

Analytical characterization and antifungal activity of *Gomutra* and *Gomutra Arka* of *Bos indicus* cattle

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ABSTRACT:

Background: *Gomutra* (GM) and its distillate (*Gomutra Arka*-GMA) are ancient medicines, but modern lifestyles and healthcare systems rely on analytical evidence to validate them. **Aim:** To determine the biochemical composition and antifungal potential of GM and GMA against *Aspergillus niger* and *Candida albicans*. **Materials and Methods:** The physicochemical composition of GM and GMA was measured according to API standards. Bioactive compound profiling was further performed using HPTLC and GC-MS. The well diffusion method was used to determine the antifungal potential of GM and GMA against *A. niger* and *C. albicans*. **Results:** Densitometric HPTLC analysis revealed that, at short-wave (254 nm), dark bands against a green background (fluorescence quenching) indicate the presence of UV-absorbing compounds such as aromatic rings, conjugated double bonds, and phenolic derivatives. At long-wave (360 nm), blue-, green-, pink- and yellow fluorescent bands were observed, indicating the presence of fluorescent constituents in GM and GMA. Furthermore, tentative GC-MS mass profiling identified 61 compounds in GM and GMA. Among them, 57 were common to both samples. Based on their reported chemical structures, the putatively identified compounds were categorized into aromatic benzoate derivatives, hydrocarbons and fatty acid derivatives. The presence of volatile fatty acids (i.e., butyric acid, propionic acid) and aromatic (i.e. 3,5-Di-tert-butylphenol) compounds may be contributing to the pungent smell of GM and GMA. The antifungal assay revealed that increasing the percentage of GM and GMA led to progressive expansion of the zone of inhibition (ZOI) against *A. niger* and *C. albicans*. Both GM and GMA showed a higher ZOI against *C. albicans* than *A. niger*. Impressively, 20% (GM) and 80% (GMA) concentrations yielded ZOI (~11.5 mm and 9.5 mm), corresponding to the standard drug Itraconazole (20 µg/mL) against *C. albicans* and *A. niger*, respectively. **Conclusion:** Although tentative mass profiling demonstrates a high (93.4%) metabolic similarity between crude GM and GMA, the antifungal assessment found that *C. albicans* and *A. niger* are more sensitive to GM. These observations might improve the scientific validation of traditional medicines. Further, *in vitro* experimental validation and clinical validation are essential to ensure safety prior to the development of GM or GMA-based antifungal formulations.

KEYWORDS: Antifungal activity, *Bos indicus*, GC-MS, *Gomutra*, *Gomutra Arka*, HPTLC

RECEIVED ON:
08-07-2026

REVISED ON:
21-09-2026

ACCEPTED ON:
11-10-2026

Access This Article Online:

Quick Response Code:



Website Link:

<https://jahm.co.in>

DOI Link:

<https://doi.org/10.70066/jahm.v14i9.2933>

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CITE THIS ARTICLE AS

Shashikala Khadabadi, Savita S Angadi, Veerayya R Hiremath, Murali Krishna Paidi. Analytical characterization and antifungal activity of *Gomutra* and *Gomutra Arka* of *Bos indicus* cattle. Journal of Ayurveda and Holistic Medicine (JAHM) 2026; 14(9):43-56.

1. INTRODUCTION

In India, cattle play a vital role in the rural economy because they are a major source of milk, manure, meat, and draught power. India has the largest and most genetically diverse cattle inventory, 308 million in 2023. [1] The two cattle subspecies, *Bos taurus* and *Bos indicus* (Zebu cattle), are the most common domestic cattle, which play an important role in modern civilization and socioeconomic development. Phenotypically, Zebu animals are also known as hump cattle (with large ears and excess skin). Typically, the *B. indicus* species was domesticated in the Indus Valley 8000 YBP (years before present). These two subspecies are well distributed across the landmasses of Africa, East Asia, Southwest Asia, and European countries between 4000 and 1300 YBP. According to a review report by Utsunomiya et al., the Food and Agriculture Organisation of the United Nations (FAO) data survey found that there are 1.48 billion cattle reared across the world. Brazil (218 million) and India (186 million) are at the top of the list. Moreover, in these two countries, the *B. indicus* subspecies are most common and abundant. [2] In Ayurveda, the cow (*Gau Mata*), whose product is called *Panchagavya* (*Amrita*), consists of milk, ghee, curd, urine, and dung.

Gomutra (GM) or *Cow urine* (CU) is composed of about 95% water and 2.5% urea, and 2.5 % others, including minerals (nitrogen, phosphate, potassium, calcium and magnesium), salts, hormones, enzymes and bioactive substances. Typically, *Gomutra* (GM) is a dark brown, natural, non-toxic, readily digestible liquid and is used in modern agriculture, medicine and electricity generation.[3] Additionally, it has been considered a natural remedy for a wide range of diseases due to the presence of minerals and bioactive compounds. Many scientific and traditional research findings suggest that CU has strong antioxidant properties [4] supports type 1 diabetes (T1D) management [5], and inhibits tumor growth. Additionally, CU has strong antimicrobial potential against bacteria, fungi, protozoa and molluscs. [6-9] In traditional Ayurvedic medicine, cow urine has been used as a therapeutic agent for leprosy, fever,

peptic ulcers, liver and kidney disorders, asthma, allergies, psoriasis, anemia, and cancer treatment. [10, 11] CU has been used as a biofertilizer in modern agriculture. [12]

Scientific investigations have demonstrated that GM can be used as a liquid fertilizer to improve plant growth rate, biochemical content and protect from pests. For example, the CU treatment on vegetable crop plants such as *Abelmoschus esculentus* (*Bhindi*) and *Trigonella foenum-graecum* (*Methi*) increased chlorophyll and protein content. [13] A field experimental application of CU with the combination of nitrogen fertilizer on rice variety *Shusk Samrat* (NDR 1045-2) increased paddy growth and number of tillers, and yield (grain and straw) compared to the control. [14] According to a review report by Katekar et al. (2024), the contemporary application range of CU increased from 1250 to 5000 pounds per acre. In terms of agriculture, consumption of CU and its distillate accounted for 51.7%, energy production (28.6%), and medicinal applications (14.3%). [3] In addition, fermentation strategies offer an eco-friendly alternative to synthetic chemical fertilizers, pesticides, reducing soil pollution and environmental toxicity. Aerobic fermentation of different formulations with commercially available microorganisms (ME-4) and CU at a 2:1 (CU: EM-4) ratio for 30 days increases the N, P and K content compared to CU: EM-4 (4:1), CU: EM-4: organic waste (4:1:1), CU: table sugar: organic waste (4:1:1), CU: EM-4: table sugar (10:5:1), and control only CU. [15]

