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Exploring the antibacterial efficacy of *Opuntia monacantha* in combatting methicillin-resistant *Staphylococcus aureus*

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Public health is seriously threatened by the rise of antibiotic-resistant strains of bacteria, especially methicillin resistant *Staphylococcus aureus* (MRSA). This study investigated the phytochemical compounds, and possible antibacterial effects of a methanolic extract of the cactus species *Opuntia monacantha* Haw. against MRSA. The powdered substance was extracted with methanol and filtered, and the filtrate was partitioned with n-hexane, chloroform and ethyl acetate. The fractions with higher percentage yields such as the n-hexane and chloroform fractions went through GC-MS analysis. They consist of various compounds that are known to have strong antioxidant and antimicrobial activities. The antimicrobial activity of the plant was measured via a diffusion assay. On the basis of these results, the *Opuntia monacantha* crude methanolic extract, and the n-hexane and chloroform fractions significantly inhibited all the MRSA strains used in the test at 100, 75 and 50 mg/mL. Furthermore, the minimum inhibitory concentrations of various fractions of the methanolic extract of *Opuntia monacantha* were also examined. Therefore, *Opuntia* could be a promising candidate for strengthening the existing formulations in pharmaceutical science for the treatment of MRSA. However, additional research is necessary before its therapeutic use is recommended. The results are intended to provide important new information for the creation of sustainable and alternative antimicrobial agents.

Keywords Antibiotic, Methanolic extract, Methicillin-resistant, Natural antimicrobials, *Staphylococcus aureus*, *Opuntia monacantha*

The World Health Organization (WHO) lists methicillin-resistant *Staphylococcus aureus* (MRSA) as a high-priority organism for the development of novel antibiotics because it exhibits resistance to multiple drug classes, including β-lactams, tetracyclines, macrolides, and fluoroquinolones[1]. MRSA continues to be a critical pathogen in healthcare and community settings, with significant implications for morbidity, mortality, and healthcare costs, despite some regions seeing a decrease in hospital prevalence due to improved infection control[2].

Direct contact is the main method of transmission, which is made easier in healthcare settings by contaminated surfaces, medical equipment, or work clothes[3]. Tens of thousands of fatalities occur each year as a result of the rapid spread of resistant genes and inappropriate antibiotic usage, which has increased the worldwide burden[4,5]. If resistance trends continue, mortality is expected to rise. Emerging resistance to existing medicines, including vancomycin, highlights the pressing need for novel antimicrobial approaches[6–8].

The hunt for synthetic antibiotic substitutes has rekindled interest in medicinal plants, which have been used for ages to treat inflammatory and infectious illnesses. Plant-derived compounds have promised antibacterial qualities and a lower risk of side effects than conventional treatments. They are also frequently more affordable and accessible. When it comes to finding bioactive substances that can solve resistance issues and enhance existing therapies, folk medicine is a useful and little-studied resource[9–12].

The prickly pear, *Opuntia monacantha*, is unique among therapeutic plants because of its phytochemical diversity and capacity to thrive in arid environments[13,14]. This plant, which belongs to the Cactaceae family,

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has antibacterial, hepatoprotective, and antioxidant qualities due to its phenolics, terpenoids, vitamins, and alkaloids[15–18]. *Opuntia monacantha* is positioned as a possible option for the development of novel antimicrobial drugs due to studies on its cladodes and stem extracts showing antibacterial activity, including actions against *S. aureus.*[19–22] From literature, we have seen that in comparison to gram-negative bacteria (*P. multocida and E. coli*), *OM* has demonstrated more consistently articulated antibacterial action against the gram-positive bacteria (*S. aureus and B. subtilis*). With the most notable zone of inhibition (9 mm and 21.5 mm, respectively) and the smallest inhibitory concentration value (90.03 mg/mL and 52.02 mg/mL, respectively), the n-butanol and chloroform fractions showed high activity against *S. aureus* in light of these data. The highest activity of the chloroform fraction and methanolic extract against *B. subtilis* was also shown, with reduced MIC values (45.15 and 70 mg/mL) and maximal zones of inhibition (19.3 and 22.8 mm). The structure of the cell membrane and the phytochemical characteristics of the plant are the reasons for showing more resistance towards gram-negative bacteria in comparison to gram-positive bacteria[23,24]. Medicinal plants produce an infinite number of secondary metabolites with strong antibacterial action, as demonstrated by in vitro tests[25–27]. These low molecular weight antibiotics derived from plants are divided into two categories: phytoanticipins, which have microbial inhibitory effects, and phytoalexins, which are primarily anti-oxidative and are created anew by plants in reaction to microbial infection[28,29]. Plant antimicrobial secondary metabolites can be broadly categorized into following groups: alkaloids, terpenes, and phenolic chemicals. Several studies have demonstrated that the plant extracts' and their active compounds' antimicrobial activity can potentially induce the production of the reactive oxygen species, promote disruption of cell wall and lysis, prevent the formation of biofilms, prevent the construction of cell walls, prevent microbial DNA replication, prevent energy synthesis, and prevent bacterial toxins from harming the host[30].

In this study, we investigated the antibacterial potential of the methanolic extract of *Opuntia monacantha* Haw. particularly against MRSA strains obtained from Ittefaq Trust Hospital, Lahore, Pakistan.

Results

Total percentage yield of extraction and fractionation

The total yield of *OM* along with its subsequent fractions is shown in Table 1 which represents CM-OM 5% however the yields of nH-OM, Chl-OM, and EA-OM were 16.1%, 9.5%, and 8.5% respectively. The yield of nH-OM was 16.1% greater than that of the other fractions.

Phytochemical screening

In accordance with the USP standards, qualitative phytochemical screening was performed to investigate the presence of secondary metabolites in the crude methanolic extract and fractions[31,32]. The outcomes are presented in Table 2.

Screening of primary and secondary metabolites

Total phenolic content

With the help of linear regression curves, the TPC was calculated via equation which was $y = 0.0109x + 0.022$ ($R^2 = 0.9995$), using gallic acid was used to create standard curve, as shown in Fig. 1. The TPC values were expressed as milligrams of gallic acid equivalent for each gram of extract. (mg GAE/g extract) and is presented in Table 3.

Total flavonoid contents (TFC)

As illustrated in Fig. 2, the TFC was determined using the equation $y = 0.0078x + 0.0018$ ($R2 = 0.9993$) derived from the linear regression curve created using quercetin as a reference. Table 4 displays the TFC results as milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

Antioxidant activity

To investigate the properties of free radical scavenging, natural compounds are usually investigated through the DPPH assay. The color of the DPPH solution was compared with the level of antioxidant potential in the tested sample. The percentage inhibition was calculated for the *OM* crude methanolic extract, different fractions and standards. nH-OM and Chl-OM had the highest percentage of inhibition with low IC_{50} values. The results are tabulated in Table 5. All the plant extracts had significant antioxidant potential. Triplicate runs of each assay were performed.

GC-MS screening

GC–MS of the active fractions n-hexane and chloroform was conducted via National Institute Standard and Technology (NIST) database for further investigation as shown in Figs. 3 and 4. The n-hexane fraction has 32

Samples	Percentage yield (g/100 g)*
CM-OM	5.0 ± 0.10
nH-OM	16.1 ± 0.05
Chl-OM	9.5 ± 0.10
EA-OM	8.5 ± 0.20

Table 1. Percentage yield of *OM* and its fractions. *Values are the mean ± SD of three separate experiments.

Phytochemical group	Identification test	CM-OM	nH-OM	Chl-OM	EA-OM
Alkaloids	Mayer's test	+	+	+	+
	Dragendrof test	+	+	+	+
	Hagers reagent	+	+	+	+
	Wagners reagent	+	+	+	+
Carbohydrate test	Molish test	+	+	+	+
	Bendict's test	+	+	+	+
Glycosides	Keller Killani test	+	+	+	+
Tannins	Lead subacetate test	+	+	+	+
	10% NaOH test	+	+	+	+
Saponins	Foam test	+	+	+	+
Flavonoids	Lead acetate test	+	+	+	+
	Aluminum chloride test	+	+	+	+
	Ferric chloride test	+	+	+	+
	Ammonia test	+	+	+	+
Terpenoids	Salkovaski test	+	+	+	+
Phenolic Compound Test	Iodine test	+	+	+	+
	Ferric chloride test	+	+	+	+
	Lead acetate test	+	+	+	+
Protein Test	Ninhydrin test	+	+	+	+
Anthraquinone	Borntrager's test	−	−	−	−
Fats and fixed oils	Spot test	+	+	+	+
Steroids		+	+	−	−

Table 2. Phytochemical screening of crude methanolic extract of the *OM* and its fractions n-hexane, chloroform and Ethyl acetate. +: Presence, −: Absence.

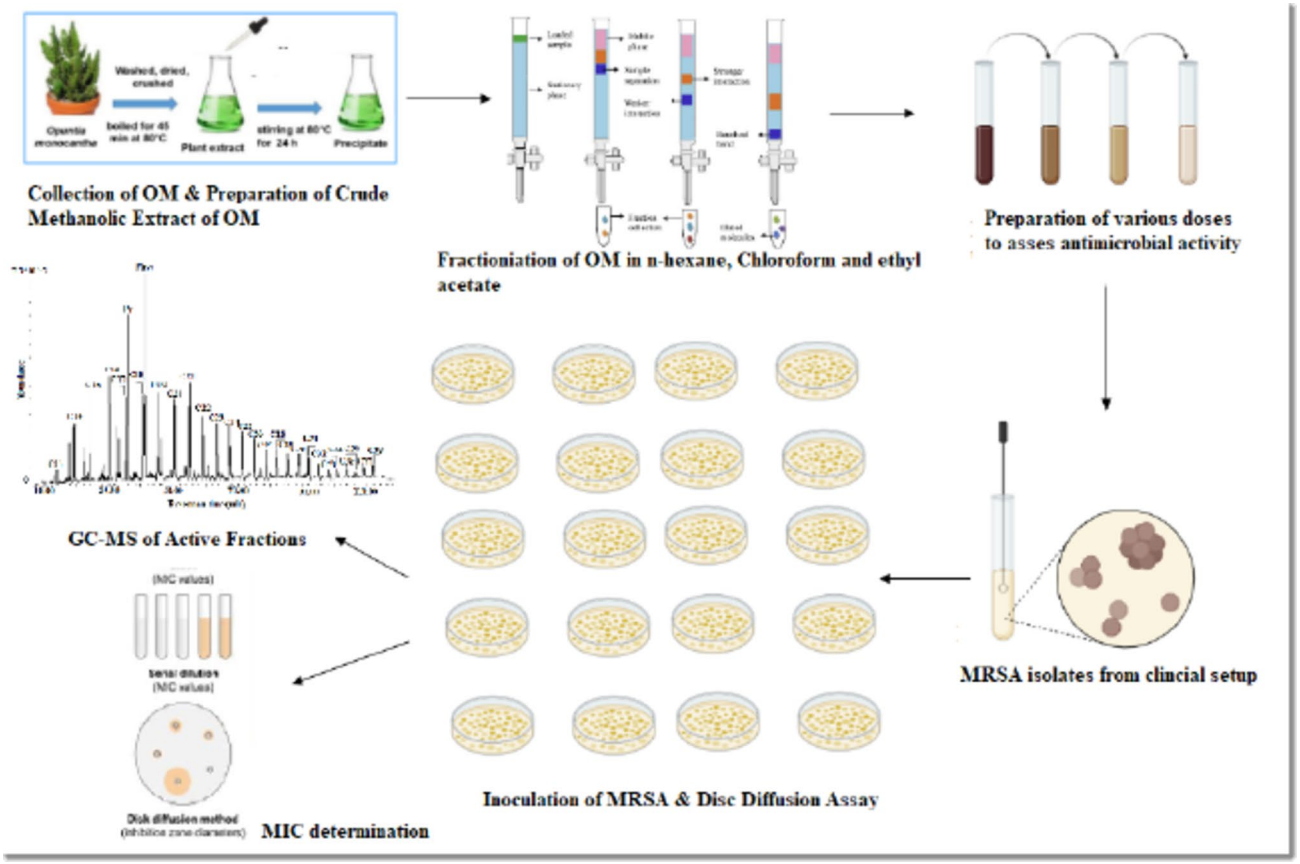

Fig. 1. Calibration curve for the Gallic acid.

