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Advances in Bioresearch

ORIGINAL ARTICLE

Unveiling the therapeutic potential of *Neptunia oleracea* and *Centella asiatica*: Antibacterial activity, phytochemical profiling and GC-MS-based identification of bioactive compounds against livestock pathogens in Purba Bardhaman district, West Bengal, India

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ABSTRACT
Antimicrobial resistance and antibiotic residues in animals may be addressed via ethno-veterinary medicine. Staphylococcus aureus, Salmonella enterica, Escherichia coli, and Pseudomonas aeruginosa are some of severe pathogens of livestock linked to diarrhea and mastitis. The present communication assessed the antibacterial activity, phytochemical profile, and bioactive compounds of leaf extracts from two medicinal plants Neptunia oleracea and Centella asiatica. Agar well diffusion was used to assess aqueous, ethanol, and methanol extracts (1.25–20 mg/ml), with ethanol extracts exhibiting the highest activity. While C. asiatica had broad-spectrum action, including against P. aeruginosa (11 ± 1 mm), N. oleracea shown greatest inhibition against S. enterica (19.66 ± 0.57 mm). The lowest MIC values were found against S. aureus and S. enterica, with values ranging from 1.25 to 5 mg/ml. Flavonoids, phenols, tannins, alkaloids, saponins, terpenoids, and phytosterols were identified by phytochemical examination. GC-MS detected bioactive substances such as γ-sitosterol in N. oleracea and phytol, madecassic acid, adipic acid ester, and eicosapentaenoic acid methyl ester in C. asiatica. These results show that C. asiatica and N. oleracea, are attractive phytobiotic options for managing bacterial infections linked to livestock.
Keywords: *Ethno-veterinary plants, livestock disease, Neptunia oleracea, Centella asiatica, antimicrobial activity, MIC, GC-MS*

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INTRODUCTION

The economic viability of livestock production is frequently undermined by the outbreak of infectious diseases, resulting in considerable financial losses and welfare concerns [1,2]. Bacterial infections constitute a major share of livestock morbidity worldwide due to their universal nature and rapid transmission. Various pathogenic bacteria are implicated in severe clinical conditions that culminate in high mortality, especially in young and immune-compromised animals. Among these, diarrhea and mastitis are the most economically devastating bacterial afflictions affecting Indian livestock. Neonatal and young ruminants are particularly susceptible to diarrhea, a multifactorial gastrointestinal syndrome. While sudden alterations in feeding regimen can predispose animals, the primary cause is often associated with enter pathogenic bacteria, notably *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*, which are widely reported as etiological agents of calf diarrhea [3,4,5]. Bovine mastitis, on the other hand, poses a severe challenge to the dairy sector. The condition, predominantly caused by *Staphylococcus aureus* in milch cows and buffaloes, is known to cause a drastic decline in milk production ranging from 50% to 65%, thereby imposing a substantial economic burden on marginal dairy farmers

[6,7]. Apart from microbial infections, health complications arising from the ingestion of toxic plants and other hazardous materials during free grazing also contribute to livestock losses [8].

In view of escalating antimicrobial resistance and the high cost of conventional veterinary drugs, there is a pressing need to explore alternative, eco-friendly and cost-effective therapeutic strategies [9,10,11].In recent decades, ethno-veterinary medicine, which utilizes traditionally employed medicinal plant for animal health, has garnered enhanced interest as a reservoir of bioactive substances with therapeutic efficacy. Ethno-veterinary medicinal plants, traditionally used by local communities, represent a promising reservoir of bioactive compounds for the management of such bacterial ailments. Effective therapeutic intervention is essential for recovery from infectious diseases and for enhancing livestock productivity. However, in agrarian regions of India, access to conventional veterinary healthcare remains inadequate, particularly in rural and peri-urban areas where diagnostic infrastructure and disease management facilities are limited [12,13]. In districts such as PurbaBardhaman of West Bengal, India, where livestock rearing is a primary source of livelihood for marginal farmers, this gap is more pronounced. Consequently, livestock keepers frequently resort to the indiscriminate and unscientific use of antibiotics and other chemotherapeutic agents. Such irrational application accelerates the emergence of antimicrobial resistance, a global health threat that may render commonly used antibiotics ineffective in the near future [9,10].

This alarming scenario has redirected scientific attention toward plant-based therapeutic alternatives rooted in traditional knowledge. The PurbaBardhaman district of West Bengal, India is known for its rich floristic diversity and long-standing ethno-veterinary practices, where local communities have conserved valuable indigenous knowledge on medicinal plants [16]. Herbal constituents derived from these plant species are increasingly recognized as a promising source of novel antibacterial agents. Numerous medicinal plants and their natural products exhibiting significant antimicrobial properties can be locally utilized for the treatment of livestock ailments [11]. Such ethno-veterinary knowledge is gaining preference over synthetic drugs in this region owing to its proven pharmacological efficacy, cost-effectiveness, easy availability, and cultural acceptability [17,18]. In contrast to conventional allopathic drugs, herbal formulations are generally considered to have fewer adverse effects and lower toxicity.

In the PurbaBardhaman district of West Bengal, India, field surveys reveal that *Neptunia oleracea*Lour. and *Centella asiatica* (L.) Urb. are among the most popularly used ethno-veterinary plants for the management of diarrhea and mastitis in livestock [15,16,18]. These plants are rich reservoirs of bioactive secondary metabolites, including alkaloids, flavonoids, phenolic compounds, tannins, and terpenoids, which are reported to possess significant activity against a wide range of pathogenic microorganisms [19,20]. These phytochemicals serve as crucial lead compounds for the development of novel therapeutic agents. In this context, the extraction efficiency and antimicrobial potential are highly dependent on the polarity of the solvents used. Previous studies have demonstrated that methanol, ethanol, and aqueous extracts often exhibit differential antibacterial efficacy due to variation in solubility of bioactive principles [21,22].Therefore, the present study was designed to systematically screen the methanol, ethanol, and distilled water extracts of *N. oleracea* and *C. asiatica* collected from PurbaBardhaman district for their antibacterial activity against major livestock pathogens such as *E. coli*, *S. enterica*, *P. aeruginosa*, and *S. aureus*, and to identify their active principles through Gas Chromatography-Mass Spectrometry (GC-MS) analysis for scientific validation of traditional knowledge.

