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Research Article

A Perspective on Male Contraceptive Therapeutics of Hydro-ethanol Extract of Seeds of *Luffa acutangula (L.) Roxb.* in Human and Albino Rat: An *In-vitro* Meta-Analysis Study

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ABSTRACT

Traditionally *Luffa acutangula (L.)* Roxb. has been utilized as an herbal contraceptive. This *in-vitro* study has been performed to search out the male contraceptive efficacy of hydro-ethanol (60:40) extract of *L. acutangula* (LAHEE) seeds in a dose-specific manner (1, 2, and 4 mg/mL of *in-vitro* media). The percentages of motile, viable, hypo-osmotic swelled (HOS), and acrosomal intact of human and rat sperms were declined significantly ($p < 0.05$) at the above-mentioned doses of LAHEE-exposed groups against the placebo group. The inhibitory concentration 50% value (IC_{50}) of LAHEE was 2.5 mg/mL in human and 1.2 mg/mL in rat spermatozoa immobilization. The Δ5, 3β and 17β hydroxysteroid dehydrogenase (HSD) activities of rat's testicular tissues were inhibited significantly at $p < 0.05$ in LAHEE-treated groups than the placebo group. Activities of superoxide dismutase (SOD) and catalase were significantly inhibited ($p < 0.05$) along with the significant increment ($p < 0.05$) in the quantity of thiobarbituric acid reactive substances (TBARS) in rat's testes and epididymis, sperm pellets of humans and rats in LAHEE-treated groups against the placebo group without any significant difference ($p > 0.05$) in above said sensors in the liver and cardiac tissues of rats. The non-toxic nature of LAHEE was indicated by no significant alterations ($p > 0.05$) in the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) after LAHEE exposure in said tissues of rats. The liquid chromatography–mass spectrometry (LC–MS) study of LAHEE identified the presence of oleic acid, dihydroquercetin, luteolin-7-glucoside, formononetin, luteolin 8-C-pentoside-6-C-hexoside, pterosin B, boldine and berberine. These findings interpreted that the LAHEE possesses spermiological inhibitory and hypo-testicular activities, which indicate a promising possibility to develop a potent herbal male contraceptive agent from this plant extract.

INTRODUCTION

The uncontrolled increment of the world population has become the biggest and most challenging issue in developing countries like India, and its consequences are undernutrition, poverty, and environmental degradation.[1] The recent projection in 2022, a prospectus of the global population, shows that the world population may exceed 8.5 billion before 2030 when India becomes the highest population-dense country by reaching 1.43 billion people.[2] Family planning is a major concern for regulating unpredictable population growth by adopting suitable contraceptive methods.[3] From this standpoint, there are significantly limited choices of contraceptives available to control male fertility with respect to females.[4] The common accessible contraceptive methods available in the market involve hormonal contraceptives such as combined hormonal contraceptives (CHC) oral pills, progestin-only pills (POP), and depot medroxyprogesterone acetate injections (DMPA-also known as Depo-Provera) and non-hormonal contraceptives includes copper intra-

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uterine devices (IUDs), barrier techniques with or without spermicides, behavioral methods, and surgical sterilization. All such contraceptives have severe adverse effects. Moreover, non-hormonal synthetic spermicidal agents like nonoxynol-9 and benzalkonium chloride, potent chemical surfactants, are linked with multiple risk factors and are vulnerable to urinary tract infection (UTI) in females that may increase viral shedding.[3]

Globally, the demand for traditional cum complementary and alternative medicine (TCAM) is increasing rapidly.[5] From that perspective, WHO decided to search out a new, oral, non-hormonal, herbal effective contraceptive regimen for fertility regulation.[6] Keeping such a view in mind, researchers at global basis are searching for another new strategy for the development of alternative contraceptives.

In India, different ethnic groups of people use different parts of *Luffa acutangula* for various health issues. In West Bengal, it is called Jhinga (Family- Cucurbitaceae) and its common names are angled loofah and ridged gourd. Numerous current pharmacological studies have been investigated to evaluate the folk applications of *L. acutangula*, and research data has been gathered to strengthen the traditional claims. A variety of constituents present in this plant, like flavonoids, anthraquinone, triterpenoids, and volatile components, indicate that this plant contains multiple biological and therapeutic activities involving antidiabetic, anticancer, antibacterial, anti-ulcer, hepatoprotective, and immunomodulatory.[7]

As per Purwaningsih 2008, *L. acutangula* has been used in Indonesia as a traditional anti-fertile regimen since ancient times.[8] Also, a literature review reported that this plant contributes to contraceptive effects, but no such scientific evidence was found in an *in-vivo* or *in-vitro* model to evaluate its male contraceptive efficacy and its mode of action.[9] Herbal medicine, the most traditional medicine is used today, solely based on experimental cum empirical experience only. Keeping this point of view, scientific approaches are adopted in this experiment to introduce this traditional herb into formal medical services.[8] In our laboratory, some *in-vitro* experiments have been focused on the spermicidal effects of some medicinal plants such as *Tinospora cordifolia* (Willd.), *Stephania hernandifolia,* and *Acyranthus aspera* in humans and rats.[10,11] Such medicinal plants are utilized as herbal contraceptives traditionally in rural regions. These plant-based spermicidal contraceptives are nature-friendly, health-friendly, easily available, and also affordable for common people.

This is the first experimental report focusing the hypo-testicular effect of *L. acutangula*. Hypo-testicular effects of different solvent extracts of the said plant were observed by our pilot experiment where hydro-ethanol (60:40) extract of mature seeds of the plant was more effective, but immature seeds had no such prominent effect. The matured and ripen fruits as well as seeds of *L. acutangula* are not considered for edible purposes. Only immature fruits and seeds are included in the meal, which raise no issue of contraceptive effect among the consumers in this perspective. Therefore, if any effective phytomolecules are detected from this plant part regarding the induction of male contraception, it will open a new dimension for male herbal contraceptive development in the pharmaceutical industry in the future without imposing any bad impact on the human food supply. Nowadays, a family planning program (FPP) has already been adopted as the 'cafeteria choice approach', where multiple new options for contraceptives are provided to the users.[12] In connection with that, this experiment was performed to explore the *in-vitro* contraceptive effects of LAHEE using human semen samples. To find out its mechanism of contraceptive action in *in-vitro* conditions, the rat's sperm, the rat's reproductive and metabolic tissues were used. Therefore, this empirical study may provide a hint to the pharmaceutical sectors for the future development of herbal male effective contraceptives.

