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Observations of Estrous Behaviour and Identification of Faecal Compounds in Umblachery Cow (*Bos indicus*) with Reference to Live Stock Production

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Abstrsct: Reproductive performance of Umblachery breed cattle (*Bos indicus*) has declined worldwide because of the complexity of female reproductive physiology and the macromolecules produced from females corresponding to hormonal regulation and physiological status. The hormonal regulation leads to reduced level of reproductive efficiency or total reproduction failure. The accurate estrus detection is essential for successful reproduction. Several attempts have been made to detect estrus but failed due to cost concerns. Many scientists reported the importance of pheromone technology for accurate detection of estrus. Based on the above information, the present study aimed to observe various estrus behavior and identify the estrus-specific compounds by gas chromatography-mass spectrometry (GC-MS) of bovine feaces during different phases of estrous cycle. In the present study fecal samples were collected during various behaviors of the estrous cows. The GC-MS results clearly showed that a total of thirteen compounds were identified in the fecal samples of different phases. Among these compounds, tetramethyl-2-hexadecenol, tetramethyl-2-hexadecene and squalene were common to all the phases. But 3-Methyl-tricyclo [4.3.2.03, 10] undecane and 2, 10-dimethyloctacosanoic acids were found only in estrus and not in other phases. Hence, the unique compounds produced at estrus phase may appear to be behaviorally important in attracting the bulls. The presence of specific compounds in estrus feaces makes the possibility to develop a biomarker for the detection of estrus in farm animals. Further research is required to understand the role of estrus-specific compounds using behavioral assay for male by which the livestock production can be improved.

Key words: Umblachery cattle · Feacal phermones · Estrus behaviors · Detection · Gas Chromatography-Mass Spectrum

INTRODUCTION

Cattle play an integral part of human society for providing food, clothing, fuel, traction and companionship and often using resources that would be of little or no value to us [1]. Milk production and reproductive performance are two major determinants of dairy cow profitability. Milk production has dramatically increased over the last several years, but the reproductive performance of Umblachery breed cattle has declined [2-4]. A wide genetic diversity was found in Indian cattle breed and most of the genetic breeds are degraded due to indiscriminate crossbreeding and irregular mating among the breeds situated in all other's vicinity. As a result, several indigenous breeds with many popular traits are in danger of extinction while others are in the path of success replaced completely by certain high-producing crossbred animals [5].

Reproductive performance is essential to effective management and production of cattle. However, a number of problems can arise with the reproductive process so that normal function is lost. This can lead to a reduced level of reproductive efficiency in some cases or total failure of reproduction [6]. Poor estrus detection is a major

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contribution to reduced reproductive efficiency in dairy cows. Usually, estrus detection was performed by visual observation for signs of mounting behavior, which is accurate, but larger herd sizes and less manpower per cow reduce opportunities for visual observation and detection to be less than 50% [7, 8]. Several methods had been used to detect the time of ovulation through tail paint, chin-ball markers, video cameras, pedometers, radio telemetry and heat-mount detectors that have been developed over the years, but these methods are far from effective and are too expensive.

Mammals release different variety of molecules into the environment, either as specific chemo-signals or metabolic by-products through vaginal fluid, urine, feaces, saliva, milk, sweat and specialized scent glands. The bovine olfactory system has the ability to recognize and discriminate thousands of structurally diverse odorants, which have access to the behavioral and physiological outputs. These chemical cues are used to pass the signals between individuals conveying information including sex, reproductive status and individual identity. The complex mixtures of molecules sensed by bovine chemosensory systems have important influences on sexual stimuli, mother–young interaction, mate choice and territorial marking [9]. Feaces can provoke a supply of chemo-signals from unicellular organisms to higher vertebrates, which contain volatile compounds with regular variations during different phases of estrous cycle [10], pregnancy [11] and lactation [12] in bovine.

Pheromones regulate reproductive behavior in many animal species and once released in the environment through excretion, these volatile substances reach other individuals of the same species and entail signaling, for instance, mating availability and strengthening ties between mother and offspring, as well as regulating social relationships. Mammalian pheromones may be a single compound or a mixture of compounds conveying specific signals related to reproductive behavior [13-16]. It was reported that the timing of the physiological events of ovulation and co-ordination of sexual behavior ensures successful fertilization [17]. Accurate estrus detection is still a major problem in bovine reproduction and similarly artificial insemination is not successful and satisfactory. Effective, accurate and economical methods are not available for large-scale field test of estrus detection. The present study discussed about the method of estrus detection, the problem associated with the activity and how they might be solved through estrus detection practice and aids. Chemical signal is a read to evaluate a method of estrus detection, which would be economic, reliable and easy so that farmers can identify the estrus phase very accurately. Based on the above information, the present study aimed to observe the behavioral patterns of Umblachery cows and the responses of bulls under a range of estrous cycles and identify the estrus-specific volatile compounds in the bovine feaces during various estrus phases of bovine.

