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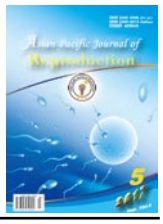

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Antifertility properties of *Centella asiatica* ethanolic extract as a contraceptive agent: Preliminary study of sperm proteomic

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ABSTRACT

Objective: To investigate the antifertility properties of *Centella asiatica* L. ethanolic extract and identify the sperm proteomic changes in treated male rats. **Methods:** A total of 32 male Sprague-Dawley rats were divided into two groups: control group (n=16) received distilled water and treatment group (n=16) which received 300 mg/kg of *Centella asiatica* L. extract for 42 d. After the treatment period, the number of implantation sites was recorded and the sperm proteomic changes were analysed by 2D gel electrophoresis. In addition, the expression of protein spots was quantified by MALDI-TOF analysis. **Results:** *Centella asiatica* L. extract resulted in low number of implantation sites in the treatment group (100.00±2.82) compared to the control group (183.00±2.14). The percentage of infertile male rats in the treatment group was higher (43.75%) compared to the control group (18.75%). Proteomic analysis showed the expression of protein spots identified in the treatment group decreased with 234 spots compared to the control group with 282 spots. **Conclusions:** The results revealed that there was antifertility activity in the male rats with the administration of *Centella asiatica* L. ethanol extract and the identified proteins could provide understanding on the adverse effect of *Centella asiatica* in male reproductive system.

1. Introduction

The world's population growth has been increasing and the rate of unintended pregnancies has also accelerated every year. This situation could cause negative impacts such as shortage of food resources and environmental degradation. Therefore, to overcome this problem, most developing countries have encouraged couples to practice family planning by using contraception. Female contraceptive could prevent fertilization, ovulation, and implantation of an embryo in the uterus while the male contraceptive could prevent sperm transportation to vas deferns, interrupts sperm function, and prevents the spermatogenesis process which could inhibit the sperm production[1].

The development of contraceptive agents that are convenient, have fewer side effects, and effective had been claimed. Recently, many studies have found the protective effect of some herbs with antifertility properties that could be developed into a contraceptive agent. Herbal medicine is believed to cause fewer side effects and beneficial to human with many advantages. Plants that possess antifertility properties could also act as contraceptive agents[2,3].

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Bacopa monnieri, *Allamanda cathartica*[4,5], *Martynia annua*[6], and *Centella asiatica* L.[7] are among the plants believed could reduce fertility.

Centella asiatica L. or pegaga is one of the popular herbs among Malaysians and Indonesians and it has been widely used as *ulam* or folk medicine in many countries. This plant contains high bioactive compounds such as madecassoside, madecassic acid, asiaticoside (glycoside) and asiatic acid (terpene acid) which are related to medicinal properties[8]. *Centella asiatica* L. extract can be used for wound healing, acts as an alternative treatment for various diseases, and has antifertility properties[9]. However, the effect of *Centella asiatica* L. on the number of implantation site and sperm proteome has not been elucidated thoroughly. Therefore, this study is conducted to investigate the effect of *Centella asiatica* L. on the number of implantation sites and sperm proteomic approaches are applied to analyse the protein expression which could reveal the antifertility activity in the male group treated with *Centella asiatica* L. extract.

2. Materials and methods

2.1. Plant extraction

Centella asiatica L. was purchased from a market in Serdang, Selangor and was verified by a botanist at Herbarium Laboratory, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The samples were washed and oven-dried for 32 h. The ethanol extract was prepared by extracting 5 000 g of *Centella asiatica* L. powder macerated with ethanol (90%) at 55 ℃ to 60 ℃ for 36 h. Then, the ethanol was removed from the extract and the extract was concentrated to dryness using a rotary evaporator (Büchi Rotavapor® R-200/205). The extracts was then weighed and preserved at 4 ℃.

2.2. Animal preparation and treatment

A total of 32 proven fertile male rats weighed 250-300 g at age of 12 wk were purchased from the Animal House, Universiti Kebangsaan Malaysia. Animal ethics approval was obtained from the Animal Ethics Committee Faculty of Medicine, Universiti Kebangsaan Malaysia. The animals were housed individually in polycarbonate cages at room temperature with alternating light and dark cycle of 12 h. They were allowed ad libitum access to food (Barastock Rat and Mouse Pellet Feed, Australia) and water. The animals were divided into two groups (n=16). The experimental groups were chosen based on the previous study done by Yunianto *et al.*[7], the high dose (300 mg/kg) of *Centella asiatica* shown the best antifertility effects compared to other two doses (100 mg/kg and 200 mg/kg). The control group received distilled water while the treatment group was orally administrated daily for 42 consecutive days with 300 mg/kg of *Centella asiatica* L. extract. The number of implantation sites was recorded and the sperm proteomic was analysed by two-dimensional (2D) gel electrophoresis followed by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) analysis.