Parameters	OM mg GAE/g
CM-OM	33.11 ± 1.31
nH-OM	29.56. ± 1.13
Chl-OM	25.13 ± 0.91
EA-OM	20.14 ± 0.61

Table 3. Total phenolic content. The Values are expressed as mean ± SEM ($n = 3$).

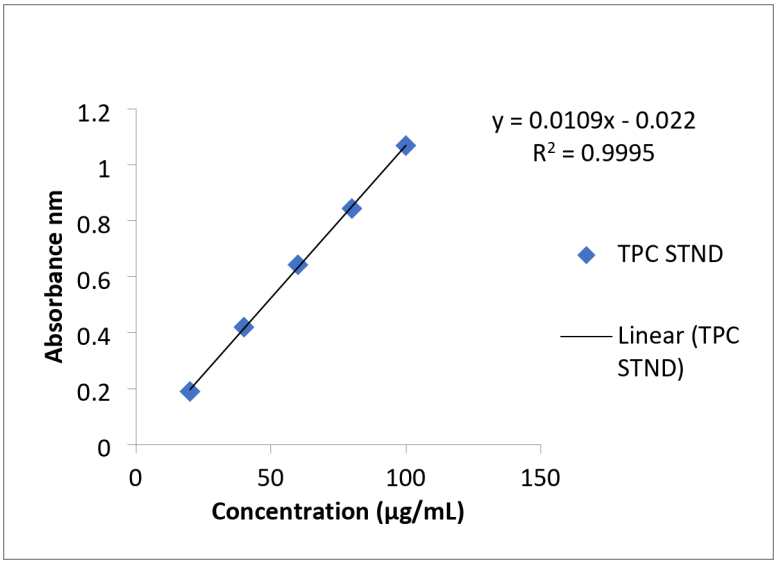

Fig. 2. Calibration curve of quercetin.

Parameters	OM mg QE/g
CM-OM	18.13 ± 0.81
nH-OM	15.24 ± 1.32
Chl-OM	13.41 ± 0.78
EA-OM	11.30 ± 1.85

Table 4. Total flavonoid contents. The value expressed as mean ± SEMs ($n = 3$).

Sr. No.	Samples	Concentration (mg/ mL)	% Inhibition (mean ± SEM)	IC_{50} (µg/ mL)
1.	CM-OM	1	86.47 ± 1.63	81.79
2.	nH-OM	1	88.43 ± 1.14	79.73
3.	Chl-OM	1	85.29 ± 3.62	83.53
4.	EA-OM	1	84.71 ± 1.15	94.02
5.	A. A	1	87.25 ± 1.08	80.05

Table 5. IC_{50} values of the OM fractions and standard. The values expressed as mean ± SEMs ($n = 3$).

compounds, and 28 compounds were found in the chloroform fraction. The compounds with the maximum area and reported antioxidant and antimicrobial effects are listed in Tables 6 and 7.

Antibacterial potential of *Opuntia monacantha* Haw. Cladode

Measurement of antibacterial activity of the extract at 100 mg/ mL

The results in Fig. 5 reveal the clearance zone from the crude methanolic extract of *OM* and its fractions at 100 mg/ mL. The crude methanolic extract had the maximum inhibitory effect on all the test strains followed by n-hexane, chloroform and ethyl acetate. The negative control did not show any change in condition. Therefore, no data is presented for it. Linezolid and vancomycin which are used as standard drugs significantly inhibited

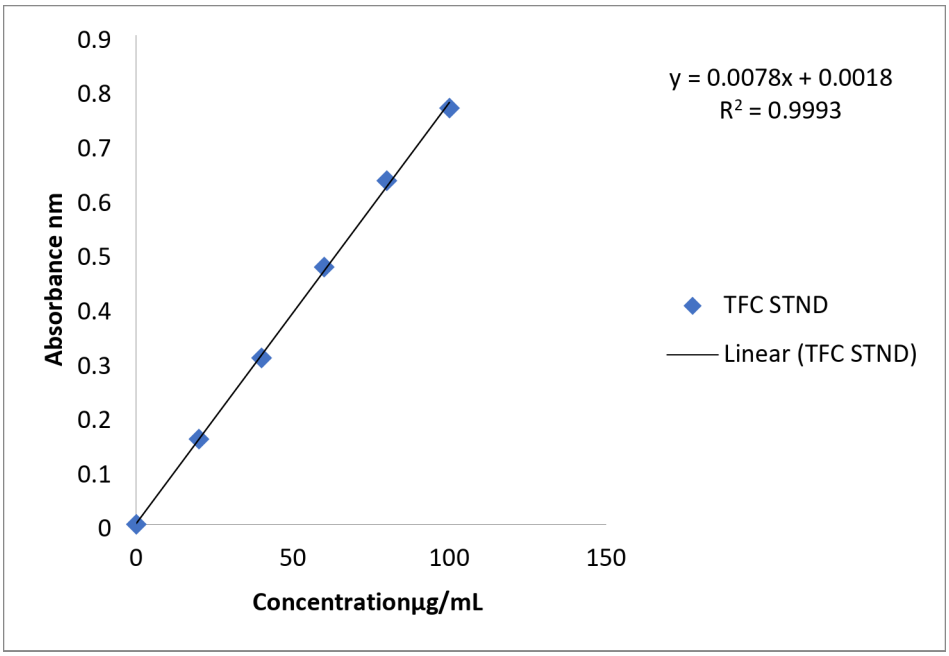

Fig. 3. GC-MS of the n-hexane fraction of *OM*.

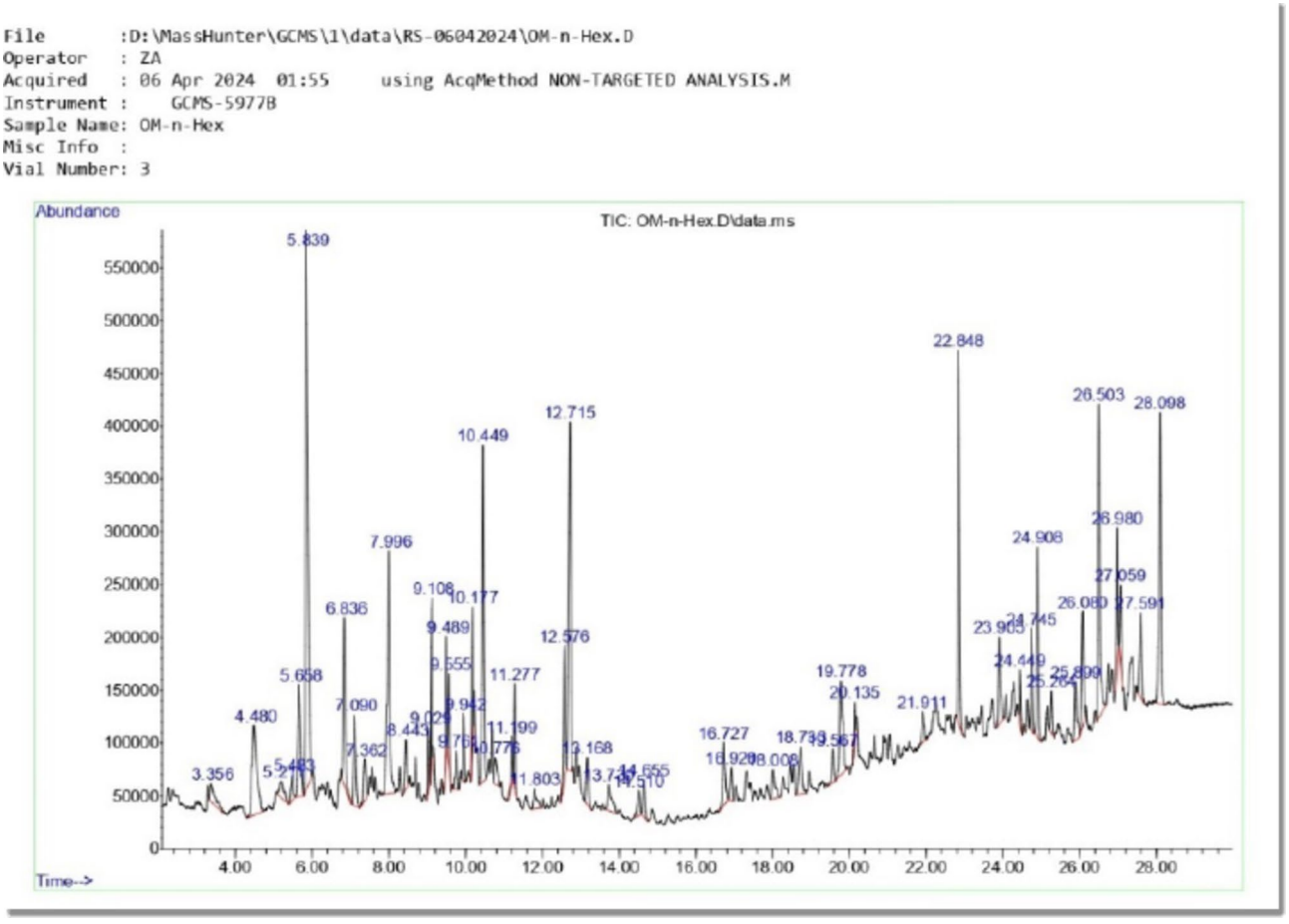

Fig. 4. GC-MS of the chloroform fraction of *OM*.