MATERIALS AND METHODS

Experimental Animals: Umblachery (*Bos indicus*) breed is an excellent draught cattle of Tamil Nadu noted for its strength and sturdiness. They are distributed in eastern coastal districts such as Thiruvarur and Nagapattinam District in Tamilnadu, India, approximately between 10° 18’ and10° 54’ N and 79° 48’ E with an estimated total area of 3500 square kilometer. The elevation of the breeding tract ranges from 0 to 50 meters above the sea level. How many???? mature cows of Umblachery breed were selected for the present study. The animals were maintained at District Livestock Farm, Korukkai, Thiruthuraipoondi, Thiruvarur District, Tamil Nadu, India and the experimental animals were fed with conventional diet (cultivated forage crops, rice straw, green fodder) and water *ad libitum.*

Changes in Physiology and Behaviors of Animals: In nonpregnant cyclic cows, physiology and behavioral responses were observed between the estrous cow and the bull. The cow physiological changes and behavioral symptoms included reduced food intake, milk secretion, weight loss, vaginal swelling, softening of valve, reddening of valve, frequent intermittent urination, salivation, tears discharge, vaginal mucus discharge and restlessness, while those of the bull included mating call., licking, self-grooming, flehmen, mounting, sniffing the vulva, copulation, penile erection, at times homosexual behavior were observed in both individuals at different phases of estrous cycle.

Estrus Phase Identification: The estrous cycle was determined with the help of the conventional estrus behaviors in female cow and fern pattern of saliva and vaginal secretions, which were checked for the confirmation of estrus phase. The length of estrous cycle was assessed through observing vaginal mucosa smear in order to know the stage of cycle to collect the vaginal fluid samples. Vaginal smears were performed and analyzed by light microscopy for the proportion of three cell types, such as leukocytes, epithelial and cornified cells [18].

Sample Collection and Preparation: The fresh fecal samples were collected during six consecutive estrous cycles of Umblachery cattle. The samples were categorized as pro-estrus, estrus and post-estrus. The samples were maintained and stored at –20°C for further analysis. In the present study, samples were extracted with nine different organic solvents viz., acetone, benzene, chloroform, dichloromethane, diethyl ether, ethanol, methanol, n-hexane and petroleum ether. The dichloromethane (DCM) extraction showed the maximum response. Consequently, DCM was used as a solvent in the present study. Each sample was thawed at room temperature prior to analysis. Then, 1g of samples in triplicate of the pooled feaces was taken and separately mixed with 2 ml of dichloromethane. The supernatant was filtered through a silica gel column (60–120 meshes), concentrated under vacuum (Temperature <30°C) for fractionation and further processed for chemical identification.

Gas Chromatography–Mass Spectrometry Analysis: The fecal samples collected from six animals were pooled and extracted with dichloromethane. Two micro liters of extract was injected into the GC-MS system on a 30-m glass capillary column with a film thickness of 0.25 mm (30 m 60.2 mm i.d. coated with UCON HB 2000) using the following temperature program: initial oven temperature of 40°C for 4 min, increased to 25°C at 158°C/ min and then held at 25°C for 10 min. GC-MS was run under computer control at 70 eV. Chemical ionization was performed using ammonia as the reagent gas at 95 eV. The solvent (Dichloromethane) peak was seen at 2.4 min during the GC-MS analysis followed by compound peaks. Identification of unknown compounds was made by probability-based matching using the system such as, WILEY Registry™95, NIST05 and NIST05s.

RESULTS

Behavioral Observation: Estrus behavioral patterns were observed in the female cow. The subsequent behaviors by bull towards female cow were also recorded. Estrus-specific behaviors were observed in female cow. Bull also exhibited some characteristic behaviors prominently toward the female cow in estrus (Table 1). Among the various behaviors exhibited by cow were restlessness, increase locomotion, weight loss, reduced feed intake, reduced milk secretion, frequent urination, vaginal mucus discharge, vaginal swelling, hyper salivation, tear discharge, bellowing, standing heat,

Table 1: Observation of estrus behaviours in bull and cow of Umblachery cattle

S.No	Behaviours	Bull	Cow
1	Flehmen	✓	X
2	Mounting	✓	X
3	Head side mounting	✓	X
4	Penile erection	✓	X
5	Body rubbing	✓	X
6	Sniffing	✓	X
7	Licking	✓	X
8	Tail raising	✓	X
9	Dominance over other bull	✓	X
10	Chasing of female	✓	X
11	Copulation	✓	X
12	Head to head interaction	✓	X
13	Lip curling	✓	✓
14	Self grooming	✓	✓
15	Allogrooming (grooming others)	✓	✓
16	Homosexual behaviour	✓	✓
17	Vaginal swelling	X	✓
18	Vaginal mucus discharge	X	✓
19	Frequent urination	X	✓
20	Hypersalivation	X	✓
21	Tear discharge	X	✓
22	Restlessness	X	✓
23	Long time walking	X	✓
24	Weight loss	X	✓
25	Body rubbing	X	✓
26	Reduced food intake	X	✓
27	Reduced milk secretion	X	✓
28	Standing heat	X	✓
29	Teasing of bull	X	✓
30	Mounting of other cow (absence of male)	X	✓
31	Flehmen (absence of male)	X	✓

"✓" - present, "X"- absent.

Table 2: Behaviors observed in cow during estrus phase

S.No	Behaviours
1	Restlessness
2	Long time walking
3	Reduced food intake
4	Reduced milk secretion
5	Weight loss
6	Vaginal swelling
7	Vaginal mucus discharge
8	Frequent urination
9	Tear discharge
10	Hypersalivation
11	Lip curling
12	Bellowing
13	Body rubbing
14	Standing heat
15	Teasing of bull
16	Homosexual behaviours
17	Mounting of other cow (absence of male)
18	Flehmen (absence of male)

