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Pharmacological evaluation & phytochemical screening of seed extracts of kaliziri (*Centratherum anthelminticum*)

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Authors' Contribution: **Khan, J.S.** set up the experiment, **A. Saleem** supervised the research. **M. Akram** prepared the draft, **M. Afzal, Lubaba, F. Jabeen** helped and support during experimentation.

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ABSTRACT

The biologically active part of medicinal plants is usually used for therapeutic treatments or as a precursor in pharmacology. Aqueous, chloroform, ethanol, methanol and Hexane were used as a solvent to extract the essential oils of kaliziri (*Centratherum anthelminticum*). Antimicrobial actity of the extracts were checked against 4 g positive (*Bacillus cereus*, *B. pumilus, B. safensis, Staphylococcus* sp.) and 8 g negative bacterial strains (*Aeromonas* sp., *Pasteurella* sp., *Escherichia coli*, *Enterobacter* sp., *Proteus* sp., *Pseudosomonas* sp., *P. aeruginosa* and *Salmonella typhi*) by agar well diffusion method. Phytochemical screening was also performed. Results showed that highest (30 mm^2) microbial zone of inhibition (MZI) observed in the extract of chloroform with *Aeromonas* sp. This followed by four strains, i.e., *Bacillus cereus*, *B. pumilus*, *Proteus* sp. and *Salmonella typhi* showed 28, 27, 25 and 24 mm^2 MZI, respectively except water extract. The minimum inhibitory concentration (MIC) of all solvent extracts varied with different bacterial strains. The range of MIC was 50 mg/mL to 500 mg/mL, where highest 500 mg/mL MIC of all extracts observed against *Pseudosomonas* sp. followed by 100-500 mg/mL MIC against *Salmonella typhi*. Presence of flavonoids, alkaloids, glycosides, phytosterols, phenol, tannin, terpenoids and saponins confirmed by qualitative analysis. High solubility of tannins and terpenoids in chloroform demonstrated the effectiveness of metabolites against bacterial strains.

Keywords: Antimicrobial activity, bacterial strains, kaliziri, minimum inhibitory concentration, solvent extracts, terpenoid.

INTRODUCTION: Infection is one of the main reasons for death of many people in the world (Parekh *et al.*, 2006). Traditionally, medicinal plants had widely been used to control the infection (WHO, 2002-2005). Natural plant products are the precursor compounds of many newly synthesized drugs (Newman and Cragg, 2016) come from plants such as digoxin (*Digitalis*), ergosterol (*Saccharomyces*), ephedrine (*Ephedra*), morphine (*Papaver somniferum*), quinine (*Cinchona officinalis*) and reserpine (*Rauwolfia*). Clinical microbiologists repeatedly screen these unique biologically active secondary metabolites therapeutically important (Silver, 2011) in different combinations for their antibacterial activity (Jain *et al.*, 2019). In Pakistan, 600-700 plants are widely been used for therapeutic purposes (Shinwari, 2010). Kaliziri extracts containing alkaloids, essential oils, flavonoids, phenolic, polyphenols, glycosides and phytosterols are active components for lowering blood sugar and cholesterol as well as used as an anti-inflammatory, anti-thrombotic, and anti-cancerous activities (Parham *et al.*, 2020). *Centratherum anthelminticum* also known as kaliziri, bitter cumin or somraj belongs to the family *Asteraceae*. It is an annual herb with upright growth habit. Naturally, it is found in the Khasi and Himalayas Mountains. It is usually used for medicinal purposes. The size of kaliziri seeds ranges from 1 to 5 mm long. Color of kaliziri seeds is brownish-black and its harvesting season is May to June (Agnihotri, 2022). Chemical constituents present in the seeds of kaliziri are volatile oils, flavonoids, phenolic, saponins, tannins, sterols, and oleic acid (Patel *et al.*, 2012). In view of medicinal activity, kaliziri has antidepresent activity (Ghosi, 2023) and reported to control blood sugar, blood pressure, and anti-inflammatory, anti-oxidant, anti-ulcer, preservative, and diuretic activities (Mudassir *et al.*, 2018). Anti-cancerous and cell growth properties of methanolic seed extract of kaliziri has been reported (Thara and Zuhara, 2016). Due to high medicinal value, the extract of kaliziri is used in various drugs and in many lifesaving medicines (Agnihotri, 2022). Many of the previously reported anti-bacterial compounds lost their effectiveness with considerable failure treatment to global public health remains a serious concern (Balouiri *et al.*, 2016).