S.no.	RT (min)	Compound name	Compound class	Molecular formula	Molecular mass g/mol	Area %
1	3.356	Tridecane	Hydrocarbon	$C_{13}H_{28}$	184.36	0.88
2	4.480	Tetradecane	Volatile Oil	$C_{14}H_{30}$	198.39	3.89
3	5.211	2-methyl dodecane	Volatile Oil	$C_{13}H_{28}$	184.36	0.71
4	5.483	10-Methyleicosane	Alkane	$C_{21}H_{44}$	296.57	0.56
5	5.658	Pentadecane	Volatile Oil	$C_{15}H_{32}$	212.41	2.69
6	5.839	2,4-Di-tert-butylphenol	Phenol	$C_{14}H_{22}O$	206.32	13.70
7	6.836	Hexadecane	Volatile Oil	$C_{16}H_{34}$	226.44	2.78
8	7.362	2-Methylnonadecane	Volatile Oil	$C_{20}H_{42}$	282.56	0.80
9	7.996	Heptadecane	Alkane	$C_{17}H_{36}$	240.47	5.47
10	9.029	1-Octadecene	Alkene	$C_{18}H_{36}$	252.46	0.71
11	9.108	Octadecane	Alkane	$C_{18}H_{38}$	254.49	1.76
12	10.177	Nonadecane	Alkane	$C_{19}H_{40}$	268.52	1.47
13	10.449	14-Methylpentadecanoic acid	Fatty Acid	$C_{16}H_{32}O_2$	256.42	4.88
14	11.199	1,2-Diethylcyclohexadecane	Volatile Oil	$C_{20}H_{40}$	280.53	0.65
15	11.277	Eicosane	Alkane	$C_{20}H_{42}$	282.55	1.23
16	12.576	9,12-Octadecadienoic acid methyl ester	Fatty Acid	$C_{19}H_{34}O_2$	294.47	2.36
17	12.715	9-Octadecenoic acid, methyl ester	Fatty Acid	$C_{19}H_{36}O_2$	296.49	6.14
18	13.168	Methyl stearate	Fatty Acid	$C_{19}H_{38}O_2$	298.50	0.86
19	13.730	11-Methylpentacosane	Fatty Acid	$C_{26}H_{54}$	366.71	0.99
20	14.510	Triacontyl pentafluoropropionate	Hydrocarbon	$C_{33}H_{61}F_5O_2$	584.83	0.60
21	14.655	Docosane	Volatile Oil	$C_{22}H_{46}$	310.6	0.73
22	16.727	Octacosyl heptafluorobutyrate	Hydrocarbon	$C_{32}H_{57}F_7O_2$	606.78	1.47
23	16.920	Tricosane	Alkane	$C_{23}H_{48}$	324.63	0.59
24	18.008	1-iodo dotriacontane	Alkane	$C_{32}H_{65}I$	576.76	0.80
25	20.135	Bis(2-ethylhexyl) phthalate	Ester	$C_{24}H_{38}O_4$	390.56	0.54
26	21.911	Heptacosane	Alkane	$C_{27}H_{56}$	380.73	0.65
27	22.848	1,5,9-Undecatriene, 2,6,10-trimethyl	Terpenoids	$C_{14}H_{24}$	192.34	4.81
28	24.908	Stigmasta-3,5-diene	Phytosterol	$C_{29}H_{48}$	396.69	2.78
29	25.899	Campesterol	Phytosterol	$C_{28}H_{48}O$	400.7	1.16
30	26.080	Stigmasterol	Phytosterol	$C_{29}H_{48}O$	412.7	1.70
31	26.503	Beta- sitosterol	Phytosterol	$C_{29}H_{50}O$	414.71	5.43
32	27.059	Alpha- amyrin	Triterpenoid	$C_{30}H_{50}O$	426.7	0.98

Table 6. Compounds detected in nH-OM by GC-MS.

the strains. The zones of inhibition values are given in Table 8 below. All the extracts had a statistically significant effect on every strain of MRSA used in the experiment, as shown in Fig. 6.

The inhibitory zone diameter (mm) includes the well diameter of 6 mm. The values are given as mean ± SDs of three different experiments. Zones of inhibition < 7 mm were considered not to have any activity, Zones between 8 and 11 mm were considered active, and Zones > 11 mm were considered very active.

Measurement of the antibacterial activity of the 75 mg/mL of extracts

The results in Fig. 7 reveal the clearance zone from the crude methanolic extract of *OM* and its fractions at 75 mg/ml. The crude methanolic extract had the greatest inhibition effect, followed by n-hexane and chloroform. Compared with the standard drugs linezolid and vancomycin, all the extracts significantly inhibited every test strain of MRSA. Unfortunately, ethyl acetate did not exhibit any inhibition at this dose. The negative control also did not show any change in its condition. Therefore, no data are presented for it. The zones of inhibition values are given in Table 9 below. The significant results are shown graphically in Fig. 8.

The inhibitory zone diameter (mm) includes the well diameter of 6 mm. The values are given as mean ± SDs of three different experiments. Zones of inhibition < 7 mm were considered not to have any activity, Zones between 8 and 11 mm were considered active, and Zones > 11 mm were considered very active.

Measurement of antibacterial activity of the extracts at 50 mg/ml

The results in Fig. 9 reveal the clearance zone from the crude methanolic extract of *OM* and its fractions at 50 mg/mL. All the extracts significantly affected every test strain of MRSA except the ethyl acetate fraction. The crude extract and n-hexane fraction showed maximum inhibition at this dose followed by the chloroform fraction. Ethyl acetate and negative control also did not show any change. Therefore, no data are presented for it. The zones of inhibition values are given in Table 10. The significant results in comparison with the standards are shown graphically in Fig. 10.

S.No.	RT (Min)	Compound Name	Compound Class	Molecular Formula	Molecular Mass g/mol	Area %
1	2.63	Bromodichloromethane	Halomethane	$CHBrCl_2$	163.83	1.32
2	3.151	3,4-Dimethylbenzaldehyde	Carbonyl compound	$C_9H_{10}O$	134.17	1.28
3	4.444	2 Methyltricosane	Hydrocarbon	$C_{24}H_{50}$	338.7	0.66
4	5.477	5-Butyldocosane	Hydrocarbon	$C_{26}H_{54}$	366.7	1.27
5	5.628	n-Hentriacontane	Hydrocarbon	$C_{31}H_{64}$	436.8	0.40
6	5.870	2,4-Di-tert-butylpenol	Phenol	$C_{14}H_{22}O$	206.32	21.56
7	6.812	2-methyltetratriacontane	Hydrocarbon	$C_{35}H_{72}$	492.9	0.90
8	7.344	2,21-Dimethyldocosane	Hydrocarbon	$C_{24}H_{50}$	338.7	0.63
9	7.537	2-Bromo dodecane	Hydrocarbon	$C_{12}H_{25}Br$	249.23	0.27
10	7.610	10-Methylicosane	Hydrocarbon	$C_{21}H_{44}$	296.6	0.20
11	7.918	5-Butylnonae	Hydrocarbon	$C_{13}H_{28}$	184.36	2.55
12	8.117	Tetracosane	Hydrocarbon	$C_{24}H_{50}$	338.7	0.27
13	8.244	1-Bromodocosane	Hydrocarbon	$C_{22}H_{45}Br$	389.5	0.69
14	8.413	13,17,21-Trimethylheptatriacontane	Hydrocarbon	$C_{40}H_{82}$	563.1	0.83
15	8.740	9-Methylheneicosane	Hydrocarbon	$C_{22}H_{46}$	310.6	0.20
16	9.084	2-methyltetratriacontane	Hydrocarbon	$C_{35}H_{72}$	492.9	0.46
17	10.214	11-Decyldocosane	Hydrocarbon	$C_{32}H_{66}$	450.9	1.14
18	10.449	Benzenepropanoic acid, 3-(1,1-dimethylethyl)-4-hydroxy-,methyl ester	Phenyl Propanes	$C_{14}H_{20}O_3$	236.31	32.95
19	11.616	Methyl 9-oxononanoate	Fatty Aldehyde	$C_{10}H_{18}O_3$	186.25	0.34
20	12.208	3-(4-nitrophenyl)-1,2,3-benzotriazin-4(3 h)-one	Heterocyclic compound	$C_{13}H_8N_4O_3$	268.23	0.26
21	12.552	9,12-Octadecadienoic acid, methyl ester	Fatty acids	$C_{19}H_{34}O_2$	294.5	1.58
22	12.661	cis-13-Octadecenoic acid, methyl ester	Fatty acid	$C_{19}H_{36}O_2$	296.5	2.40
23	12.770	7-Octadecenoic acid methyl ester	Fatty acid	$C_{19}H_{36}O_2$	296.5	0.46
24	13.144	Methyl stearate	Fatty acid	$C_{19}H_{38}O_2$	298.5	7.54
25	18.932	1,12-Tridecadiene	Hydrocarbon	$C_{13}H_{24}$	180.33	0.45
26	19.778	Heptadecyl heptadecanoate	Esters	$C_{34}H_{68}O_2$	508.9	0.73
27	22.171	Methyl-18-propylhenicosanoate	Esters	$C_{25}H_{50}O_2$	382.7	0.60
28	22.811	4-Butyl-5-(3-methylbutyl)-6-(1-methylethenyl)-2 H-pyran-2-one	Ester	$C_{17}H_{26}O_2$	262.4	0.22

Table 7. Compounds detected in Chl-*OM* by GC-MS.

The inhibitory zone diameter (mm) includes the well diameter of 6 mm. The values are given as mean ± SDs of three different experiments. Zones of inhibition < 7 mm were considered not to have any activity, Zones between 8 and 11 mm were considered active, and Zones > 11 mm were considered very active.

Measurement of mics of the crude methanolic *Opuntia monacantha* Haw. and its fractions against MRSA

The MICs of the fractions and the crude methanolic extract of *OM* were investigated via well diffusion assay. The negative control (distilled water) had no inhibitory effect. The crude methanolic acid had the strongest inhibitory effect against all strains with an MIC value of 3.12 mg/mL against MRSA 1, MRSA 2, MRSA 3 and MRSA 4 as shown in Table 11. The n hexane fraction extract had an MIC value of 6.25 mg/mL whereas the chloroform fraction of *OM* had MIC value of 12.5 mg/mL and the ethyl acetate fraction had an MIC value of 100 mg/mL against MRSA strains.

Discussion

Because of advancements in the medicinal effects of plants and herbs, the importance of natural products has steadily increased in recent decades. Pakistan is home to a wide variety of plants, and the local populace has long used many of these naturally occurring herbs and plants for their medicinal properties. Data show that approximately 25% of various pharmaceutical drugs are generated globally based on the understanding of traditional usage of medicinal plants[33]. The chemical makeup of the natural source, which is based on the availability of secondary metabolites with significant medicinal value, determines the therapeutic efficacy of these pharmaceutical products. The goal has been to create novel pharmacological entities with the greatest possible therapeutic effects and the least or minimum adverse effects possible. However, as herbal formulations are typically delivered in the form of extracts, teas, juices, and decoctions, use of these goods rationally depends on product quality assurance, which is a prerequisite step in the validation and identification of natural products in any medicinal system. As a result, it is crucial that every component of the plant be genuine and devoid of any adulteration or contamination[34]. The WHO is consistently highlighting the need for a standardized mechanism for all commercial herbal medicines, given the significant variance between formulation batches[35].

