Original Article

Synergistics Effect Of Cow Urine And Essential Oils Against Bacterial Isolates

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Abstract

Cow urine shows a synergistic effect with various naturally occurring oils and herbs. In this study, we analyzed how the antibacterial activity of cow urine gets enhanced in the presence of essential oils. The observations suggested that 0.5% or 1% essential oil in combination with cow urine makes cow urine activity better than raw cow urine. Essential oils contain active components and phenolic-like compounds as active ingredients that enhance their antibacterial properties. These active components degrade the bacterial cell wall and inhibit bacterial survival in the oil's environment.

A total of 15 samples were prepared from cow urine (CU+0.5% E.O. and CU+1% E.O.). The mixture of cow urine and essential oils were processed and shows better activities than raw cow urine. The activity of all these samples is because of phenolic groups present in cow urine and essential oils.

The activity is carried out by agar well diffusion technique against various bacterial isolates and the zone of inhibition was measured in mm. The essential oils of Ginger, Spearmint, Star anise, and Clove were found highly effective in the presence of Cow Urine against the Escherichia coli and Staphylococcus aureus followed by Klebsiella pneumoniae and Bacillus subtilis. It has been concluded from the present study that the (treated) cow urine enhances the activity of essential oils and showed a synergistic effect against the bacterial isolates.

Keywords: Cow urine, cinnamon oil, cumin oil, clove oil, garlic oil, Bacterial isolates.

Introduction

Cow (Bos indica) urine is described in Ayurveda for the treatment of several diseases and is regarded as an immunomodulator and elixir. It is widely used in cardiac, respiratory, and renal diseases, indigestion, stomachache, diarrhea, edema, jaundice, anemia, hemorrhoids, and skin diseases. Cow urine is a very complex mixture containing several inorganic salts of calcium, iron, magnesium, and about fifteen others. It also has organic compounds in varying amounts, including enzymes, hormones, cytokines, amino acids, vitamins, etc. [1,2].

Research has shown that essential oils exhibit various properties including bactericidal, fungicidal, virucidal, insecticidal, antiparasitic, medicinal, and cosmetic applications. They are also being used nowadays as flavor enhancers in the pharmaceuticals, cosmetic, sanitary, agricultural, and food industries. They also act as antioxidants [3-5]. They have been used either alone or in combination with other preservation methods [3].

Essential oils are volatile in nature due to the presence of different kinds of volatile molecules like terpenes, terpenoids, phenol-derived aromatic components, and aliphatic components [3,4]. Most of the essential oils show the presence of chemicals like dipentene /limonene, alpha-terpineol, linalool, 4-terpineol, eucalyptol, gamma-terpinene, alpha-terpineol, and linalyl acetate. The most active antibacterial chemical constituent is 1,8-cineole in a few essential oils [5-7].

The purpose of this research work was to enhance the antibacterial effects of essential oils by combining them with treated cow urine (pH – 3.8) and to preserve, deodorize, and flavor it to make it palatable using natural products only.

Materials and Methods

1. Collection of CU (Cow Urine)

Fresh cow urine was collected in a sterile container from Gau gram organic products situated at Kota, Rajasthan. The urine was filtered through 5-micron filter paper to get rid of debris and precipitated material.

2. Isolation and identification of microorganisms

The strains of Escherichia coli, Staphylococcus aureus, Klebsiella sp., and Bacillus were isolated from various sources. Bacteria were identified by colony morphology (shape, structure, color, pattern, size) gram staining, microscopical observations, and biochemical testing (indole production, Catalase, Lactose, Methyl red) [4].

Ethical Statement: No ethical statement is required as all the laboratory isolates were used in the study.

3. Preparation of inoculums

The bacterial isolates were maintained on nutrient agar and subcultured every three days. Each bacterial isolate was suspended in 5 ml of saline solution and adjusted to give a concentration of bacterial cells equivalent to 0.5% McFarland turbidity standard prior to the antibacterial testing [5]

4. Treatment of CU

To a fresh Cow urine sample (pH 8.2), 2% Citric Acid is added to bring its pH from 8.2 to 4.9. To the above-prepared sample, 0.8% Ascorbic Acid (Vitamin C) and 1.5% Lactic Acid is added to make it pH.3.8. Now this prepared sample is filtered through a 5-micron filter. Non-caloric sweeteners and food color are added afterward.