Materials And Methods

Seed Extraction

Mature seeds of *L. acutangula* were procured from Sealdah seed house (Kolkata-700009, West Bengal), validated (voucher number *L. acutangula*/VU/BIO/07/22) by the taxonomist of Botany department of our university, Midnapore (Pin-721102,). After purchasing the seeds, they were cleaned using distilled water (DW) and shade-dried for 48 hours. Dried seeds were coarsely ground, and 100 g of such grounded seeds were drenched in 2 liters of hydro-ethanol (60:40) solvent and left to soak for 48 hours at room temperature with intermittent shaking for extraction purpose. Whatman filter paper (No. 1) was used to filter the solvent. The solvent extract was evaporated using a rotary evaporator to collect the powder form of that extract and stored properly for experimental use in the time course.[13]

Experimental Design

Six healthy, normospermic, fertile adult males (25–30 years) were selected as per standard instructions.[14] On the other hand, six fertile male albino rats (Wistar strain) with 110 to 120 g body weight (BW) were bought from a Committee for Control and Supervision of Experiments on Animals (CCSEA), Govt. of India authorized vendor. Rats were acclimatized in cages at 25 ± 2°C for ten days with food and water *ad libitum*.

In-vitro Study Protocol

In-vitro media was prepared by using Kreb's Ringer bicarbonate buffer (KRB) solution with providing a proper gaseous mixture (95% O_2 and 5% CO_2) and transferred into separate clear test tubes.[15] Each test tube contains 10 mL KRB solution.

Human sperm were taken after three to four days of abstinence from mating or masturbation, as per the WHO guidelines. Semen samples were subjected to allow for spontaneous coagulation and re-liquefaction within one hour in an incubator at 37°C as per standard protocol.[16] About 1-mL of re-liquefied semen sample was directly placed into the 10 mL of KRB solution at 1:10 ratio to examine different spermiological sensors covering the percentages of motile, viable, hypo-osmotic swelled sperm (HOS) and sperm with acrosomal intactness status (AIS). After the sacrifice of all the rats, epididymal sperm, testes, epididymis, liver, and cardiac tissues were collected, sperm suspension was prepared by washing the epididymis using pre-warmed normal saline solution (37°C) with an equal ratio of weight/volume of the epididymis. About 1-mL of epididymal washed sample was transferred into the 10 mL of KRB solution at 1:10 ratio for measuring the above-mentioned spermiological sensors. To check the oxidative stress parameters of sperm, 75% volume of human liquefied semen samples and same volume of rat's epididymal-wash samples were allowed for centrifugation to obtain sperm pellets. The prepared sperm pellet and collected tissues of different groups in this experiment were directly transferred into the 10 mL KRB solution.

Human and rat sperm samples were processed in *in-vitro* test tubes as spermatozoa suspension but sperm pellets of human and rat samples were used separately for exposure to different doses of LAHEE for different periods to study spermatozoa-based enzyme activities. Three doses of LAHEE, i.e., 1, 2, 4 mg/mL of *in-vitro* media were used here as extract exposed groups along with the placebo group. For the assessment of spermiological sensors of both human and rat, the spermatozoa suspensions were placed separately in marked test tubes. The test tubes were exposed to the extract for 20 seconds, 15 and 30 minutes. In contrast, for the enzymatic study, sperm pellets of humans as well as rat in *in-vitro* media and interested tissue samples of rat were incubated in *in-vitro* media at different doses of extract along with a placebo for 2 hours.

Placebo group

Human and rat spermatozoa suspension samples were allowed for incubation separately for 20 seconds, 15 and 30 minutes. Already prepared rat's and human sperm pellets, slices of reproductive and metabolic tissues were placed in separate test tubes without extract at standard conditions of temperature and gas mixture supply for 2 hours in connection with enzyme activity assessment.

1-mg/mL LAHEE-exposed group

In this group, spermatozoa suspension samples of humans and rats were exposed separately at the dose of 1-mg of LAHEE/mL of *in-vitro* media for the aforementioned three durations of incubation. Human and rat sperm pellets, which were already prepared and the slices of said reproductive and metabolic tissues of rats were directly charged with 1-mg of LAHEE/mL, incubated for 2 hours at 37°C with a proper mixture of O_2 and CO_2 supply.

2 mg/mL LAHEE-exposed group

Human and rat sperm suspension samples were exposed separately in the presence of LAHEE at 2 mg dose/mL of *in-vitro* media and allowing for incubation for above three durations like previous groups. Prepared sperm pellets of humans and rats, said reproductive and metabolic tissues of rats were directly exposed in presence of 2 mg LAHEE/mL of *in-vitro* media, allowed for 2 hours incubation at 37°C, and processed as previous groups.

4 mg/mL LAHEE-exposed group

Sperm suspension samples of humans and rats were separately charged with LAHEE at 4 mg dose/mL of *in-vitro* media for three separate durations of incubations. The aforementioned reproductive and metabolic tissues of rats, sperm pellets of humans and rats were exposed to 4 mg LAHEE/mL of *in-vitro* media and processed for 2 hours as other groups.

Sperm Motility

Sperm motility was checked from dose-dependent LAHEE-exposed sperm samples and a placebo group of humans and rats at different incubation periods.[17] One drop of the sperm sample with *in-vitro* mixture was placed on separate grease-free glass slides and covered by clean glass coverslips. Prepared slides were examined under the light microscope (400X) to count the motile sperm from at least five fields and then the collected data was expressed in percentage.

Inhibitory Concentration 50% of LAHEE

The Inhibitory Concentration 50% value of the LAHEE for sperm immobilization assessment indicating 50% of sperm become immotile due to direct exposure to a specific dose of LAHEE. This was evaluated by exposing the sperms to doses of 1, 2, and 4 mg/mL, and the values were placed graphically for plotting the best-fitting graph. By extrapolating the graph, IC_{50} of LAHEE for sperm immobilization was determined.[18]

Sperm Viability

The percentage of viable sperm was counted both from LAHEE-exposed sperm samples and placebo group of humans and rats at different durations by the eosin nigrosine [E-N] staining method.[17] Smears on glass slides were prepared by using the mixture of 50 µL of *in-vitro* media exposed sperm sample of different groups, 50 µL of eosin Y (1%), and 100 µL of nigrosine (10%). About 50 µL of the said mixture was used for smear preparation. Count of viable and non-viable sperms was done from the prepared slides under a microscope (400X) and obtained data was transformed into the percentage value.

