Comparative Thin-layer chromatography profiling of deoni cow urine and Acalypha indica leaf extracts using an in vitro multi-solvent approach

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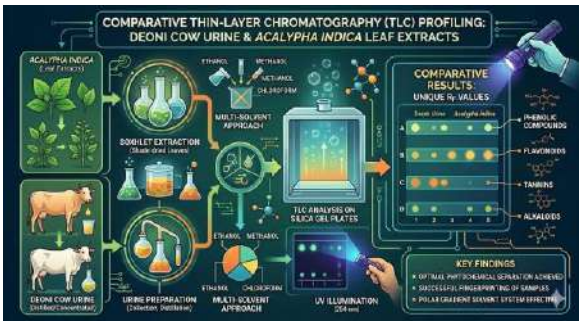
Abstract

In this investigation, a new planar chromatography method was developed for fingerprinting the extracts of Deoni cattle urine and Acalypha indica leaf samples. The researchers used a polar solvent gradient that included various combinations of ethanol, methanol, and chloroform. Optimal phytochemical separation was achieved on thin-layer chromatography silica gel plates. Freshly collected urine samples were distilled and concentrated, whereas shade-dried plant samples were subjected to Soxhlet extraction. Under UV illumination (254 nm), unique Rf values were obtained, thus indicating a significant amount of phenolic compounds, flavonoids, tannins, and alkaloids.

Graphical Abstract



Keywords: Cow urine *Acalypha indica*, Solvent systems, TLC, R_F, Bioactive components.

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Introduction

Thin-layer chromatography (TLC), remains a versatile and cost-effective sophisticated modern analytical technique because of its simplicity, speed of separation, and high sensitivity. The standardization of the main active constituents and providing a chromatographic plant extracts fingerprint and it should be considered for evaluating the overall quality and safety of herbal medicines. It is used to monitor the reaction progression, identification of the components of the mixture and the determination of the purity of the mixture. The behaviour of an individual compound in TLC is characterized by a quantity known as (R_F). The R_F is calculated by dividing the distance the compound travelled from the original position by the distance the solvent travelled from the original position (the solvent front). These R_F values obtained from the phytochemicals provide the important information about their polarity and important clues for the separation of these phytochemical in the separation process. Different R_F values of the compound also reflect an idea about their polarity by the use of the various solvent systems for TLC studies could be important for the selection of the appropriate solvent system. Its applicability in phytochemical analysis has made it an essential tool in natural product research, drug discovery, and traditional medicine validation. The increasing interest in characterizing bioactive constituents from ethno-veterinary resources has led to a renewed focus on developing TLC-based profiling methods capable of capturing diverse chemical classes by employing multiple solvent systems.

Cow urine (animal based by-product), an important component in traditional Indian medicine and

ethno-veterinary practices, is reported to contain a wide spectrum of metabolites including volatile fatty acids, phenols, urea derivatives, organic acids, and trace elements. Among indigenous breeds, *Deoni* cattle hold particular agricultural and medicinal importance due to their adaptability, disease resistance, and reported biochemical richness of their urine. Despite documented therapeutic attributes-such as anti-bacterial (Raad *et al.,* 2013), anti-oxidant (Krishnamurthy *et al.,* 2004), anti-inflammatory (Rachana and Sreepada, 2019) and wound healing (Hirapara, 2016). The topical application of cow urine and its potent forms cow urine distillate or ark, cow urine concentrate proved potentially effective for wound healing on small animals (Wate *et al.,* 2011) that is why in Ayurveda, concisely it is called as 'Sanjivani' (multidimensional Ayurvedic drug) because of its multiple therapeutic roles. The chemical fingerprint of *Deoni* cow urine remains insufficiently explored, especially with respect to chromatographic separation patterns under different polarity-based solvent systems.

Acalypha indica Linn is an annual erect herb belongs to family *Euphorbiaceae* and also known as 'Indian Copperleaf (Kuppaimeni)' is a tropical medicinal plant found worldwide. The leaves are the most abundant part and 64% of ethno-medicinal practices consume only the leaf parts of the plant, followed by the whole plant (24%) and the root (12%). It is widely distributed medicinal herb (plant based) known for its antimicrobial, anti-inflammatory (Govindarajan *et al.,* 2008), antioxidant (Sudhakar Chekuri *et al.*, 2016) and wound healing (Laut *et al.,* 2019; Ng Kit Yeng *et al.,* 2019) activities, also contains diverse secondary metabolites such as

flavonoids, tannins, alkaloids, terpenoids, saponins, and phenolic acids. The plant is frequently used in ethno-veterinary formulations, often in combination with cow-derived products, to manage wound infections, skin disorders, and inflammation in livestock. However, systematic comparative chromatographic profiling of *Acalypha indica* extracts alongside cow urine is very limited.

Using multiple solvent systems of varying polarity in planar chromatography provides a comprehensive approach to resolve chemically diverse compounds present in these biological materials. Such profiling facilitates qualitative assessment, preliminary identification of metabolite classes, and supports standardization of ethno-veterinary formulations. Moreover, comparative TLC fingerprints provide a foundation for correlating chemical constituents with observed bioactivities in subsequent *in vitro* assays.

Therefore, in the current study aims for the first time to develop and evaluate planar chromatographic profiles of *Deoni* cow urine and *Acalypha indica* leaf extracts using a series of solvent systems. This work seeks to elucidate the chemical diversity, optimize solvent-based separation, and provide baseline chromatographic fingerprints that support future phytochemical and biological investigations, particularly in the context of ethno-veterinary wound healing-research.