OBJECTIVES: Therefore, aim of the present study was to determine the chemical composition and anti-bacterial activities of the extract of kaliziri (*C. anthelminticum*) by using different extraction methods.

MATERIAL AND METHODS: **Plant material:** Seeds (1000g) of kaliziri were procured from local market. Fresh specimens of the seeds were air dried at room temperature at 25°C. Debris and pebbles were removed and each specimen was ground into fine powder by using grinding mill and kept in polythene bag for subsequent examinations.

Preparation of volatile seed extracts of kaliziri: Clevenger method: Seed powder (100g) and water (100 mL) was heated gently causing the water to boil. The volatile oils was collected in the new flask. Volatile oil was collected and stored in an airtight glass tube (Clevenger, 1928). The volatile oil obtained from Clevenger method was mixed again in 25mL hexane and then allowed the solvent to evaporate to get traces of volatile oil in a glass bottle.

Hexane extract method: Seed powder (10g) was mixed in n-hexane (25mL) and placed on orbital shaker for 1h. That material was filtered through Whatman filter paper (Das *et al.*, 2010).

Water extracts method: Seed powder (10g) was mixed in water (10mL) taken in a 100mL capacity conical flask and placed on orbital shaker, heated to 100°C constant temperature. That material was filtered through filter paper (whatman No.1) after 1 h. (Remington, 2006).

Different solvent methods: Sequential extraction was employed to obtain various extracts from kaliziri seed powder. For chloroform extraction, 20g of the seed powder were mixed with 100mL of chloroform and placed on an orbital shaker for 7 days. The same amount of seed powder was soaked in 100mL of 96% ethanol and subjected to agitation on an orbital shaker for a week. Following a parallel procedure, hexane extraction was conducted by adding 20 g of seed powder to 100mL of hexane. Methanol extraction involved mixing 20 g of seed powder with 100mL of methanol, left to extract for a week. Lastly, a distilled water extract was obtained by shaking 25 g of seed powder in 100mL of distilled water for a week. Through these methods, a variety of extracts were obtained, each potentially containing different compounds of interest.

Preparation of agar media plates: For the preparation of LB medium (Tryptone 10g, yeast extract 5g, NaCl 5g, agar 15g and pH 7) were dissolved in a 1L. of distilled water, sterilized at 15 $lbs/inch^2$ pressure and 121°C for 15min. and poured (Wood and Krieg, 1989). After cooling, 20 ml sterilized medium was poured into pre-sterilized 90 mm capacity petri plates in sterile conditions of laminar flow cabinet and kept under the culture room conditions until use.

Procurement of bacterial strains: Three strains *Aeromonas* sp, *Pasteurella* sp and *Staphylococcus* sp. were obtained from Microbiology Lab., Institute of Botany, University of the Punjab Lahore and *Bacillus cereus*, *B. pumilus, B. safensis, Escherichia coli*, *Enterobacter* sp, *Proteus* sp, *Pseudomonas* sp, *P. aeruginosa* and *Salmonella typhi* were procured from Microbiology Lab, Sheikh Zayed Hospital, Lahore, Pakistan.