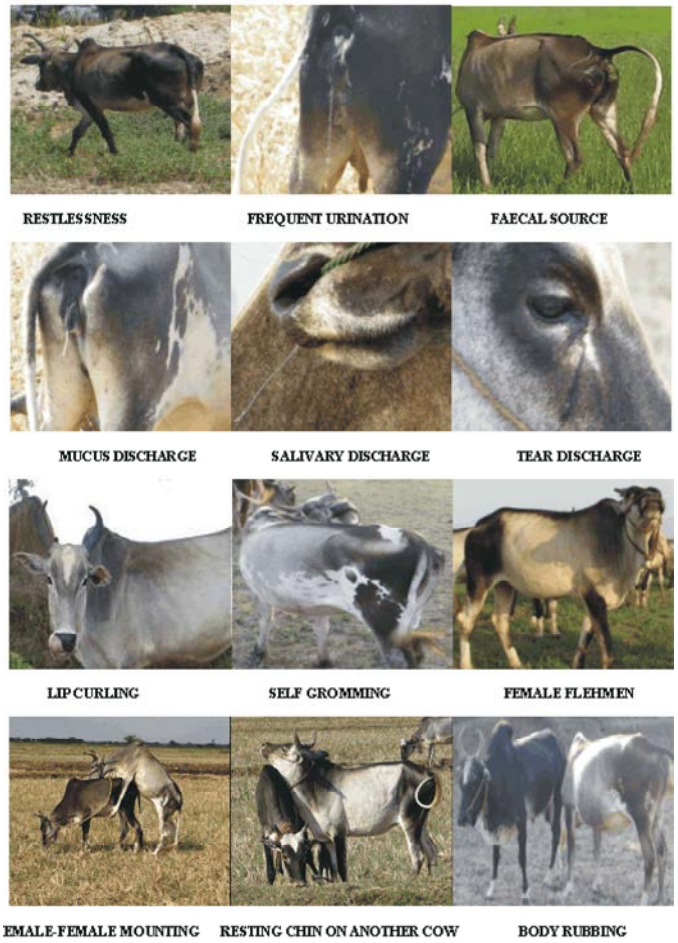


Fig. 1: Observation of behaviors in Umblachery cow during estrous cycle

self-grooming and allogrooming were intense during estrus. At times, the female cow was involved in the homosexual behavior like flehmen and mounting. The behaviors exhibited by female cows were categorized based on the intensity of expression and are given in (Fig. 1; Table 2). The behaviors like body rubbing, licking, sniffing, flehmen, mounting, penis erection and copulation were observed in bull, which encountered with cow in estrus are given in (Fig. 2).

GC-MS Analysis: GC-MS analysis was carried out using bovine feaces of three different stages of estrous cycle (Pro-estrus, estrus and post-estrus). GC-MS results showed that thirteen compounds were identified in the various fecal samples of bovine. Among this, six compounds were obtained in pro-estrus, nine compounds in estrus and eight compounds in post-estrus stages. The compounds like 6-methyl tetrathiadodecadiene (I), tetramethyl-2-hexadecenol (II), tetramethyl-2 hexadecene (III), citronellyl propionate (IV), dimethyl eicosadienoic acid (V), squalene (VI), phthalic acid (VII), 3-methyl-tricyclo [4.3.2.03, 10] undecane (VIII), hexadecanoic acid (IX), 2, 10-dimethyloctacosanoic acid (X), tetracontane (XI), octadecane (XII) and octadecanoic acid (XIII) are shown in Table.3. The identified compounds belong to alkane, alkene, phytol and acid groups. The alkane and acid groups were mostly present. The compounds Tetramethyl-2-hexadecenol, Tetramethyl-2-hexadecene and Squalene are found in all stages and citronellyl propionate is found in pro-estrus and estrus. In particular, 6-methyl tetrathiadodecadiene and dimethyl eicosadienoic acid were found only in pro-estrus stage, not in other stages. 3-methyl-tricyclo [4.3.2.03, 10] undecane and 2, 10 dimethyloctacosanoic acid were specifically present in the estrus stage not in other stages. Phthalic acid, hexadecanoic acid and tetracontane were present in estrus and post-estrus stage not in pro-estrus stage. Octadecane and Octadecanoic acid are present only in post-estrus not in estrus and pro-estrus stages.