HOS test

The solution for the HOS test was prepared by dissolving fructose (1 g) and sodium citrate (0.735 g) in 100 mL of DW. About 100 µL of LAHEE-exposed sperm samples of humans and rats were mixed separately with 1000 µL of HOS solution (pre-warmed at 37°C) at a 1:10 ratio, then incubated at 37°C for 2 hours. The same procedure was followed for placebo group. The percentage of tail curling sperms was computed after collecting the data under the microscope (400X). Sperm tail curling indicating sperm membrane integrity and intactness.[19]

AIS test

Gelatine-coated glass slides were prepared for the acrosomal intactness status (AIS) test and after 24 hours of the slide preparation, the gelatine-coated slides were fixed by using glutaraldehyde solution (0.05%). In 1-mL of phosphate buffer saline D-glucose was used here with 100 µL of processed sperm samples at a 10:1 ratio. Diluted sperms (20 µL) were placed separately on such slides; then, the smear was prepared and allowed for incubation (37°C) for 2 hours. The spermatozoa with holes in the gelatine coat was counted and transformed the data into percentages, indicating the intact functional acrosome status against the placebo group.[20]

Activities of Testicular Δ5,3β and 17β-Hydroxysteroid Dehydrogenase (HSD)

After 2 hours incubation, LAHEE-exposed testis of rats were homogenized at 100 mg tissue/mL of potassium phosphate and EDTA mixture at the strength of 5 and 1-mM. Centrifugation was done at 10000 rpm for 30 minutes at 4°C. About 1-mL supernatant part was collected to measure for each of enzyme activity. Absorbance was recorded at 30 seconds intervals for successive 3 minutes at 340 nm.[21,22]

Activities of Antioxidant Enzymes

After completing 2 hours of incubation, the testes, epididymis, liver, and cardiac tissues of rats were homogenized at 50 mg tissue/mL in phosphate buffer solution (PBS) (pH-7.4) at the strength of 0.1 M for the measurement of superoxide dismutase (SOD) and catalase activities. Human and rat sperm precipitates were prepared separately by centrifugation after being homogenized it in same media. All homogenates were allowed for centrifugation of the *in-vitro* media containing extract or placebo-exposed sperm pellet and then these were homogenized as other tissues. All homogenates were allowed for centrifugation at 10000 rpm for 10 minutes at 4°C. For SOD activity, 2.04 mL of tris buffer (pH-8.0), 20 µL of supernatant, and 20 µL of pyrogallol were taken in a cuvette, and then absorbance was noted at 30-second intervals for successive 3 minutes at 420 nm.[23]

For estimation of catalase activity, spectrophotometric cuvette was filled with 500 µL of H_2O_2, 2.5 mL of DW, 40 µL of supernatant, and then absorbance was recorded at 30-second intervals for 3 minutes successively at 240 nm.[24]

Measurement of Thiobarbituric Acid Reactive Substances (TBARS) Level

Pre-mentioned tissues of rats as well as sperm pellets of humans and rats were homogenized after completion of 2 hours incubation separately at 50 mg/mL concentration in PBS (pH-7.4) at the strength of 0.1 M. About 500 µL

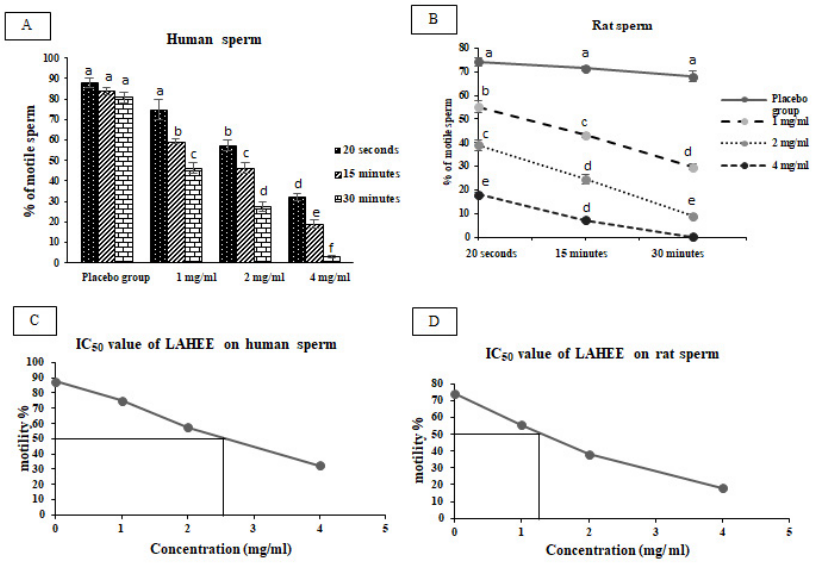

Fig. 1: Effects of the different doses of LAHEE with various incubation durations on sperm motility and its IC_{50} value i.e., (A, C) for humans and (B, D) for rats. Presented data indicated mean ± SEM (n = 6), statistical analysis for a significant study conducted by the Multiple-Comparison Student's two-tail *'t'*-test after ANOVA. Superscripts (a, b, c, d, e, f, g) on bars and points on line diagrams differ from one another significantly, $p < 0.05$

homogenate was taken in a screw cap centrifuge tube where 500 µL of normal saline solution and 2 mL of thiobarbituric acid-trichloroacetic acid (TBA-TCA) solution were added. All the preparations were boiled at 100°C, and centrifuged at 4000 rpm for 10 minutes. Absorbance was recorded at 535 nm.[25]

Assay of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities

The aforementioned tissues were homogenized in PBS (pH-7.4) separately after 2 hours of incubation. The tissue concentration was 50 mg/mL in PBS. About 200 µL of tissue homogenate was mixed with the prepared substrate solution. After that, 1-mL of 2,4-dinitrophenyl hydrazine (DNPH) was used to stop the reaction. Then 10 mL NaOH of 0.4 (N) was added to develop the color. Absorbance was noted at 520 nm against blank.[26]

Qualitative Analysis of Phytomolecules

Qualitative analysis for identification of nature of phyto-ingredients present in LAHEE was performed using the standard method.[27]

Liquid Chromatography-mass Spectrometry Analysis

The liquid chromatography-mass spectrometry analysis (LC-MS) study was conducted by using QuattroMicro^{TM}API mass spectrometer (Waters, Milford, Massachusetts, USA).[28] The liquid chromatographic apparatus contained a quaternary pump, an online vacuum degasser, an autosampler, and a thermostatic column compartment that were attached to the semiconductor device as a detection unit (Waters 2998). MassLynx 4.1 software was considered to acquire and analyze the data. About 10 µL of the sample was injected here by an autosampler. The mobile phase consisted of 94% water, 2% acetonitrile, and 4% methanol. All the compounds were detected in the range of 190 to 690 nm. Within candidate mass (m/z) 100 to 1900, spectra were captured in both positive and negative ionization mode with a 0.4-second scan time, and a 0.01-second inter-scan delay period.

