, control without MHA

P. aeruginosa treated with 100µg of MHA



P. aeruginosa treated with 600µg of MHA



S. aureus, control without MHA

S. aureus treated with 100µg of MHA

S. aureus treated with 600µg of MHA

FIG. 6: *IN-VITRO* INHIBITORY ASSAYS OF MHA BY DISC DIFFUSION, AGAR WELL, MODIFIED AGAR WELL DIFFUSION, SUPPLEMENTARY OR COMPLEMENTARY EFFECT ON ANTIBIOTICS, SPREAD PLATE METHOD

Thin Layer Chromatography (TLC): The TLC results present around two number of phytocompounds within the range of 0.19 - 0.28 to 0.64 - 0.93 of Rf value for Hexane: Ethanol and four phytocompounds within the range of Rf 0.6-0.65 to 0.91-0.95 in Diethyl ether: Ethyl acetate, which probably are the major bioactive compounds, probably regulating the therapeutic properties **Fig. 7**. These compounds can be further isolated for detailed studies.

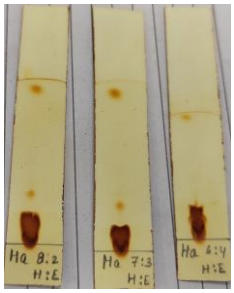
Hexane: Ethanol chamber	Possible components with Rf values	Diethyl ether: Ethyl acetate chamber	Possible components with Rf values
	Two prominent components : 0.19 to 0.28 0.64 to 0.93		Three prominent components: 0.6 to 0.65 0.8 to 0.84 0.91 to 0.95

FIG. 7: TLC PLATES OF DIFFERENT CHAMBERS PRESENTING THE COMPOUNDS WITH THE CALCULATED RF VALUES

DISCUSSION: Aqueous extract of *H. auriculata* root exhibited protective effect against CCl_4 ion induced liver damage which may be due to its anti-lipid peroxidative and free radical scavenging properties [19]. The potent aphrodisiac, antiurolithic [20], antidiabetic, antioxidant [21] and antidiuretic activities [22] of the plant is well established by traditional claims and scientific studies. The neuroprotective effect of crude terpenoids from *H. auriculata* against tGCI induced oxidative stress, which improves impaired neurological deficit and sensory motor function and treatment of CNS related diseases [7]. Bioactive compounds from the plant have been found to possess antimicrobial, anthelmintic, antitermite, nephroprotective, hepatoprotective, central nervous system protective, antitumour, antidiabetic, anticataract, antioxidant, haematopoietic, diuretic, antinociceptive, anti-inflammatory, antipyretic, antimotility, aphrodisiac, neuroprotection, anti-endotoxin and anti-urolithiatic activities [23].

The phytochemical studies conducted so far confirm the presence of various phytoconstituents like alkaloids, reducing and non-reducing sugars, polyphenolics (flavonoids and tannins), steroids, saponins, proteins, amino acids and triterpenes along with bioactive compounds like lupeol and stigmasterol in methanolic extract of *H. auriculata* [19, 20, 22, 24]. Ethyl acetate extract of root of the plant was found with profuse alkaloids and proteins, small quantity of terpenoid, saponin, steroids and absence of tannin and quinines [25].

In previous investigations, around 9.91 ± 0.44 mg/g of moisture, 3.33 ± 0.39 mg/g of lipid, 8.01 ± 0.03 mg/g of protein,10.60 ± 0.39 mg/g of ash, 50.76 ± 0.26 mg/g of total carbohydrate, 17.36 ± 0.24 mg/g of fibers and 13.55 ±0.06 mg/g of Potassium (K), 12.82 ±0.03 of Nitrogen (N_2) content, 1.04 ±0.03mg/g of Phosphorus (P), 21.52 ±0.34 mg/g of Sodium (Na) are been recorded in the plant [26]. Preliminary phytochemical screening of aqueous, alcoholic, n-butanolic and ethyl acetate fractions revealed the presence of flavonoids, terpenoids, tannins and phenolic compounds, rendering the diuretic effects [24].

A list of secondary metabolites like flavonoids (apigenin, luteolin), alkaloids (asteracanthine and asteracanthicine), triterpenes (lupeol, betulin, hentriacontane) aliphatic esters, sterols (β-sitosterol, stigmasterol), along with essential oils, minerals, vitamins (ascorbic acid, nicotinic acid), carbohydrates (maltose, xylose, rhamnose), sterols, amino acids (histidine, phenylalanine, lysine), fatty acids (myristic acid, palmitic acid, stearic acid,) and other compounds like vannilic acid, syringic acid are the probable phytocompounds present in leaves, flowers, whole plant, providing the anticancerous properties [27].

Standard qualitative screening of MHA confirmed the presence of bioactive compounds like alkaloids (Mayer's test, Wagner's test), carbohydrates (Fehling's Test, Molisch Test), coumarin, flavonoids, phenol compounds ($FeCl_3$ test, Potassium dichromate test), proteins (Ninhydrin solution test), steroids, tannins. But tests for anthocyanin, anthraquinones, glycosides (Liebermann's test, Acetic acid Test), leucoanthocyanins, saponins (Foam test with Water and $NaHCO_3$), terpenoids gave negative results indicating their absence in MHA.