MATERIAL AND METHODS

Plant collection

Fresh leaves of *Neptunia oleracea* Lour. and *Centella asiatica* (L.) Urb. were collected from their natural habitat at Katwa in PurbaBardhaman district, West Bengal, India (23°38′ N latitude and 88°07′ E longitude) in October 2025. The specimens were identified and authenticated by a taxonomist and voucher specimens were deposited in the herbarium for future reference. After collection, the leaves were thoroughly washed with tap water to remove dust and other contaminants and were shade-dried at room temperature for 14 days. The completely dried leaf samples were pulverized separately into fine powder using an electric grinder, sealed in airtight containers and stored at 4°C in a refrigerator until further experimental use.

Antibacterial assay of Pant extracts

Preparation of Plant Extracts for Antibacterial Assay

Two types of solvent systems were employed for extraction based on polarity: distilled water as an aqueous polar solvent, and methanol and ethanol as polar organic solvents. For aqueous extraction, 2 g of fine leaf powder of each plant (*N. oleracea* and *C.asiatica*) was mixed with 100 mL of sterile distilled water in a beaker. The suspension was incubated in a water bath at 45-50°C with continuous stirring for 20-30

minutes. After incubation, the mixture was filtered through Whatman filter paper No. 1 to obtain a clear filtrate with a concentration of 20 mg/mL, considered as the stock solution. Lower concentrations (10, 5, 2.5, and 1.25 mg/mL) were prepared by serial dilution of the stock with sterile distilled water. The aqueous extracts were stored at 4°C for subsequent antibacterial screening. In case of organic extraction,100 g of shade-dried leaf powder of each plant (*N. oleracea* and *C.asiatica*) was separately extracted with 80% methanol (v/v) and 95% ethanol (v/v) by cold percolation method. The powdered material was soaked in the respective solvent and kept overnight with occasional shaking. The mixture was then filtered through Whatman filter paper No. 1. The filtrate was concentrated to dryness under reduced pressure using a rotary evaporator at 40°C. The dry crude extracts were weighed and the extraction yield was calculated. Stock solutions were prepared by dissolving the dried extracts in their respective solvents and further diluted to obtain working concentrations of 20, 10, 5, 2.5, and 1.25 mg/mL. All extracts were stored at 4°C until further use.

Collection and Maintenance of Test Microorganisms

Four livestock-associated pathogenic bacterial strains, namely *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from the Department of Microbiology, West Bengal University of Animal and Fishery Sciences (WBUAFS), Kolkata, West Bengal, India. Among these, *E. coli*, *P. aeruginosa* and *S. enterica* are reported as major etiological agents of diarrhea in calves, goats, cattle and buffaloes [23,24], whereas *S. aureus* is primarily associated with mastitis in dairy cows and buffaloes [25]. All bacterial cultures were sub-cultured on Nutrient Agar (NA) medium at weekly intervals to maintain viability and preserved at 4°C for further experimental use.

Preparation of Bacterial Inoculum

Each pathogenic bacterial strain was inoculated separately into sterile Nutrient Broth (NB) and incubated overnight at 37 ± 2°C. After 18-20 h of incubation, visible turbidity indicated active bacterial growth. The broth cultures were then used as inoculum for further antimicrobial assays after adjusting to 0.5 McFarland standard (approx. 1.5×108 CFU/mL).

Determination of Antibacterial Activity

The antibacterial activity of leaf extracts against diarrhea and mastitis-causing pathogenic strains was evaluated by the agar well diffusion method [26]. Sterile Mueller-Hinton Agar (MHA) was poured into sterile Petri dishes under laminar airflow and allowed to solidify. After solidification, 100 µL of standardized bacterial inoculum (0.5 McFarland standard, ~1.5×10^8 CFU/mL) was spread uniformly over the agar surface using a sterile L-shaped spreader. Six wells, each 6 mm in diameter, were bored into the agar medium using a sterile cork borer. Among them, five wells were filled with 100 µL of plant extract at different concentrations (20, 10, 5, 2.5, and 1.25 mg/mL), while the sixth well received an equal volume of respective solvent serving as a negative control. The plates were kept undisturbed for 45-60 min at room temperature to allow pre-diffusion of the extracts. Subsequently, the plates were incubated in a BOD incubator at 37 ± 2°C for 24 h. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (ZOI) around each well in millimeters (mm).

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the plant extract that inhibits visible growth of the test microorganism after 24-48 h of incubation. In preliminary screening, most of the extracts showed maximum antibacterial activity at 20 mg/mL. Therefore, this concentration was considered as the initial concentration for MIC determination against the diarrhea-causing pathogens. A two-fold serial dilution technique was employed to prepare different concentrations (10, 5, 2.5, and 1.25 mg/mL) from the stock extract. The antibacterial activity of each diluted concentration was evaluated by the agar well diffusion method as described by Das *et al*. (2013). The lowest concentration producing a measurable zone of inhibition was recorded as the MIC value, which indicates the efficacy of the leaf extracts in controlling livestock-associated bacterial infections.

Phytochemical Analysis

Qualitative Preliminary Phytochemical Screening

Preliminary phytochemical screening of the leaf extracts was carried out to detect the presence of major bioactive constituents including flavonoids, phenols, alkaloids, tannins, terpenoids, saponins, phytosterols, carbohydrates, reducing sugars, and steroids. All tests were performed following the standard protocols described by Shaikh and Patil [27].