Statistics

Findings were presented as mean ± SEM, (n = 6). Model one variance analysis (ANOVA) followed by both directional *'t'*-test for Multiple-Comparison was adopted to test the significant differences at $p < 0.05$.[29]

Results

Sperm Motility

The reference cut-off of motile sperm is 40% for male fertility as per WHO guidelines.[30] Considering this borderline, the fertility capacity of sperm in the LAHEE-exposed group was analyzed in humans and rats. In humans, exposure to 2 mg LAHEE, the percentage of motile sperm went below the WHO reference limit after 30 minutes of incubation, while in 1-mg/mL LAHEE-exposed group, the percentage of motile sperm was above the said cut-off value after all incubation periods (after 20 seconds, 15 and 30 minutes). In 4 mg/mL LAHEE-exposed group, the motility percentages were reached below 40% after all said incubation periods (Fig. 1A).

In case of rat, less than 40% of motile sperm were noted in 1-mg/mL LAHEE-exposed groups after 30 minutes of incubation. Also, in 2 and 4 mg/mL LAHEE-exposed group, the motility percentage was seen below the WHO reference value after all pre-mentioned incubation times (Fig. 1B). Significant declination ($p < 0.05$) was seen in the percentages of motile sperms (humans and rats) in dose and duration-specific exposure of LAHEE against the placebo group (Figs 1A and B).

IC_{50} Value

The IC_{50} values for sperm immobilization by LAHEE was noted at 2.5 mg/mL in human spermatozoa and 1.2 mg LAHEE/mL in rat spermatozoa (Figs 1C and 1D).

Sperm Viability

The reference value of sperm viability is 58% of an individual for maintaining fertility as per WHO guidelines.[30] In humans, exposure to 4 mg/mL LAHEE, below 58% of viable sperm, was found after all said incubation periods. While, the percentage of viable sperm was above the WHO reference value after all the incubation times in 1 and 2 mg/mL LAHEE-exposed groups (Fig. 2A). Considering the WHO reference value of humans, the percentage of viable sperm was analysed in rats. Exposure to 2 and 4 mg/mL LAHEE, the viable sperm attained less than the WHO reference limit after completing all above incubation times. Less than 58% viable sperm were not observed at any incubation time of 1-mg/mL LAHEE-exposed group (Fig. 2B).

After comparing all the LAHEE-exposed groups in contrast to the placebo group, a significant declination ($p < 0.05$) was displayed in the percentages of viable spermatozoa of humans and rats with different durations and dose-specific exposure of LAHEE (Figs 2A and B).

Hypo-osmotic Swelled Sperm (HOS-sperm)

WHO reference value of HOS-positive sperm is above 58% which is necessary for human fertility.[30] In humans, below 58% of HOS-positive sperm was noted after 15 and 30 minutes of incubation of 2 mg/mL LAHEE-exposed group, but less than 58% of HOS-positive sperm was not observed in 1-mg/mL LAHEE at any incubation period. Also, in 4 mg/mL LAHEE-exposed group, the percentages of HOS-positive sperm remained lower than WHO reference value after all said incubation periods (Fig. 2C). In rats, the percentages of HOS-positive sperm in the LAHEE-exposed group were estimated in contrast to the WHO reference value of human sperm. Below 58% of HOS-