With these considerations in mind, this research was conducted to evaluate the antibacterial efficacy of *OM* to determine its therapeutic importance and establish scientific documentation. The plant was collected and after

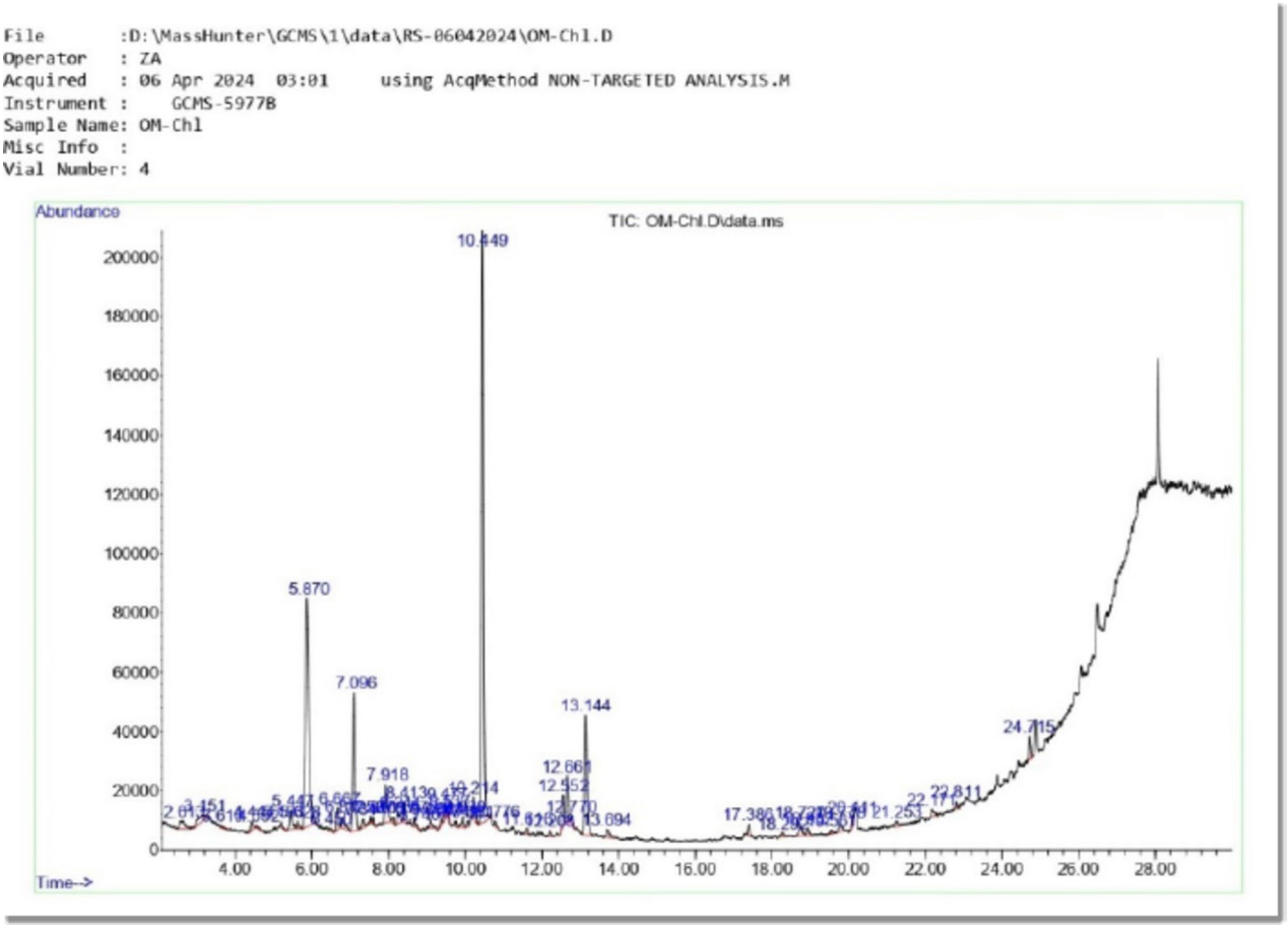

Fig. 5. Zone of inhibition (ZOI) of the crude methanolic extract of *Opuntia monacantha* (CM-OM) and its fractions n-hexane (nH-OM), chloroform (Chl-OM), and ethyl acetate (EA-OM) against MRSA strains at 100 mg/ mL along with the negative control (distilled water) and positive control linezolid (LZ) (30 µg) and vancomycin (VAN) (30 µg), (**A–D**) represents the ZOI of 100 mg of crude methanolic extract and its fractions on MRSA 1, (**E–F**) represents the ZOI of 100 mg of crude extract and its fractions on MRSA 2, (**I–L**) represents the ZOI of 100 mg of crude extract and its fractions on MRSA 3 and (**M–P**) represents the ZOI of 100 mg of the crude extract and its fractions on MRSA 4.

Microbial Strains Inhibition Zone (mm)[2]								
	MRSA 1				MRSA 2			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-Om	Chl-OM	EA-OM
Extract 100 mg/ mL	15.2 ± 0.1	12.0 ± 0.1	11.3 ± 0.15	5.1 ± 0.10	15.1 ± 0.1	15.1 ± 0.1	13.6 ± 0.1	4.2 ± 0.05
Linezolid (30 µg)	15.4 ± 0.05	12.2 ± 0.05	20.16 ± 0.05	15.1 ± 0.11	20.1 ± 0.15	17.0 ± 0.15	15.2 ± 0.05	20.1 ± 0.1
Vancomycin (30 µg)	10.0 ± 0.15	10.1 ± 0.15	10.2 ± 0.05	10.0 ± 0.05	10.2 ± 0.05	10.1 ± 0.05	10.0 ± 0.05	10.1 ± 0.1
	MRSA 3				MRSA 4			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-OM	Chl-OM	EA-OM
Extract 100 mg/ mL	13.4 ± 0.05	15.1 ± 0.1	13.2 ± 0.1	3.1 ± 0.05	15.2 ± 0.05	12.0 ± 0.1	10.2 ± 0.05	4.16 ± 0.15
Linezolid (30 µg)	20.1 ± 0.1	17.1 ± 0.1	15.3 ± 0.1	20.0 ± 0.05	15.3 ± 0.15	15.2 ± 0.05	13.1 ± 0.05	20.1 ± 0.05
Vancomycin (30 µg)	12.0 ± 0.05	10.1 ± 0.05	10.2 ± 0.05	9.1 ± 0.1	10.1 ± 0.1	10.2 ± 0.11	10.1 ± 0.1	9.1 ± 0.11

Table 8. Antibacterial potential of *Opuntia monacantha* cladodes by the well diffusion method at 100 mg/ml.

drying it was subjected to the phytoconstituents extraction via cold maceration involving methanol as a solvent. The fractionation of extracted crude methanolic extract was performed with n-hexane, chloroform and ethyl acetate in the sequence of increasing polarity. The total yield of CM-OM was 5% followed by the subsequent fractions nH-OM, Chl-OM, and EA-OM, which were 16.1%, 9.5%, and 8.5% respectively. The yield of EA was the lowest among all fractions. The nature and volume of solvent mixed during the extraction process determine

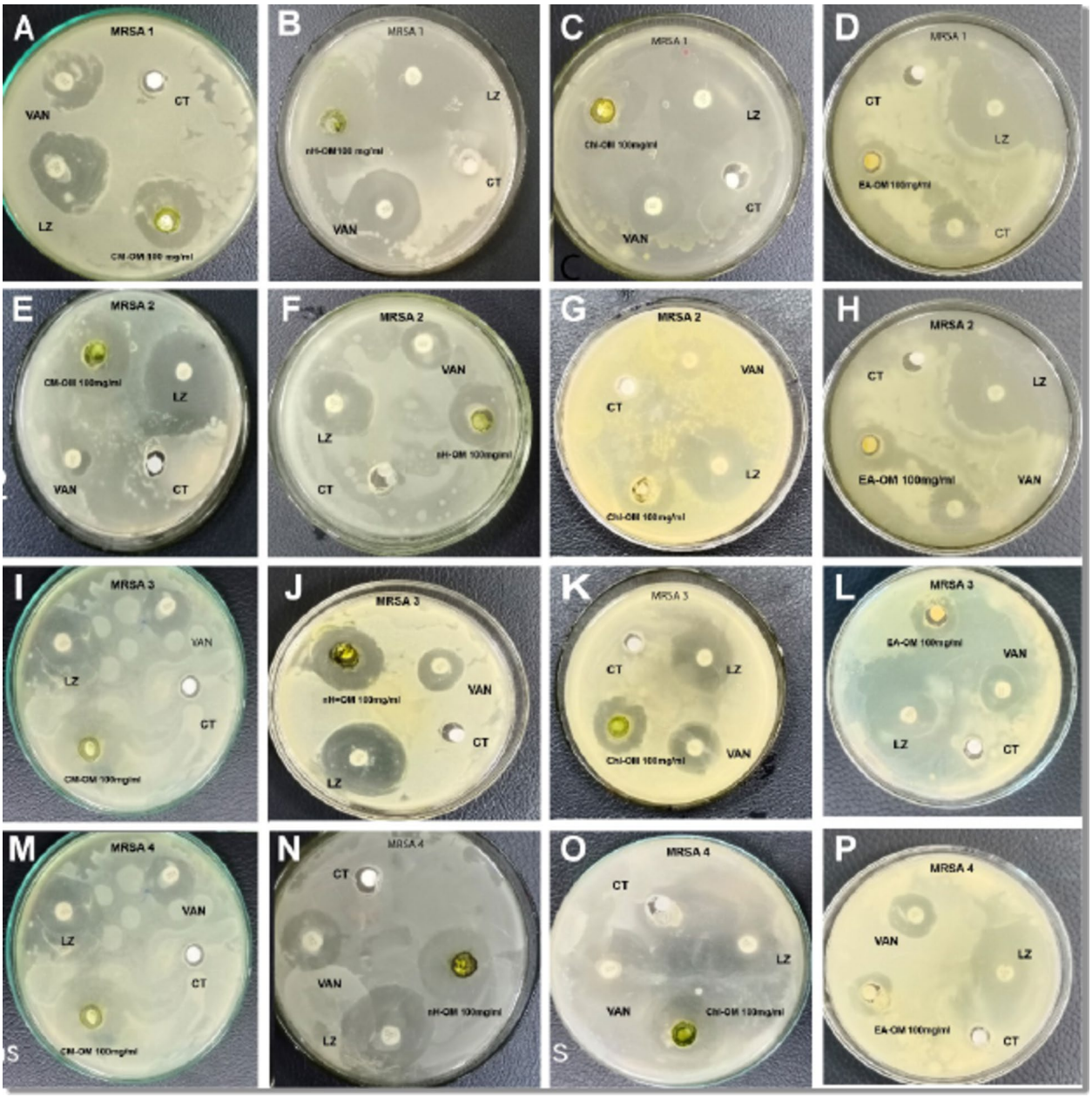

Fig. 6. Antibacterial activity of crude methanolic extract of Opuntia monacantha (CM-OM), its fractions nH: n-hexane, Chl: Chloroform EA: ethyl acetate at 100 mg/mL and the commercially available antibiotic discs LZ: Linezolid 30 µg/disk and VAN: vancomycin 30 µg/disk. (**a–d**) Different strains of methicillin resistant *Staphylococcus aureus*. The mean zones of inhibition (mm) created by the respective treatments against each bacterial strain were recorded. The data presented are the means of three independent experiments ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$ ns $P > 0.05$ (for comparisons of all treatments by one-way ANOVA, followed by Tukey's Test).