GC-MS Analysis

For GC-MS analysis, 5% (w/v) methanol extracts were prepared using methanol of 99.8% purity. The suspensions were subjected to continuous stirring at 50°C for 7-8 h on a magnetic stirrer to ensure complete extraction. The resulting solutions were filtered through Whatman No. 1 filter paper. The filtrates were transferred to open beakers and allowed to evaporate to dryness at room temperature for

removal of residual solvent. The dried residues were then stored and used for GC-MS analysis.GC-MS analysis of methanol leaf extracts was performed using a PerkinElmer Clarus 680 Gas Chromatograph coupled with a Clarus 600C Mass Spectrometer. Separation was achieved on an HP-5MS capillary column (5% phenyl-95 percentage methylpolysiloxane), known for its low-bleed characteristics and high inertness. The samples were vaporized in the injection port at 300°C and carried through the column by high-purity helium (99.99%) as the carrier gas. Inside the column, compounds were separated based on their boiling points and affinity towards the stationary phase, eluting at different retention times. Upon exiting the GC column, the separated compounds entered the mass spectrometer where they were subjected to electron ionization at 70 eV, resulting in fragmentation into charged ions. The ions were subsequently accelerated through an electromagnetic field and separated according to their mass-to-charge (m/z) ratio, generating a mass spectrum for each compound. Phytochemicals were identified based on their mass fragmentation pattern, retention time, and relative peak area percentage by comparing the obtained mass spectra with the standard reference spectra available in the NIST (National Institute of Standards and Technology) digital library. All tests were performed in triplicate. Statistical analysis was performed with descriptive analysis to Microsoft Excel 2021 and data were expressed as mean ± SD.

RESULT

Antimicrobial Activity of Plant Extracts

The antimicrobial activity of the two plant species (*N. oleracea* and *C.asiatica*) was evaluated by agar well diffusion method against four bacterial strains. Among them, three were Gram-negative bacteria, namely *S. typhi, P. aeruginosa* and *E. coli* and one was Gram-positive *S. aureus*. The antibacterial potential of *N. oleracea* was evaluated using different solvent extracts (distilled water, ethanol, and methanol) at concentrations ranging from 1.25 to 20 mg/mL against four pathogenic bacteria. The zone of inhibition including well diameter (6 mm) was recorded and presented in Table 1 and Fig 1.

Table:1 Antibacterial activity (zone of inhibition) of *N. oleracea* against 4 pathogenic bacteria

Pathogen	Extract	1.25mg/ml	2.5mg/ml	5mg/ml	10mg/ml	20mg/ml
SA	Distilled water	6±0	6±0	6±0	6±0	7.33±0.57
	Ethanol	6.33±0.47	7.33±1.15	9±1	11±0	13.33±0.57
	Methanol	6±0	6±0	7.33±0.57	7.66±0.57	10±1
SE	Distilled water	6±0	6±0	6±0	6±0	6±0
	Ethanol	6.33±0.57	7.33±0.57	9±0	13±0	19.66±0.57
	Methanol	6±0	6±0	9.33±0.57	9.33±0.57	12.33±0.57
PA	Distilled water	NI	NI	NI	NI	NI
	Ethanol	NI	NI	NI	NI	NI
	Methanol	NI	NI	NI	NI	NI
EC	Distilled water	6±0	6±0	6±0	6±0	6±0
	Ethanol	6±0	6±0	7.66±0.57	9.33±0.57	11.67±0.57
	Methanol	6±0	6±0	6±0	8±1	10±1

Values are mean ± SD of triplicate experiments. SA= *Staphylococcus aureus*, SE= *Salmonella enterica*, PA= *Pseudomonas aeruginosa*, EC= *Escherichia coli*, NI= No zone of inhibition. Zone of inhibition (in mm) including the diameter of well 6 mm.

The results indicated a clear dose-dependent increase in antibacterial activity, with maximum inhibition observed at 20 mg/mL in all susceptible strains. Among the solvents tested, ethanol extract exhibited the highest antibacterial efficacy, followed by methanol extract, whereas aqueous extract showed negligible to low activity. Against S*. enterica*, ethanol extract demonstrated the strongest activity with a zone of inhibition of 19.66±0.57 mm at 20 mg/mL, which was significantly higher than methanol extract (12.33±0.57 mm) and aqueous extract (6±0 mm). Similarly, against *S. aureus*, the ethanol extract produced a maximum zone of 13.33±0.57 mm at 20 mg/mL compared to 10±1 mm for methanol extract. In case of *Escherichia coli*, moderate activity was observed, with ethanol and methanol extracts showing zones of 11.67±0.57 mm and 10±1 mm respectively at the highest concentration. Notably, none of the extracts of *N. oleracea* exhibited any inhibitory effect against *Pseudomonas aeruginosa* at any tested concentration, indicating resistance of this strain. Overall, the ethanol extract of *N. oleracea* at 20 mg/mL was found to be most effective, particularly against *S. enterica*, suggesting its potential as a source of antibacterial agents.

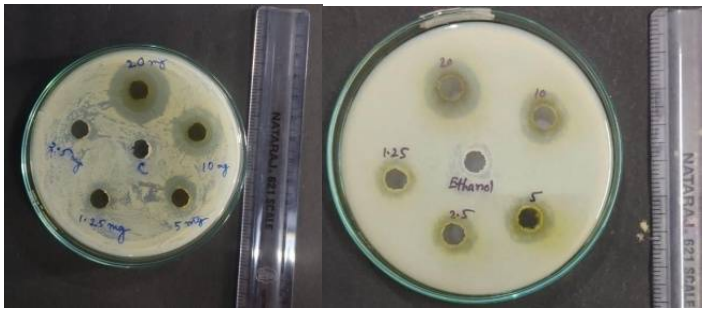


Fig: 1. Zone of inhibition of *N. oleracea* against *Staphylococcus aureus*& *Salmonella enterica*

The antibacterial potential of *C. asiatica* was assessed using aqueous, ethanol and methanol extracts at concentrations ranging from 1.25 to 20 mg/mL. The inhibitory activity was determined in terms of zone of inhibition (mm), including the well diameter of 6 mm, against four clinically important pathogens (Table 2 and Figure 2).