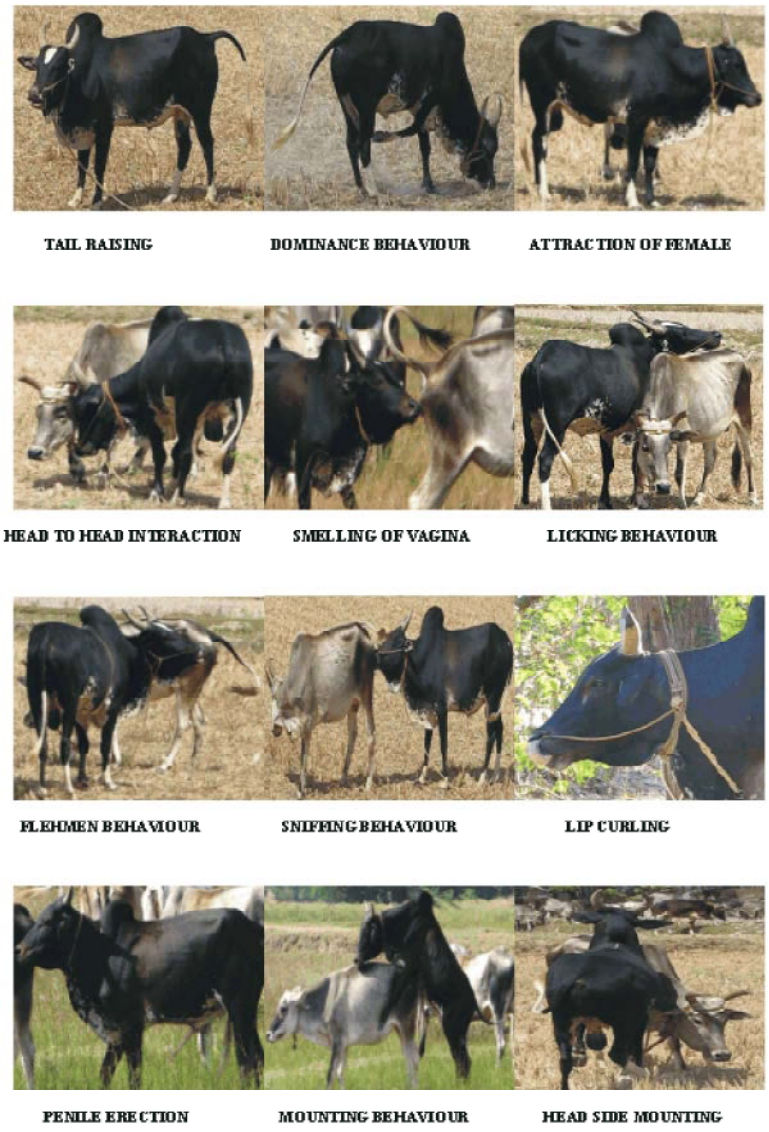


Fig. 2: Estrus behaviors exhibited by bull towards cow

Table 3: Identification of faecal volatile compounds in different phases of estrus Umblachery cow.

Peak No.	Compounds	Nature of compounds	M.F	M.W	Pro estrus	Estrus	Post Estrus
I	6-Methyl tetrathiadodecadiene	Alkene	$C_9H_{16}S_4$	252	✓	X	X
II	Tetramethyl-2-hexadecenol	Phytol	$C_{20}H_{40}O$	296	✓	✓	✓
III	Tetramethyl-2-hexadecene	Alkene	$C_{20}H_{40}$	280	✓	✓	✓
IV	Citronellyl propionate		$C_{13}H_{24}O_2$	212	✓	✓	X
V	Dimethyl eicosadienoic acid	Acid	$C_{23}H_{42}O_2$	350	✓	X	X
VI	Squalene		$C_{30}H_{50}$	410	✓	✓	✓
VII	Phthalic acid	Acid	$C_{17}H_{24}O_4$	292	X	✓	✓
VIII	3-Methyl-tricyclo[4.3.2.03, 10]undecane	Alkane	$C_{12}H_{20}$	164	X	✓	X
IX	Hexadecanoic acid	Acid	$C_{20}H_{40}O_2$	312	X	✓	✓
X	2, 10-Dimethyloctacosanoic acid	Acid	$C_{27}H_{55}Br$	458	X	✓	X
XI	Tetracontane	Alkane	$C_{40}H_{82}$	562	X	✓	✓
XII	Octadecane	Alkane	$C_{18}H_{38}$	254	X	X	✓
XIII	Octadecanoic acid	Acid	$C_{22}H_{44}O_2$	340	X	X	✓

Present " " absent "X" M.F – molecular formula M.W – molecular weight

Please cite the Figures 3, 4, 5 and table3 in the text of the result section!!!!!!