Among the aforesaid bioactive compounds, the phenolic compounds, flavonoids and tannins provides more therapeutic properties to plant parts, which needs quantification from the standard

calibration curves. Previous research works have estimated a TFC of 128.43±5.10mg/mg RE and DPPH free radical scavenging potential as 156.71µg/mL in terms of IC_{50} of *H. auriculata* [26]. In the present study, The MHA was estimated as 20.42±1.2 mg/g GAE of TPC, whereas TFC was recorded as 348±8.6 mg/g RE. The free radical scavenging capacity of the seed extract in terms of IC_{50} value was calculated as 58.37µg/mL with ascorbic acid as a standard.

The inhibition of bacterial growth was dose-dependent since the inhibitory action of the methanolic extract was evidenced to increase with an increase in concentration. The highest concentration of 30µL of methanolic extract of plant showed maximum inhibition activity against species such as *S. aureus* (17.66±1.52), moderate activity against *E. coli* (17.3±0.57) and comparatively least activity of 15.8±1.0 mm through agar well diffusion method [28]. Gram-positive bacterial strains have more susceptibility to plant extracts of *H. auriculata* than Gram-negative bacteria, where the sensitivity is attributed by its permeability of peptidoglycan layer, the difference in cell wall composition and thickness [29]. The medicinal plant extract possesses around 8, 11 and 20mm of ZOI at a concentration of 50µL, 100µL and 150µL, respectively. But, it was ineffective against the bacterial strain of *Enterobacter faecalis* [26]. Whereas, the ethanolic extract of leaves of *H. auriculata* is effective against *E. coli* with ZOI of 8, 11, 14mm at 50µL,100µL and 200µL, respectively in well diffusion, whereas no apparent inhibition is marked against *Citrobacter divergens, Enterobacter faecalis, Klebsiella pneumoniae* and *Serratia marcescens* [30]. According to this *in-vitro* antibacterial piece of research work, 400µg of MHA has highest bactericidal effect against the *S. aureus* followed by *P. aeruginosa* and *E. faecalis* in disc diffusion. Similarly, a dose of 1200µg of MHA is best effective against *P. vulgaris*, followed by *P. aeruginosa* and *S. aureus* in swabbing and pour plate method of agar well diffusion. *S. aureus* was found to be the most vulnerable strain when treated with MHA in modified agar well diffusion method followed by *P. vulgaris* and *E. coli.* It was noted that, the antibacterial efficacies of the traditional antibiotics are supplemented and complemented to variable extents against the pathogenic strains, when treated with MHA. This proves that, the seed extracts can be directly effective against the strains or the phytocompounds may add up to the inhibition rate along with the antibiotic disc components. A maximum of 90.4% of increased inhibition is recorded in cfu/mL of *S. aureus* at a higher concentration of 600µg MHA as compared to a lower dose of MHA of 100µg, followed by *P. aeruginosa.*

CONCLUSION: The results drawn from the present scientific study concludes that, the methanolic extract of *Hygrophila auriculata* (K. Schum) Heine possess potential antibacterial efficacies against the uropathogens like *E. coli, E. faecalis, P. aeruginosa, P. vulgaris, S. aureus.* The possible phytochemical constituents of MHA are identified as alkaloids, carbohydrates, coumarin, flavonoids, phenols, proteins, steroid, tannins and absence of anthocyanins, anthraquinone, glycosides, leucoanthocyanins, saponins, terpenoids. The therapeutic phytocompounds are quantified, which can be extracted further for supplementing the pharmaceuticals against the uropathogens. Further isolation of these compounds may be proved useful in synthesizing the evolved antibiotics of plant origin, most probably against aforesaid uropathogens, especially *P. vulgaris* followed by *P. aeruginosa,* which are noted to be the most vulnerable uropathogens.

As a pilot investigation on the complementary or supplemental antibacterial, antioxidant and antibiotic actions of *H. auriculata* seed extract, there is not enough literature to compare and contrast our results with those of earlier studies. However, this current study will open up new avenues for researchers to investigate novel plant-based treatments against uropathogenesis and urinary tract diseases, which are highly widespread and frequently a bothersome issue notably in females of all ages. Similarly, the efficacy of the plant as a formulated feed in aquaculture, for the well-being of the *Cirrhinus mrigalah* as already been proven [31], which opens the future research opportunity to explore the effectiveness of the plant as the nutraceuticals for improving human health.

STATEMENTS & DECLARATIONS:
Funding: No funding is received for this work.

ACKNOWLEDGEMENT: We would like to thank all officials of Microbiology Department of Maharaja Krushna Chandra Gajapati Medical College, Berhampur, Odisha, India for their kind support in providing us the strains of uropathogens, which are collected and cultured from patients.

We extend our gratitude to Dr. S. S. Mahapatra of Department of Biotechnology, Berhampur University for gifting us the strains of *Escherichia coli* and *Pseudomonas aeruginosa*. We also are thankful to the Central Instrumentation Centre (Center of Excellence, wing-2), Berhampur University for their kind support.

CONFLICTS OF INTEREST: The present paper is an original piece of research work, which has no conflict of interest with anyone.

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How to cite this article:
Tripathy SR and Das S: *In-vitro* antioxidant, antibiotic complementary or supplementary effects and bactericidal activities of the seeds of a weed: *Hygrophila auriculata* (k. schum) heine against uropathogens. Int J Pharm Sci & Res 2024; 15(1): 153-65. doi: 10.13040/IJPSR.0975-8232.15(1).153-65.