Antimicrobial activity: Agar cup method was used for analysis antibacterial activities of seed extracts. For this purpose, 4mm broad wells 6 in number in each plate were prepared with the help of sterilized spatula. Then 100µL of soulution of each tested bacterial strain was swabbed thoroughly on agar solidified media. About 80µL of kaliziri extract of 05, 10, 20, 30, 40 or 50 mg/mL were poured into each well of agar plates and placed in the incubator for 24 h. at 25°C and diameter of inhibition zone was calculated (Selvamohan *et al.*, 2012). Minimum inhibitory concentration (MIC) was measured as the lowest amount of extract that inhibits microbial growth (Andrews, 2001). The MIC was also compared with commercially available disk of antibiotics such as ampicillin, ciprofloxacin, penicillin and gentamicin.

Photochemical analysis of seeds extract: Photochemical analyses were carried out for seed extracts to examine different metabolites as per standard methods described previously. Metabolite tests were performed for all plant extracts by using the following methods.

Glycosides: The extract (1g) was mixed in water (1mL), yellow color appeared on adding aqueous solution of NaOH was the indication of glycosides. The chemical composition of the seed extracts was investigated using a series of tests. Tannins were detected by mixing 0.5mL of extract with 1mL of water and adding 2 drops of ferric chloride solution, resulting in the production of a green-black color indicative of tannins (Nortjie *et al.*, 2022). Terpenoids were identified by combining 2mL of chloroform with 5mL of extract in a test tube, followed by the addition of 3mL of concentrated sulfuric acid, leading to the development of a reddish-brown color at the interface (Nortjie *et al.*, 2022). The presence of saponins was confirmed using the Froth Test, where shaking 5mL of extract in 15mL of water for 15 min. in a cylinder produced a 1cm thick layer of foam, indicating the presence of saponins (Tiwari *et al.*, 2011). Additionally, phytosterols were identified through Salkowski's test, where 1 mL of chloroform was added to the seed extract, filtered, and then treated with a few drops of concentrated H_2SO_4, resulting in a golden yellow color. Phenols were detected using the Ferric chloride test, which produced a bluish-black color upon the addition of 3-4 drops of ferric chloride solution to the extract. Flavonoids were identified through the Alkaline reagent test, wherein 5 drops of 1% NaOH were mixed with the extract solution, producing an intense yellow color that became colorless upon the addition of any dilute acid. Finally, proteins and amino acids were detected using the Ninhydrin test, where the presence of these compounds was indicated by the development of a blue tint upon boiling the extract with ninhydrin reagent (0.25% w/v) for a few minutes. These tests provided comprehensive insights into the chemical composition of the seed extracts..

RESULTS AND DISCUSSION: In the present investigation, 12 bacterial strains (table 1) were tested on agar plates (figure 1a-j) against nine extracts type of kaliziri (table 2) for the determination of zone of inhibition (MZI) (table 3) and minimum inhibitory concentration (MIC) (table 4). Organic solvents used had different solubility and color change of kaliziri metabolites (figure 2a-c).