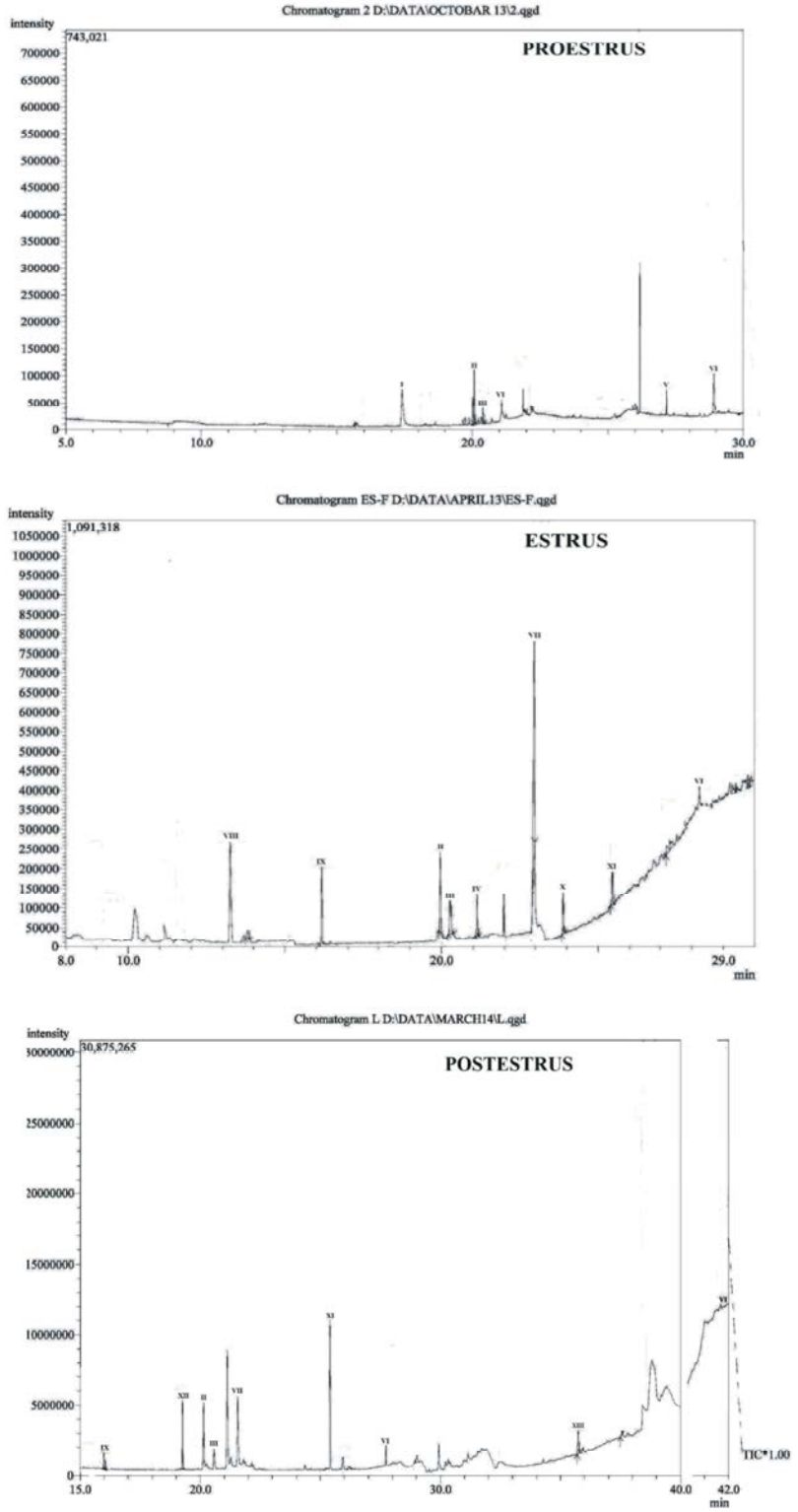


Fig. 3: Gas chromatography analysis of faecal volatile compounds from different phases of Umblachery