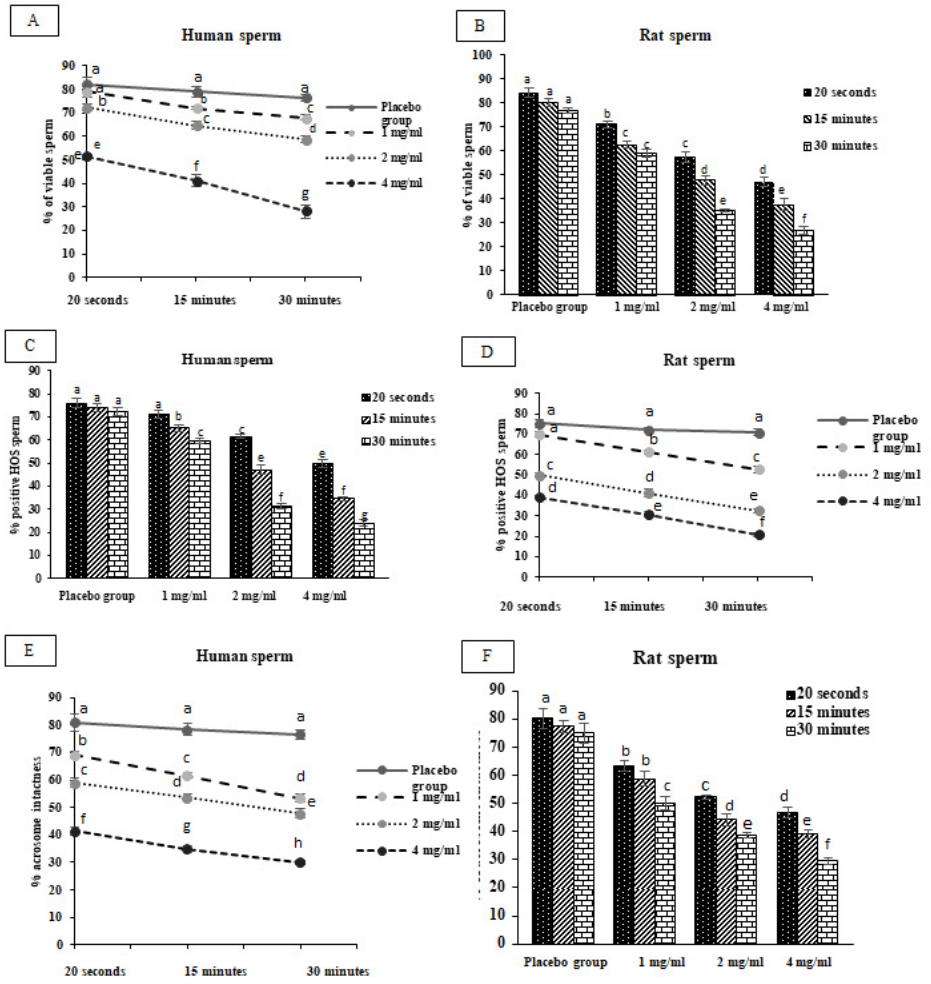

Fig. 2: Dose-dependent direct effects of LAHEE with various incubation durations on the percentage of viable sperm, positive HOS sperm, and intact acrosome sperm, i.e., (A, C, E) for humans and (B, D, F) for rats. Values were demonstrated as mean ± SEM (n = 6). ANOVA, followed by the Multiple-Comparison Student's two-tail *'t'*-test, was used. Bar and line diagrams with distinct superscripts (a, b, c, d, e, f, g, h) differ significantly from one another, $p < 0.05$

positive sperm were found in 1-mg/mL LAHEE-exposed group after 30 minutes of incubation. After completing all three incubation times, less than 58% HOS-positive sperm was noted in 2 and 4 mg/mL LAHEE-exposed groups, respectively (Fig. 2D).

Considering the reduction percentages of HOS-positive sperms of humans and rats in LAHEE-exposed groups in contrast to the placebo group, significant declination ($p < 0.05$) was seen at different periods and dose-specific exposure of LAHEE (Figs 2C and D).

Acrosomal Intactness Status

In humans, the acrosomal intactness status (AIS)-spermatozoa is less than 40%, indicating infertility.[31] In humans, 4 mg/mL LAHEE-exposed group went below 40% of AIS-sperms after 15 and 30 minutes of incubation. While, below 40% of AIS-sperm was not observed in 1 and 2 mg/mL LAHEE-exposed groups at any time of incubation (Fig. 2E).

The percentage of AIS in rat sperm was estimated considering the human reference value. Less than 40% of AIS-sperm was seen after 30 minutes of incubation time in 2 mg/mL LAHEE-exposed group. While, exposure to 1-mg/mL LAHEE, the percentage of AIS was above the pre-mentioned reference value. Also, 4 mg/mL LAHEE-exposed group showed below 40% of AIS-sperm after 15 and 30 minutes of incubation (Fig. 2F).

In contrast to the placebo group, significant diminutions ($p < 0.05$) were seen in the percentages of AIS-sperm of humans and rats at various doses and duration-dependent exposure to LAHEE (Figs 2E and F).

Activities of Δ5, 3β and 17β-HSD

In respect to the placebo group, a non-significant ($p > 0.05$) reduction of these enzymes' activities was noticed in 1-mg/mL LAHEE-exposed groups, while after exposure to 2 and 4 mg/mL LAHEE, a significant inhibition ($p < 0.05$) was seen in contrast to 1-mg dose of exposure and placebo

group. The Δ5, 3β-HSD activities were 21.81% (2 mg/mL), 24.78% (4 mg/mL), and 17β-HSD activities were 24.92% (2 mg/mL), 28.57% (4 mg/mL) significantly inhibited ($p < 0.05$) than the placebo group. Statistically, a non-significant reduction ($p > 0.05$) was noted among 2 and 4 mg/mL LAHEE-exposed groups (Fig. 3).

Activities of Antioxidant Enzymes

Dose-specific significant inhibition ($p < 0.05$) was recorded in SOD and catalase activities in LAHEE-exposed sperm pellets of humans and rats as well as reproductive tissues of rats against the placebo group. In human sperm pellets, 2 and 4 mg/mL LAHEE-exposed groups demonstrated significant inhibition ($p < 0.05$) in these enzymes' activities where non-significant reduction ($p > 0.05$) was noted in 1-mg/mL of LAHEE-exposed group against to the placebo group (Table 1). In rat sperm pellets, significant inhibition ($p < 0.05$) in SOD and catalase activities was noted in all said three doses of LAHEE-exposed groups than the placebo group (Table 2).

In rat's reproductive tissues, the activities of the above sensors were declined significantly at $p > 0.05$ after exposure to 2 and 4 mg/mL LAHEE, respectively. Compared to the placebo group, no significant inhibition ($p > 0.05$) was seen in 1-mg/mL LAHEE-exposed group (Table 2). In rat's metabolic tissues, a non-significant downturn ($p > 0.05$) was found in said LAHEE-exposed groups than the placebo group (Table 2).

Quantification of TBARS

Quantification of TBARS was significantly elevated ($p < 0.05$) in sperm pellets of humans and rats, reproductive tissues of rats in contrast to the placebo group at a dose-specific exposure of LAHEE. In sperm pellets of humans, significant increment ($p > 0.05$) was noted after exposure to 2 and 4 mg/mL LAHEE in respect to the placebo group. The elevation of TBARS levels was noted at 1-mg dose/mL of LAHEE-exposed group in respect to the placebo group is not significant ($p > 0.05$) (Table 1). In case of rat's sperm pellets, all LAHEE-exposed groups were showed significant elevation ($p < 0.05$) in said sensor against the placebo group (Table 3).

Another elevation in TBARS level of rat's testes and epididymis was seen significantly ($p < 0.05$) after exposure to 2 and 4 mg/mL LAHEE against the placebo group. Also, a non-significant elevation ($p > 0.05$) was found between the placebo and 1-mg/mL LAHEE-exposed groups (Table 3). After comparison with the placebo group, the TBARS level was elevated non-significantly ($p > 0.05$) in the metabolic tissues of rats in all three doses of LAHEE-exposed groups (Table 3).

Activities of AST and ALT

In comparison with the placebo group, LAHEE-exposed groups showed non-significant ($p > 0.05$) changes in AST and ALT activities in the testicular, epididymal, liver, and cardiac tissues of rats (Table 4).

Qualitative Analysis of Phytomolecules

Qualitative analysis of phytomolecules resulted that, LAHEE contains flavonoids, alkaloids, and triterpenoids (Table 5).