Materials and Methods

Materials

Deoni cow urine samples were collected from the cattle yard of the Southern Regional Station, National

Dairy Research Institute (SRS-NDRI), Bengaluru, India. *Acalypha indica L.* plants were obtained from the Gudiyatham region in Tamil Nadu, India. Whatman filter papers were purchased from Amazon Private Limited, Bengaluru. Medi-Test Combi 10^R VET strips were procured from MACHEREY-NAGEL, Germany.

Materials and Methods

Collection of cow urine, its distillation and concentration

Indigenous *Deoni cows* housed at the Livestock Research Centre of the National Dairy Research Institute in Bengaluru, Karnataka, India (latitude:12.972442, longitude:77.580643), were selected for this study. These cows were maintained under uniform farm conditions, stall-fed with seasonal green fodder (hybrid napier grass), dry fodder (ragi straw), and a mineral mixture concentrate from the Karnataka Milk Federation. Urine samples from ten healthy cows of the *Deoni* breed in their late lactation stage were collected randomly in a clean containers during the early morning hours. Samples were filtered with sterile Whatman filter paper and screened for health parameters using "Medi-Test Combi 10^R VET" dip sticks (examination of urine) and pooled in equal volume within 1-2 hours. The filtered cow urine was subjected to distillation to obtain cow urine distillate, the residue left after the reduction of volume to $1/10^{th}$ by distillation procedure was designated as cow urine concentrate (Fig. 1).

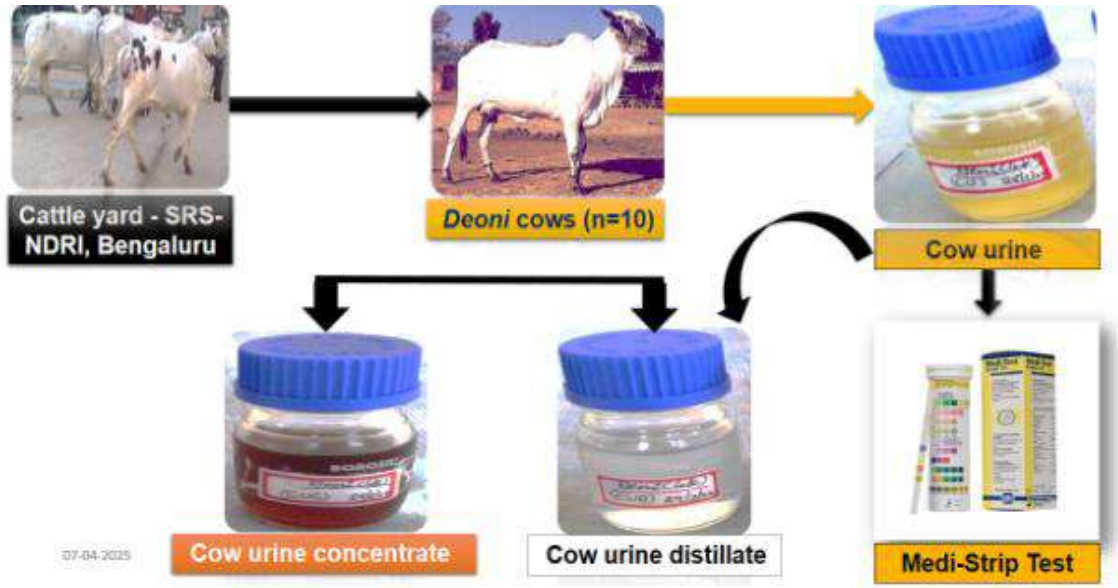

Fig. 1. Collection of cow urine samples and its cow urine distillate and concentrate preparation

Fig 2. Flow diagram of Soxhlet solvent extraction of *Acalypha indica* plant leaf extracts

Acalypha indica plants leaf powder and its extracts

Acalypha indica L. plants were collected from the fields of Gudiyatham, Tamil Nadu, India. The leaves were separated and thoroughly washed with tap water, followed by distilled water for the removal of dust and soil particles in a tray 2-3 times and shade dried for 3-5 days. Dried leaves were milled to obtain a fine powder. Extracts of *Acalypha indica* plant leaves was prepared by Soxhlet extraction method (Fig. 2). In a thimble, 10g of *Acalypha indica* leaves fine powder was extracted with 150 ml of 70% ethanol and 70% methanol solvents independently. The extraction was carried out for 13 hours continuously until the transparency of the extracts was obtained, and the obtained extracts were thereafter concentrated by evaporation in a rotary-vacuum. The obtained dry forms of extracts were used to prepare the samples. Finally, the leaf extraction yield was calculated as;

(% Yield = Mass of total extracted compound/Mass of raw material × 100)

Phytochemical screening of *Deoni* cow urine and *Acalypha indica plant* leaf extracts

The freshly prepared *Deoni* cow urine samples and *Acalypha indica* leaf extracts (both methanolic and ethanolic) were subjected to qualitative phytochemical screening to test for the presence of the phyto-constituents/active principles such as alkaloids, tannins, saponins, phenolics, flavonoids, and terpenoids following standard procedures to identify the constituents as described by Edeoga (2005), Sofowara (1993) and Harborne (1973).