cow estrous cycle Peak no. references to Table 3

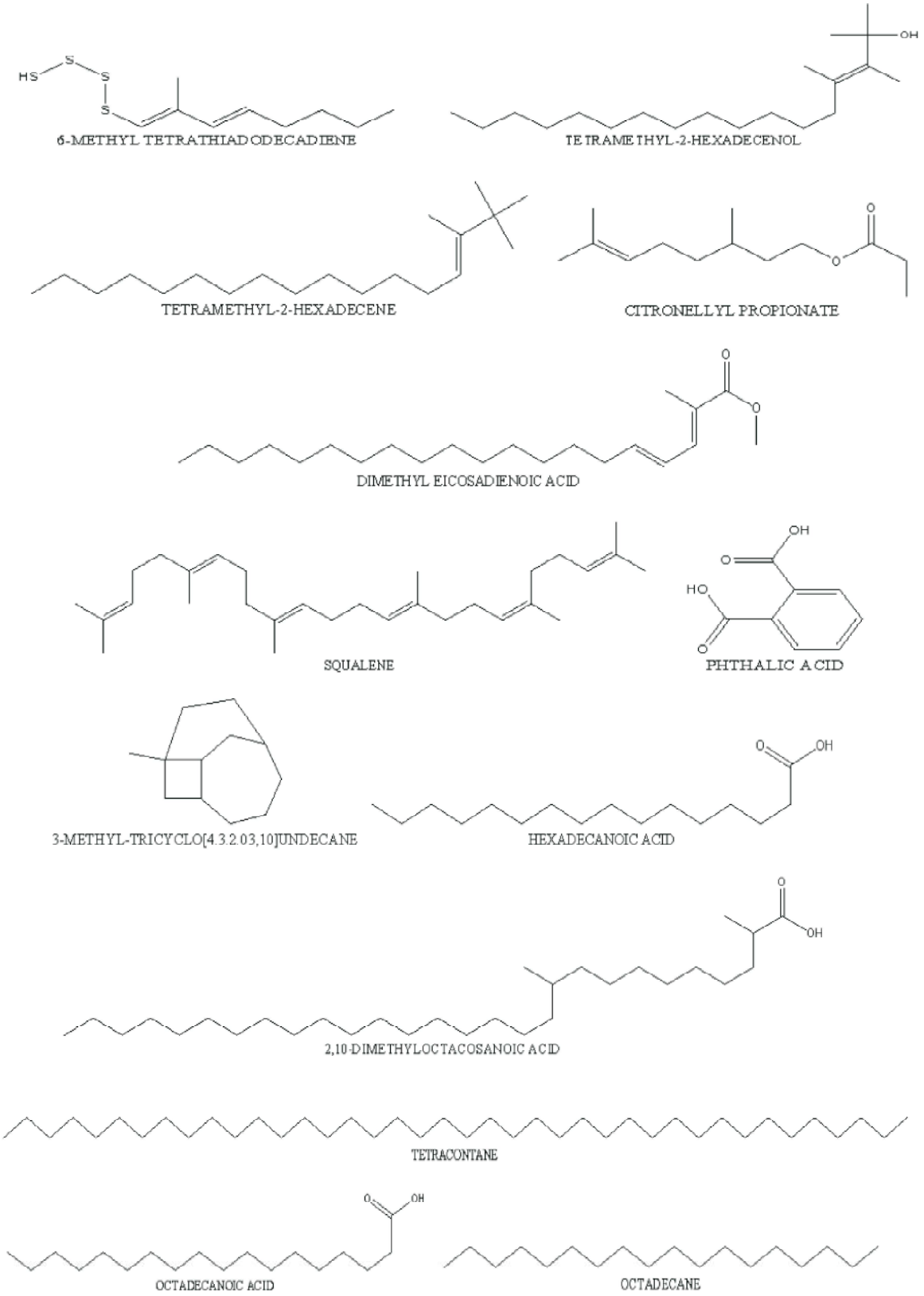
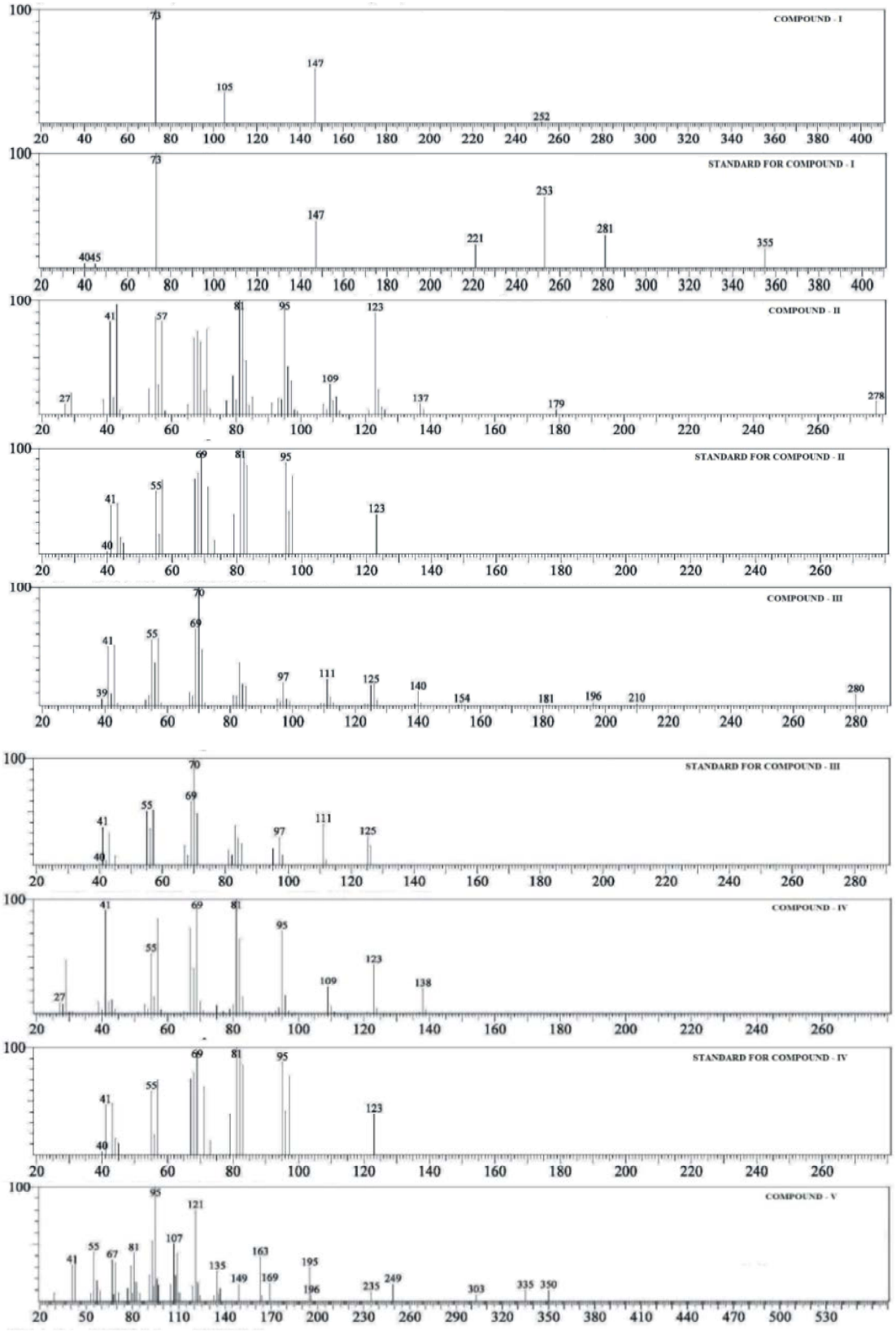