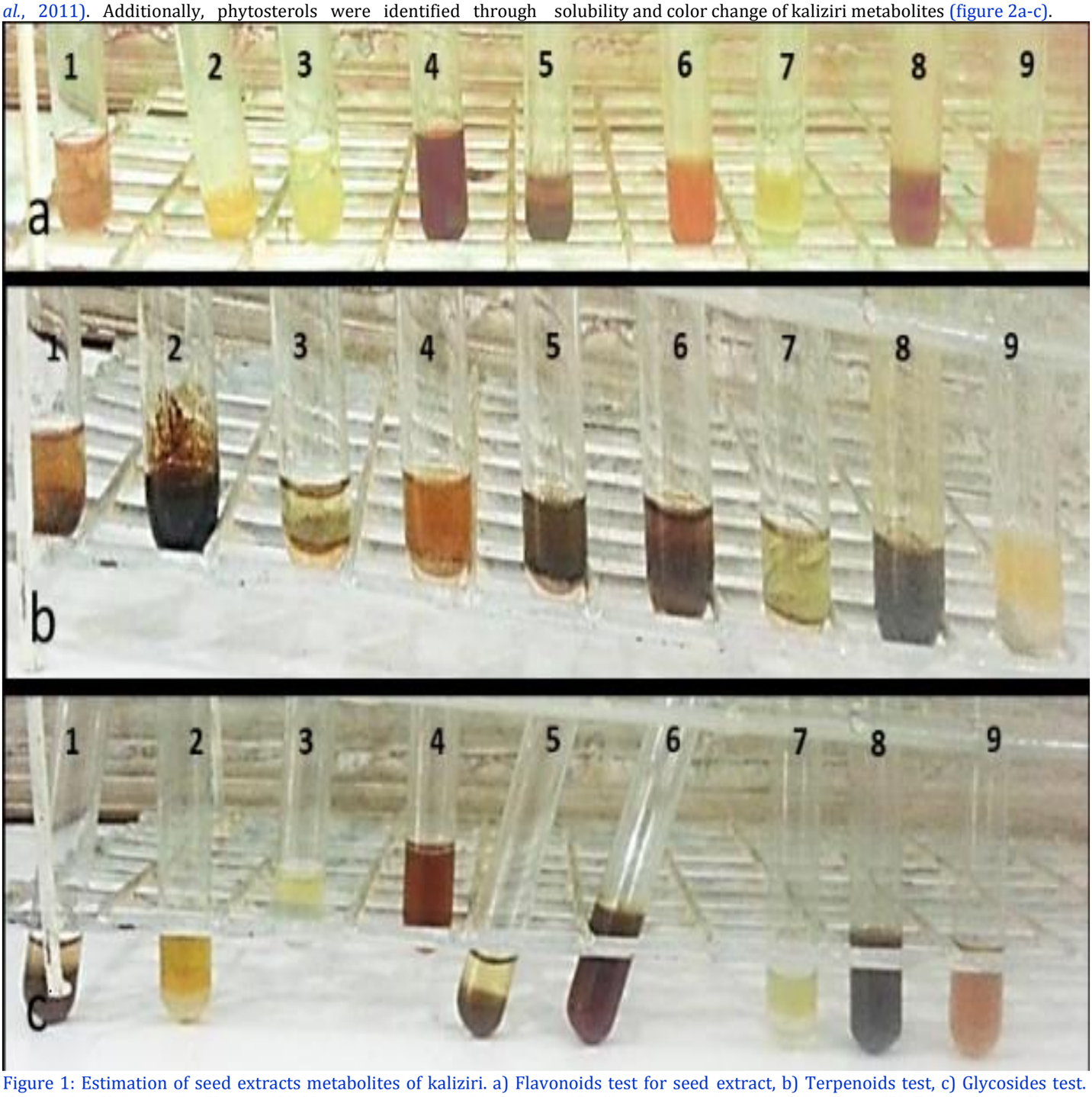

Figure 1: Estimation of seed extracts metabolites of kaliziri. a) Flavonoids test for seed extract, b) Terpenoids test, c) Glycosides test. Volatile (1), Volatile oil + Hexane (2), Hexane extract on orbital shaker (3), Clevenger method (4), Chloroform (5), Ethanolic (6), Hexane (7), Methanolic (8), Extract of distilled water (9).

Based on various natural chemicals in kaliziri seeds that had antimicrobial effect, previous reports also demonstrated the similar work in other species by using different solvent extracts including ethanol (Ethiraj and Balasundaram, 2016; Mehta *et al.*, 2016; Thouri *et al.*, 2017; Kavital and Hiremath, 2023). Mehta *et al.* (2016) reported that ethanol extract of kaliziri seeds played significant role against all tested human pathogenic bacteria that may be due to the presence of terpenoid like subtances. Similalry, different concentrations of cucurbita seed extract in acetone also shown anti-bacterial activity against *Pseudomonas aeruginosa, Staphylococcus aureus, B. subtilis, B. cereus, and E. coli* attributed to high tannins amount in seed extracts (Ethiraj and Balasundaram, 2016). The

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bioactive compounds of *Syzygium cumini* acetone seed extract had an extraordinary antibacterial effect (16.67mm MZI) against *Staphylococcus aureus, E. coli, and Klebsiella pneumoniae* that may be due to the presense of phenols and flavonoids in the seed metabolites (Kavital and Hiremath, 2023).