Table:2 Antibacterial activity (zone of inhibition) of *C. asiatica* against four pathogenic bacteria

Pathogen	Extract	1.25mg/ml	2.5mg/ml	5mg/ml	10mg/ml	20mg/ml
SA	Distilled water	6±0	6±0	6±0	6±0	6±0
	Ethanol	6±0	6.33±0.57	8.66±0.57	12±1	16±1
	Methanol	6±0	6.33±0.27	9.66±0.57	11.66±0.57	13.66±0.57
SE	Distilled water	6±0	6±0	6±0	6.33±0.57	7.33±0.57
	Ethanol	6±0	6±0	8±0	10.33±1.54	16.33±0.57
	Methanol	6±0	6±0	6±0	7±0	9.66±0.57
PA	Distilled water	6±0	6±0	6±0	6±0	6±0
	Ethanol	6±0	6±0	6.66±0.57	9±0	11±1
	Methanol	6±0	6±0	6±0	6.33±0.57	7.66±0.57
EC	Distilled water	6±0	6±0	6±0	7.66±0.57	9.33±0.57
	Ethanol	6±0	7±0	9±1	11.33±0.57	14.66±0.57
	Methanol	6±0	6±0	6±0	8±0	10±0

Values are mean ± SD of triplicate experiments. SA= *Staphylococcus aureus*, SE= *Salmonella enterica*, PA= *Pseudomonas aeruginosa*, EC= *Escherichia coli*. Zone of inhibition (in mm) including the diameter of well 6 mm.

All the extracts exhibited dose-dependent inhibition, with the highest activity observed at 20 mg/mL. Of the solvents evaluated, ethanol proved to be the most effective extracting solvent, followed by methanol, while distilled water showed comparatively weak activity. The ethanol extract was highly active against Gram-positive *S. aureus*, producing an inhibition zone of 16±1 mm at 20 mg/mL whereas the methanol and aqueous extracts at the same concentration showed 13.66±0.57 mm and no inhibition (6±0 mm) respectively. Against Gram-negative *S. enterica*, a similar trend was observed, with maximum inhibition of 16.33±0.57 mm in ethanol extract at 20 mg/mL, which was markedly higher than that of methanol extract (9.66±0.57 mm).For*E. coli*, the ethanol extract displayed a zone of 14.66±0.57 mm at the highest dose, compared to 10±0 mm and 9.33±0.57 mm for methanol and aqueous extracts. Notably, *C. asiatica* showed inhibitory effect against *P. aeruginosa*, with ethanol extract recording 11±1 mm at 20 mg/mL, while methanol and aqueous extracts exhibited only marginal activity (7.66±0.57 mm and 6±0 mm).

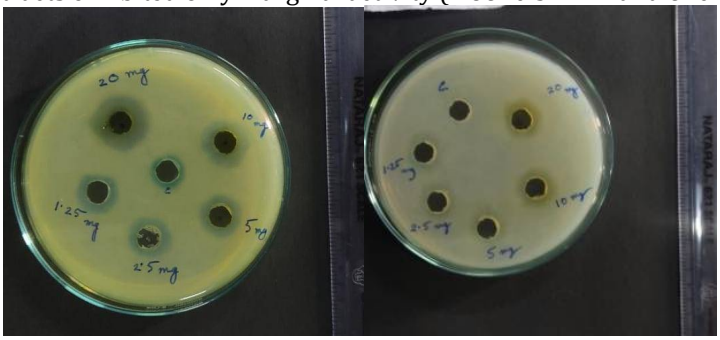


Fig: 2 . Zone of inhibition of *C. asiatica* against *Staphylococcus aureus* & *Pseudomonas aeruginosa*

Among all tested extracts of *C. asiatica*, ethanol extract demonstrated broad-spectrum and highest antibacterial activity, particularly against *S. aureus* and *S. enterica* indicating its therapeutic potential.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined to evaluate the lowest effective dose of the plant extracts required to inhibit bacterial growth (Table: 3). The assay was based on agar well diffusion method where the lowest concentration producing a measurable zone of inhibition was considered as MIC, following the protocol of Das *et al*. [26]. For *N. oleracea*, ethanol extract exhibited the most potent activity with an MIC of 1.25 mg/ml against both *S. aureus* and *S. enterica*, indicating that even at very low concentration it can inhibit these pathogens. Its methanol extract showed moderate efficacy with MIC of 5 mg/ml against the same organisms. Against *E. coli,* the MIC was recorded as 5 mg/ml for ethanol and 10 mg/ml for methanol extract, suggesting comparatively higher dose requirement. Distilled water extract showed weak activity with MIC of 20 mg/ml for *S. aureus* only while no MIC was observed for *S. enterica* and *E. coli* up to 20 mg/ml. Importantly, no MIC could be established against *P. aeruginosa* for any extract of *N. oleracea* due to complete resistance (NI). For *C. asiatica*, a broad-spectrum MIC pattern was observed. Ethanol extract showed lowest MIC of 2.5 mg/ml against S. aureus and E. coli, and 5 mg/ml against *S. enterica* and *P. aeruginosa*, demonstrating its effectiveness even against the intrinsically resistant *P. aeruginosa.* Methanol extract showed MIC of 2.5 mg/ml against *S. aureus* and 10 mg/ml against the other 3 pathogens. Aqueous extract was least effective, with MIC of 10 mg/ml against *S. enterica* and *E. coli* and no activity against *S. aureus* and *P. aeruginosa* at tested doses. Overall, lower MIC values (1.25-2.5 mg/ml) for ethanol extracts correlate with larger zones of inhibition and indicate high antibacterial potency at low doses, supporting their potential use as alternative antimicrobial agents for livestock bacterial infections. Higher MIC values (10-20 mg/ml or >20 mg/ml) for aqueous extracts reflect poor solubility of active principles in water.

Table: 3. Zone of inhibition

Pathogen	Plant	Extract	MIC
S. aureus	*N. oleracea*	Distilled water	20
		Ethanol	1.25
		Methanol	5
	C. asiatica	Distilled water	20
		Ethanol	2.5
		Methanol	2.5
S. enterica	*N. oleracea*	Distilled water	20
		Ethanol	1.25
		Methanol	5
	C. asiatica	Distilled water	10
		Ethanol	5
		Methanol	10
P. aeruginosa	*N. oleracea*	Distilled water	NI
		Ethanol	
		Methanol	
	C. asiatica	Distilled water	20
		Ethanol	5
		Methanol	10
E. coli	*N. oleracea*	Distilled water	20
		Ethanol	5
		Methanol	10
	C. asiatica	Distilled water	10
		Ethanol	2.5
		Methanol	10