Gomutra (Cow Urine) and its distillate (*Gomutra Arka*) have gained attention for therapeutic applications. [16] Arka is the distilled, purified and concentrated essence of CU. Arka or CUD is more palatable, has a longer shelf life, and has a lower concentration of ammonia than CU. Many in-vitro experiments found that cow urine distillate (CUD) has antimicrobial potential, which depends on concentration and test organisms. For example, 15% CUD shows the highest zone of inhibition (ZOI), ranging from 13 to 24 mm, and the minimum inhibitory concentration (MIC) range is 12.5 to 50 µg/mL against *Staphylococcus aureus* and *Salmonella typhi*. [17] Moreover, Hoh and Dhanashree's

study reveals that 20-50% of CU distillate exhibits antifungal potential (75.7%) on 37 clinical isolates of *Candida* species. [18] In the modern era, experimental studies suggest that GM is one of the important medicines used to control various diseases. Gosavi et al. (2011) investigated that GMA shows antioxidant and immunomodulatory effects in Adult Wistar Albino rats. [19] CUD and Arka are used as chemotherapeutic agents to prevent the development of drug resistance and enhance the effect of standard antibiotics. [20] Arka is used as an antimicrobial and antiproliferative agent to control breast cancer cell lines (MCF-7). [6]

Although CU and CUD are clinically proven as potential agents for various diseases, they can also be used as a bio-fertilizer and natural pesticide to improve nutrient uptake and crop yield. Additionally, advanced analytical approaches have been employed to characterize CU and CUD at the molecular level. Metabolic profiling of urine from diverse cattle breeds such as *Gir*, *Sahiwal*, *Gangatiri*, *Hariana*, *Kankrej*, *Rathi*, *Gaolao* and *Jersey* has been carried out using gas chromatography mass spectrometry (GC-MS), which reveals the bioactive compound chemistry. [21, 22] Similarly, physicochemical characterization of GM and GMA through thin-layer chromatography (TLC), FT-IR and GC-MS suggests bioactive potential. [4, 23]

Aim: To evaluate the physicochemical and antifungal potential of *Gomutra* and *Gomutra Arka* against *Aspergillus niger* and *Candida albicans*.

Hypothesis: *Gomutra* and *Gomutra Arka* can influence the growth of *Aspergillus niger* and *Candida albicans*.

Objectives:

- Physicochemical characterization and antifungal potential of cow urine and its distillate (*Arka*) of local desi cow (*Hallikar cattle* or *Bos indicus*) using HPTLC and GC-MS.
- The antifungal activity of GM and GMA was evaluated against *Aspergillus niger* and *Candida albicans*.

2. MATERIALS AND METHODS

Collection of *Gomutra* and preparation of *Gomutra Arka*

To prepare *Gomutra Arka* (cow urine distillate), a fresh *Gomutra* (GM) sample (8 L) was procured from *Hallikaar cattle* (Desi cow) at the Shree Mahaveer Jain Goushala, Koppala. The collected samples were filtered using a muslin cloth, then subjected to conventional distillation (Fig. 1). *Arka* was prepared by the distillation method using an ISKO Borosilicate glass assembly distillation unit (ISKO, Gupta Scientific and Glass Works, India). In this process, the GM was transferred into the *Arka yantra*, which consists of a round-bottom distillation flask and a collecting flask. The distillation flask was exposed to heating, and the vapors were condensed through a condensation tube at room temperature. The resulting distilled liquid was collected into a flask and stored at 4°C for further analysis.

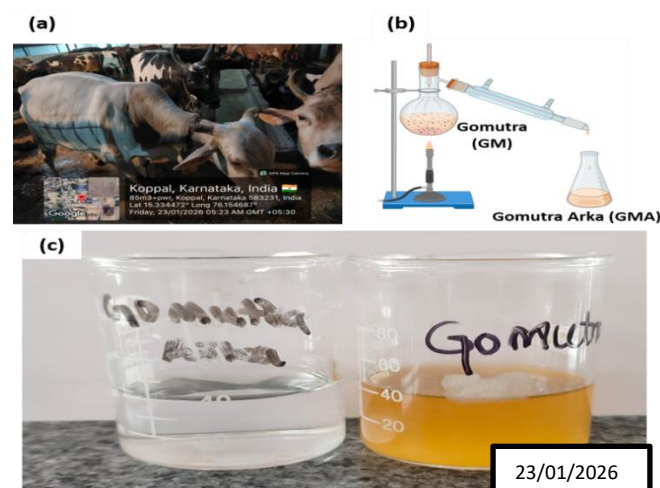


Figure 1: Collection of *Gomutra* samples from a desi cow (*Hallikaar variety*) at Shree Mahaveer Jain Goushala, Koppala (a), Conventional distillation unit representing *Gomutra* distillate (*Arka*) production (b), and physical appearance of *Gomutra* (left side image in pale yellow or amber) and *Gomutra Arka* (right side image in transparent).

Physicochemical characteristics of GM and GMA

Physical properties of GM and GMA, including color and solubility properties, were evaluated based on a visual evaluation method. The odor and taste were determined by sensory evaluation. [24] Physicochemical properties, such as dryness, pH, ash content, acid, alcohol and water solubility, specific gravity and viscosity, were evaluated

according to API standards in an analytical laboratory at Aviskara Central Research Laboratory, SJGAMC, Gavimath campus, Koppal, Karnataka. [25, 26] The dry weight was calculated based on the loss of Dryness (LOD %) formula:

$$\text{LOD (\% w/L)} = \frac{W_i - W_d}{W_i} \times 100$$

Where:

W_i – initial sample weight of samples (1.12 kg or 1L)

W_d – weight after drying

Thereafter, ash content (non-volatile) was determined using the dried residue or dry-weight basis from the following formula:

$$\text{Total Ash (\% w/w)} = \frac{W_{ash}}{W_d} \times 100$$

Similarly, acid (acid-insoluble %) was determined using the formula: $W_{\text{acid-insoluble}}/W_{\text{dry-ash}} \times 100$. The alcohol and water-soluble extract of dried residue (W_d) was calculated from the following formula:

Alcohol or Water soluble extract (% w/w)

$$= \frac{W_{aex(or)wex}}{W_d} \times 100$$

Where:

W_{aex} – Dry weight of alcohol extract of dried residue (W_d)

W_{wex} – Dry weight of Water extract of dried residue (W_d)