5. Sources of Essential oil

Essential oils are obtained from Himani International Delhi.

S. No.	Essential Oils	Active Component
1.	Carom oil	Phenolic carvacrol & thymol
2.	Cinnamon oil	Cinnamaldehyde, Transcinnamaldehyde
3.	Clove oil	Eugenol, eugenyl acetate, caryophyllene
4.	Cumin oil	Cuminaldehyde
5.	Garlic oil	Diallyl thiosulfonate, diallyl sulfide, diallyl disulfide, diallyl trisulfide, E/Z - ajoene, S - allyl – cysteine, S – allyl – cysteine sulfoxide
6.	Ginger oil	Gingerol & paradol, shogaols & zingerone
7.	Iso Eugenol	Iso Eugenol
8.	Orange oil	d-limonene (a monocyclic monoterpene)
9.		
10.	Oregano oil	Carvacol, thymol, p-cymene, γ-terpinene, caryophyllene
11.	Rosemary oil	1,8-cineol, camphor, α-pinene, limonene, camphene, and linalool

12.	Rose Water	Phenyl Ethyl Alcohol
13.	Spearmint oil	Carvone and limonene
14.	Star Anise oil	Trans – anethole
15.	Tea Tree oil	Terpinen-4-ol, γ-terpinene, 1,8-cineole, α-terpinene, α-terpineol, p-cymene, and α-pinene

Table 1: Representation of several essential oils with their active components

6. Samples Preparation

Four types of samples were prepared.

- **Sample 1 (CU):** Treated cow urine at pH 3.8.
- **Sample 2 (EO):** 90µl of essential oil mixed with 10µl of tween 20.
- **Sample 3 (EOCU01):** 985µl treated cow urine mixed with 0.5% (5µl) essential oil and 10µl of tween 20.
- **Sample 4 (EOCU02):** 980µl treated cow urine mixed with 1%(10µl) essential oil and 10µl of tween 20.

7. Antibacterial screening of the treated cow urine and combination of Cow urine with Essential oil

Antimicrobial activity was evaluated by the Agar well diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) [6]. The zones of inhibition (ZOI) were reported in millimeters (mm) and used as references for the antimicrobial assay. The assay was performed and Nutrient agar plates were inoculated with bacterial strain under aseptic conditions. Subsequently, wells of 6mm diameter were punched with a sterile cork borer, and a volume of 100 μL of all types of samples prepared (as mentioned in section 6) is introduced into the well and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 37°C for 24 h. For comparison, raw cow urine alone and Essential oil alone were used as negative controls. Antibiotics, Ciprofloxacin were used as a positive control. The antibacterial activity was assessed in agar plates based on the size of the inhibition zone diameter surrounding the wells. For each test, three replicates were performed, and the results were calculated as mean±SD.

Results and Discussions

The samples obtained from various sources were processed in the laboratory. The characterization of microorganisms was performed to know the bacterial genera present in the samples obtained (Table 2).

Table:2. Characterization of Bacterial Strains

S. No.	Bacteria	Sources	Characterization
1.	E. coli	Cow Dung	Microscopic observations: Gram -ve Rod Shaped Bacilli. Cultural Characteristics: Greenish colonies on EMB Agar Media. Biochemical Characteristics: Indole +ve
2.	Klebsiella sp.	Urine	Microscopic observations: Gram -ve Rod Shaped Bacilli. Cultural Characteristics: Pinkish Round colonies on MacConkey Agar media. Biochemical Characteristics: Indole -ve

3.	Staphylococcus aureus	Abscess and other Skin Lesions	Microscopic observations: Gram +ve Rod Shaped Cocci in Bunches Cultural Characteristics: Golden Yellow colonies on Nutrient Agar media Biochemical Characteristics: Catalase +ve
4.	Bacillus sp.	Soil	Microscopic observations: Gram +ve Rod Shaped Bacilli. Cultural Characteristics: Grey-white, granular colonies with a less wavy edge on Nutrient Agar media. Spore staining: Endospore-forming bacilli