Photochemical analysis of kaliziri seeds: Based on color variations, flavonoids (figure 1a), terpenoids (figure 1b), and glycosides (figure 1c) were identified with solvent chemical reactions. Nine different solvents (figure 1) demonstrated that kaliziri seeds were rich in terpenoids in seven different solvents except hexane and distilled water, whereas the amount of tannins were higher in six solvent extracts (table 2). Glycosides and flavonoids were observed in five solvent extracts. Amino acids + proteins were only present in volatile oil as well as distilled water extract. Amongst individual solvents, chloroform and water were most effective for maximum number of metabolite extraction from seeds of kaliziri in the present investigation. Chloroform is soluble with the most of organic metabolites as reported in other plant seeds including black cumin seed powder (Alam *et al.*, 2010). However, Odo *et al.* (2013) reported that chloroform alone was not active until methanol was mixed for seed extraction of *Persea americana.* Photochemical screening shows the presence of biological active substances of glycosides, phenols, alkaloids, amino acid (figure 1). Tannin is toxic to bacteria, yeast, and filamentous fungi (Chung *et al.*, 1998). Chemicals that we identified are familiar in several pharmacological activities, such as alkaloids generally used as antibacterial, anti-malarial, cytotoxic, and anti-cancer agents (Wirasathien *et al.*, 2006). Saponins are important as fungicidal, antibiotic, insecticidal (Sparg *et al.*, 2004). Miller and Ruiz-Larrea (2002) reported that flavonoids have strong effect against inflammation, insects, pathogenic bacteria, allergy, virus as well as antioxidants, and vasodilatory activities.

Microbial zone of inhibition (MZI): The MZI observed by two bacterial strains namely *Aeromonas* sp. (AS) and *Staphylococcus* sp. (SS) against all seed extracts with 12-30 mm^2 and 7-21 mm^2 MZI, respectively. The highest (30 mm^2) MZI was observed in the extract of chloroform with AS strain. This was followed by four strains, i.e., *Bacillus cereus* (BC), *Bacillus pumilus* (BP), *Proteus* sp. (PrS) and *Salmonella typhi* (ST) showed 28, 27, 25 and 24 mm^2 MZI, respectively except water extract (table 3, figures. 2 a,b).