Determination of Urea and Uric Acid: The content of urea and uric acid in *Arka* was determined by the Urease and Berthelot endpoint assay by the Murray analytical method. [27, 28]

Determination of Heavy Metals: To examine the presence of heavy metals in GM and GMA, different precipitation reactions were performed according to macro and semimicro qualitative inorganic analytical protocols by Svehla (1978). [29]

Phytochemical assessment: The aqueous extract and alcoholic extract of *Gomutra* and *Gomutra Arka* were used to determine the phytochemical composition. The extracts were subjected to preliminary qualitative analysis, including Mayer's test for alkaloids, Molisch's test for carbohydrates, Alkaline reagent test for flavonoids, Libermann-Burchard's test for phytosterols, Millon's test for protein, Ninhydrin

test for amino acids, Ferric chloride test for phenolic compounds and tannins, Borntrager's test for glycosides, and foam tests for saponins. [30] The presence of quinones, anthraquinones, and anthocyanins was determined by Harborne's phytochemical methods. [31]. The results of the biochemical tests indicated the presence (+) or absence (-) of colour precipitation.

High-Performance Thin-Layer Chromatography (HPTLC)

analysis: For metabolite screening, a semi-automatic High-Performance Thin-Layer Chromatography (Serial No. 100632, CAMAG Linomat 5, Muttenz, Switzerland) was used. Test samples were labelled KA1 and prepared in ethanol. Then loaded onto stationary phase, i.e. pre-coated silica gel 60 F₂₅₄ HPTLC plates (50 × 100 mm; Merck, Darmstadt, Germany) in varying volumes (5.0, 6.0, 8.0, and 9.0 µL) using a semi-automatic applicator. Sample bands were applied 8.0 mm from the lower edge of the plate, with a band length of 8.0 mm and an inter-track distance of 10.4 mm. The dosage speed was set at 100 nL/s, and a pre-dosage volume of 0.20 µL was used to ensure accurate sample delivery.

Chromatographic development was performed in a twin-trough glass chamber (20 × 10 cm) pre-saturated for 20 minutes with a mobile phase of Chloroform: Methanol: Water (v/v/v) (70:30:4). Saturation was carried out using appropriate filter paper liners to ensure consistent vapor-phase conditions. Plates were developed up to a solvent front distance of 70 mm, followed by drying at ambient room temperature for 5 minutes. The plate was also derivatized with anisaldehyde-sulphuric acid reagent and heated at 105 °C for 5 min. The derivatized plates were documented under visible light to visualize colored bands. Densitometric evaluation of the plates was conducted using a CAMAG TLC Scanner 3 (Serial No. 140507, CAMAG, Mulund, Mumbai, India), operated under Vision CATS software version 3.2.23095.1 (CAMAG, Linomat 5 Switzerland). Scanning was performed in absorbance mode at multiple wavelengths, 254 nm and 366 nm, using deuterium and tungsten lamps. The scanner was configured

with automatic detector mode, a scanning speed of 20 mm/s, a resolution of 100 $\mu\text{m}/\text{step}$, and a slit dimension of $5 \times 0.45 \text{ mm}$.

Data acquisition and processing included Savitzky-Golay smoothing (window size 7), baseline correction using the lowest slope method, and peak detection using a Gaussian legacy algorithm with sensitivity set to 0.1, peak separation of 1, and a threshold of 0.1. Integration was carried out over an RF range of 0.00 to 1.00. Each sample volume yielded consistent peak patterns with reproducible RF values, supporting the method's robustness. All operations were performed in accordance with CAMAG standard practices and HPTLC analytical guidelines described in the literature.

GC-MS analysis of Gomutra and Gomutra Arka

Preprocessing of samples: For GC-MS analysis, 10 mL of *Gomutra* (GM) and *Gomutra Arka* (GMA) samples were filtered through a syringe filter (Nylon 13 mm 0.2 μm) into 50 mL tubes separately. Then 10 mL of ethyl acetate was added and mixed by mechanical shaking for 5 min. Subsequently, 20 mL of analytical-grade hexane was added to the mixtures, and the tubes were vortexed for 10 min. Thereafter, the tubes were kept standing in a test tube stand for phase separation. The resulting upper organic phase (hexane) was collected into a fresh tube and evaporated at room temperature under dark conditions. The dried organic residual powder (10 mg) was resuspended in 50 μL of hexane (HPLC grade) and stored at 4°C until GC-MS analysis.

GC-MS Instrumentation and Analytical Conditions: For metabolomics mass profiling, the Agilent 7890 A GC (Agilent 7890 A GC, Agilent Technologies, Andheri East, Mumbai, India) was used. GC was coupled with a mass selective detector (5975C MSD), and a triple-axis detector system was pre-optimized under optimal conditions. A capillary column DB-5MS (30 m $\times$ 0.250 mm diameter $\times$ 0.25 μm thickness) was used as a stationary phase for the separation of bioactive compounds in GM and GMA. The injection volume was kept constant at 2 μL , and the system

was operated in splitless mode. Helium gas (99.9995%) was used as the carrier gas at a flow rate of 1 mL/min. For better separation, the GC oven temperature was programmed. The initial temperature was 40°C, held for 2 min. After that, the temperature was gradually increased to 150°C at 10°C/min hold for 5 min, followed by the oven temperature was increased up to 280°C at 10°C/min hold for 10 min. The mass spectrometry (MS) analysis was performed in the EI (electron impact) mode with 70 eV of ionization energy. The resulting mass spectral data were annotated using integrated spectral processing GC solution software, and the spectra were matched against a standard mass spectral database (NIST-08 spectral library).

Antifungal activity of GM and GMA

Material and sterilization: For the antimicrobial test, the autoclavable plastic Petri plates (90 mm), inoculation loop, stainless steel cork borer (8.1 mm), pipette tips (Torson 1 mL, 200 mL and 10 mL), and two measuring cylinders (100 mL) were washed in diluted liquid soap water and subjected to autoclaving at 121°C for 15 minutes under 15 psi pressure, then dried in an oven at 60°C.

Potato dextrose agar (PDA) plate preparation: To prepare PDA plates, commercially available PDA powder (39 g) was taken into a 2 L conical flask containing 1 L of distilled water and dissolved with the help of a magnetic stirrer at 100 rpm. The mixture was sterilized by autoclaving at 121°C for 15 minutes under 15 psi pressures. After sterilization, the medium was allowed to cool to room temperature. The sterile medium (~20 mL) was poured aseptically into sterile Petri plates inside a laminar airflow chamber. The plates were solidified at RT and sealed with Parafilm, then stored at 4°C for further subculturing and antifungal activity tests.