The Essential oils of Ginger, Spearmint, and Star anise were found highly effective against all the isolates obtained from several sources (Tables 3 and 4). The zone of inhibition was found maximum in ginger oil+ CU (25 mm) against S. aureus followed by spearmint oil (25 mm). The inhibitory effect of spearmint oil (23mm) and star anise oil (24.33mm) was obtained against B. subtilis. The effectiveness of spearmint oil (25mm) was found against E. coli and ginger oil (22.33mm) against Klebsiella pneumoniae. The results also showed the effective inhibitory effect of Clove oil and Cinnamon oil in combination with cow urine against the bacterial isolates (Tables 3 and 4). Similar studies were reported by Zemek et al., 1979 and Laekeman et al., 1990[7,8]. Their findings stated that the antibacterial activity of eugenol present in clove oil is due to its free hydroxyl group and its position of double bonds in α, and β positions of the side chain, and a methyl group in the γ position. Cinnamon Oil has been reported to inhibit bacteria through anti-quorum sensing effects; inhibiting cell division, ATPase, biofilm formation membrane porin, and mobility; altering the lipid profile and thereby acting cell membrane producing lumps and autoaggregation [9],[10]. The effect of oregano oil was found less effective as compared to other Essential oils, although some studies reported that Carvacrol present in oregano oil possessed a good antibacterial activity by permeabilization and depolarization of the cytoplasmic membrane [11].

In Carom oil phenols especially thymol and some carvacrol provide antimicrobial properties by obstructing the peroxidation of liposome phospholipids in a concentration-dependent manner [12]. However, in our studies, the findings were not suitable due to the less inhibitory effect of EO. The ginger oils have analgesic, sedative, antipyretic, antibacterial, and gastrointestinal tract motility effects. [13]. Many studies indicate that limonene is the most significant ingredient that plays an essential role in orange peel oil consistency, and demonstrates its antimicrobial, antioxidant, and toxic agents [15]. Similar results of the highly effective Antibacterial activity of Ginger oil were obtained from the findings of the present study. The mechanism of action of Cumin oil in some bacterial cells can be represented by cell elongation, repression of capsule expression in some bacteria, and inhibition of Urease activity [16,17]. Our findings reported less inhibitory effect of Cumin EO on bacterial isolates. The antimicrobial activities of anise extracts may be attributed to their phenolic contents since numerous phytochemical studies indicated the presence of noticeable amounts of phenolic compounds in anise [18],[19]. In the present study, the effectivity of Anise oil was found less inhibitory against the laboratory isolates. Allicin, diallyl disulfide, and allicin, which are responsible for the strong smell of garlic and have antibacterial and antioxidant properties [20,21], were positively not found high in the present study.

The star anise EO showed a high inhibitory effect against the bacterial isolates, especially E. coli (Table 3) and S. aureus (Table 4). The mechanism of action of carvone on the growth microorganisms includes the destabilization of the phospholipid bilayer structure, interaction with membrane enzymes and proteins, and its act as a proton exchanger reducing the pH gradient across the membrane [22]. The antimicrobial activity of star anise extract may be attributed to its essential oils, particularly the anethole present in the dried fruit [23],[24]. Tea tree oil exhibits a broad-spectrum antimicrobial activity that can be principally attributed to terpinen-4-ol [25-27], while the present study reported less inhibitory action of tea tree oil. Phenethyl alcohol or phenyl ethyl alcohol (PEA) is a naturally occurring aromatic compound that is found in various flowers including rose, hyacinth, neroli, ylang-ylang, geranium, and champaca [26-28]]. The bacteriostatic activity of PEA was primarily reported by Lilley and Brewer [26], later studies revealed that PEA inhibits DNA synthesis [27-29].