Thin layer chromatography (TLC) Profiling of *Deoni* cow urine and *Acalypha indica plant* leaf extracts

Thin layer chromatography profile was assessed by the method given by Nautiyal *et al*. (2021) to separate different bioactive compounds present in *Deoni* cow urine samples and *Acalypha indica* leaf extracts. 15µl of *Acalypha indica* leaf extract was spotted by a capillary tube on pre-coated silica gel chromatography plates (Merck, TLC grade) as stationary phase, whereas the different solvent systems were used as the mobile phase. The best separation was achieved by using the following solvent systems such as; ethanol/water (80:20 v/v), methanol/acetic acid (80:20 v/v), methanol/water (80:20 v/v) and chloroform/acetic acid (80:20 v/v). After the development of chromatograms, the plates were removed and allowed to dry.

Detection of the spots

All the plates were dried properly, position of different compound spots were detected on plate by visualizing directly under UV light at 254 nm. The movement of the active compound was expressed by the retention factor (R_F). The retention factors (R_F) were calculated for each spot using the following formula:

R_F = (Distance travelled by solute/Distance travelled by solvent)

Results and Discussion

Collection of cow urine samples and its preparation of distillate and concentrate

Urine samples were successfully collected from ten healthy *Deoni* cows (n=10) in the late lactation stage, were obtained during the early morning hours from the cattle yard at the Southern Regional Station (SRS) of ICAR-NDRI, Bengaluru. Early morning, first-voided cow urine is considered more sterile and enriched with macro and micronutrients, enzymes, and urea, making it potentially more effective for therapeutic and

experimental applications (Pescheck-Böhmer and Schreiber, 1999). Following collection and preliminary screening, two potent forms of the urine: cow urine distillate and cow urine concentrate were prepared according to standard protocols. The preparation process is illustrated in (Fig. 1).

Preparation of *Acalypha indica* leaf extracts

The extracts of *Acalypha indica* plant leaves was prepared by Soxhlet extraction method using 70% methanol and ethanol as solvents and obtained a good yield of extracts. The collection and preparation of extracts were mentioned in (Fig.2).

Active principle of samples

Phyto-chemical screening of cow urine sample and its concentrate and solvent extracts of herb demonstrated that cow urine samples contained phenols, alkaloids, flavonoids; cow urine concentrate contain phenols, alkaloids, flavonoids, saponins, terpenoids, tannins and steroids; leaf extracts contained alkaloids, tannins, saponins, phenolics, flavonoids and terpenoids. These findings are summarized in (Table 1).

Thin Layer Chromatography profiles of *Deoni* cow urine samples

The thin layer chromatography system was used to separate different bioactive compounds based on their R_F values present in the *Deoni* cow urine and its potent forms. In our study, we have chosen three different solvent systems such as; Methanol/Acetic acid (80:20 v/v), Chloroform/Acetic acid (80:20 v/v) and Ethanol/Water (80:20 v/v) based on literature to achieve for the best separation of active compounds from cow urine and its potent forms based on R_F values on TLC plate by using TLC method.

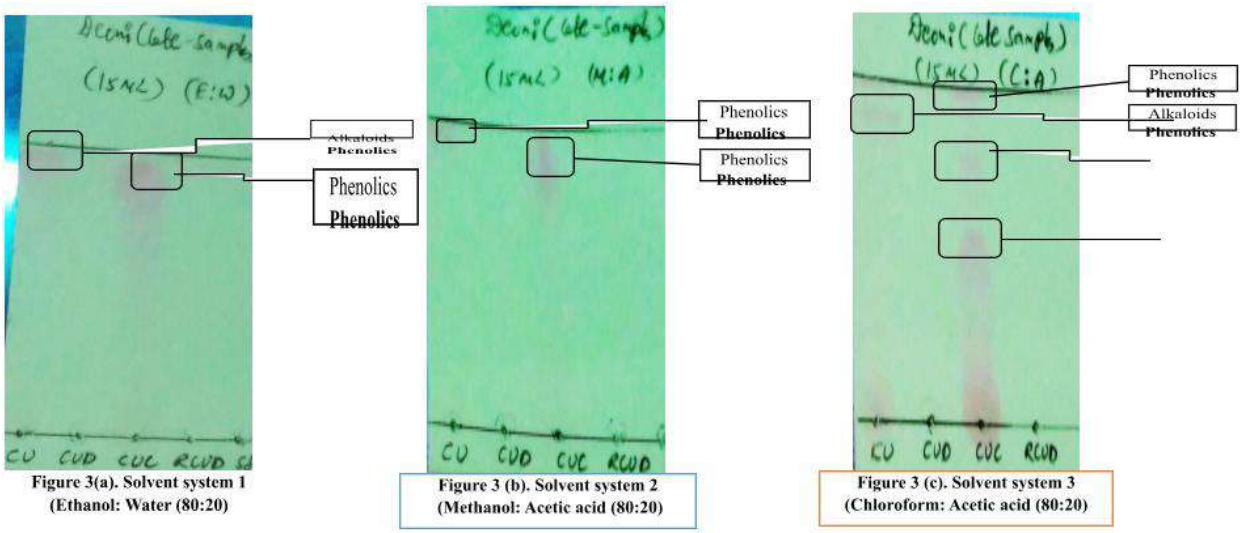

Figure 3(a). Solvent system 1 (Ethanol: Water (80:20)

Figure 3 (b). Solvent system 2 (Methanol: Acetic acid (80:20)

Figure 3 (c). Solvent system 3 (Chloroform: Acetic acid (80:20)

[**CU:** Cow urine; **CUD:** Cow urine distillate; **CUC:** Cow urine concentrate]

Fig. 3. Thin Layer Chromatography profiling of *Deoni* cow urine samples

Solvent system 1 - Methanol/Acetic acid (80:20 v/v)

The thin layer chromatography of the urine samples in solvent system 1- Ethanol: Water (80:20 v/v) was used to separate the active components and two visible spots were obtained with the R_F values of 1.0 (phenols) and 0.95 (alkaloids) respectively. No active principle in cow urine distillate samples was observed (Fig. 3a).