Fungal strains and inoculum preparation: Two fungal isolates, *C. albaicans* (pseudohyphae) and *Aspergillus niger* (filamentous), were used as test microbes in this study. Initially, a loop of selected isolates was transferred into 1.5 mL sterile tubes containing 1.0 mL of liquid potato dextrose broth (PDB) and vortexed at 20 rpm for 5 min. Then incubate the cultures at 30°C for 24 h. Culture turbidity was

adjusted to 0.5 McFarland standards (approximately 10^6 CFU/mL).

Detection of MIC of GM and GMA for *Aspergillus niger* and *Candida albicans*: The GA and GMA samples were filter-sterilized using a 0.25 μ m syringe filter. The resulting filtrate was stored at -20°C until antimicrobial activity was assessed. The well diffusion method was employed to determine the minimum inhibitory concentration (MIC) potential of GM and GMA against *Aspergillus niger* and *Candida albicans* strains. [18] About a 10 μ L aliquot of each inoculum was transferred to pre-prepared PDA plates and spread using a sterile glass spreader. Subsequently, 8 mm wells were created using a sterile stainless steel cork borer (8 mm diameter). Different concentrations of test samples were prepared (Table 1), and 50 μ L of samples were loaded into each well. At the same time, a positive control (Itraconazole and Fluconazole) was also loaded into the respective. Similarly, a negative control well was maintained with sterile water. All experimental plates were incubated at 30 $\pm$ 2°C for 48 hours, and the MIC was defined based on the visible growth.

3. RESULTS

Physicochemical characteristics of GM and GMA: Physically Gomutra (GM) samples were pale yellow or amber in color. During the distillation procedure, GM color was diluted and appeared transparent (Fig. 1c). Physicochemical analysis revealed that GM and GMA exhibited quite different characteristics. GM samples are pale yellow to orange-brown in color with an unpleasant aromatic odor. Whereas the GMA samples were clear with a strong astringent odor. These are quite similar to the previous findings by Raut et al. (2023). The fresh GM samples from Sarvodaya Goshala Charitable Trust, Padhegaon, Wardha. They noticed that the collected fresh GM samples were pungent smell, dark brown in color before the onset fermentation. After the fermentation, a mild alcoholic, Gomutrasava was produced. [32] GM and GMA were water-soluble with a pungent, bitter taste (Table 1). The weight loss on drying was higher for GMA (91.79%) compared to GM (89%). This represents a

high % of moisture or volatile content. Similarly, total ash content was higher (27%) in GM than in GMA (8.03%). The acid-insoluble ash (18%) was higher in the case of GM than in GMA (8%), indicating the presence of inorganic residues. Whereas, GMA exhibits a high % of alcohol-soluble extract (60%) and water-soluble extract (66%) compared to GM (6% and 4.80%, respectively). This indicates that the solvent-based extraction process concentrates the highly soluble organic fractions more than water extracts. These results indicate a richer presence of soluble bioactive constituents in GMA. The alkaline pH (8), specific gravity (1.03 and 1.007) and viscosity (0.987 and 0.92) were almost similar. Additionally, biochemical qualitative analysis confirmed the presence of urea and uric acid in both GM and GMA, while chloride was negative. Heavy metal analysis showed the presence of lead, copper, arsenic, and zinc in the GM, whereas the GMA contained only copper (Table 2).

Table 1: Physicochemical properties of Gomutra and Gomutra Arka.

Parameter	Gomutra (GM)	Gomutra Arka (GMA)
Physical properties		
Color	Yellowish orange brown	Clear Liquid
Odour	Unpleasant with aromatic	Strong Astringent
Taste	Pungent, Bitter	Pungent, Bitter
Solubility	Water soluble	Water soluble
Physicochemical properties		
pH	7.95	8.01
Specific gravity	1.03	1.007
Viscosity	0.987	0.92
Loss on drying (%)	89%	91.79%
Dry matter weight (%)	11% (11 g)	8.21% (8.21 g)
Total Ash value (% w/w)	27% (2.97 g)	8.03% (0.66 g)
Acid-insoluble ash (%) w/w)	18% (0.53 g)	8% (0.12 g)
Water-soluble ash (%) w/w)	6% (0.18 g)	4% (0.026 g)

Alcohol soluble extract of	60% (6.6 g)	66% (5.42 g)
W _d (% w/w)		
Water-soluble extract of	4.80% (0.53g)	2.48% (0.203 g)
W _d (% w/w)		
Biochemical tests		
Urea	+	+
Uric acid	+	+
Chloride test	-	-
Heavy metals		
Lead (Pb)	+	-
Silver (Ag)	-	-
Copper (Cu)	+	+
Mercury (Hg)	-	-
Cadmium (Cd)	-	-
Arsenic (As)	+	-
Zinc (Zn)	+	-

Note: the symbols “+/-” indicate the presence or absence of respective biochemicals and heavy metals in the samples.

Phytochemical composition of GM and GMA:

Phytochemical analysis of GM and GMA revealed the presence of carbohydrates, tannins, phytosterols, proteins and amino acids, anthraquinones, and glycosides (Table 2). Among them, the presence of saponins was noticed only in GMA. This indicates that during the distillation process (heating), the solubility of saponins increased and they were extracted into water. The presence of tannins, glycosides, and anthraquinones tests were positive for both, suggesting that both GM and GMA can be used as antimicrobial and antioxidant agents. In addition, the presence of phytosterols, proteins and amino acids can be used for therapeutic or nutritional purposes. Whereas the tests for flavonoids, alkaloids, quinones, phenols, and anthocyanins were negative in both GM and GMA. These findings suggest that these compounds were either absent or present below the detection limits of the respective qualitative tests. From the phytochemical qualitative profiling, we noticed that GM and GMA compositions were almost similar, but further untargeted metabolic profiling is essential to decode the composition.

Table 2: Phytochemical composition of Gomutra and Gomutra Arka.

Parameter	Gomutra (GM)		Gomutra Arka (GMA)	
	Aqueous Extract	Alcoholic extract	Aqueous Extract	Alcoholic extract
Carbohydrates	+	+	+	+
Saponin	-	-	+	-
Tannins	+	+	+	+
Flavonoid	-	-	-	-
Phytosterols	+	+	+	+
Alkaloid	-	-	-	-
Proteins and Amino acids	+	+	+	+
Quinone	-	-	-	-
Anthraquinone	+	+	+	+
Phenol	-	-	-	-
Glycoside	+	+	+	+
Anthocyanins	-	-	-	-

Note: the symbols “+/-” indicate the presence or absence of respective phytochemicals in the samples.