The results for all extracts were more effective against the Gram-positive bacteria (S. aureus) compared to the results for the Gram-negative ones. The higher resistance of the Gram-negative bacteria could be due to the complexity of the cell wall of this group of microorganisms. Indeed, the external membrane of Gram-negative bacteria renders highly hydrophilic surfaces whereas the negative charge of the surface of the Gram-positive wall may reduce their resistance to antibacterial compounds []. The significant data for studies were considered by using the efficient biostatistics analysis [30]. Several researchers are working on the antimicrobial activity of essential oils [31-32] and natural phytocompounds against bacterial as well as fungal isolates [33-34]. Nagarajan, 2022 advanced his findings in the field of virology [35].

Table 3: - Antibacterial activity of Cow Urine with essential oils against Staphylococcus aureus and Bacillus subtilis

	Staphylococcus aureus (mm)						**Bacillus subtilis (mm)**				
SAMPLE	**ANTIBIOTIC (mm)**	**EO**	**CU**	**EOCU01**	**EOCU02**		**ANTIBIOTIC (mm)**	**EO**	**CU**	**EOCU01**	**EOCU02**
Cinnamon oil	23.33±0.66	21.66±0.85	19±0.57	22±0.52	24±1.15		23.33±0.66	19.66±0.85	18±0.57	20±0.57	21±1.15
Clove oil	23.33±0.66	20.66±2.04	19±0.57	21±0.33	22±0.57		23.33±0.66	18.66±2.04	18±0.57	20±0.57	22±1.15
Carom oil	23.33±0.66	19.33±3.75	19±0.57	20±0.88	21±0.88		23.33±0.66	18.33±3.75	18±0.57	20±0.57	22±1.15
Oregano oil	23.33±0.66	18.33±0.66	19±0.57	19±0.57	20±1.15		23.33±0.66	16±0.66	18±0.57	18±0.57	22±0.57
Ginger oil	23.33±0.66	20.33±0.53	19±0.57	23±0.37	25±1.15		23.33±0.66	19.33±0.53	18±0.57	20±0.57	22.33±0.88
Orange oil	23.33±0.66	17±0.47	19±0.57	18±0.77	21±1.15		23.33±0.66	17±0.47	18±0.57	20±0.57	21±1.15
Cumin oil	23.33±0.66	20±0.57	19±0.57	21.33±0.33	23±1.15		23.33±0.66	19±0.57	18±0.57	21±0.57	23±1.15
Anise Bush oil	23.33±0.66	16±0.33	19±0.57	17±0.57	20±1.15		23.33±0.66	15±0.33	18±0.57	18±0.57	20±1.15
Garlic oil	23.33±0.66	19±0.57	19±0.57	20±0.33	22±0.88		23.33±0.66	18±0.57	18±0.57	18±0.57	19.33±0.88
Spearmint oil	23.33±0.66	22±0.57	19±0.57	23±0.57	25±0.66		23.33±0.66	20±0.57	18±0.57	21±0.57	23±0.57
Star Anise oil	23.33±0.66	21±0.57	19±0.57	22±0.33	24±0.88		23.33±0.66	20±0.57	18±0.57	21±0.57	24.33±0.88
Tea Tree oil	23.33±0.66	19±0.57	19±0.57	20±0.88	22±1.15		23.33±0.66	18±0.57	18±0.57	18±0.57	19±1.15
Rosemary oil	23.33±0.66	18±0.57	19±0.57	20±0.57	22±0.45		23.33±0.66	17±0.57	18±0.57	17±0.57	19.33±0.88
Phenyl Ethyl Alcohol	23.33±0.66	16±0.57	19±0.57	18±0.57	21±1.15		23.33±0.66	15±0.57	18±0.57	18.33±0.66	21.66±0.88
Iso Eugenol	23.33±0.66	18±0.57	19±0.57	20±1.15	21±1.15		23.33±0.66	18±0.33	18±0.57	20±0.88	21.33±0.88

CU: - Cow Urine (Treated at pH 3.8); EO=Essential Oil; EOCU01:- Essential oil (0.5%) + Cow urine; EOCU02= Essential oil (1%) + Cow urine; ANTIBIOTIC: - Ciprofloxacin (16mg/ds)

Table 4: - Antibacterial activity of Cow Urine with essential oils against Escherichia coli & Klebsiella sp.