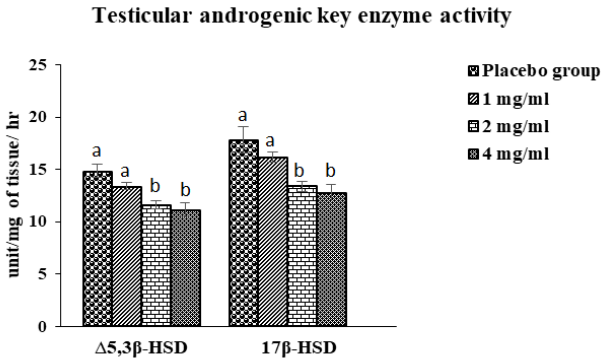

Fig. 3: Direct effects of LAHEE on the activities of Δ5, 3β-HSD and 17β-HSD. All data were manifested as mean ± SEM (n=6). ANOVA, followed by the Multiple-Comparison Student's two-tail *'t'*-test. Bars with distinct superscripts (a, b) differ significantly from one another, $p < 0.05$

Table 1: Dose-dependent effects of LAHEE on oxidative stress biomarkers in human sperm pellets

Experimental groups	*Oxidative stress biomarkers (Human sperm pellet)*		
	SOD (Unit/mg of tissue)	*Catalase (μM H_2O_2 consumed/mg of tissue/min)*	*TBARS (nM/mg of tissue)*
Placebo group	8.00 ± 0.57[a]	33.67 ± 1.85[a]	36.11 ± 1.73[a]
1 mg/mL LAHEE-exposed group	7.50 ± 0.4[a] (6.25%↓)	32.00 ± 1.63[a] (4.95%↓)	37.16 ± 1.66[a] (2.9%↑)
2 mg/mL LAHEE-exposed group	6.28 ± 0.17[b] (21.5%↓)	27.00 ± 0.93[b] (19.78%↓)	42.14 ± 1.2[b] (16.69%↑)
4 mg/mL LAHEE-exposed group	5.66 ± 0.21[c] (29.25%↓)	23.66 ± 0.8[c] (29.7%↓)	45.78 ± 0.82[c] (26.77%↑)

Values were expressed as mean ± SEM (n = 6). ANOVA, followed by the Multiple-Comparison Student's two-tail 't'-test. Values with distinct superscripts (a, b, c) in the column differ from each other significantly, $p < 0.05$, the percentage given in parenthesis'↑' denotes increase and '↓' indicates diminution than the placebo group.

Table 2: Dose-dependent effects of LAHEE on SOD and catalase activities in reproductive, metabolic tissues and sperm pellets in rats

Experimental groups	*SOD (Unit/mg of tissue)*					*Catalase (μM H_2O_2 consumed/mg of tissue/min)*				
	Testes	*Epididymis*	*Sperm pellet*	*Liver*	*Cardiac*	*Testes*	*Epididymis*	*Sperm pellet*	*Liver*	*Cardiac*
Placebo group	0.55 ± 0.02[a]	0.68 ± 0.01[a]	1.35 ± 0.1[a]	2.61 ± 0.1[a]	2.93 ± 0.1[a]	2.26 ± 0.08[a]	0.97 ± 0.07[a]	1.68 ± 0.07[a]	9.00 ± 0.37[a]	7.39 ± 0.29[a]
1 mg/mL LAHEE-exposed group	0.49 ± 0.02[a] (10.9%↓)	0.62 ± 0.01[a] (8.82%↓)	0.71 ± 0.02[b] (47.4%↓)	2.60 ± 0.06[a]	2.92 ± 0.1[a]	2.05 ± 0.04[a] (9.29%↓)	0.89 ± 0.01[a] (8.24%↓)	1.03 ± 0.03[b] (38.69%↓)	8.99 ± 0.4[a]	7.35 ± 0.32[a]
2 mg/mL LAHEE-exposed group	0.38 ± 0.01[b] (30.9%↓)	0.54 ± 0.02[b] (20.58%↓)	0.54 ± 0.01[c] (60%↓)	2.57 ± 0.08[a]	2.85 ± 0.09[a]	1.85 ± 0.03[b] (18.14%↓)	0.64 ± 0.01[b] (34.02%↓)	0.88 ± 0.02[c] (47.61%↓)	8.88 ± 0.43[a]	7.32 ± 0.4[a]
4 mg/mL LAHEE-exposed group	0.35 ± 0.04[b] (36.36%↓)	0.53 ± 0.01[b] (22.05%↓)	0.51 ± 0.01[c] (62.22%↓)	2.55 ± 0.14[a]	2.83 ± 0.06[a]	1.80 ± 0.04[b] (20.35%↓)	0.58 ± 0.02[b] (40.2%↓)	0.83 ± 0.03[c] (50.59%↓)	8.85 ± 0.27[a]	6.31 ± 0.51[a]

Values were expressed as mean ± SEM (n = 6). ANOVA, followed by the Multiple-Comparison Student's two-tail *'t'*-test. Values with the distinct superscripts (a, b, c) in the columns differ from each other significantly, $p < 0.05$, percentage given in parenthesis'↓' indicates diminution against the placebo group.

Table 3: Dose-dependent effects of LAHEE on the quantity of TBARS in reproductive, metabolic tissues and sperm pellets in rats

Experimental groups	*TBARS (nM/mg of tissue)*				
	Testes	*Epididymis*	*Sperm pellet*	*Liver*	*Cardiac*
Placebo group	18.15 ± 0.60[a]	21.30 ± 0.47[a]	35.59 ± 0.96[a]	150.33 ± 2.45[a]	157.00 ± 1.65[a]
1 mg/mL LAHEE-exposed group	19.25 ± 0.58[a] (6.06%↑)	23.01 ± 0.6[a] (8.02%↑)	43.99 ± 1.4[b] (23.6%↑)	153.33 ± 1.89[a]	158.66 ± 1.99[a]
2 mg/mL LAHEE-exposed group	22.49 ± 0.8[b] (23.91%↑)	26.33 ± 0.6[b] (23.61%↑)	49.93 ± 1.5[c] (40.29%↑)	155.33 ± 1.74[a]	160.83 ± 1.62[a]
4 mg/mL LAHEE-exposed group	22.83 ± 0.7[b] (25.78%↑)	26.67 ± 0.51[b] (25.21%↑)	51.80 ± 1.1[c] (45.54%↑)	157.83 ± 2.37[a]	162.33 ± 1.54[a]

Values were expressed as mean ± SEM (n = 6). ANOVA, followed by the Multiple-Comparison Student's two-tail *'t'*-test. Values with distinct superscripts (a, b, c) in the column differ from each other significantly, $p < 0.05$, and the percentage given in parenthesis'↑' indicates elevation in contrast to the placebo group.