HPTLC analysis: High-Performance Thin-layer chromatographic (HPTLC) analysis of GM and GMA exhibits three prominent dark bands against a green background at short-wave (UV 254 nm), indicating fluorescence quenching. These bands appeared at a lower R_f value, indicating the presence of UV-active compounds (Fig. 2). TLC scan at the long-wave (UV 366 nm) region showed faint fluorescent color bands, including blue-, green-, pink- and yellow-fluorescent bands, indicating the presence of fluorescent constituents in GM and GMA. HPTLC scan of the derivatized sample shows weak and diffuse bands at 540 nm, indicating a low concentration of polar compounds and volatile compounds. From these observations, GMA contains less metabolic diversity.

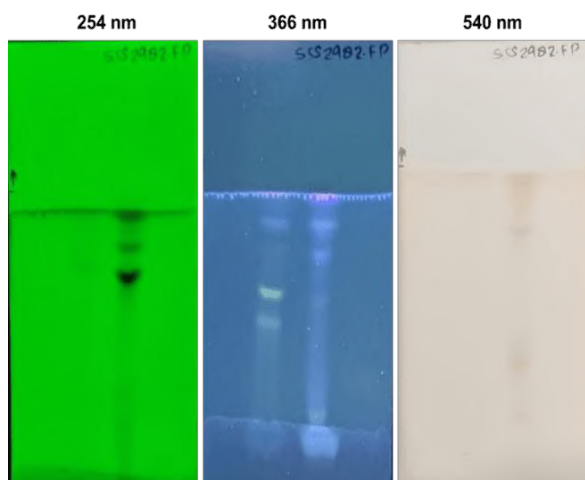


Figure 2: HPTLC analysis of *Gomutra* (first lane from the left side in TLC) and *Gomutra Arka*. Plates scanned at 254 nm, 366 nm, and 540 nm.

Densitometric HPTLC analysis at UV-254 nm and UV-366 nm revealed qualitative differences between GM and GMA. Under UV-254 nm, GM contains a single dominant peak near $R_f \sim 0.98$, indicating the presence of a major UV-absorbing compound (Fig. 3a). Whereas GMA (Fig. 3b) showed three distinct peaks at R_f values of 0.80, 0.88, and 0.97. These peaks represent the UV-active compounds. Under UV-366 nm, both GM and GMA contain a single sharp peak at R_f 0.98 (Fig. 3c), indicating the presence of fluorescent active compounds in both. Derivatized GM densitometric analysis of the TLC scan at 540 nm represents the three minor peaks in the R_f region (~ 0.96 to 1.00) (Fig. 4a). In contrast, GMA (Fig. 4b) shows a peak at R_f 0.05, indicating the presence of a polar compound, and peaks at R_f 0.97 and 1.00 correspond to the non-polar compounds. From these observations, the distillation of GM might extract and enrich specific non-polar and UV-active compounds.

Gas Chromatography analysis of GM and GMA: From GC-MS profiling, a total of 61 metabolites were identified from GM and GMA. Out of them, 57 compounds are commonly detected in both GM and GMA (Fig. 5). The diversity of bioactive compounds was higher in GM than in GMA. Further, the mass spectral data acquisition and annotation results confirmed that GM and GMA contain volatile hydrocarbon derivatives (Butyric acid, hexanol,

cyclopropane and propionic acid), aromatic derivatives (i.e. phenolic or benzene derivatives), and fatty acid derivatives (Table 2). The phenol, 3,5-bis(1,1-dimethylethyl)-1,2,4,5-cyclohexanetetrol, and (5-benzoyloxy-4,6-dihydroxy-3-methoxycyclohexen-1-yl)methyl benzoate were uniquely detected in GM. The methylated sugar derivative, such as methyl- α -D-glucopyranoside, was detected in GMA (Fig. 5c).

Among 61 compounds, 1,2,4,5-Cyclohexanetetrol (10.79%), (5-benzoyloxy-4,6-dihydroxy-3-methoxycyclohexen-1-yl)methyl-benzoate (10.75%), Beta-Lactose 6.24%, N-Benzyleamide (5.32%), Palmitic acid (4.96%), n-Hexadecanoic acid (3.77%), Palmitoleic acid (2.88%), Eicosapentaenoic acid (2.69%) and Tris(2,4-di-tert-butylphenyl) phosphate (2.48%) were quantitatively higher (Fig. 6).

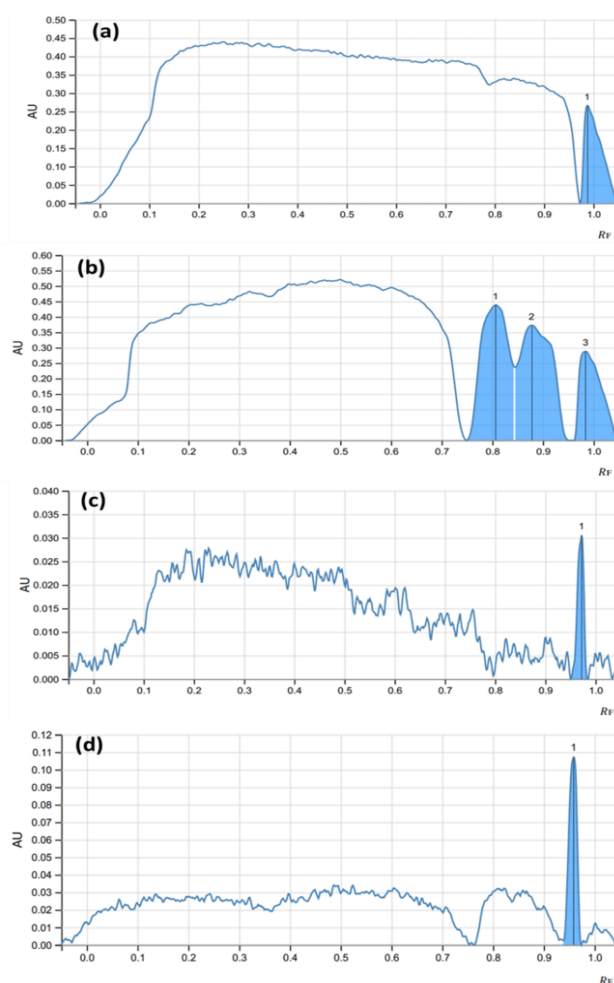


Figure 3: Evaluation of TLC plate scans of *Gomutra* and *Gomutra Arka* at 254 nm (a&b), TLC scan at 366 nm (c&d).