Solvent system 2 - Methanol: Acetic acid (80:20 v/v)

TLC profile of the urine samples in solvent system 2- Methanol: Acetic acid (80:20 v/v) separated two visible spots at R_F values 1.0 (phenols) and 0.97 (phenols) respectively (Fig.3b). No active principle in cow urine distillate samples was observed.

Solvent system 3 - Chloroform: Acetic acid (80:20 v/v)

TLC analysis of the urine samples of solvent system 3 (Chloroform: Acetic acid (80:20 v/v) revealed four visible spots at R_F values 0.92 (alkaloids), 1.0 (phenols), 0.78 (flavonoids) and 0.54 (alkaloids) respectively (Fig. 3c). No active principle was observed in cow urine distillate samples. The Thin layer chromatography profile of cow urine samples in solvent system – 1, 2 and 3 are mentioned in Table 2.

Thin Layer Chromatography profiles of *Acalypha indica* leaf extracts

The thin layer chromatography system was used to separate different bioactive compounds based on their R_F values present in the *Acalypha indica* leaf extracts. In our study, we have chosen four different solvent systems such as; Methanol: Water (80:20), Methanol/Acetic acid (80:20 v/v), Chloroform/Acetic acid (80:20 v/v) and Ethanol/Water (80:20 v/v) based on literature to achieve for the best separation of active compounds from cow urine and its potent forms based on R_F values on TLC plate by using TLC method.

In our study, we have chosen four different solvent systems such as; Ethanol/Water (80:20 v/v), Methanol/Acetic acid (80:20 v/v), Chloroform/Acetic acid (80:20 v/v) & Methanol/Water (80:20 v/v) respectively to achieve best separation based on their R_F values on TLC Plate by using TLC method. 15µl of 70% methanolic and 70% ethanolic leaf extracts samples were used for spotting purposes along with Reference standard - Gallic acid.

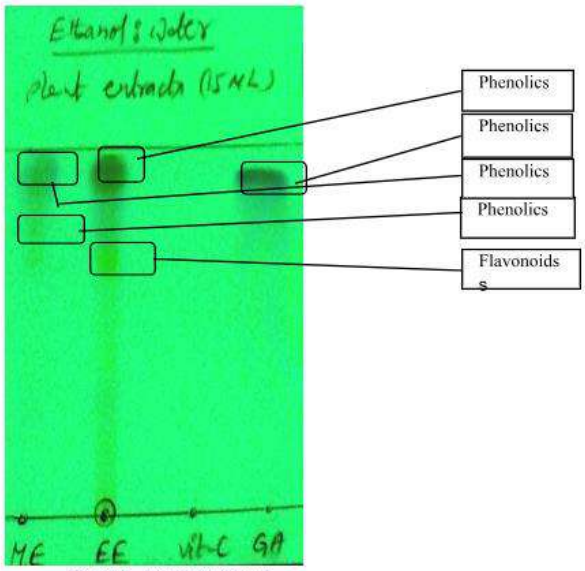

Fig. 4(a). Solent system 1
(Ethanol: Water (80:20)

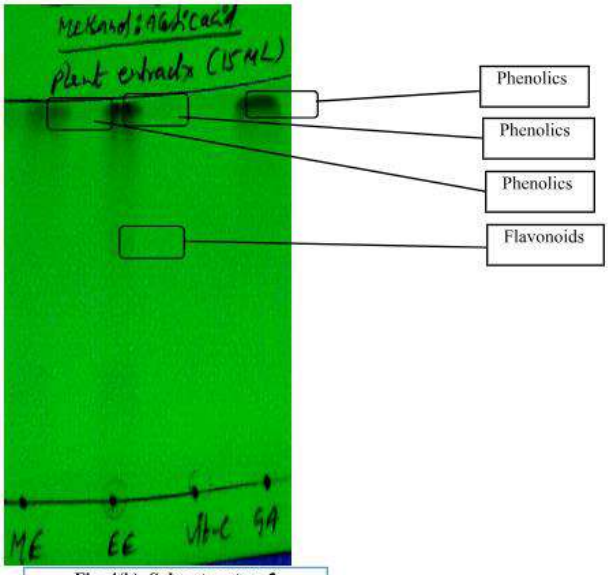

Fig. 4(b). Solent system 2
(Methanol: Acetic acid (80:20)