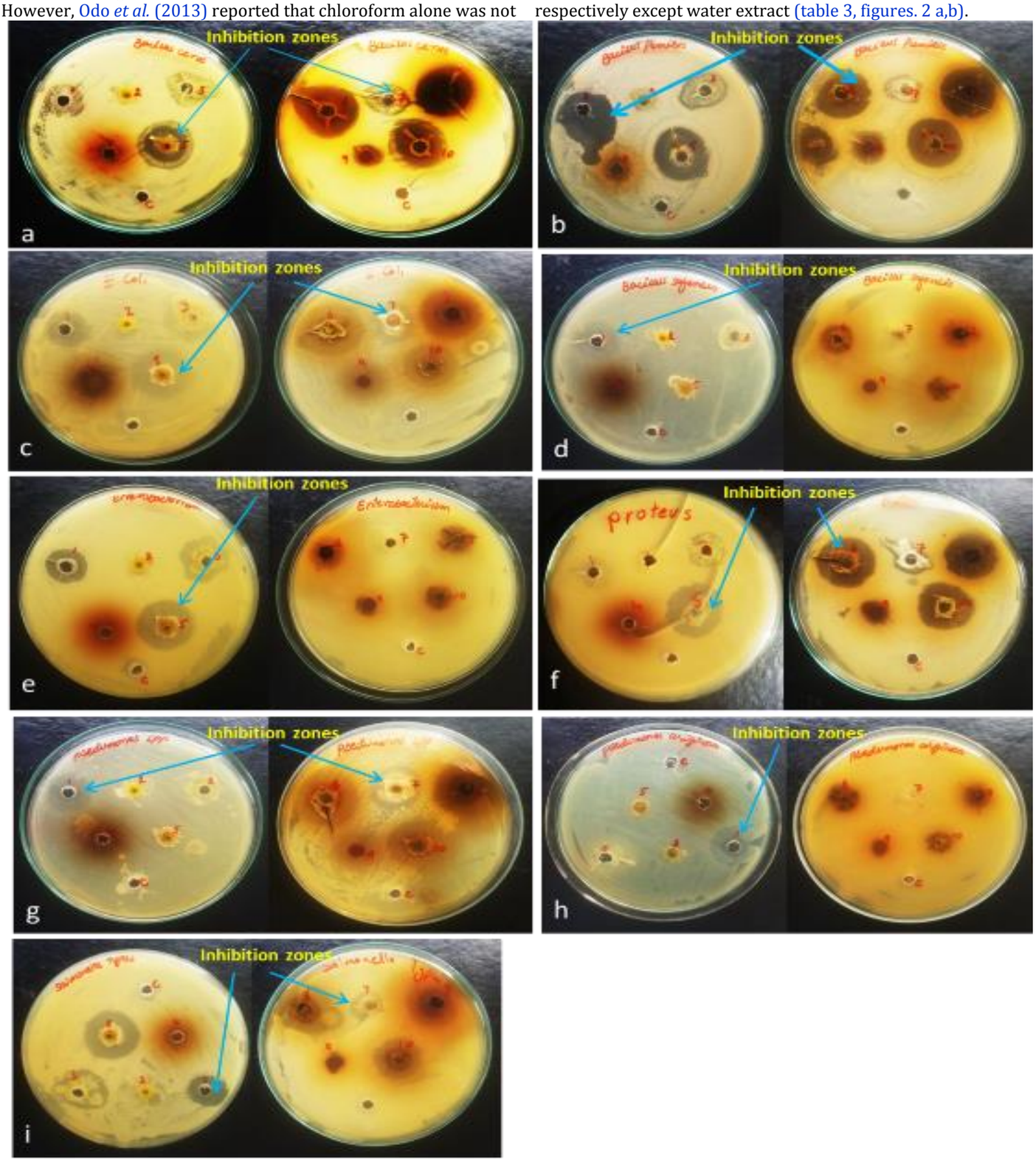

Figure 2: Antimicrobial activity of different solvent extracts of kaliziri against different bacterial strains on agar plate. a) *Bacillus cereus (BC),* b) *Bacillus pumillus (BS)*, c) *Escherichia coli (EC),* d) *Bacillus safensis (BS)*, e) *Enterobacter sp. (ES)*, f) *Proteus* sp. (PrS), g) *Pseudomonas* sp. *(PdS)*, h) *Pseudosomonas aeruginosa (PA)*, i) *Salmonella typhi (ST).*

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The EC (figures 2c) was resistant against most of the solvent extracts as compared to BS (figure 2d). *Pseudomonas* sp. (PdS), *Pseudomonas aeruginosa* (PA) and *Bacillus safensis* (BS) were resistant against seed + hexane extract, Clevenger, water and chloroform extracts and showed 13-18, 17-24 and 7-25 mm^2 MZI, respectively (figures 2 d,g,h). Similar results have been reported by Sharma *et al.* (2016) by using two bacterial strains *Pseudosomonas* sp., *P. aeruginosa*. These bacterial strains are very resistant against plant extracts and difficult to control their growth on the agar media. Similar to our work, Negi *et al.* (2014) also reported the chloroform extract active against G-ve bacteria *Pseudomonas* sp., *Bacillus subtilis* and *E. coli*. On the other hand, ethanolic extract of kaliziri was most effective against all bacterial strains at a very low MIC (Patel *et al.*, 2012). Tannins and terpenoids are the common metabolites of all extracts of the present study (table 2) with high MZI. This might be due to have antimicrobial properties of tannins and terpenoids (Mahizan *et al.*, 2019; Farha *et al.*, 2020). Moreover, antimicrobial activity in nine bacterial strains of *Diplococcus peunoniae, Escherichia coli, Pseudomonas aeruginosa, P. pyocyneaus, Klebisella aerogenes, Staphylococcus albus, S. saureus, S. heamolyticus* and *Vibrio cholera* has been reported (Mehta *et al.*, 2016).