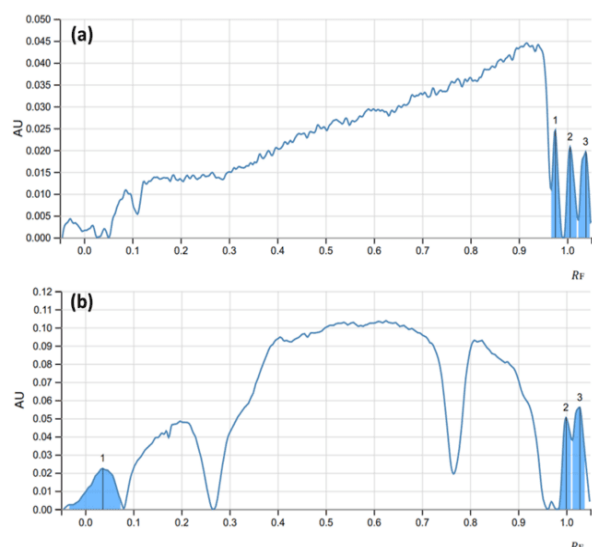


Figure 4: Evaluation of TLC scans of derivatized *Gomutra* and *Gomutra Arka* at 540 nm (a&b).

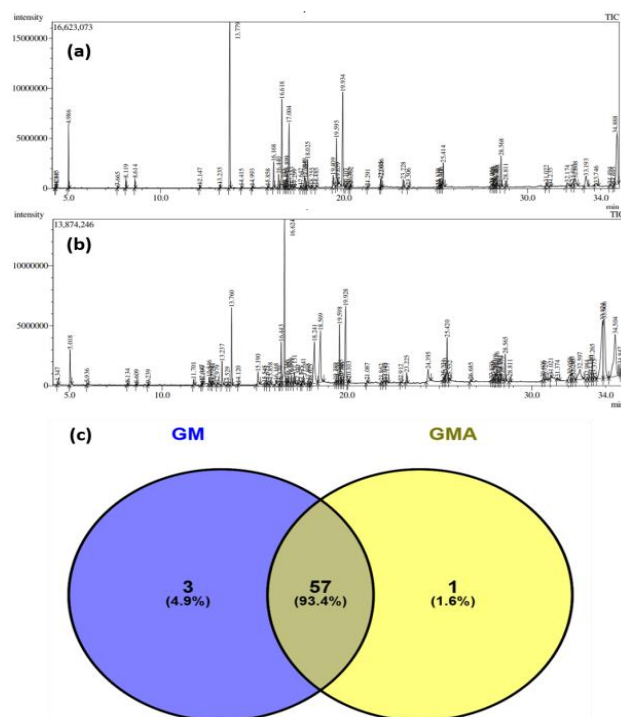


Figure 5: GC-MS-based tentative mass profiling of *Gomutra* (GM) and *Gomutra Arka* (GMA). (a&b) TIC Chromatogram of GM and GMA, and (c) total metabolites identified and distribution.

Antifungal potential of GM and GMA: Antifungal test results revealed that *Gomutra* and *Gomutra Arka* samples exhibited stronger growth-inhibitory potential against *Candida albicans* than against *Aspergillus niger*. The zone of inhibition (ZOI) increased linearly with increasing percentages of GM.

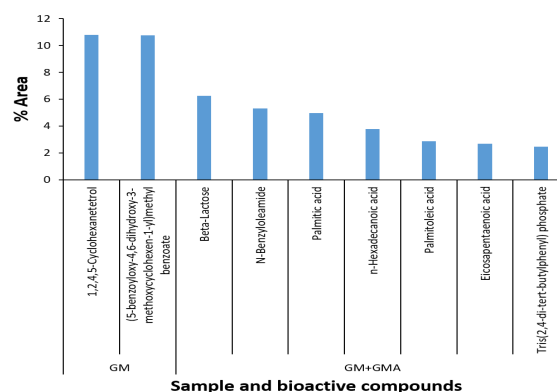


Figure 6: Most abundant (>2% area) bioactive compounds detected in GC-MS analysis of the *Gomutra* and *Gomutra Arka* samples.

Whereas, GMA treatment ZOI did not follow the same linear trend. A clear zone formation was absent around the negative control wells, which were loaded only with sterile water (Fig. 7 and 8). GM-loaded wells exhibited the lowest ZOI against *A. niger* exposed to 20% (1.5 mm), 40% (2.0 mm), 60% (3.2 mm), 80% (10.6 mm), and 100% (22.8 mm) (Fig. 7A and c). GMA-treatment against *A. niger* showed the highest ZOI values of 3.1 mm (20%), 11.9 mm (40%), 13.75 mm (60%), 14.8 mm (80%), and 20.35 mm (100%), respectively, compared to GM plates (Fig. 7B and D). Similarly, with GM treatment against *C. albicans*, the ZOI increased in the order of 20% (11.5 mm) < 40% (13.5 mm) < 60% (14.5 mm) < 80% (17.5 mm) < 100% (43.1 mm), respectively (Fig. 8A and C). However, GMA treatment against *C. albicans* showed an increased ZOI, but comparatively it was smaller than GM treatment and non-linear (Fig. 8 B and D). The ZOI order follows 20% (9.5 mm) < 40% (9.75 mm) < 80% (10.6 mm) < 60% (11.5 mm) < 100% (41.65 mm) (Table 3). From these observations, *C. albicans* was highly sensitive to GM or GMA.

The test organisms, *A. niger* and *C. albicans*, showed a clearer ZOI in the presence of itraconazole (Tz) than fluconazole (Fz). Comparatively, the Tz treatment showed the highest ZOI on *C. albicans* plates as compared to *A. niger* plates (Table 3). The ZOI increased with increasing concentration of Tz, 10.8 mm (10 µg/mL), 11.5 mm (20 µg/mL), 12.2 mm (30 µg/mL), and 14.5 mm (40 µg/mL).

Whereas, with fluconazole (Fz) treatment, both organisms showed resistance at lower concentrations (10 µg/mL to 20 µg/mL). At higher concentrations (30 to 40 µg/mL), it exhibited a clear ZOI of 5.5 mm and 6.2 mm against *C. albicans*, respectively. In the case of *A. niger* plates, ZOI was unclear and remained lower (1.1 mm and 2.3 mm) (Fig. 5F and 6F). From these observations, Tz has better antifungal potential than Fz. The test samples GM and GMA (20% and 80 % v/v) induced zone of inhibition (ZOI) values corresponding to the standard drug Tz (20 µg/mL) against *C. albicans* and *A. niger*, respectively.