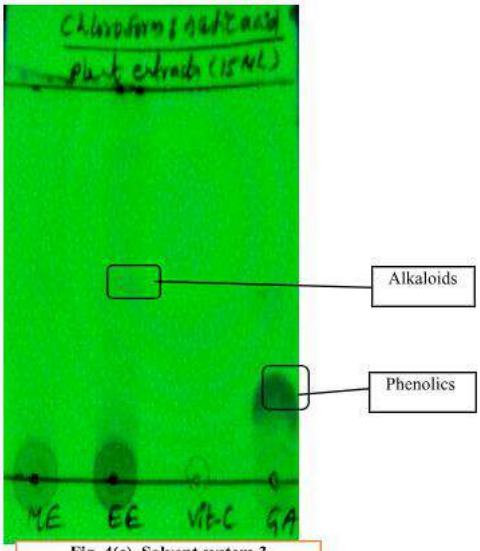

Fig. 4(c). Solent system 3
(Chloroform: Acetic acid (80:20)

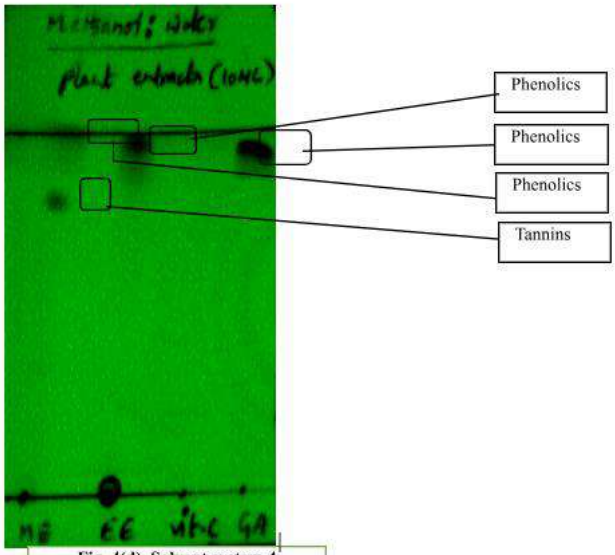

Fig. 4(d). Solent system 4
(Methanol: Water (80:20)

[**ME:** Methanolic leaf extracts **EE:** Ethanolic leaf extracts (Test samples); **GA:** Gallic acid (Standard); **Vit-C:** Vitamin-C]

Fig. 4 Thin Layer Chromatography profiling of *Acalypha indica* leaf extracts

Solvent system I (Ethanol: Water (80:20 v/v) and Solvent system II (Methanol: Acetic acid (80:20 v/v)

TLC study of the 70% methanolic and 70% ethanolic leaf extracts samples of solvent system I (Ethanol: Water (80:20 v/v) was used to separate the active components and 5 visible spots were obtained and the R_F values are: 1.0 (phenolics), 0.8 (phenolics) in methanolic leaf extract; 1.0 (phenolics), 0.78 (flavonoids) in ethanolic leaf extract & 0.92 (Gallic acid - standard) respectively which was described in (Fig. 4a).

TLC analysis of the 70% methanolic and 70% ethanolic leaf extracts samples of solvent system II (Methanol: Acetic acid (80:20 v/v) was used to separate the active components and 4 visible spots were obtained and the R_F values were; 1.0 (phenolics) in methanolic leaf extract; 1.0 (phenolics), 0.71 (flavonoids) in ethanolic leaf extract & 0.97 (Gallic acid- standard) respectively which was described in (Fig. 4b). The thin layer chromatography profiling of separated active principles along with its R_F values in solvent system - I & II were depicted in (Table 3).

Solvent system III (Chloroform: Acetic acid (80:20 v/v) and Solvent system IV (Methanol: Water (80:20 v/v)

TLC profile of the 70% methanolic and 70% ethanolic leaf extracts samples of solvent system III (Choloroform: Acetic acid (80:20 v/v) was used to separate the active components and 2 visible spots were obtained and the R_F values were; 0.57 (alkaloids) in ethanolic leaf extract & 0.28 (Gallic acid-standard) respectively. No Active principles were observed in methanolic leaf extract and which was mentioned in (Fig. 4c).

TLC analysis of the 70% methanolic and 70% ethanolic leaf extracts

samples of solvent system IV (Methanol: Water (80:20 v/v) was used to separate the active components and 4 visible spots were obtained and the R_F values were; 0.85 (tannins), 1.0 (phenolics) in methanolic leaf extract; 1.0 (phenolics) in ethanolic leaf extract & 0.97 (Gallic acid-standard) respectively which was represented in (Fig. 4d). The thin layer chromatography profiling of separated active principles along with its R_F values in solvent system – III & IV were depicted in (Table 3).

In the present investigation we performed and explored the thin layer chromatography profiles of *Deoni* cow urine and *Acalypha indica* leaf extracts samples and their phytochemicals identification, separation and detection for the first time *in vitro* using multi-solvent systematic approach.

Urine samples from ten healthy cows of the *Deoni* breed in their late lactation stage were collected randomly in a clean containers and were filtered with sterile Whatman filter paper and the filtered cow urine was subjected to distillation, the residue left after the reduction of volume to $1/10^{th}$ by distillation procedure was designated as 'Cow urine concentrate' (Wate *et al.,* 2011) (Fig. 1).