Phytochemical profiling

The preliminary qualitative phytochemical analysis of leaf extracts of *N. oleracea* and *C. asiatica* was carried out to evaluate the presence of major classes of secondary metabolites in different solvent systems (Fig 3). The findings are presented in Table 4A, 4B &4C. The analysis revealed that both plant species are rich sources of diverse bioactive phytoconstituents. Among the three solvents employed, methanol extract showed the greatest diversity of phytochemicals, followed by ethanol and aqueous extracts. This indicates that methanol, due to its high polarity and superior solubilizing capacity is more

effective in extracting a wide range of polar and moderately polar secondary metabolites. Flavonoids, phenols, carbohydrates and reducing sugars were detected in almost all extracts of both plants, indicating their ubiquitous distribution. Alkaloids, tannins and saponins were predominantly present in methanol and ethanol extracts whereas they were absent or detected less frequently in aqueous extracts. Phytosterols, steroids and terpenoids were mainly recovered in organic solvent extracts and were largely absent in aqueous extracts, which may be attributed to their non-polar nature and poor water solubility. Overall, the results indicate that *N. oleracea* and *C. asiatica* are potential sources of diverse phytoconstituents with organic solvents, particularly methanol, being more suitable for their extraction.

Table: 4A Phytochemical screening of leaf extracts in ethanol solvent

Phytochemicals	*Neptunia oleracea*	*Centella asiatica*
Flavonoids	+	+
Phenols	+	+
Alkaloids	+	+
Tannins	+	-
Terpenoids	-	+
Saponins	+	+
Phytosterols	-	+
Steroids	+	+
Carbohydrates	+	+
Reducing Sugar	+	+

Table: 4B Phytochemical screening of leaf extracts in methanol solvent

Phytochemicals	*Neptunia oleracea*	*Centella asiatica*
Flavonoids	+	+
Phenols	+	+
Alkaloids	+	+
Tannins	+	+
Terpenoids	+	+
Saponins	+	+
Phytosterols	+	+
Steroids	-	+
Carbohydrates	+	+
Reducing Sugar	+	+

Table: 4C Phytochemical screening of leaf extracts in aqueous solvent

Phytochemicals	*Neptunia oleracea*	*Centella asiatica*
Flavonoids	-	+
Phenols	+	+
Alkaloids	-	+
Tannins	+	+
Terpenoids	-	-
Saponins	+	+
Phytosterols	-	-
Steroids	-	-
Carbohydrates	+	+
Reducing Sugar	+	+

Note:+ = Present, - = Absent

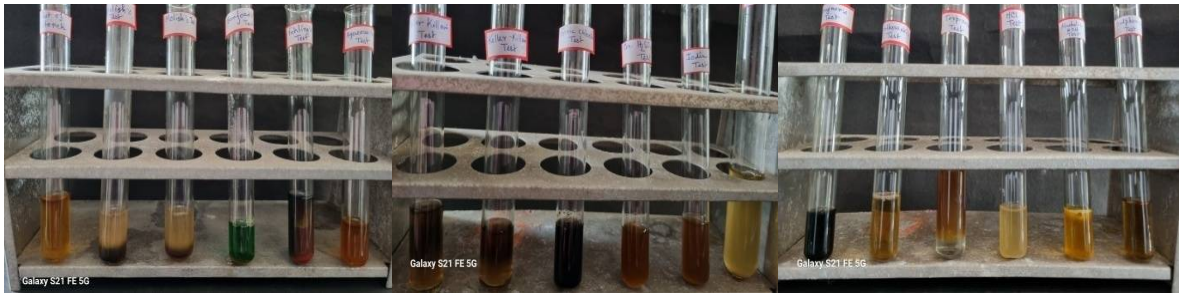


Fig: 3. Qualitative tests for preliminary phytochemical screening of two selected leaf extracts

GC-MS Analysis of Leaf Extracts

To characterize and identify the bioactive compounds contributing to the pharmacological potential, GC-MS analysis was carried out. Based on preliminary phytochemical screening, methanol leaf extracts of *N. oleracea* and *C. asiatica*, which exhibited the maximum diversity of secondary metabolites, were selected for GC-MS profiling. The analysis was performed by Gas Chromatography-Mass Spectrometry and the compounds were identified by comparing their mass spectral fragmentation patterns with the NIST library. The GC-MS chromatograms displayed numerous peaks at different retention times corresponding to various bioactive phytoconstituents with their peak area percentage indicating relative abundance. The detailed chemical profiles of the individual plant extracts are elaborated in the following sections.

The GC-MS analysis of methanol leaf extract of *N. oleracea* revealed the presence of ten major bioactive phytocompounds (Table 5). The compounds were identified based on retention time, peak area (%), molecular formula and NIST library match (>85% similarity).

The chromatogram (Fig 4) was dominated by phytosterols and polyunsaturated fatty acids. γ-sitosterol was detected as the predominant compound with highest peak area of 11.25% at RT 35.58 min ($C_{29}H_{50}O$, MW 414.71), followed by 9,12,15-octadecatrienoic acid (Z,Z,Z)- (α-linolenic acid, an omega-3 PUFA) with 5.877% peak area at RT 31.93 min ($C_{18}H_{30}O_2$, MW 278.43). Another significant sterol identified was stigmasta-5,22-dien-3β-ol (stigmasterol) at RT 36.28 min (0.664%, $C_{29}H_{48}O$, MW 412.69). The extract also contained saturated and branched fatty acids including pentadecanecarboxylic acid (hexadecanoic acid), octadecanoic acid (stearic acid) and methyl 14-methyl-pentadecanoate, along with a diterpene alcohol, 3,7,11,15-tetramethylhexadec-2-en-1-ol (phytol) at RT 33.97 min, and a fatty alcohol acetate (11-tetradecen-1-ol, acetate). Minor acetylenic fatty acid, 2-hexynoic acid was detected in trace amount (0.05%). The presence of phytosterols, omega-3 fatty acids and phytol indicate the therapeutic potential of the methanol extract and supports its antioxidant, anti-inflammatory and antidiabetic activities.