LC-MS Analysis

The LC-MS spectrum of LAHEE identified five peaks with m/z at positive ion mode, i.e., 218.54, 304.48, 269.36, 580.07 and 336.44, and three peaks with m/z at negative ion mode, i.e., 281.27, 446.01 and 328.17 (Fig. 4). The retention time (RT) along with the nature of each detected phyto-ingredients are presented in Table 6.

Discussion

According to folklore medicine, many plants have been extensively utilized since ancient times for their spermicidal, abortifacient, and contraceptive efficacies.[32] However, scientific investigation is necessary to validate and to potentiate the therapeutic effects by active molecule isolation of herbal plants. Though local folklore medicinal practitioners have used this plant from date back but still scientific experiments on this plant have not been explored properly from the view of male contraception.[8] On this perspective, this experiment has been conducted to check the anti-spermiological and hypo-testicular effects of LAHEE in the dose and duration-dependent fashion on sperm samples of humans and rats as well as reproductive and metabolic tissues of rats in this *in-vitro* study, the primary and direct study in the specific concern. The WHO guideline of human semen analysis was selected as a reference value to determine the anti-spermiological efficacy of said extract for the assessment of its male contraceptive potentialities. Fertility-relevant different spermiological sensors, i.e., sperm motility, viability, and HOS were considered here and evaluated following standard methods, after comparing with the WHO reference values. As there is no such reference value for rat spermiological sensors, so, translation of the human reference value for the study on rat spermiology was adopted in this aspect.

The activity cum motility of sperm depends primarily on mitochondria located in the midpiece region. Adenosine triphosphate (ATP) is the principal source of energy produced mainly by the mitochondrial oxidative phosphorylation pathway (OXPHOS) in sperm cells in contrast to glycolysis.[33] Various ion channels (Ca^{2+}, K^{+}) are distributed on the plasma membrane and are participated in sperm capacitation, motility, acrosome reaction (AR), and hyperactivation.[34] Sperm motility is

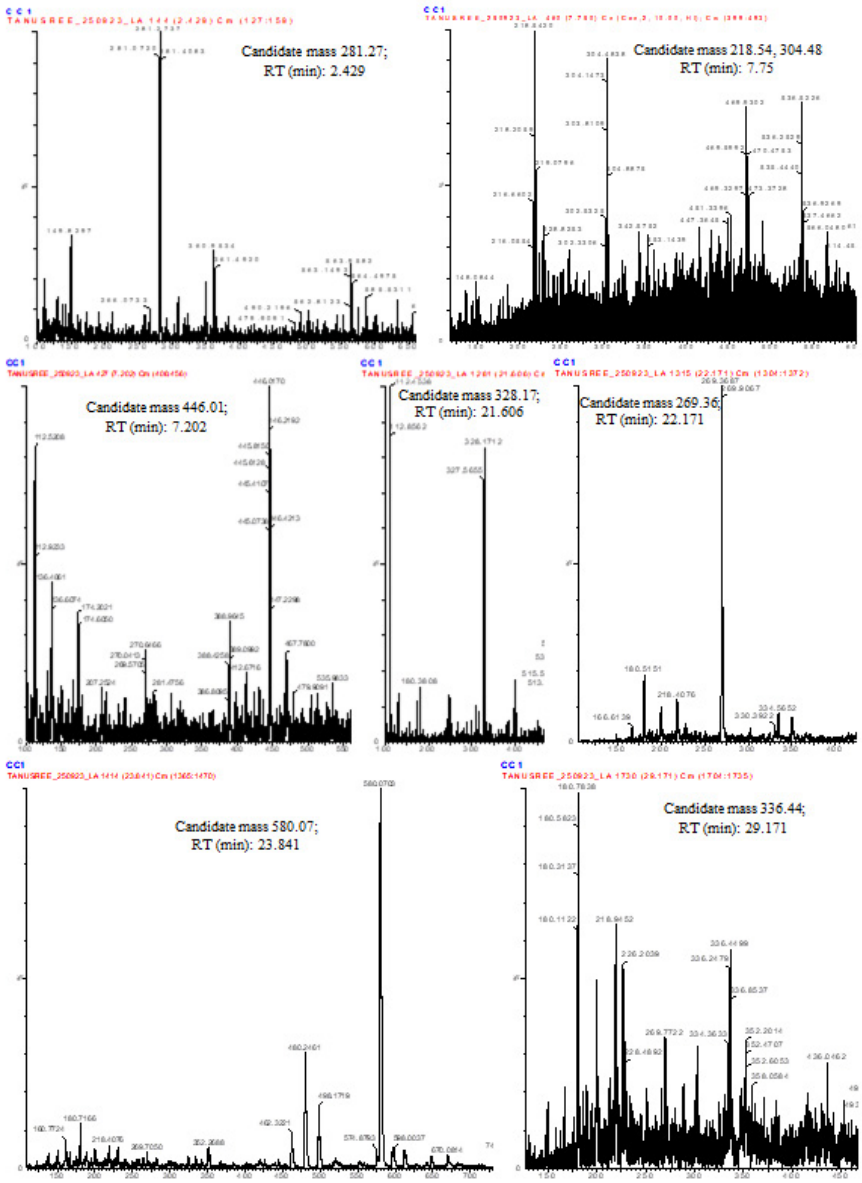

Fig. 4: Eight compounds in LAHEE by LC-MS study with candidate mass (m/z) at positive ions 304.48, 218.54, 269.36, 580.07 and 336.44; negative ions 281.27, 446.01 and 328.17

Table 4: Activities of AST and ALT in reproductive and metabolic tissue samples after exposure to various doses of LAHEE in rats