how much amount can be recovered from a plant, and variance in the extracted components might occur from sample to sample[24,36]. Pharmacognostic studies are always a crucial for standardizing crude herbal drugs and establishing quality control standards[37]. According to official guidelines, common standardization methods include physicochemical tests, chromatographic fingerprinting, DNA profiling, macroscopic/microscopic characterizations, chemical tests, quantitative determination of specific compounds or markers or a group of compounds, and tests for microorganisms and chemical contaminants[38].

Phytochemical analysis of the crude methanolic extract of *OM* and its fractions revealed the presence of alkaloids, tannins, saponins and flavonoids whereas steroids were detected only in the crude extract and n-hexane fraction. However anthraquinones were absent[39].

The total phenolic and flavonoid content of the *OM* extract along with its fractions were analyzed via linear regression equation with gallic acid and catechins used as standards as shown in Figs. 1 and 2 respectively.

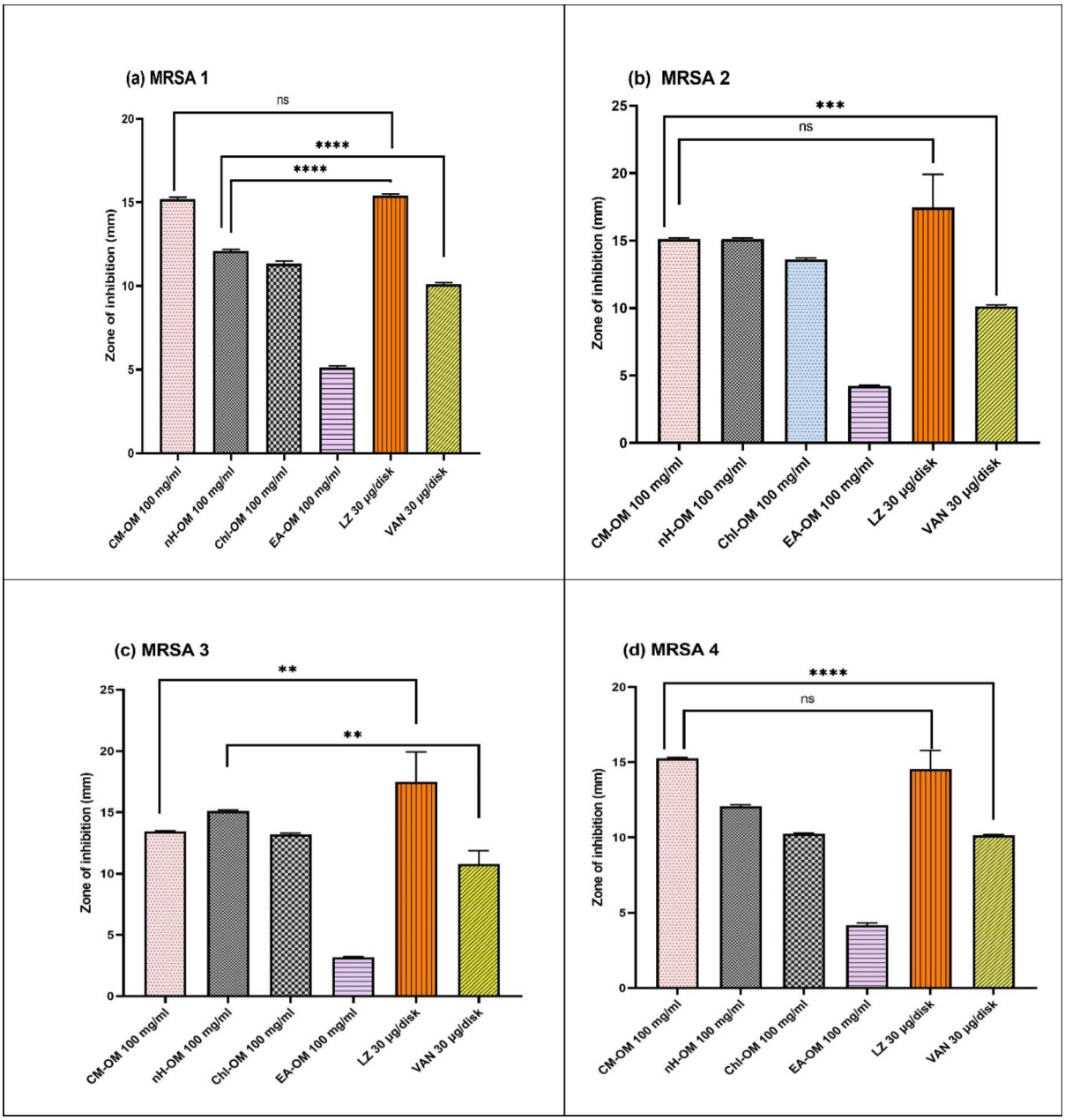

Fig. 7. Zone of inhibition (ZOI) of the crude methanolic extract of *Opuntia monacantha* (CM-OM) and its fractions n-hexane (nH-OM), chloroform (Chl-OM), and ethyl acetate (EA-OM) against MRSA strains at 75 mg/mL along with negative control (distilled water) and positive control linezolid (LZ) (30 µg) and vancomycin (VAN) (30 µg), (**A–D**) represents the ZOI of 75 mg of crude extract and its fractions on MRSA 1, (**E–F**) represents the ZOI of 75 mg of crude extract and its fractions on MRSA 2, (**I–L**) represents the ZOI of 75 mg of crude extract and its fractions on MRSA 3 and (**M–P**) represents the ZOI of 75 mg of crude extract and its fractions on MRSA 4.

The results revealed that plant extract contained phenolic compounds, flavonoids and their derivatives[40,41]. Flavonoids, one of the group of phenolic compounds are also known to have antioxidant and antimicrobial activity[42]. Antioxidant activity was quantified via DPPH assay which revealed that the percentage inhibition by the n-hexane fraction was the highest (88.43 ± 1.14), with low IC_{50} value of 79.73 µg/mL. According to the findings, phenolic chemicals made a substantial contribution to medicinal plants' antioxidant potential[43]. We can say that plants that are rich in phenolics, have hydroxyl groups that enable them to scavenge free radicals and have antioxidant properties[21,44]. The various active ingredients found in herbal plants, pharmaceutical drugs or food industry, forensic, and environmental applications, such as, alkaloids, organic acids, alcohols, esters, steroids and long chain hydrocarbons, should be tested. One of the best fast and accurate techniques for the analysis of the obtained extracts is GC-MS[45]. This method uses gas chromatography to separate the constituents from the mixture, and mass spectrometry to examine each component independently[46]. The

Microbial Strains Inhibition Zone (mm)[2]								
	MRSA 1				MRSA 2			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-OM	Chl-OM	EA-OM
Extract 75 mg/mL	10.1 ± 0.05	10.1 ± 0.1	6.2 ± 0.1	0.0 ± 0.0	10.3 ± 0.1	9.6 ± 0.1	8.8 ± 0.1	0.0
Linezolid (30 µg)	25.1 ± 0.1	28.0 ± 0.05	20.0 ± 0.1	20.0 ± 0.05	20.1 ± 0.1	15.1 ± 0.05	16 ± 0.1	20.0 ± 0.05
Vancomycin (30 µg)	17.1 ± 0.1	15.2 ± 0.05	9.2 ± 0.2	10.1 ± 0.1	5.46 ± 0.2	7.1 ± 0.1	6.3 ± 0.1	10.1 ± 0.05
	MRSA 3				MRS 4			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-OM	Chl-OM	EA-OM
Extract 75 mg/mL	9.4 ± 0.05	12.4 ± 0.05	10.5 ± 0.05	0.0 ± 0.0	10.3 ± 0.15	9.4 ± 0.1	8.2 ± 0.1	0.0 ± 0.0
Linezolid (30 µg)	20.1 ± 0.1	20.0 ± 0.05	16.1 ± 0.1	15.1 ± 0.1	16.5 ± 0.05	20.0 ± 0.1	20.1 ± 0.15	20.0 ± 0.05
Vancomycin (30 µg)	12.0 ± 0.05	9.6 ± 0.05	7.2 ± 0.05	10.1 ± 0.1	15.1 ± 0.15	10.1 ± 0.1	14.5 ± 0.05	10.1 ± 0.1

Table 9. Antibacterial potential of *Opuntia monacantha* cladodes by the well diffusion method at 75 mg/ml.

samples were subjected to GC-MS analysis, and the results were compared with public data from the NIST library as shown above in Tables 6 and 7. Chemical studies have shown that *OM* mainly contains phenols, alkanes, fatty acids, terpenoids, etc., The major compounds identified in the n-hexane fraction are tetradecane, 2,4-di-tert-butylphenol, 14-methylpentadecanoic acid, octacosyl heptafluorobutyrate, stigmasta-3,5-diene and beta-sitosterol. All these compounds have been reported in the literature to possess antimicrobial and antioxidant activities. Studies performed by Sallam and Abed 2022 and Badar and Shabban in 2011 reported tetradecane as an antibacterial compound[47,48]. In other studies performed by Aissaoui and Mahjoubi in 2018, 2,4-di-tert-butylphenol was characterized as an antibacterial compound. Octacosyl heptaflurobutyrate has been reported as a major compound with high antioxidant capacity done by Elwekeel and Hassan 2023[49]. Other compounds such as 14-methylpentadecanoic acid,, stigmasta-3,5-diene, and beta-sitosterol have also been reported as antimicrobial agents[50–52]. The compounds isolated from the chloroform fraction were 2,4-Di-tert-butylphenol, 3,4, dimethylbezadledehyde, tetracosane, benzenepropanoic acid, 3-(1,1-dimethylethyl)-4-hydroxy-, methyl ester and methyl stearate. 2,4-di-tert-butylphenol is one of the prominent compounds in this study and possesses an anti-MRSA activity, as reported previously[53]. Polyphenol such as benzenepropanoic acid, 3-(1,1-dimethylethyl)-4-hydroxy-,methyl ester known to have strong antioxidant and antifungal activities[54]. Other compounds found in our study such as 3,4, dimethyl benzaldehyde, tetracosane, and methyl stearate also possess antibacterial activity[55–57]. However, follow-up experiments is required to isolate and evaluate these compounds individually to determine their MIC values against MRSA.