Table 3: Antifungal potential of Gomutra and Gomutra Arka against *Aspergillus niger* and *Candida albicans*.

% of test sample	Sample	Average Zone of inhibition (in mm)	
		<i>Aspergillus niger</i> (48 h)	<i>Candida albicans</i> (48 h)
0 (-ve Control)	GM	0	0
	GMA	0	0
20	GM	1.5	11.5
	GMA	3.1	9.5
40	GM	2.0	13.5
	GMA	11.9	9.75
60	GM	3.2	14.65
	GMA	13.75	11.5
80	GM	10.6	17.35
	GMA	14.8	10.75
100	GM	22.8	43.1
	GMA	20.35	41.65

and GMA, (C&D) GM and GMA in gradients 20% (1), 40% (2), 60% (3) and 80% (4) (v/v)); (E&F) wells with positive control Itraconazole (Tz) and Fluconazole (Fz) in gradients from 10 µg/mL to 40 µg/mL (1 to 4).

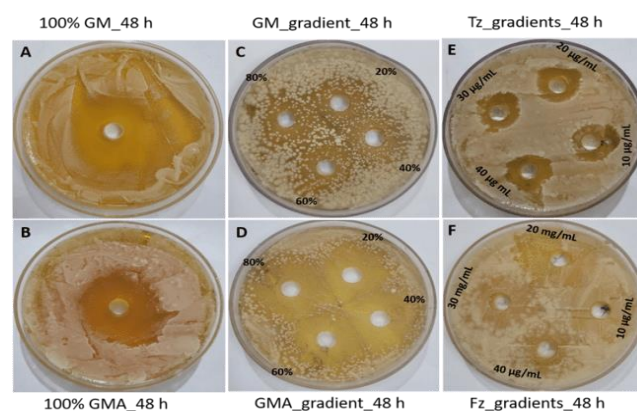


Figure 8: Antifungal activity of Gomutra (GM) and Gomutra Arka (GMA) against *Candida albicans*. (A&B) 100% test samples GM and GMA, (C&D) wells loaded with GM and GMA in gradients of 20% (1), 40% (2), 60% (3) and 80% (4) (v/v), respectively, and (E&F) positive control: Itraconazole (Tz) and Fluconazole (Fz) in gradients from 10 µg/mL to 40 µg/mL.

4. DISCUSSION

Gomutra, or cow urine, has been described as *Amrita* (beverage of immunity). GM has been proved as a cost-effective medicine with fewer adverse effects compared to modern allopathic medicine. [23] The hydro-distillation of cow urine is used as a therapeutic agent against HIV, hepatitis B and as a prodrug formulation to treat cancer.[3] In the present study, a conventional distillation unit was used for the preparation of *Gomutra Arka* (Distillate of *Gomutra*). During this process, GM colour (amber or pale yellow) was dispersed, and a transparent distillate was formed, called *Gomutra Arka* (GMA). The alcohol-soluble and water-soluble extract was higher in GMA compared with GM. Indicates that the organic solvent-based extraction process can concentrate the highly soluble organic compounds in GMA. (Table 1) Further, phytochemical tests suggest that GM and GMA contain tannins, glycosides, and anthraquinones. (Table 2)

HPTLC is an advanced version of traditional TLC. TLC relies on larger particles (silica) as a stationary phase and manual capillary sample loading methods. In standard TLC, bands

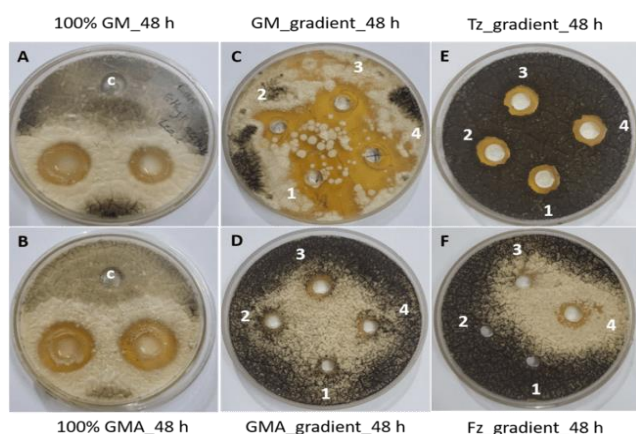


Figure 7: Antifungal activity of Gomutra (GM) and Gomutra Arka (GMA) against *Aspergillus niger*. (A&B) 100% test samples GM

are primarily observed with the naked eye under a UV cabinet (at 254 nm and 366 nm). Whereas HPTLC evolved with fine micro-particle stationary plates and automated sampling. The reduced particle size increases ultra-band separation of organic mixtures and precision.[33] HPTLC is coupled with a specialized scanning densitometer to measure accurate absorbance or fluorescence. [34] The solid-phase extraction HPTLC method was used in the detection of urinary morphine. [35] Due to its advanced features, HPTLC has been used in several biological and non-biological organic compound detections. Currently, HPTLC analysis of GM and GMA revealed that UV-active substances are present in both samples. Densitometric TLC scanning under UV region at 254 nm showing a single dominant peak with $R_f \sim 0.98$ for GM. Whereas, GMA showed three distinct peaks at R_f values of 0.80, 0.88, and 0.97. ([Fig. 3a and b](#)). This banding pattern and diversity of densitometric peaks represent the formation of new UV-active compounds during production of GMA. These results correspond to the Ashok et al. (2024) investigation. According to them, the HPTLC analysis of cow urine and *Gomutra Ghana* noticed one extra peak noticed in *Gomutra Ghana* compared to cow urine. In indicating the formation of a new compound during *Gomutra Ghana* preparation. [26]