Minimum inhibitory concentration (MIC): MIC may be defined as a lowest concentration of seed extract that may have inhibitory power against visible growth of bacteria. In the present study, all solvent extracts had different effects against different bacterial strains. The range of MIC was 50 mg/mL to 500 mg/mL. The results demonstrated that highest 500 mg/mL MIC was observed against PdS (figure 2g) followed by 100-500 mg/mL MIC against ST (figure 2i). The lowest 50 mg/mL MIC was recorded against PS. Higher the MIC indicates that extract has maximum capacity to kill the bacterial strains and vice versa. Hexane extract showed MIC against all bacterial strains except BS (figure 2d) whereas water extract showed MIC against only three strains of AS, PS and SS (table 4). Chloroform and ethanolic extracts have lowest MIC (50 mg/mL). Similarly, ethanolic extract has strong activity against bacterial strains *Pesudomonas aurigenousa* and *Klebsiella pneumoniae* of 2 mg/mL and 2.5 mg/mL of seed extract, respectively (Patel *et al.*, 2012). The remaining extracts of the present study were also effective but in the mid-range, MIC was observed against the bacterial strains. Whereas, methanolic extract of kaliziri with 3 mg/mL MIC was effective against *Diplococcus peunoniae*, *EC, PA, P. pyocyneau, Klebisella aerogenes, Staphylococcus albus, S. aureus, S. heamolyticus* and *Vibrio cholera* (Mehta *et al.*, 2016). Prominent anticancer activity of the methanolic seed extract of kaliziri with 30 mg/mL MIC occurs in inhibiting cell line growth and least toxic to normal cell-line and on cancer cells to have an anti-proliferative effect (Thara and Zuhara, 2016).

CONCLUSION: The present research demonstrated that kaliziri seeds might be used for successfully controlling some pathogenic bacterial species of human. Chloroform and ethanol extracts with 50 to 100 MIC was active against the bacteria due to the presence of some tannins and terpenoids. Others extracts were also effective but their effect varied depending upon the presence of active ingredients against bacterial growth. This project may be expanded on larger scale by using some latest techniques.

S No.	Bacterial strain	Growth on L-broth	Elevation	Form	Color	Margins
1	*Bacillus cereus*	Pellicle	Raised	Circular	Off white	Entire
2	*Bacillus pumilus*	Sediment	Raised	Irregular	Orange	Undulate
3	*Bacillus safensis*	Sediment	Convex	Circular	Off white	Entire
4	*Staphylococcus* sp.	Pellicle	Convex	Circular	Translucent white	Entire
5	*Aeromonas* sp.	Flocculent	Raised	Circular	Creamy orange	Entire
6	*Escherichia coli*	Pellicle	Raised	Circular	Off white	Entire
7	*Enterobacter* sp.	Uniform turbidity	Raised	Circular	Translucent white	Entire
8	*Pasteurella* sp.	Flocculent	Raised	Circular	Translucent white	Entire
9	*Proteus* sp.	Uniform turbidity	Raised	Circular	Off white	Entire
10	*Pseudomonas aeruginosa*	Uniform turbidity	Raised	Circular	Greenish brown	Entire
11	*Pseudomonas* sp.	Uniform turbidity	Convex	Circular	Off white	Entire
12	*Salmonella typhi*	Sediment	Umbonate	Circular	Translucent white	Entire

Table 1: Morphological characterization of resistant pathological bacterial strains.