Fig. 4: Chemical structures of identified faecal compounds of Umblachery cow in various stages of estrous cycle



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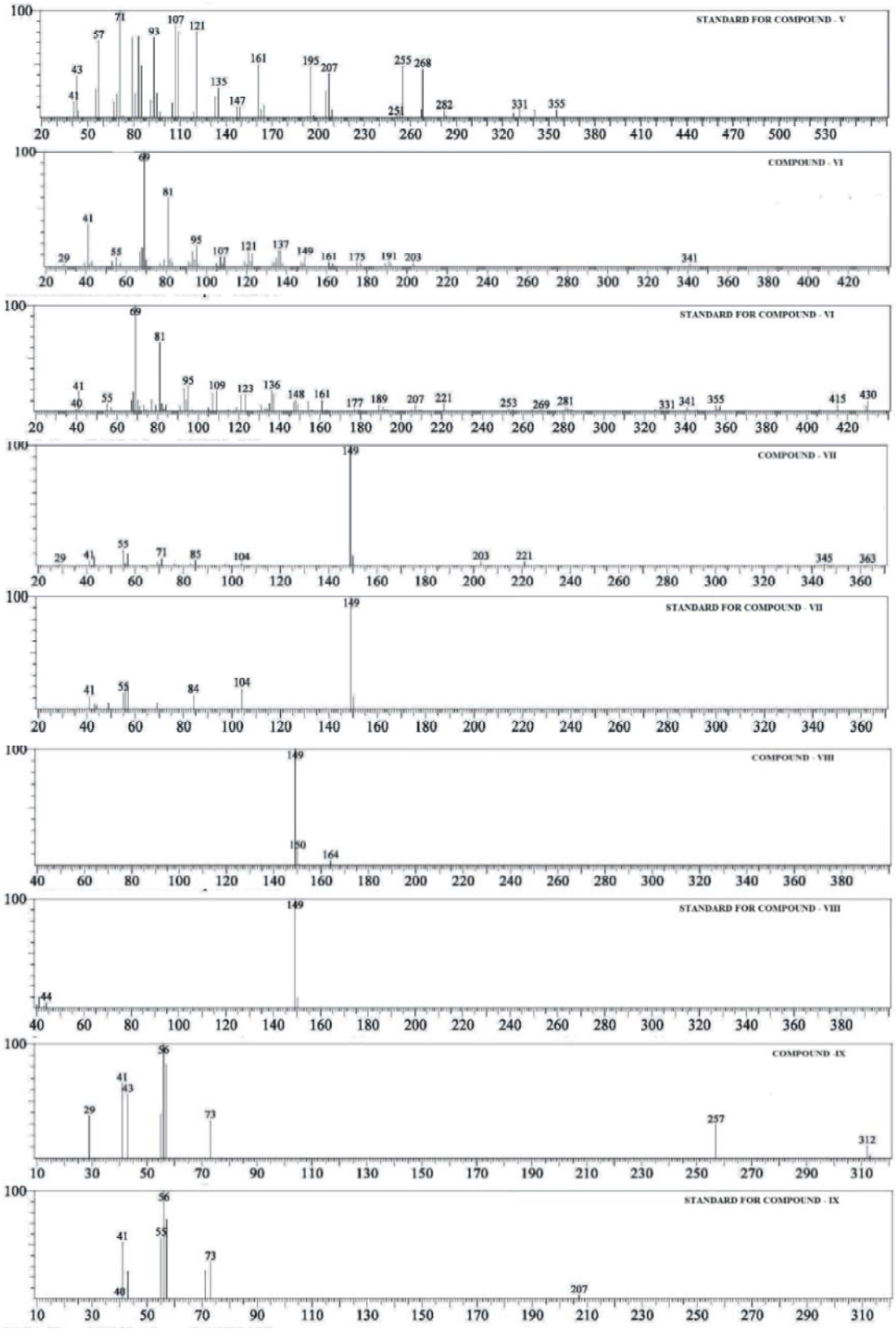

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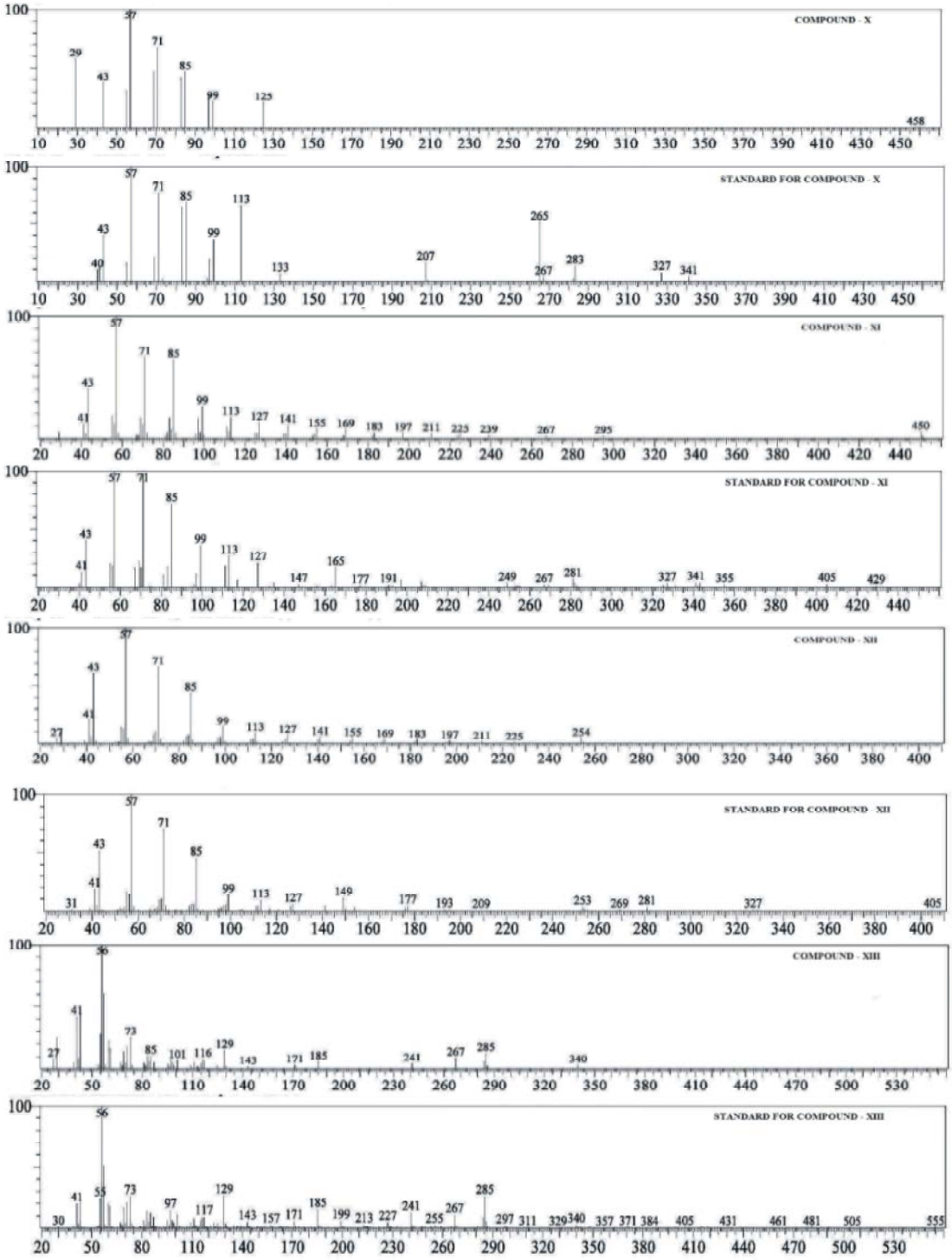