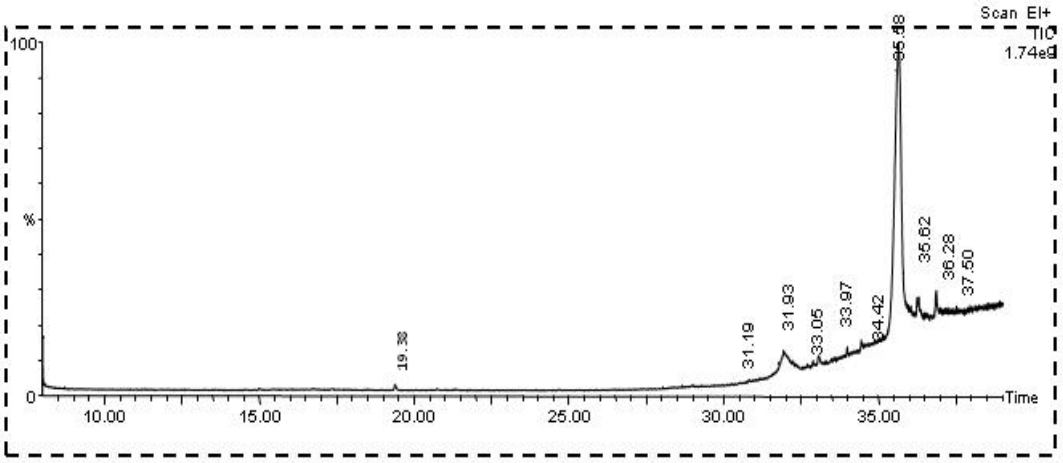


Fig: 4. GC-MS chromatogram of *N. oleracea* leaf in methanol extracts

Table 5: Major phytochemical compounds identified in methanol extracts of *N. oleracea* leaves by GC-MS analysis

Sl no.	Retention time	Peak area (%)	Compound Name	Type of Compound / Chemical Class	Molecular Formula	Molecular weight
1	19.38	0.050	2-hexynoic acid	Acetylenic fatty acid	$C_6H_8O_2$	112
2	31.19	0.083	11-tetradecan-1- ol, acetate,(Z)-	Fatty alcohol acetate	$C_{16}H_{30}O_2$	254
3	31.93	5.877	9,12,15-octadecatrienoic acid, (z,z,z)-	Omega-3 polyunsaturated fatty acid	$C_{18}H_{30}O_2$	278
4	33.05	0.870	9,12,15-Octadecatrienoic acid, methyl ester	Fatty acid methyl ester	$C_{19}H_{32}O_2$	292
5	33.97	0.040	3,7,11,15-tetramethylhexadec-2-en-1-ol	Diterpene alcohol	$C_{20}H_{40}O$	296
6	34.42	0.614	Methyl 14-methyl-pentadecanoate	Long-chain fatty amide	$C_{17}H_{34}O_2$	270
7	35.58	11.25	γ-sitosterol	Phytosterol	$C_{29}H_{50}O$	414
8	35.62	0.350	Pentadecanecarboxylic acid	Saturated fatty acid	$C_{16}H_{32}O_2$	256
9	36.28	0.664	Stigmasta-5,22-dien-3β-ol	Phytosterol	$C_{29}H_{48}O$	412
10	37.50	0.094	Octadecanoic acid	Saturated fatty acid	$C_{18}H_{36}O_2$	284

The GC-MS phytochemical profiling of the methanol leaf extract revealed the presence of nine major bioactive compounds (Table 5). The compounds were identified based on retention time, peak area percentage and NIST library match. The chromatogram (Fig 5) was dominated by sesquiterpenoid and fatty acid esters. Longipinenepoxide, a sesquiterpene epoxide, was found as the major constituent with highest peak area of 15.35% at RT 32.23 min ($C_{15}H_{24}O$, MW 220), followed by 5,8,11,14,17-eicosapentaenoic acid, methyl ester (EPA ester) at 9.09% (RT 32.66 min, $C_{21}H_{32}O_2$, MW 316) and the diterpene alcohol, phytol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol at 5.16% (RT 34.76 min, $C_{20}H_{40}O$, MW 296). The extract also contained saturated fatty acids such as eicosanoic acid (0.85%) and n-hexadecanoic acid (2.76%), polyunsaturated fatty acid ester methyl stearidonate (2.74%) and diterpene alkene 3-methylene-7,11,15-trimethylhexadec-1-ene (1.22%). Notably, the presence of pentacyclic triterpenoids, asiatic acid (0.86%, RT 35.83 min, $C_{30}H_{48}O_5$, MW 488) and madecassic acid (0.07%, RT 36.22 min, $C_{30}H_{48}O_6$, MW 504) confirms the characteristic triterpenoid profile of the plant. The co-occurrence of phytol, sesquiterpene epoxide and bioactive triterpenoids support its therapeutic potential.

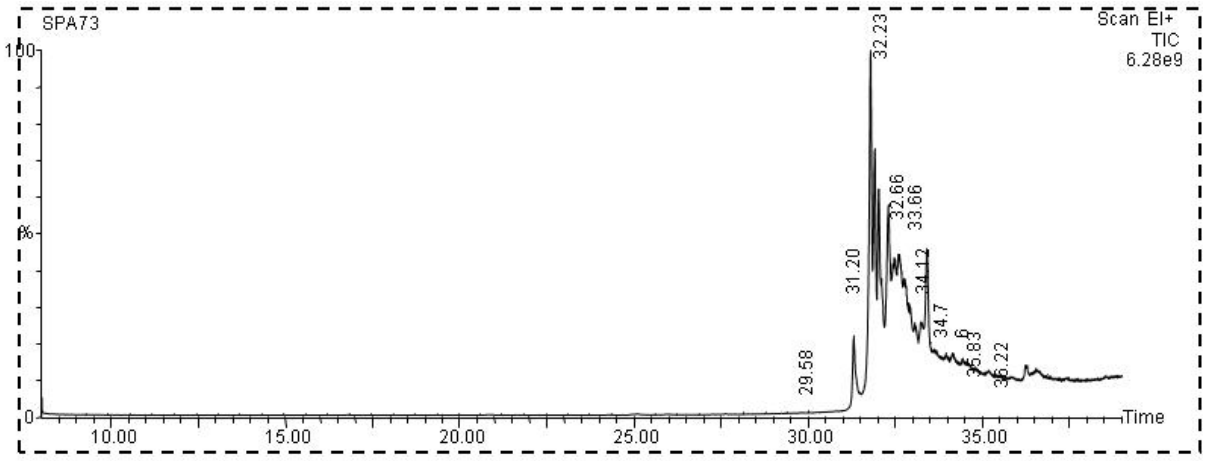


Fig: 5. GC-MS chromatogram of *C. asiatica* leaf in methanolextracts

Table 6: Major phytochemical compounds identified in methanol extracts of *C.asiatica* leaves by GC-MS analysis