Raw cow urine is highly dilute and complex biological fluid is mostly water (about 95-98%), with only 2-5% solids which include urea, creatinine, phenols, volatile fatty acids, enzymes, various minerals and with small amounts of organic and inorganic solutes are present in trace amounts. Early morning first voided cow urine is more sterile and has more macro and micronutrients along with other enzyme/urea content could be more effective. The main of cow urine is

phenols. Phenols are bactericidal to *gram positive* and *gram negative* bacteria. Therefore, the presence of phenols in cow urine has a potent anti-microbial activity. The fresh cow urine contains more amount of phenol and hence has a better anti-microbial activity (Jarald *et al.,* 2008). Fresh cow urine decomposes quickly due to microbial growth and enzymatic reactions (like urease breaking down Urea to Ammonia and CO_2). Cow urine has different fractions like anti-microbial activity due to the presence of certain components like volatile and non-volatile ones (Jarald *et al.,* 2008). Presence of creatinine, urea, swarnakshar (aurum hydroxide), phenols, carbolic acid, calcium and manganese has strongly explained the germicidal and anti-microbial properties of cow urine. Presence of amino acids and urinary peptides may enhance the bactericidal effect by increasing the bacterial cell surface hydrophobicity.

Fresh cow urine contains higher amounts of phenols makes it more effective against microbes. After photo-activation, few biogenic volatile inorganic and organic compounds such as; CO_2, NH_3, CH_4, methanol, propanol, acetone and some metabolic secondary nitrogenous products are also formed (Singh *et al.,* 2012). Cow urine contains phenolic acids (gallic, caffeic, ferulic, o-coumaric, cinnamic and salicylic acids) which have anti-fungal characteristics (Upadhyay *et al.,* 2010). When we want to analyse active compounds, direct analysis of raw urine is not effective. Hence, distillation and concentration are essential preparatory steps for separating organic compounds of interest.

Distillation helps separate volatile (phenols, aldehydes, short chain acids) and non-volatile substances, removing unwanted interfering substances before testing for analysis. Distillation and concentration of urine samples are crucial to remove water and impurities, enrich the organic constituents, and improve the accuracy and sensitivity of bioactive compound analysis. Distillation (evaporation) removes the bulk water, leaving behind a concentrated residue containing the. This concentration step improves the detection sensitivity of various analysis. Without concentration, the extraction efficiency is poor. After concentration, FTIR signals become stronger and clearer, making it possible to detect specific active compounds.

By concentrating urine (through evaporation or distillation; the bioactive compounds (urea, uric acid, volatile fatty acids, phenols and hippuric acid) become more potent per unit volume. This improves the biological activity and therapeutic efficacy in formulations. The concentrated urine reduces the volume needed for storage and transport and allows easier incorporation into the formulations. Concentration helps to standardize the active fractions and allow for quantitative estimation of key biomarkers for batch-batch consistency. Concentrated urine acts as a bio-enhancer (increasing absorption or activity of metabolites). Concentration-especially after mild heating or distillation –inactivates enzymes and reduces microbial load, adjusting P^H improves stability. Concentration removes volatile ammonia and offensive odour (partially), microbial contaminants and organic debris. Thus making it more acceptable and safer for

pharmaceutical or veterinary formulations.

The phytochemical screening confirmed the presence of bioactive compounds such as alkaloids, phenolic compounds, terpenoids, tannins, flavonoids and saponins in *Deoni* cow urine samples (Table 1). Previous studies have also reported the presence of similar bioactive compounds such as alkaloids, phenolic compounds, carbohydrates, proteins, steroids, terpenoids, and cardiac glycosides in cow urine from other Indian indigenous breeds like the *Junagadh Gir* (Farida and Minocheherhomji, 2016).

The thin layer chromatography profiling of *Deoni* cow urine samples using multi-solvent systems were performed for the first time to investigate the bioactive constituent's identification and separation. The *Deoni* cow urine samples exhibited characteristic spots that corresponds to volatile fatty acids, phenolic acids, urea derivatives, and trace alkaloid-like compounds. The occurrence of strong spots under UV-254 nm suggests the presence of aromatic compounds and conjugated systems (dark spots). The multi-solvent approach also revealed distinct R_F patterns suggestive of:

The solvent system – I: ethanol: water in (80:20) ratio generally provides an ideal polarity balance, enabling efficient extraction of broad spectrum of phytochemicals while maintaining compound stability and safety. It is widely extracting flavonoids, phenolic acids, saponins, tannins, alkaloids and terpenoids. But in case of *Deoni* cow urine samples, it extracted only phenols and alkaloids.

The solvent system – II: methanol: acetic acid in (80:20) ratio gives the broad spectrum extraction, good

stability and recovery of active principles. In cow urine samples, it only extracting phenols.

The solvent system – III: chloroform: acetic acid in (80:20) ratio is used to obtain acid-stable, non-polar to moderately polar compounds especially alkaloids, terpenoids and lipids. The acid improves stability, while chloroform ensures efficient extraction of lipophilic compounds. In cow urine samples, it only extracted alkaloids, phenols and flavonoids.

The diverse of bands detected supports the known medicinal properties of cow urine-including antimicrobial and antioxidant activities through multiple natural metabolites acting synergistically. The TLC profiles of urine samples (Fig. 3a, 3b, 3c and Table 2).

Similarly, *Acalypha indica L.* plants were collected from the fields of Gudiyatham, Tamilnadu, India. The leaves were separated and dried leaves were milled to obtain a fine powder. Extracts of *Acalypha indica* plant leaves was prepared by Soxhlet extraction method using 70% methanol and 70% ethanol as solvents and obtained a good yield of extracts (Fig. 2).