GC-MS is a well-known analytical technique used to determine the composition of complex mixtures. In this study, the tentative metabolic composition of GM and GMA was evaluated by GC-MS. The TIC illustrates that raw GM samples contained more diverse metabolites than GMA. A total of 61 compounds were identified; out of them, 57 compounds were commonly detected in both. Volatile short-chain and long-chain hydrocarbons were predominant in both GM and GMA ([Table 2](#)). The aromatic compounds, including phenol, 1,2,4,5-cyclohexanetetrol, and (5-benzoyloxy-4,6-dihydroxy-3-methoxycyclohexen-1-yl)methyl benzoate, were uniquely detected in GM only, indicating that raw *Gomutra* has more antioxidant and antimicrobial potential. Whereas n-benzyleamide,

palmitic acid, n-hexadecanoic acid, palmitoleic acid, eicosapentaenoic acid, and tris(2,4-di-tert-butylphenyl) phosphate were predominantly detected in both GM and GMA. The presence of volatile hydrocarbons (Butyric acid, Propionic acid) and aromatic (3,5-Di-tert-butylphenol) derivatives causes a pungent smell in GM and GMA. Phenolic compounds are most active and induce microbial cell wall damage, denature proteins, and induce oxidative stress, leading to inhibition of antimicrobial potential. [36] Moreover, the aliphatic hydrocarbons, such as pentadecanoic acid, stearic acid, stigmasterol, arachidonic acid, palmitelaidic acid, alpha-linolenic acid, 9-hexadecenoic acid, and dodecanoic acid, were detected in both GM and GMA samples. These may contribute to altering membrane permeability, enhancing the penetration of exogenous compounds, and acting as a carrier for lipophilic antifungal agents. [37] These observations indicate that the chemical signature of raw *Gomutra* and *Gomutra Arka* overlaps with the previous investigation. Previously, several studies have found that phenolic compounds and their derivatives are among the most abundant constituents in Indian cow urine samples. [16, 38] According to Raut et al. (2023), analytical characterisation (GC-MS profiling) of *Gomutra* and *Gomutrasava* revealed that the phenol 2-methyl and phenol 3-methyl compounds are diverse and abundant. [32] FTIR and GMS-MS analysis of cow urine demonstrate the presence of 12 bioactive compounds. Among them, 1-heneicosanol was the dominant compound. These compounds might exhibit antibacterial activity. [4] GC-MS based metabolite profiling of various breeds of cow urine revealed a wide range of bioactive compounds, including ethenone, cresol, bis(2-ethylhexyl) phthalate, phenol, eicosane, pentanol, isobutyl ester, ethyl ester, binapacryl, trifluoroacetate, xylene, amylene hydrate, dibutyl ester, and formamide. Out of them, bis(2-ethylhexyl) phthalate and xylene were constantly detected in almost all indigenous breeds such as Gir, Sahiwal, Gangatiri, Kankrej, Haryana, Gaolao and Jersey cows. However, the dominance

of eicosane and pentanol was noticed in Gangatiri and Haryana breeds. [22]

The antifungal assay results suggest that GM and GMA showed a zone of inhibition (ZOI) which gradually increased with increasing percentages from 20-100% of test samples. (Table 3). These results correspond to the previous reports by Jandaik et al. (2015). They found that 15% of cow urine showed 78.5% and 78.37% growth inhibition of *Fusarium oxysporum* and *Rhizoctonia solani*, respectively. [13] In another study, 75.7% of clinical isolates of *Candida* spp. including *C. albicans* ATCC14053, *C. parapsilosis* ATCC22019, *C. glabrata*, and *C. tropicalis* were susceptible to cow urine at 20 to 50%. [18]

Scope and Limitations: This study explored the preliminary assessment of the biochemical, phytochemical and heavy metal composition of raw *Gomutra* and *Gomutra Arka*. However, certain limitations should be noted. The physicochemical, biochemical, HPTLC and heavy metal assessments were performed as single determinations and were intended primarily for qualitative screening rather than quantitative evaluation. Therefore, replicate measurements and mean $\pm$ SD statistical analysis were not performed. GC-MS mass profiling remains tentative, as compound annotations were based on NIST-08 library mass spectral matching without validation from certified reference standards. Other limitations include the lack of physicochemical replication, the absence of individual compound-specific antifungal testing, and an inability to establish that GC-MS-detected compounds caused the observed antifungal activity. Further studies incorporating validated quantitative methods in replicates along with certified reference standards are warranted to substantiate the reproducibility of present findings. These steps strengthen the current findings and generate robust evidence of chemical composition, safety and potential biological significance of GM and GMA.

5. CONCLUSION

Gomutra (GM) and *Gomutra Arka* (GMA) are traditional medicines. The current study evaluated the

physicochemical and metabolic composition of GM and GMA using HPTLC and GC-MS. HPTLC analysis showed that GM and GMA contained UV-active and fluorescent substances. Densitometer TLC scanning confirmed the presence of UV-active and fluorescent substances in both GM and GMA. GC-MS analysis illustrates that GM and GMA contain 57 common active compounds. Among them, N-benzyleamide, pentadecanoic acid, stearic acid, arachidonic acid, palmitelaidic acid, alpha-linolenic acid, 9-hexadecenoic acid, dodecanoic acid and stigmasterol were prominent. Antifungal assays demonstrated that GM and GMA showed the highest zone of inhibition against *C. albicans* compared to *A. niger*. At 20% and 80 % (v/v), GM and GMA induced ZOIs against *C. albicans* and *A. niger* that were comparable to the standard drug Tz (20 μ g/mL), respectively. From these observed ZOIs, *C. albicans* was more susceptible (sensitive) to GM and GMA than *A. niger*. These findings suggest the potential antifungal activity of raw GM and distillate (GMA) and provide a basis for further investigation of their application in natural products-based formulation. However, further quantitative analysis using certified standards are required before their therapeutic applications.

Abbreviations:

GM – *Gomutra*; GMA- *Gomutra Arka*; CU – Cow Urine

A. niger – *Aspergillus niger*; *C. albicans* – *Candida albicans*

HPTLC – High-Performance Thin-Layer Chromatography

GC-MS – Gas Chromatography Mass Spectrometry; Fz - Fluconazole; Tz –

Itraconazole; ZOI – Zone of inhibition; TIC – Total Ion Chromatogram; nm – nanometer; mL – milliliter; μ L – microliter; L – litre

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Acknowledgements – The authors sincerely thank Avishkara Central Research Laboratory, Shree Jagadguru Gavisiddheswara Ayurveda Medical College, for providing the resources and facilities required for this study. The authors are also grateful to Dr Shivaleela Biradar, CAMR, BLDE (Deemed to be University) and Dr Nagthan, SSM College of Pharmacy & Research Centre, BLDE, for their support in conducting the antifungal activity assays. The authors further acknowledge Biobeinfo (R&D Support Startup) for manuscript drafting and data analysis.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statement: This study did not involve human participants or experimental animals; therefore, Institutional Ethics Committee approval and informed consent were not applicable.

Declaration of Generative AI: The authors declare that this manuscript was written without the use of generative artificial intelligence tools. All the content, including text generation, data analysis and references, was developed and reviewed by the authors without assistance from AI technologies.

Conflict of Interest – The authors declare no conflicts of interest.

Source of Support – The authors declare no source of support.

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