Sl no.	Retention time	Peak area (%)	Compound Name	Type of Compound / Chemical Class	Molecular Formula	Molecular weight
1	29.58	0.85	Eicosanoic acid	Saturated fatty acid	$C_{20}H_{40}O_2$	312
2	31.20	2.74	methyl stearidonate	Polyunsaturated fatty acid ester (Omega-3)	$C_{19}H_{30}O_2$	290
3	32.23	15.35	longipinenepoxide	Sesquiterpene epoxide	$C_{15}H_{24}O$	220
4	32.66	9.09	5,8,11,14,17-eicosapentaenoic acid, methyl ester, (all-z)-	Polyunsaturated fatty acid ester (EPA)	$C_{21}H_{32}O_2$	316
5	33.66	2.76	n-Hexadecanoic acid	Saturated fatty acid	$C_{16}H_{32}O_2$	256
6	34.12	1.22	3-Methylene-7,11,15-trimethylhexadec-1-ene	Diterpene alkene	$C_{20}H_{38}$	278
7	34.76	5.16	Phytol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol	Diterpene alcohol	$C_{20}H_{40}O$	296
8	35.83	0.86	Asiatic acid	Pentacyclic triterpenoid	$C_{30}H_{48}O_5$	488
9	36.22	0.07	Madecassic acid	Pentacyclic triterpenoid	$C_{30}H_{48}O_6$	504

DISCUSSION

A variety of herbal products have been shown to possess advantageous effects and therapeutic attributes, such as antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory functions, while not negatively impacting growth and feed efficiency, thus serving as growth-promoting feed additives in animal husbandry [28]. The present study was designed to scientifically validate the ethno-veterinary claims of*N. oleracea* and *C. asiatica* through systematic evaluation of antibacterial activity, qualitative phytochemical screening and GC-MS based metabolite profiling. These plants were usually used as fodder for many domestic and wild animals. The results obtained provide substantial evidence for their potential as alternative antimicrobial agents against common veterinary pathogens. In present communication, the antibacterial activity of *N. oleracea* and *C. asiatica* leaf extracts was evaluated using the agar well diffusion method against four clinically important bacteria, including three Gram-negative organisms *S. enterica*, *E. coli*, *P. aeruginosa* and one Gram-positive organism *S. aureus*. The zone of inhibition exhibited a clear dose-dependent response with maximum activity at 20 mg/mL, which is in agreement with earlier reports on concentration-dependent activity of plant extracts [22,29]. Among the three solvents tested, ethanol extract displayed the highest efficacy, followed by methanol and aqueous extracts. This pattern can be explained by the polarity and extraction efficiency of solvents. Ethanol, being less polar than methanol and water, efficiently extracts both polar polyphenols and moderately non-polar compounds such as sterols, terpenoids and fatty acid esters that possess strong antimicrobial properties [30]. Similar observations have been reported where ethanolic extracts of *Neptunia* spp. showed higher inhibition compared to aqueous extracts [31].For *N. oleracea*, ethanol extract showed the most potent activity against *S. enterica* with a zone of 19.66 ± 0.57 mm at 20 mg/mL and a minimum inhibitory concentration (MIC) of 1.25 mg/mL. This is of significant veterinary importance, as *Salmonella* is a major cause of enteritis and septicemia in poultry, cattle and pigs and is often associated with antibiotic resistance [32]. The strong activity against *S. aureus* (13.33 ± 0.57 mm, MIC 1.25 mg/mL) also correlates with previous findings were*N. oleracea* shoot extract inhibited *S. aureus* with MIC of 2 mg/mL [19]. Moderate activity against *E. coli* (11.67 ± 0.57 mm) was also noted while complete resistance was observed for *P. aeruginosa*. The intrinsic resistance of *P. aeruginosa* is well documented and attributed to its low outer membrane permeability and overexpression of MexAB-OprM efflux pumps [33,34].In contrast, *C. asiatica* exhibited a broader spectrum of activity. Its ethanol extract inhibited all four pathogens, including *P. aeruginosa* (11 ± 1 mm at 20 mg/mL, MIC 5 mg/mL), which was resistant to *N. oleracea*. Maximum activity was observed against *S. aureus* (16 ± 1 mm, MIC 2.5 mg/mL) and *S. enterica* (16.33 ± 0.57 mm, MIC 5 mg/mL). The ability of *C. asiatica* to inhibit *P. aeruginosa* is noteworthy and can be attributed to its triterpenoid constituents which act as efflux pump inhibitors [35,36]. Earlier studies have reported that *C. asiatica* extracts possess anti-pseudomonal activity with zones ranging from 10-18 mm, supporting our findings [37,38].

Overall, a consistent trend of antibacterial activity in the order *S. aureus*, *S. enterica*, *E. coli*, and *P. aeruginosa* was observed for both extracts, reflecting a typical susceptibility pattern. Gram-positive bacteria are more susceptible due to direct interaction of hydrophobic compounds with the peptidoglycan layer whereas Gram-negative bacteria possess an additional lipopolysaccharide (LPS) outer membrane that acts as a permeability barrier [39]. The low MIC values of 1.25-2.5 mg/mL for ethanol extracts indicate high potency at low doses, which is desirable for development of phytotherapeutics and is consistent with the criteria of strong antibacterial activity defined as MIC < 5 mg/mL [40]. Preliminary phytochemical screening provides an overview of secondary metabolite diversity and helps in selecting appropriate solvents for detailed profiling.