Soxhlet extraction is preferred in our present study because it provides a reproducible, exhaustive, and solvent-efficient sequential extraction way to isolate active compounds allowing precise identification and characterization of functional groups. It also allows continuous washing of the sample with fresh solvent, ensuring that all extractable compounds are completely removed from the matrix. This exhaustive extraction is essential for accurate functional group analysis. The solvent collected after Soxhlet extraction is relatively pure and concentrated, containing only the soluble active

molecules. This facilitates accurate analysis of compounds. It also offers high reproducibility compared to other methods.

We used 70% methanol as a solvent for Soxhlet extraction, because it provides balanced polarity, capable of extracting a wider spectrum of plant bioactive compounds both polar and moderately non-polar. This methanol penetrates through plant cell wall more effectively and dissolve intracellular metabolites. This synergy enhances overall extraction efficiency. The boiling point of 70% methanol is slightly higher (70ºC) than pure methanol (65ºC), which provides a milder extraction temperature and helps preserve heat-sensitive compounds (flavonoids and vitamins). 70% methanol gives high phenolic and flavonoid contents and shows better antioxidant and antimicrobial activities. In phytochemical analysis alkaloids and phenols are more in methanol, saponins are present in only methanol. Methanol has a good affinity towards alkaloids, phenols and flavonoids and low affinity towards saponins and amino acids. Methanol extract has the more active compounds. Phytochemical analysis shows that most of the phytochemicals got dissolved in methanol solvent (Sudhakar Chekuri *et al.,* 2016). The phytochemical constituents commonly present in both the leaf extracts include saponins, flavonoids, terpenoids and cardiac glycosides. Overall, the 70% methanol is used in Soxhlet extraction because the water-methanol combination provides optimal polarity, enhances cell wall swelling, improves solubility of diverse phytochemicals, and preserves thermo-labile bioactive compounds resulting in maximum yield, high reproducibility and functional bioactivities of extracted compounds.

We selected 70% ethanol as a solvent for Soxhlet extraction, because it provides balanced polarity, capable of extracting a wider and broad spectrum of plant bioactive compounds both polar (phenolics, flavonoids, some alkaloids) and moderately non-polar (essential oils and terpenoids). This ethanol penetrates through plant cell wall more efficiently and dissolve intracellular metabolites. This synergy enhances overall extraction efficiency. Many bioactive compounds (especially polyphenols, flavonoids and saponins) are more soluble in aqueous ethanol than absolute ethanol. The boiling point of 70% ethanol is slightly higher (78-83ºC) than pure ethanol (78ºC), which provides a milder extraction temperature and helps preserve heat-sensitive compounds (flavonoids and vitamins). 70% ethanol gives high phenolic and flavonoid contents and shows better antioxidant and antimicrobial activities. Overall, the 70% ethanol is used in Soxhlet extraction because the water-ethanol combination provides optimal polarity, enhances cell wall swelling, improves solubility of diverse phytochemicals, and preserves thermo-labile bioactive compounds resulting in maximum yield, high reproducibility and functional bioactivities of extracted compounds.

Qualitative phytochemical analysis were conducted to confirm the presence of active principles in *Acalypha indica* leaf extracts exhibited a broader range of phytochemicals, including phenols, alkaloids, flavonoids, saponins, terpenoids, and tannins (Table 1). These phyto-constituents are known for their significant antioxidant, antibacterial, and anti-inflammatory properties, indicating the potential therapeutic efficacy strongly supports in the plant

based novel formulation in veterinary applications.

In the present study; we used different solvent systems for the first time to detect various phytochemicals in *Acalypha indica* plant leaf extracts using thin layer chromatography. These solvent systems used because to separate broad spectrum of phytochemicals with good stability and safety. Generally, these solvent systems gives the following phytochemicals while running on TLC in the given below:

The solvent system – I: methanol: acetic acid in (80:20) ratio gives the broad spectrum extraction, good stability and recovery of active principles.

The solvent system – II: methanol: water in (80:20) ratio is especially effective; phenolic compounds and flavonoids, saponins, moderately polar alkaloids and polyphenols.

The solvent system – III: ethanol: water in (80:20) ratio provides an ideal polarity balance, enabling efficient extraction of broad spectrum of phytochemicals while maintaining compound stability and safety. It is widely extracting flavonoids, phenolic acids, saponins, tannins, alkaloids and terpenoids.

The solvent system – IV: chloroform: acetic acid in (80:20) ratio is used to obtain acid-stable, non-polar to moderately polar compounds especially alkaloids, terpenoids and lipids. The acid improves stability, while chloroform ensures efficient extraction of lipophilic compounds.

We performed the thin layer chromatography for the first time to identify and detect bioactive compounds in *Acalypha indica* leaf extracts using different solvent systems mentioned above. We also observed the movement of

phytochemicals and how the phytochemicals are separated and detected on TLC plate (Fig. 4a, 4b, 4c, 4d and Table 3). The movement of solvent systems are in the following order of separation of phytochemicals based on their polarity and we observed more precisely;

M: AA (80:20) > M: W (80:20) > C: AA (80:20) > E: W (80:20

(Fastest) (Medium) (Slower)

TLC profiling of the extracted active compounds from both *Acalypha indica* plant leaf extracts gives an impressive result that directing towards the presence of number of phytochemicals and showed the good sensitivity and separation. In all four solvent systems used, based on TLC profiling, we identified and confirmed that phenolics are more in both *Acalypha indica* leaf extracts followed by flavonoids, alkaloids and tannins (Table 3). The previous findings also clearly reported that (very few reports available); TLC is used for identification of compounds from the crude extract of *A. indica*, four major spots were observed with petroleum ether, ethyl acetate (4.2:0.8) with the R_F value of 0.18, 0.36, 0.70 and 0.96; indicating the presence of tannin, alkaloid, flavonoid and phenol (V. Thamil Priya *et al.,* 2020). Also reported four compounds (pale yellow, green yellow, green) were identified in alcohol and aqueous crude extract of *A. indica* (Thenmozhi and Rajan, 2012).