The main objective of our investigation was to assess the possible antibacterial activity of *Opuntia monacantha* against MRSA. To our knowledge, it is the first report of antibacterial activity against MRSA by *OM* species present in Pakistan. The crude methanolic extract of the *OM* extract had the greatest inhibitory effect on all the MRSA strains. This significant inhibitory activity of the methanolic extract has also been observed against resistant *S. aureus* by other species of *Opuntia*[21,58,59]. Significant inhibitory activity was also observed with the n-hexane fraction, followed by the chloroform fraction as shown in Tables 8 and 9, and 10. These findings are also consistent with the studies of Ennouri et al., 2014 and Elkady et al., 2022 who measured the antibacterial activity of *O. inermis* hexane extract and *O. ficus indica* against *S. aureus*, respectively. They also reported marked significant inhibition of *Staphylococcus* bacteria by these species[60,61]. However, the ethyl acetate fraction had a non-significant effect on all the MRSA strains. This finding is not consistent with the literature, where ethyl acetate is known to inhibit *S. aureus*[58,62,63]. It is possible that the difference in extraction method and various environmental factors affects the phytoconstituents present in the *OM* account for their different capacities toward strains of MRSA.

Previous studies have also shown that *OM* species have a significant effect on the inhibition of *S. aureus*[20,24,64]. Our findings are consistent with several earlier studies that demonstrated that phenolic compounds from natural extracts have greater inhibitory effects on gram-positive bacteria than on gram-negative bacteria. The properties of the drug (hydrophobicity or hydrosolubility) and the makeup of the microbial membrane determine how susceptible bacteria are to medications[65]. Extracts from *Opuntia* cladodes may have antimicrobial properties because of their high polyphenol content[66]. A number of polyphenols, including phenolic acids, tannins, lignans, stilbenes, flavonoids (particularly flavonols), and combinations of these in botanical mixtures, have demonstrated strong antibacterial action against both resistant and non-resistant Gram-positive bacteria at low µg/mL range MIC values. Cell walls, lipid membranes, membrane receptors, ion channels, bacterial metabolites, and biofilm formation are all targets of their varied mode of action. Certain combinations of polyphenols and antibiotics were also shown to have synergistic effects[23].

The effectiveness of *OM* and its fractions in reducing MRSA growth was reported in terms of MIC. According to the MIC values as shown in Table 11, CM-OM, even at the lowest measured concentration (3.12 mg/mL), it appears to be effective at preventing the growth of MRSA strains because organisms are absent from all tested concentrations. The n-hexane (nH-OM) and chloroform (Chl-OM) fractions exhibit variable degrees of inhibition and partial effectiveness. nH-*OM* has shown antibacterial potential between 3.15 and 6.25 mg/mL. For Chl-OM organisms are absent at concentrations above 6.25 mg/mL but present at both 3.12 and 6.25 mg/mL, indicating a MIC slightly above 3.12 mg/mL. However, within the studied concentration range, the ethyl acetate fraction (EA-OM) has no inhibitory effect on all MRSA strains. The results of this examination clearly

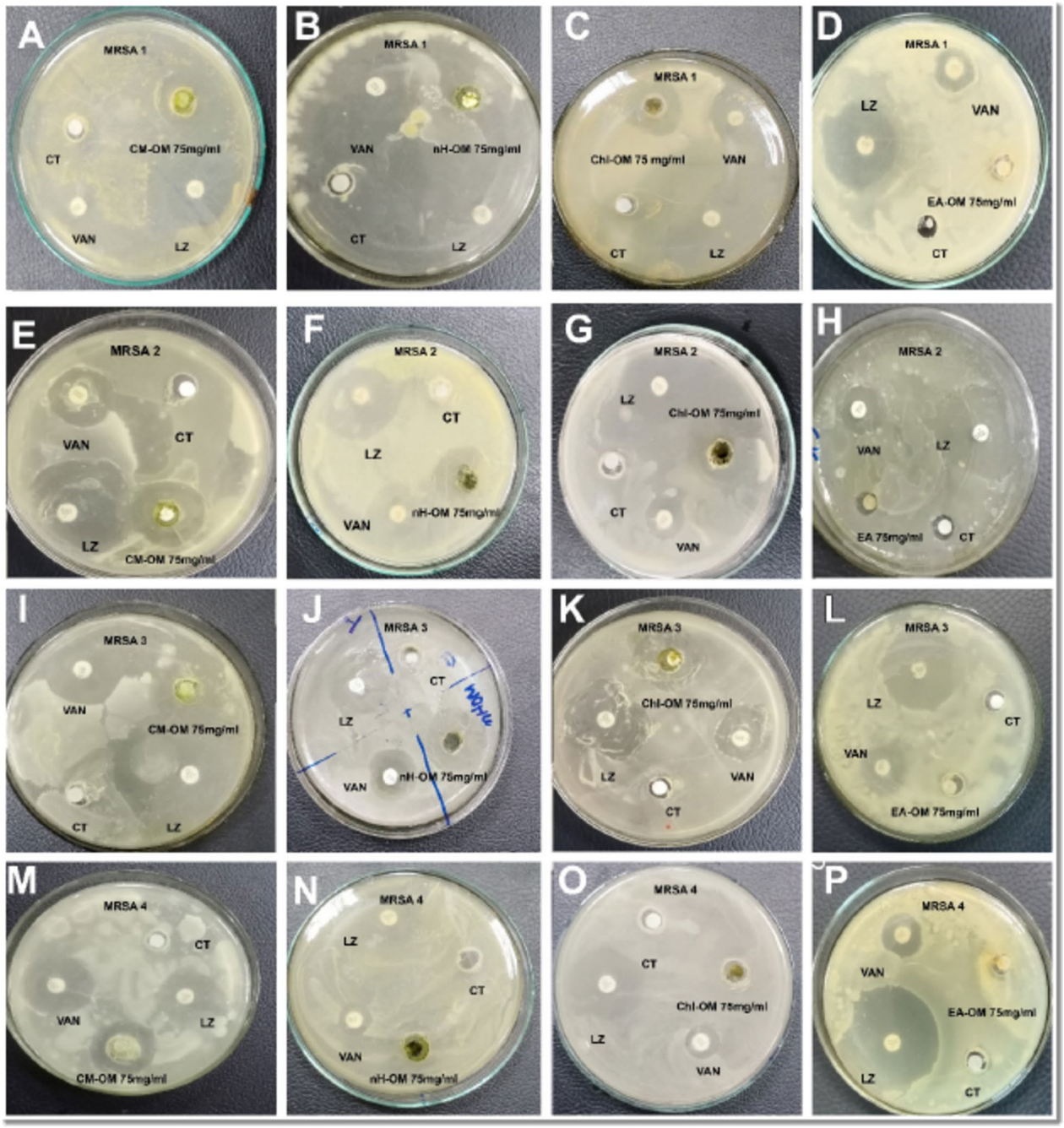

Fig. 8. Antibacterial activity of the crude methanolic extract of Opuntia monacantha (CM-OM), its fractions nH: n-hexane, Chl: chloroform at 75 mg/mL and commercially available antibiotic discs LZ: linezolid 30 µg/disk and VAN: vancomycin 30 µg/disk. (**a–d**) Different strains of methicillin resistant *Staphylococcus aureus*. The mean zones of inhibition (mm) created by the respective treatments against each bacterial strain were recorded. The data presented are the means of three independent experiments ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$ ns $P > 0.05$ (for comparisons of all treatments by one-way ANOVA, followed by Tukey's Test).

show that CM-OM is the most promising candidate with high antibacterial activity for additional development in antimicrobials, whereas EA-OM shows little to no activity at the tested dosages.

According to published data, plants with high potential as antimicrobials against bacteria typically have a low minimum inhibitory concentration (MIC), and vice versa. To combat increasing medication resistance in humans, the development of new antimicrobial metabolites from medicinal plants such as *OM* is crucial. Plant extracts with established antibacterial qualities can play a significant role in medical interventions. *Opuntia* species been utilized for long to treat various harmful bacterial infections[67].