SAMPLE	Escherichia coli (mm)						Klebsiella sp. (mm)				
	ANTIBIOTIC (mm)	EO	CU	EOCU01	EOCU02		ANTIBIOTIC (mm)	EO	CU	EOCU01	EOCU02
Cinnamon oil	22.06±0.52	20±0.57	18.33±0.33	21±0.57	22±1.15		23±0.57	18±0.57	17±0.57	19±0.57	22±1.15
Clove oil	22.07±0.52	19±1.52	18.33±0.33	20±0.57	22±0.57		23±0.57	16±0.57	17±0.57	20±0.57	21±1.15
Carom oil	22.07±0.52	18±0.57	18.33±0.33	19±0.57	21±0.88		23±0.57	18±0.57	17±0.57	19±0.57	22±1.15
Oregano oil	22.07±0.52	15±0.57	18.33±0.33	16±0.57	19±1.15		23±0.57	16±0.57	17±0.57	18±0.57	20±0.57
Ginger oil	22.07±0.52	20±0.57	18.33±0.33	22±0.57	24±1.15		23±0.57	18±0.57	17±0.57	20±0.57	22.33±0.88
Orange oil	22.07±0.52	16±0.57	18.33±0.33	17±0.57	19±1.15		23±0.57	18±0.57	17±0.57	19±0.57	20±1.15
Cumin oil	22.07±0.52	18±0.57	18.33±0.33	18.33±0.33	20±1.15		23±0.57	20±0.57	17±0.57	21±0.57	22±1.15
Anise Bush oil	22.07±0.52	14±0.57	18.33±0.33	16±0.57	19±1.15		23±0.57	18±0.57	17±0.57	18±0.57	20±1.15
Garlic oil	22.07±0.52	18±0.57	18.33±0.33	18.33±0.33	19±0.88		23±0.57	19±0.57	17±0.57	19±0.57	20±0.88
Spearmint oil	22.07±0.52	21±0.57	18.33±0.33	22±0.57	25±0.66		23±0.57	19±0.57	17±0.57	20±0.57	22±0.57
Star Anise oil	22.07±0.52	20±0.57	18.33±0.33	21±0.57	23±0.88		23±0.57	15±0.57	17±0.57	18±0.57	20.33±0.88
Tea Tree oil	22.07±0.52	17±0.57	18.33±0.33	18±0.57	21±1.15		23±0.57	11±0.57	17±0.57	16±0.57	19±1.15
Rosemary oil	22.07±0.52	17±0.57	18.33±0.33	19±0.57	22±1.15		23±0.57	16±0.57	17±0.57	17±0.57	19.33±0.88
Phenyl Ethyl Alcohol	22.07±0.52	16±0.57	18.33±0.33	17±0.57	19±1.15		23±0.57	16±0.57	17±0.57	19±0.57	20.66±0.88
Iso Eugenol	22.07±0.52	16±0.57	18.33±0.33	18.33±0.88	20±1.20		23±0.57	14±0.57	17±0.57	18±0.57	19.33±0.88

CU: - Cow Urine (Treated at pH 3.8); EO=Essential Oil; EOCU01:- Essential oil (0.5%) + Cow urine; EOCU02= Essential oil (1%) + Cow urine; ANTIBIOTIC: - Ciprofloxacin (16mg/ds)

Conclusions

Our results confirmed that commercial essential oils with CU cause significant growth-inhibiting effects on Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria. The above studies show that EOs have high antibacterial properties under specific pH values. Their bactericidal activity may be attributed to the presence of some bioactive molecules in them. These molecules are mainly low mol. wt. phenolic compounds, aldehydes, ketones, ethers, lactones, and phenylpropanoids. The monoterpenoids present in these EOs are also highly antibacterial at a pH below 4.5. EOs or their combinations may be developed into a wide-spectrum natural antibiotic. The antibacterial activity of active compounds from the essential oils needed to be further investigated in external applications.

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