Conclusion

In the current study successfully established a systematic multi-solvent TLC profiling method of *Deoni* cow urine samples and *Acalypha indica* leaf extracts, enabling clear visualization and differentiation of their phytochemical compounds. Sequential extraction using solvents of increasing polarity proved effective

in maximizing compound recovery, reproducible resolution of bands corresponding to diverse secondary metabolites. It highlight its effectiveness for systematic separation and identification of diversity of bioactive compounds, supporting quality evaluation and preliminary identification of phytochemicals.

Solvents with varying polarities extract different classes of phytochemicals, demonstrating that solvent selection is critical and crucial step for efficient recovery of target compounds. Polar solvents such as methanol and ethanol yielding high amounts of phenolics and flavonoids, where as non-polar solvents like chloroform is more effective in extracting terpenoids and fatty acids. The TLC fingerprints obtained in this study revealed the presence of multiple phytochemical constituents-including phenolics, flavonoids, terpenoids, tannins, saponins and alkaloids-supporting the traditional therapeutic relevance of both *Deoni* cow urine and *Acalypha indica.* The distinct banding patterns across solvents highlight the complementary chemical profiles of the two natural products and suggest potential synergistic applications in ethno-veterinary or biomedical formulations.

This profiling strategy serves as a reliable method for rapid screening of natural products by systematic and robust approach for comprehensive phytochemical analysis. Overall, this multi-solvent TLC approach offers a simple, rapid, cost-effective, and reproducible method for preliminary phytochemical screening, standardization, quality control, and future bioactivity-guided fractionation.

Declaration

Conflict of interest statement

The author (s) clearly certify that there is no conflict of interest in any manner with regards to the content/research work presented in the manuscript briefly as Original Article for submission to Curevita Research International Nexus. Peer-reviewed International Journal.

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Author contribution declaration

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Supplementary Sheet

Table 1. Phytochemical screening of *Deoni* cow urine samples and *Acalypha indica* leaf extracts

Qualitative test	Active principles	Cow urine	Cow urine Concentrate	Methanolic leaf extract	Ethanolic leaf extract
Wagner's	Alkaloids	+	+	+	+
Ferric Chloride	Tannins	-	+	+	+
Foam test	Saponins	+	+	+	+
Ferric Chloride	Phenolics	+	+	+	+
Shinoda/Lead acetate	Flavonoids	+	+	+	+
Liberman Burchard	Terpenoids	-	+	+	+

Table 2. Thin Layer Chromatography Profiling of *Deoni* cow urine samples

(-); indicates absence of active principle, (+) indicates presence; (-) indicates absence of phyto-chemicals

Solvent systems	Test samples *Deoni* cow urine and its potent forms	R_F values (cm)	Active Principles
1. Ethanol: Water (80:20 v/v)	Cow urine	7 / 7 = 1	Phenols
	Cow urine distillate	-	-
	Cow urine concentrate	6.7 / 7 = 0.95	Alkaloids
2. Methanol: Acetic acid (80:20 v/v)	Cow urine	7 / 7 = 1	Phenols
	Cow urine distillate	-	-
	Cow urine concentrate	6.8 / 7 = 0.97	Phenols
3. Chloroform: Acetic acid (80:20 v/v)	Cow urine	6.5 / 7 = 0.92	Alkaloids
	Cow urine distillate	-	-
	Cow urine concentrate	7 / 7 = 1 5.5 / 7 = 0.78 3.8 / 7 = 0.54	Phenols Flavonoids Alkaloids

Table 3. Thin Layer Chromatography profiling of *Acalypha indica* plant leaf extracts

Solvent systems	*Acalypha indica* plant leaf extracts	R_F values (cm)	Active Principles
1. Ethanol :Water (80:20 v/v)	Methanolic leaf extract	7 / 7 = 1 5.6 / 7 = 0.8	Phenolics Phenolics
	Ethanolic leaf extract	7 / 7 = 1 5.5 / 7 = 0.78	Phenolics Flavonoids
	Gallic acid (standard)	6.5 / 7 = 0.92	Phenolics
2. Methanol : Acetic acid (80:20 v/v)	Methanolic leaf extract	7 / 7 = 1	Phenolics
	Ethanolic leaf extract	7 / 7 = 1 5 / 7 = 0.71	Phenolics Flavonoids
	Gallic acid	6.8 / 7 = 0.97	Phenolics
3. Chloroform : Acetic acid (80:20 v/v)	Methanolic leaf extract	-	-
	Ethanolic leaf extract	4 / 7 = 0.57	Alkaloids
	Gallic acid	2 / 7 = 0.28	Phenolics
4. Methanol: Water (80:20 v/v)	Methanolic leaf extract	6 / 7 = 0.85 7 / 7 = 1	Tannins Phenolics
	Ethanolic leaf extract	7 / 7 = 1	Phenolics
	Gallic acid	6.8 / 7 = 0.97	Phenolics

(-); indicates absence of active principle