Fig. 5: Mass spectrum of Umblachery faecal compounds identified in various stages of estrous cycle

DISCUSSION

Animal's behavior can provide an extremely important approach to wildlife conservation because of their tendency to examine individual differences, variability and different behavioral strategies. Problems in animal management are a subset of the universal green trouble, which are of prime interest in conservation

biology. Major biological problems include the extensive loss of species through the modification of global biogeochemical cycles. Information corresponding to animal behavior are not the solitary solution to solving overall conservation problems [19]. The female elephant dung may contain chemo-signals that could preferentially change the attraction of male towards female feaces during the ovulatory phases [10]. Similarly, in our study, the Umblachery cow feaces attract the bull during three different phases of estrous cycle. The following behaviors, such as, sniffing the female vagina, licking, dominant over other bull, flehmen, mounting, head-side mounting, chasing of females, penile erection and copulation were observed only on the estrus cow and not on the cow at other phases. Sequences of behavior require animals to change from one behavior to another. A similar pattern of behaviors were observed in goats during the onset of estrus in which bleating, restlessness and vaginal mucus discharge were prominent [20, 21]. Homosexual behaviors of estrus females are very often observed as expressed in flehmen and mounting behavior towards other females. This observation is consistent with the report of Sankar *et al*. [22] who reported that the homosexual behaviors as one of the most important behaviors in estrus phase. Homosexual behavior may be defined as informal or stable sexual interaction between two or more individuals from the same gender [23]. Homosexual behavior also been reported in several species, including sheep [24] goats [21, 25] bison [26] giraffes, antelopes, musk ox [27] and some deer species [27, 28]. Homosexual was frequently observed in males [29] and in estrus females [30]. In addition, vaginal mucus discharge is one of the standard criteria in the identification of estrus in female dairy cattle [31]. In this study, clear vaginal mucus discharge was observed during estrus phase of the Umblachery cow. Behavioral expression is a major aspect of animal communication and would easily reflect the internal status to the other members. Small-to-large mammals display considerable variation in the exhibition of behaviors during different physiological states [32]. Bull exhibit different behaviors of which the flehmen and mounting are prominent [33].

Excretory sources are excellent indicators of the physiological status of animals. For this explanation, animals communicate better through the chemical signals that are present in the excretory products. Among the excretory products, the feaces excreted by an animal in visually conspicuous sites act as a good indicator of communication. Furthermore, the volatile organic compounds present in the excretory sources could be used as markers for the identification of the physiological status of the animals [34].

The considerable range of molecules acting as pheromones across the animal territory suggests that almost any kind of molecule can evolve into a pheromone if it gives selective advantage [35]. Pheromones are a specific group of chemical attractants produced by animals to stimulate other animals. They are present in all body fluids and there are many different types in cattle, including alcohols, diols, alkanes, alkenes, ethers, diethers, ketones, primary amines and aromatic alkanes. The estrous pheromones are mainly released from the body surface, particularly in the hindquarters and genital region, rather than in urine, feaces or vaginal mucus [22]. In the present study, thirteen different volatile compounds were identified in the feaces of cow at different stages of estrous cycle, which qualitatively differed from one stage to the other. Most of the researchers reported that the mammalian pheromones are excreted through body fluids. Also, it was reported that acetic acid, propionic acid and 1-iodo undecane appeared during estrus phase, but were not found in the other reproductive phases. Among the compounds identified in estrus feaces, acetic and propionic acids belong to acids and 1-iodo undecane belongs to alkane group. 1-iodo undecane in the urin during estrus showed a significant precopulatory behavior in bull [36]. Gnanamuthu and Rameshkumar [9] reported that fatty acids such as valeric acid, caproic acid, myristic acid, gadoleic acid and pelargonic acid were found only in the fecal samples during estrus but not during either pro- or post-estrus of Umblachery cattle. Achiraman and Archunan [37] reported that the compound, 1-iodo-2methyl undecane may be the putative chemical signal appearing prior to estrus phase and excreted through estrus. The present study publicize that the chemical compounds 3-methyl tricycle [4.3.2.03, 10] undecane and 2, 10 dimethyloctacosanoic acid are identified in the estrus bovine feaces. To the best of our knowledge, these compounds were not reported in the animal or insect pheromone list and 3-methyl tricycle [4.3.2.03, 10], undecane and 2, 10 dimethyloctacosanoic acid recorded in the current study are considered newly well-known pheromone compounds from Umblachery cattle feaces as it arouse the reproduction of this animal. An important finding in the present study is that the identification of specific compounds during estrus in the feaces and conformation of identified compounds with bull by behavioral studies using synthetic compounds.

CONCLUSION

The present study concluded that the compounds 3-methyl tricycle [4.3.2.03, 10] undecane and 2, 10 dimethyloctacosanoic acid only present in estrus stage may be possibly considered as the biostimulants that stimulate the bull's sexual activity in order to improve the reproductive status of Umblachery cattle. This bio-stimulation offers a potentially useful and practical way to improve reproductive efficiency in livestock species. Further study is required to prove the role of identified estrous-specific compound(s) with the behavior of bull and it would be reliable indicators of estrus and pave the way to develop a biomarker for the detection of estrus in Umblachery cattle.

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