In the present study, screening of ethanol, methanol and aqueous extracts revealed presence of flavonoids, phenols, carbohydrates and reducing sugars in almost all extracts, indicating their ubiquitous nature (Table 4A, 4B, 4C). Methanol extract showed the greatest diversity, containing flavonoids, phenols, alkaloids, tannins, terpenoids, saponins, phytosterols, steroids, carbohydrates and reducing sugars in both plants. Ethanol extract showed slightly less diversity, while aqueous extract showed the least. The superior extraction efficiency in the order methanol, followed by ethanol and aqueous is well documented, as methanol with high polarity index (5.1) and small molecular size can efficiently solvate both polar and moderately non-polar metabolites [41,42]. Similar results were reported in *C. asiatica* where methanol recovered maximum phytochemicals compared to water [43]. The presence of alkaloids, tannins and saponins predominantly in organic extracts is significant from antimicrobial perspective. Alkaloids intercalate with bacterial DNA and inhibit cell division, tannins inactivate microbial adhesins and enzymes through protein precipitation, while saponins cause membrane disruption through interaction with cholesterol [22,44]. Flavonoids and phenols, detected in all extracts, are potent antimicrobial agents that inhibit nucleic acid synthesis and energy metabolism. Notably, phytosterols, steroids and terpenoids were absent in aqueous extracts but present in methanol and ethanol extracts of both plants. This is attributed to their non-polar nature and poor water solubility. Since these compounds are major contributors to antibacterial activity through membrane disruption, their absence explains the weak activity of aqueous extracts observed in antimicrobial assay [45]. The rich phytochemical diversity in methanol extract justified its selection for GC-MS analysis to identify specific bioactive molecules responsible for antibacterial effects.

GC-MS analysis was carried out for methanol extracts based on maximum phytochemical diversity. GC-MS can quickly determine volatiles and gases produced by plant tissues or organs [46]. The identification was performed using NIST library with >85% similarity. Ten major compounds were identified (Table 5, Fig. 4), dominated by phytosterols and polyunsaturated fatty acids. γ-sitosterol (11.25% at RT 35.58 min) was the predominant compound. γ-sitosterol is a documented antimicrobial phytosterol that disrupts bacterial membrane fluidity, causing leakage of potassium ions and ATP [47,48]. Stigmasterol (0.664%) has been shown to inhibit biofilm formation in *S. aureus* [49]. The second major group 9,12,15-octadecatrienoic acid [α-linolenic acid, 5.87%] and its methyl ester are omega-3 PUFAs that inhibit enoyl-acyl carrier protein reductase, a key enzyme in bacterial fatty acid synthesis, particularly effective against *Salmonella* and *E. coli* [50]. The diterpene alcohol phytol induces oxidative stress and acts as an efflux pump inhibitor in multidrug-resistant *P. aeruginosa* [51,52]. Saturated fatty acids such as pentadecanecarboxylic acid and octadecanoic acid cause membrane depolarization [53]. The synergistic action of these metabolites explains the strong activity against *S. enterica* and *S. aureus* and moderate activity against *E. coli*, while resistance of *P. aeruginosa* may be due to efficient efflux.

Nine compounds were identified (Table 6; Fig. 5), dominated by longipinenepoxide (15.35%), EPA methyl ester (9.09%) and phytol (5.16%). Longipinenepoxide, a sesquiterpene epoxide, possesses a reactive epoxide ring that alkylates bacterial proteins [54]. The most important finding is detection of asiatic acid (0.86%) and madecassic acid (0.07%), pentacyclic triterpenoids that are chemotaxonomic markers of *C. asiatica*[55]. Asiatic acid inhibits NorA efflux pump and biofilm formation in *S. aureus*[56], while madecassic acid inhibits DNA gyrase and FtsZ cell division protein in Gram-negative bacteria [57]. The higher susceptibility of *S. aureus* is due to absence of LPS barrier. Phytol and methyl stearidonate act as natural permeabilizers, facilitating entry of triterpenoids into *P. aeruginosa* and *E. coli*, explaining its broad-spectrum activity including anti-pseudomonal effect [33,58]. The presence of n-hexadecanoic acid (2.76%) further contributes through quorum sensing inhibition [59]. In conclusion, the comprehensive antibacterial, phytochemical and GC-MS studies validate the ethno-veterinary use of *N. oleracea* and *C. asiatica*. The identified phytosterols, omega-3 PUFAs, phytol, sesquiterpene epoxide and pentacyclic triterpenoids exert antimicrobial action through multi-target mechanisms including membrane disruption, FabI and DNA gyrase inhibition, anti-biofilm and efflux pump inhibition [60]. This makes them

promising candidates for development of herbal antimicrobial formulations to address antibiotic resistance in livestock.

CONCLUSION

The present investigation validates the ethno-veterinary importance of *N. oleracea* and *C. asiatica* as promising sources of natural antibacterial agents against major livestock pathogens. Ethanolic leaf extracts demonstrated potent, dose-dependent activity with low MIC values (1.25-2.5 mg/mL), with *N. oleracea* being highly effective against *S. enterica* and *S. aureus*, while *C. asiatica* exhibited broad-spectrum activity including against *P. aeruginosa*. Phytochemical screening and GC-MS profiling revealed that methanol extracts contain maximum diversity of bioactive compounds with γ-sitosterol, α-linolenic acid, phytol, longipinenepoxide, asiatic acid and madecassic acid as key antibacterial principles acting through multi-target mechanisms. The findings provide scientific justification for traditional use of these plants and highlight their potential for development of herbal formulations and antibiotic adjuvants to combat antimicrobial resistance in veterinary medicine. Future research should focus on bioassay-guided fractionation, safety evaluation, bactericidal dynamics and in vivo studies to translate these findings into standardized herbal formulations for veterinary application.

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Competing interests

The authors have no conflict of interest to declare.

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Authors' contribution

Each author has made substantial effort to complete this research work. Suvendu Pal: Literature study, design of work, data curation, statistical analysis, writing- original draft, review & editing; Somen Dey: Resources, writing review and editing; Pritish Mitra: Resources, writing review & editing; Dr. Anup Kumar Sarkar: Resources, writing review & editing;Dr. Asish Mandal: Conceptualization, supervision, validation, writing- review & editing. All authors have read and approved the final manuscript.

Data availability

All the generated or analysed data of this study are included in manuscript.

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