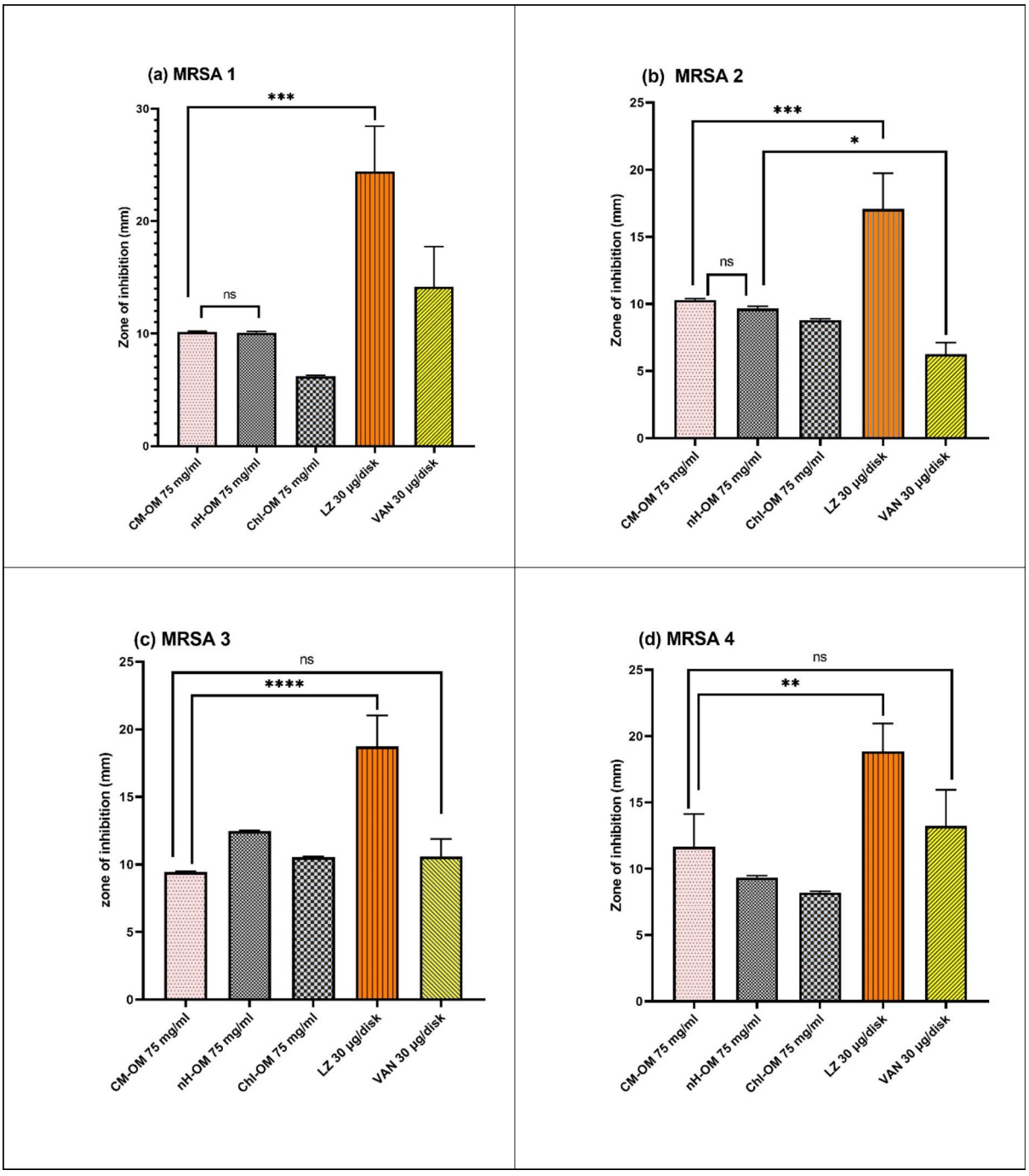

Fig. 9. Zones of inhibition (ZOI) of crude methanolic extract of *Opuntia monacantha* (CM-OM) and its fractions n-hexane (nH-OM), chloroform (Chl-OM), and ethyl acetate (EA-OM) against MRSA strains at 50 mg/mL along with negative control (distilled water) and positive control linezolid (LZ) (30 µg)and vancomycin (VAN) (30 µg), (**A–D**) represents the ZOI of 50 mg of crude methanolic extract and its fractions on MRSA 1, (**E–F**) represents the ZOI of 50 mg of crude extract and its fractions on MRSA 2, (**I–L**) represents the ZOI of 50 mg of crude extract and its fractions on MRSA 3 and (**M–P**) represents the ZOI of 50 mg of crude extract and its fractions on MRSA 4.

Conclusion & future prospective

Many pharmacognostics studies and kinetics of medicinal plants have demonstrated that plant-derived bioactive chemicals and crude extracts may increase the effectiveness of conventional antimicrobial agents, which may be less expensive, cause fewer side effects, and result in better treatment outcomes. The findings of our research revealed that *Opuntia monacantha* Haw. methanolic extract and its fractions (n-hexane and chloroform) had antibacterial effects on methicillin-resistant *Staphylococcus aureus* strains that had been clinically isolated at

Microbial Strains Inhibition Zone (mm)2								
	MRSA 1				MRSA 2			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-OM	Chl-OM	EA-OM
Extract 50 mg/mL	9.5 ± 0.1	9.1 ± 0.15	8.2 ± 0.15	0.0 ± 0.0	9.9 ± 0.1	8.9 ± 0.2	8.1 ± 0.15	0.0 ± 0.0
Linezolid (30 µg)	15 ± 0.1	15.2 ± 0.2	25.1 ± 4.9	20.2 ± 0.1	15.1 ± 0.1	15.1 ± 0.1	20.1 ± 0.05	19.6 ± 0.4
Vancomycin (30 µg)	10.1 ± 0.1	10 ± 0.17	10.4 ± 0.05	10.3 ± 0.2	10.3 ± 0.2	10.1 ± 0.1	10.2 ± 0.05	10.2 ± 0.1
	MRSA 3				MRSA 4			
	CM-OM	nH-OM	Chl-OM	EA-OM	CM-OM	nH-OM	Chl-OM	EA-OM
Extract 50 mg/mL	10.1 ± 0.1	8.4 ± 0.1	7.4 ± 0.05	0.0 ± 0.0	10.2 ± 0.05	7.4 ± 0.16	6.43 ± 0.05	0.0 ± 0.0
Linezolid (30 µg)	15.1 ± 0.05	15.1 ± 0.05	15.03 ± 0.05	16.0 ± 0.05	15.2 ± 0.05	10.03 ± 0.05	10.1 ± 0.05	20.0 ± 0.05
Vancomycin (30 µg)	10.03 ± 0.2	10.1 ± 0.05	10.1 ± 0.1	10.06 ± 0.05	10.06 ± 0.05	6.0 ± 0.1	6.0 ± 0.05	10.3 ± 0.20

Table 10. Antibacterial potential of *Opuntia monacantha* cladodes by the well diffusion method at 50 mg/ml.

different concentrations. The activity of crude extract was greater than that of other three extracts because the synergistic properties of other extracts decreased after partitioning. This study validates its historic use in treating bacterial illness. On the basis of these findings, medicinal plants such as *Opuntia monacantha* Haw. are recommended for use as an alternative therapy for infections caused by bacteria in developing countries. As shown in this study, numerous compounds with antimicrobial and antioxidant activities, the *OM* extract can be used in pharmaceuticals as an alternative for resistant bacterial strains. However, there are also some study limitations such as limited MRSA strains were tested, potential variability in *OM* composition from different environments.

Further exploration is required such as in in-vivo studies, toxicity profile using animal models, and in-silico studies along with molecular docking is essential for the isolation and characterization of the active compounds responsible for the antibacterial activity of the extract to explore the binding potential of the ligands to their target binding sites.

Materials and methods

Plant material collection and pretreatment

The cladodes of *Opuntia monacantha* Haw. (Cactaceae) as shown in Fig. 11 was collected from the Lahore to Islamabad Motorway (M1-Motorway, Kalarkhar) Punjab, Pakistan in May and June 2023. The identification and authentication of sample was performed by taxonomist Dr. Uzma Hanif, Botany Department of Government College University (GCU), Lahore, Pakistan under the voucher No: GC. Herb. Bot. 4053 for O*puntia monacantha* Haw *and* plant specimen was deposited in the herbarium of Department of Botany, Government College University Lahore, Pakistan with GC. Herb. Bot No.4053. The collection of plants for this study is conducted solely for educational research purposes, adhering strictly to the general publication standards and ethical guidelines of the University of Central Punjab, Lahore, Pakistan. As per these guidelines, no specific permission is required for plant collection in such contexts, provided the research is conducted responsibly and does not involve endangered or protected species or violate local environmental laws. All efforts are made to ensure ethical and sustainable practices during the collection process. This is according to gee After *Opuntia monacantha* Haw. was collected it was washed carefully with distilled water for surface cleaning and elimination of dust particles as well as contaminants. The plant cladodes were left under shade for air drying at the Pharmacology Research Lab, University of Central Punjab, for 20 days. The dried form was pulverized into fine powder via electrical blender and stored within an airtight container. The study design methodology has been shown in Fig. 12.

Preparation of extract

Using the cold maceration procedure, the powdered plant material (400 g) was precisely weighed and then dipped into 1.3 L of methanol at room temperature (RT) intended for a duration of one whole week. Once the methanolic extract was filtered, the same process was performed three times. The filtrate was subjected to drying via a rotary evaporator set to 40–45 °C at reduced pressure. After that, the residue was collected, weighed and combined with distilled water. Next, it is fractionated by using various solvents (the n-hexane, chloroform, ethyl acetate) that increase in polarity[68].

All the fractions were then dried and weighed and calculated the percentage yield of each fraction with the formula given below.

$$\text{Percentage yield } \% = \frac{\text{Weight of concentrate obtained after drying}}{\text{initial weight of plant used}} \text{x100}$$

Microorganisms

The microorganisms, used in the study namely methicillin resistant *Staphylococcus aureus* (MRSA 1, MRSA 2, MRSA 3, MRSA 4) used in the study were confirmed clinical isolate provided by the microbiology department, Ittefaq Trust Hospital, Lahore by Dr. Roman. Phenotype resistance of the strains was maintained throughout the study by keeping control strains with known phenotype resistance as a benchmark to prepare fresh subcultures to avoid the genetic drift.