Experimental groups	*AST activity (Unit/mg of tissue)*				*ALT activity (Unit/mg of tissue)*			
	Liver	*Cardiac*	*Testes*	*Epididymis*	*Liver*	*Cardiac*	*Testes*	*Epididymis*
Placebo group	27.48 ± 0.97[a]	25.90 ± 0.82[a]	20.01 ± 0.74[a]	22.59 ± 0.6[a]	22.50 ± 0.62[a]	16.21 ± 0.76[a]	21.69 ± 0.56[a]	23.25 ± 0.69[a]
1 mg/mL LAHEE-exposed group	28.00 ± 0.63[a]	26.11 ± 0.93[a]	21.58 ± 0.76[a]	23.04 ± 0.38[a]	24.47 ± 1.14[a]	17.10 ± 0.87[a]	22.29 ± 0.45[a]	23.50 ± 0.73[a]
2 mg/mL LAHEE-exposed group	28.32 ± 0.74[a]	26.60 ± 0.65[a]	21.76 ± 0.81[a]	23.21 ± 0.77[a]	24.73 ± 1.15[a]	17.69 ± 0.34[a]	22.46 ± 0.51[a]	24.71 ± 1.03[a]
4 mg/mL LAHEE-exposed group	28.78 ± 0.83[a]	27.79 ± 0.86[a]	21.89 ± 0.45[a]	23.80 ± 0.34[a]	25.11 ± 1.14[a]	18.04 ± 0.36[a]	23.18 ± 0.69[a]	25.47 ± 1.5[a]

Values were manifested as mean ± SEM (n = 6). ANOVA, followed by the Multiple-Comparison Student's two-tail *'t'*-test. Data with the same superscript (a) in each column did not differ from each other significantly, $p > 0.05$.

Table 5: Qualitative screening of phytomolecules present in LAHEE

Phytochemicals	*Result*
Flavonoids	+
Alkaloids	+
Phenols	-
Triterpenoids	+
Tannins	-
Glycosides	-
Saponins	-
Steroids	-

Result '+' denotes the presence, and '-' denotes the absence of concerned phytocomponents.

an essential sensor for the assessment of male fertility. In this experiment, sperm motility eventually decreased in humans and rats following various doses and durations of LAHEE exposure, possibly due to the mitochondrial dysfunction by phytocompounds present in LAHEE that may results in less production of ATP through the uncoupling of OXPHOS.[35,36] Also, direct interference with the above-said ion channels by the phytocompounds may diminish sperm motility.[37,38] Another hypothesis for the declination in sperm motility is the imposition of reactive oxygen species (ROS) by LAHEE that hamper sperm mitochondrial functions. Similarly, after LAHEE exposure, sperm viability and HOS-positive sperm were drastically declined which indicate the architectural destruction of the spermatic plasma membrane, may be due to the ROS-induced oxidative damage, membrane phospholipid oxidation and peroxidation chain reaction by spermicidal compounds present in LAHEE.[10,39] As high quantities of phospholipids and saturated fatty acids are present in sperm cell membranes, which makes them highly vulnerable to oxidative injury by ROS than other cells.[40] The imposition of ROS was further confirmed by the target-specific oxidative stress-inducing actions of LAHEE on spermatozoa cells, which has been resulted by elevation of TBARS (end product of lipid peroxidation) level and inhibition of antioxidant enzymes (SOD, catalase) activities in reproductive tissues like testes and epididymis. No such reduction was noticed in the activities of said antioxidant enzymes and the elevation in TBARS level in metabolic tissue like the liver and cardiac tissue of rats by LAHEE. Such target-specific effects of LAHEE on sperm cells without imposing any stress cum toxicity on metabolic organs highlighting the possibility of male contraceptive efficacy of LAHEE in more safer way. The spermicidal effects of LAHEE were further confirmed by its IC_{50} value, which supported it as the effective spermicidal activity of the extract as this value indicating the sperm immobilization. A remarkable reduction in the percentage of holes on gelatine-coated slides further indicates the low fertility efficacy of LAHEE exposed human's and rat's sperms, as acrosomal enzymes are mainly involved in fertilization. Phyto-ingredients can act in multiple ways to lose the AIS of sperm cells. One possible way is the suppressive efficacy of LAHEE on the activities of membrane-specific key enzymes such as acrosin and hyaluronidase, mainly involved for penetration of spermatozoa through oocyte coverings.[41] Another is the deterioration of outer acrosomal membrane integrity, ATP leakage, and generation of ROS by spermicidal phytocompounds, all of which may be associated with the decline in AIS in sperm cells.[10,41,42]

The hypo-testicular effect was seen in all LAHEE-exposed groups by inhibiting the activities of key androgenic enzymes in rats (Δ5,3β and 17β-HSD).[43] Due to suppression of such enzymes' activities, testicular testosterone is decreased which may suppress the spermatogenesis as well as other sperm sensors.[44] Bioactive phytochemicals may possess competitive or non-competitive inhibition by binding the isosteric site of the targeted biocatalyst or by altering the configuration of isosteric site of the biocatalyst. Such suppression of testicular steroidogenic enzyme activities by competitive or non-competitive modes of inhibition have been noted using other plants.[45,46] Non-significant alterations in the activities of AST and ALT in pre-mentioned tissues further indicated that the LAHEE might be non-toxic and safe for use.

Dose-dependent contraceptive actions of LAHEE may be explained from the viewpoint of the spare-receptor concept.[47] In rats, 1-mg/mL dose of LAHEE delivers a submaximal effect due to the insufficient concentration

Table 6: The list of phytocompounds identified in LAHEE by LC-MS study

S. No.	*RT (min)*	*Observed m/z*	*Ion mode*	*Reference m/z*	*Proposed compound*	*Nature of the compound*
1.	2.429	281.27	$[M - H]^-$	281.13	Oleic acid	Fatty acid
2.	7.75	218.54	$[M + H]^+$	218.1	Pterosin B	Terpenoid
3.	7.75	304.48	$[M + H]^+$	304.25	Dihydroquercetin	Flavonoid
4.	7.202	446.01	$[M - H]^-$	447	Luteolin-7-Glucoside	Flavonoid
5.	21.606	328.17	$[M - H]^-$	328.15	Boldine	Alkaloid
6.	22.171	269.36	$[M + H]^+$	269	Formononetin	Flavonoid
7.	23.841	580.07	$[M + H]^+$	580.49	Luteolin 8-C-pentoside-6- C-hexoside	Flavonoid
8.	29.171	336.44	$[M + H]^+$	336.12	Berberine	Alkaloid