Sr. No	Type of extracts	Metabolites of Kaliziri seeds							
		Amino acids and Proteins	Flavonoids	Glycosides	Phenols	Phytosterols	Saponins	Tannins	Terpenoids
1	Volatile oil	+	-	+	-	-	-	+	+
2	Seed + Hexane	-	+	+	-	+	+	-	-
3	Clevenger	-	+	+	-	-	-	+	+
4	Water	-	-	-	+	+	+	+	+
5	Chloroform	-	+	+	-	+	-	+	+
6	Ethanolic	-	-	-	+	-	-	+	+
7	Hexane	-	+	+	-	-	+	-	+
8	Methanolic	-	+	-	+	-	-	+	+
9	Distilled water	+	-	-	+	+	+	-	-

Table 2: Photochemical analysis for the determination of secondary metabolites in kaliziri seed extracts

Sr. No.	Extracts	Microbial zones of inhibition (mm^2) against different bacterial strains											
		AS	BC	BP	BS	EC	PS	EB	PrS	PdS	PA	ST	SS
1	Volatile oil	15	16	28	-	17	-	16	13	17	18	18	7
2	Seed + Hexane	12	12	12	-	-	-	10	10	-	-	7	8
3	Clevenger	23	17	20	25	24	13	20	18	-	-	23	14
4	Water	12	-	-	-	-	12	-	-	-	-	-	7
5	Chloroform	30	25	25	7	23	25	25	21	-	-	24	21
6	Ethanolic	23	27	26	24	28	26	17	25	24	13	23	19
7	Hexane	23	15	17	-	18	16	17	13	21	14	16	15
8	Methanolic	24	23	25	17	25	18	14	22	24	-	21	16
9	Distilled water	17	14	14	-	23	8	-	10	18	-	9	11

Table 3: Microbial zones of inhibition (mm^2) of kaliziri seed extracts (500 mg/mL) against human pathogenic bacterial strains.
'-' indicates zone of inhibition was not found. AS (*Aeromonas* sp.), BC (*Bacillus cereus*), BP (*Bacillus pumilus*), BS (*Bacillus safensis*), EC (*Escherichia coli*), PS (*Pasteurella* sp), EB (*Enterobacter* sp.), PrS (*Proteus* sp.), PdS (*Pseudosomonas* sp.), PA (*Pseudomonas aeruginosa*), ST (*Salmonella typhi*), SS (*Staphylococcus* sp.)

Sr. No.	Extracts	Minimum inhibitory concentration (mg/mL) against different bacterial strains											
		AS	BC	BP	BS	EC	PS	EB	PrS	PdS	PA	ST	SS
1	Volatile oil	200	500	300	-	300	-	500	300	500	400	400	500
2	Seed + Hexane	400	100	300	-	-	-	500	400	-	-	400	400
3	Clevenger	100	100	100	200	300	100	300	100	-	-	500	200
4	Water	500	-	-	-	-	500	-	-	-	-	-	400

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5	Chloroform	-	-	50	300	50	50	100	-	-	-	500	-
6	Ethanolic	-	-	-	50	-	50	-	-	500	500	-	-
7	Hexane	100	100	100		100	100	200	100	500	300	300	100
8	Methanolic	-	100	-	300	-	50	400	-	500	-	100	500
9	Distilled water	200	200	100	-	200	200	-	200	500	-	500	300

Table 4: Minimum inhibitory concentration (MIC) (mg/mL) Percentage of plant extracts against human pathogenic bacteria.
'-'indicates MIC was not observed. AS (*Aeromonas* sp.), BC (*Bacillus cereus*), BP (*Bacillus pumilus*), BS (*Bacillus safensis*), EC (*Escherichia coli*), PS (*Pasteurella* sp), EB (*Enterobacter* sp.), PrS (*Proteus* sp.), PdS (*Pseudosomonas* sp.), PA (*Pseudosomonas aeruginosa*), ST (*Salmonella typhi*), SS (*Staphylococcus* sp.).

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ETHICAL RESPONSIBILITY: This manuscript is original research, and it is not submitted in whole or in parts to another journal for publication.

INFORMED CONSENT: The author(s) have reviewed the whole manuscript and approved the final version of the manuscript before submission.

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