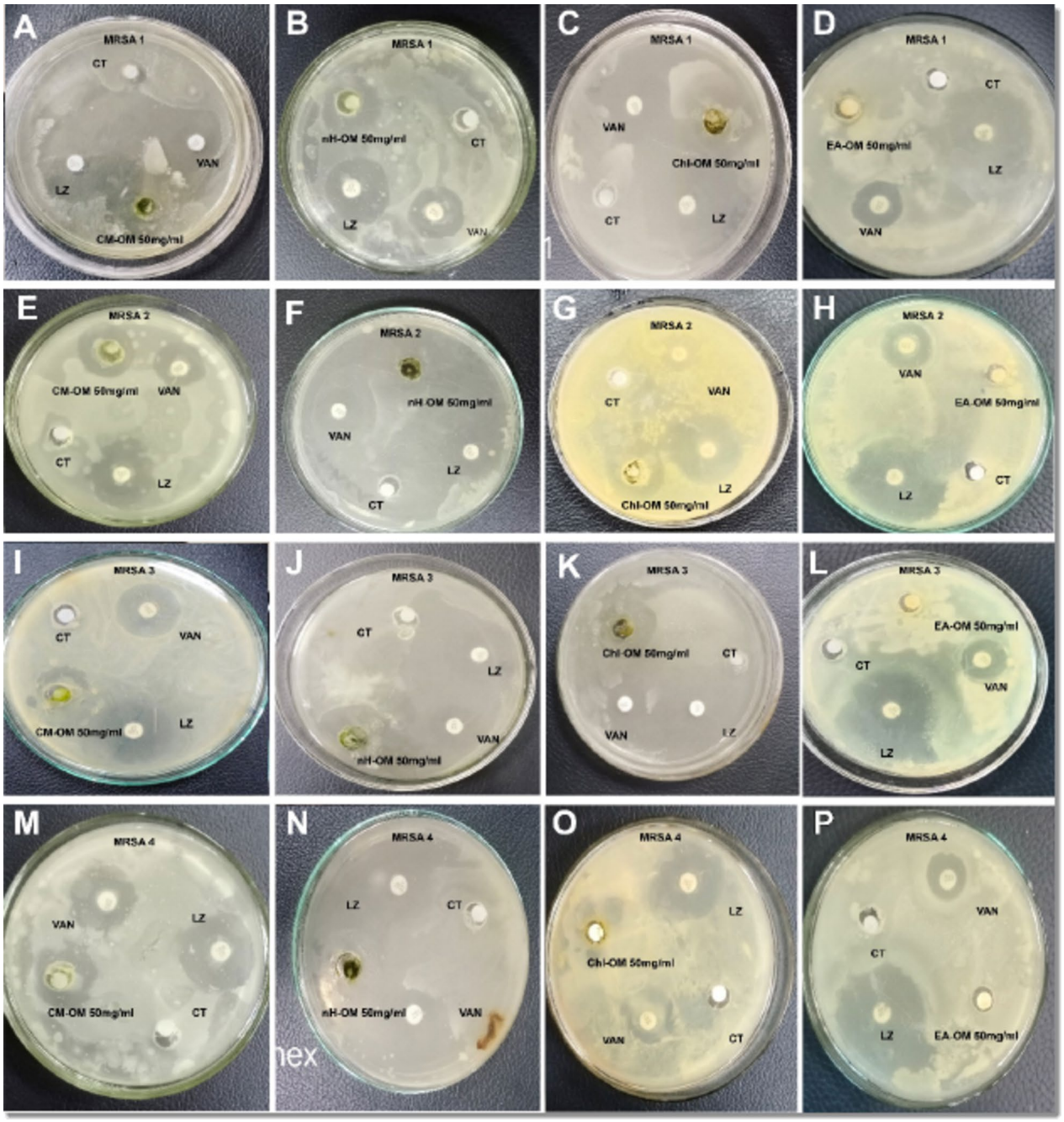

Fig. 10. Antibacterial activity of the crude methanolic extract of Opuntia monacantha (CM-OM), its fractions nH: n-hexane, Chl: chloroform at 50 mg/mL and the commercially available antibiotic discs LZ: linezolid 30 µg/disk and VAN: vancomycin 30 µg/disk. (**a–d**) Different strains of methicillin resistant *Staphylococcus aureus*. The mean zones of inhibition (mm) created by the respective treatments against each bacterial strain were recorded. The data presented are the means of three independent experiments ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$ ns = $P > 0.05$ (for comparisons of all treatments by one-way ANOVA, followed by Tukey's Test).

Media preparation

The bacterial media prepared for the antimicrobial assay. Mueller Hinton agar was used to grow bacteria. In one liter of distilled water, 38 g of powder were dissolved. The mixture was heated until it dissolved completely, and then autoclaved it for 15 min at 121 ˚C (15 Ibs) pressure to sanitize it. Once the mixture had cooled to 45–50˚C, it was transferred into petri dishes.

Strains	CM-OM mg/mL	nH-OM mg/mL	Chl-OM mg/mL	EA-OM mg/mL
MRSA 1	3.12	6.25	12.5	75.0
MRSA 2	3.12	6.25	12.5	75.0
MRSA 3	3.12	6.25	12.5	75.0
MRSA 4	3.12	6.25	12.5	75.0

Table 11. MIC values of crude *OM* and its fractions.

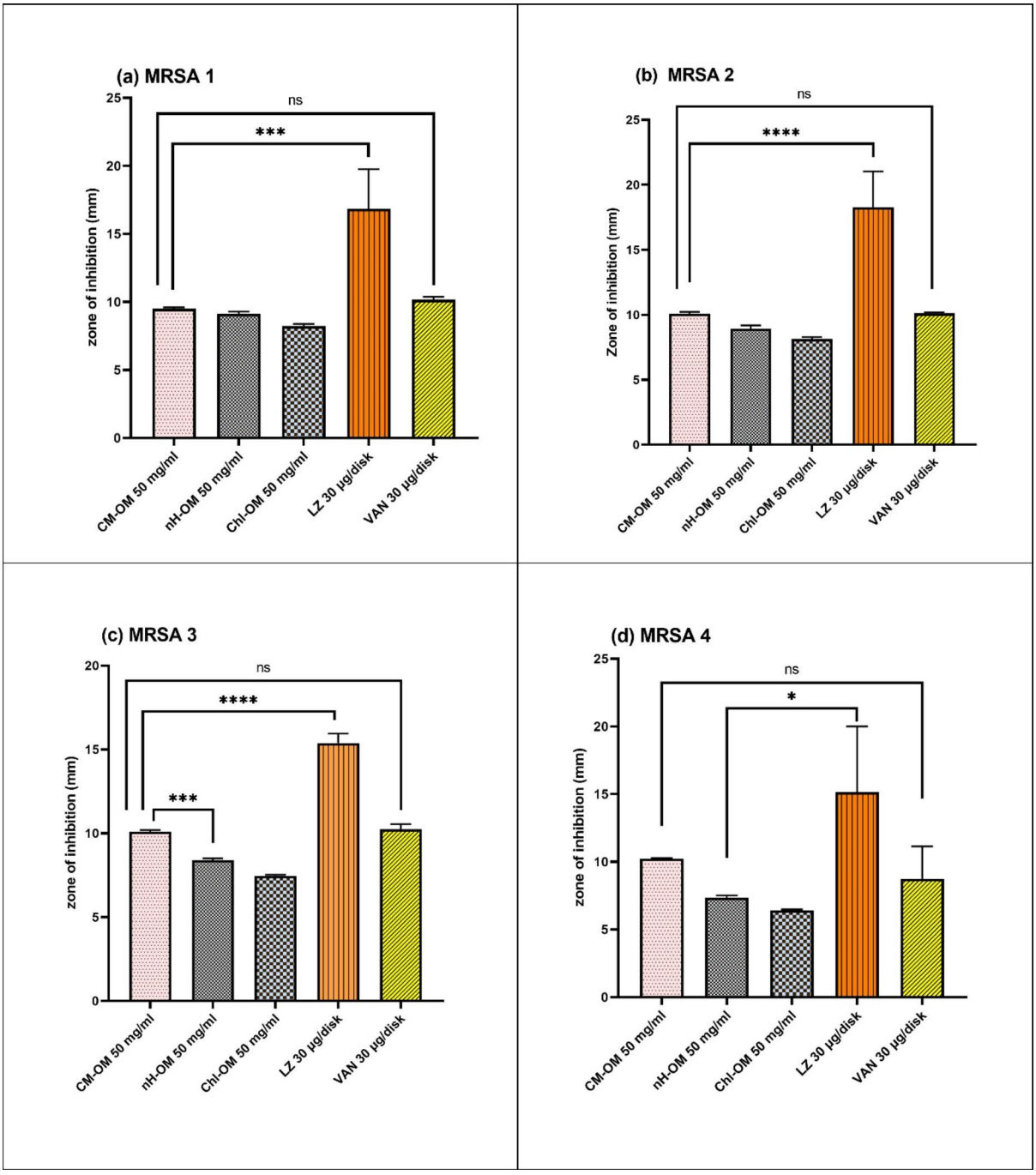

Fig. 11. *Opuntia monacantha* Haw.

Fig. 12. Study design methodology for evaluating the antibacterial effect of *Opuntia monacantha* Haw.

Well diffusion method

The assay was run to investigate antimicrobial potential. Mueller-Hinton agar media was prepared and sterilized according to standard protocols. The agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then a circular holes 6 mm are made aseptically into the agar using a sterile cork borer, and a suitable volume 100 µL of the sample solution at the chosen concentration is applied into the well and then incubated it for 24 h[69]. The assay was performed in triplicate and zones of inhibition around the wells were calculated at three doses (100, 75 and 50 mg/mL)[70,71]. Doses were selected with reference to already established anti MRSA activity of another species of *Opuntia* known as *Opuntia ficus indica*.[21] Compared to manufactured antibiotics, substances produced from plants may have less effective antibacterial action. But with high concentration, molecules with low activity can be evaluated successfully, capturing any possible therapeutic benefit.

Measurement of the minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of the crude methanolic extract and the n-hexane, chloroform and ethyl acetate fractions were determined via the well diffusion method. This method was selected according to the availability of resources[72]. Concentrations of 25, 12.5, 6.25, and 3.12 mg/mL of *Opuntia monacantha* methanolic extract and its fractions were formed. Negative controls for the experiment were formed using distilled water alone. Zones of inhibition diameter were used to calculate the antibacterial activity of each sample[73].

Antioxidant activity

Opuntia monacantha and its fractions were interpreted via DPPH assay as explained by[74,75] for their antioxidant activity. The stock solutions (1 mg/mL) of OM and the fractions were made in methanol. Various concentrations (50, 100, 150, 200 and 250 µg/mL) of OM, fractions and ascorbic acid (standard compound) were prepared. The standards and samples were incubated for 30 min at room temperature. A UV-Vis spectrophotometer used to measure absorbance at 517 nm. The assay was performed in triplicates and absorbance decrease was recorded. The DPPH activity was calculated with formula given below.

$$(\%)\ \text{Inhibition of DPPH activity} = [(\text{Ac} - \text{As}) \div \text{Ac}] \times 100$$

Where Ac is control absorption and As is sample absorbance respectively.

Gas chromatography-mass spectrometry (GC-MS) analysis

An Agilent 19091-433HP, USA gas chromatograph system and mass spectrophotometer were used to perform the GC-MS analysis. The apparatus was equipped with a column known as HP-5 MS fused silica column (consists of 5% phenyl and 95% methyl siloxane) interfaced with a 5675 C. Mass selective detector (inert) with Triple-Axis detector. Velocity flow of 1.0 milliliters per minute was present in a column, the carrier gas helium was used.

The additional GC-MS parameters included an ion source of 250 °C; Interface with 300 °C for; 16.2 psi; 1.8 mm for the outer time; and an injector of 1 µL of sample in split mode with a split ratio of 1:50 with 300 °C injection temperature. The temperature in the column rose to 150 °V at a rate of 4 °C per minute after five minutes at 36 °C. The temperature was raised to 250 °C at a rate of 20 °C per minute, and it was then held

there for five minutes. Elution took 47.5 min in total. The proportional percentage of every component was ascertained in comparison with its average peak area to the total area[76].

Statistical analysis

The mean ± SEM was used to express the data and analyzed via GraphPad Prism software 5.00 (San Diego, USA), compared via one way ANOVA with Tukey test and plotted. Data was considered significant at $P < 0.05$.

Data availability

All data is mentioned in the manuscript.

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S.J: Conceptualization, Methodology, Formal analysis, Writing - original draft. Data curation, Investigation; M.A.K: Conceptualization, Supervision, Project administration, Investigation; A.A.: Formal analysis, Data curation, Writing - review & editing; H.H.: Methodology, Validation, Visualization, Writing - review & editing; S.U.R: Data curation, Visualization; review & editing, A.I.; Formal analysis, Funding acquisition, Visualization, Writing - review & editing; G.A.S: Data curation; Funding acquisition, Investigation, Writing - review & editing; Y.A.B.J: Funding acquisition, Formal analysis, Visualization; Project administration, Writing - review & editing. All authors reviewed, commented and agreed on the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The collection of plants for this study is conducted solely for educational research purposes, adhering strictly to the general publication standards and ethical guidelines of the University of Central Punjab, Lahore, Pakistan. As per these guidelines, no specific permission is required for plant collection in such contexts, provided the research is conducted responsibly and does not involve endangered or protected species or violate local environmental laws. All efforts are made to ensure ethical and sustainable practices during the collection process.

Additional information
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