2.3. Assessment of implantation sites

Female Sprague-Dawley rats were placed individually in a cage with a treated male rat for five days. On the 6th day, vaginal smear was performed to determine the presence of sperm. The day that the sperm appeared in the vaginal smear (under microscope observation) was considered as day 1 of pregnancy. On the 17th day, the animals were laparotomized under light ether anesthesia and the number of implants in the uterine horns was recorded.

2.4. Preparation of sperm protein extraction

The cauda epididymis was minced into small pieces in 10 mL Biggers, Whitten & Whittingham medium. It was kept in a 5% CO_2 incubator for 15 min to allow the sperm to swim up. The sperm suspension was centrifuged at 4 000 r/min for 15 min. The supernatant was removed while the pellet was homogenized for 1 h in a glass homogenizer containing 200 μL of lysis buffer {7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.8% immobilized pH gradient (IPG) buffer and 1 mM phenylmethanesulfonyl fluoride}. The samples were then centrifuged (1 500 r/min, 20 min, 4 ℃) and the supernatant was mixed with 60 mM diothreitol before stored at -20 ℃. The protein concentration was determined by using the Bradford protein assay.

2.4.1. Isoelectric focusing

The protein samples (160 μL) were applied on the 7 cm of IPG strips by placing the strips downward touching the samples. Mineral oil (300 μL) was added to the IPG Strips and the strips were placed on the Ettan IPGPhor isoelectric focusing (GE Healthcare). The isoelectric focusing was carried out at 50 μA per strip for 8 h at 20 ℃ with three stages including 250 V/h, 500 V/h, and 8 333 V/h. Then, the strips were equilibrated by adding equilibration buffer (2% sodium dodecyl sulphate, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol and 0.002% bromophenol blue) supplemented with 65 mM diothreitol followed by the addition of second equilibration buffer (0.135 M iodoacetamide) for 15 min by using a 3D rotator machine (Lab-Line).

2.4.2. Sodium dodecyl sulphate-PAGE gel electrophoresis and MALDI-TOF

The preparation of 12.5 % polyacrylamide gels was according to the method described by Laemmli. The gel was stained with Coomassie Brilliant Blue R-250 and scanned using Image Scanner ⅢⅢ (GE Healthcare). The gel was analysed using ImageMaster 2D Platinum version 6.0 (GE Healthcare). Then, the protein spots

obtained were excised, digested and analysed by MALDI-TOF. The Mascott search engine was used to determine the selected protein spots (upregulated, downregulated, and disappeared) in the treatment group compared to the control group.

2.5. Statistical analysis

Data were expressed in terms of mean±SEM. The data of the experimental groups were compared to the control group subjected to one-way ANOVA using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) and then followed by LSD. Values were considered significantly different at $P<0.05$.

3. Results

3.1. Assessment of implantation site and sperm quality

The number of implantation sites in the female rats that underwent fertility test with male that have been treated with *Centella asiatica* L. extract is less than the control group. The number of implants in the uterine horns was observed and it was showed the number of implantation sites in the treatment group (100.00±2.82) compared with the control group (183.00±2.14). The percentage of infertile male rats in the treatment group was 43.75% while the control group was 18.75%. No toxic effect was observed in the animals during the experiment.

In addition, abnormal sperm morphology was found in the treatment group (flattened head and bent tail) while the control group showed normal sperm morphology as observed under scanning electron microscope (Figure 1).

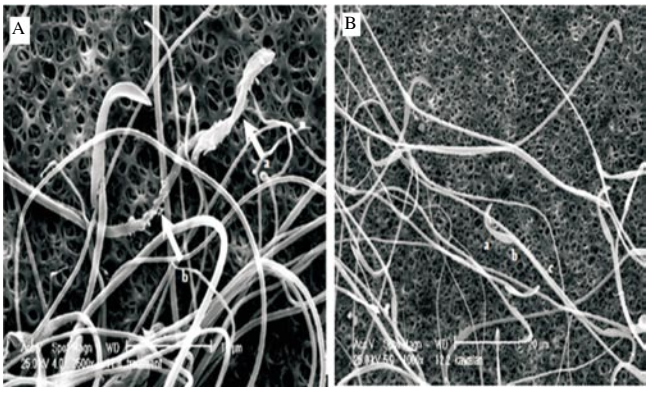

Figure 1. Scanning electron microscope of sperm morphology.
A: Abnormal sperm morphology showed by the treatment group with 300 mg/kg of *Centella asiatica* (2 500 × magnification); (a) flattened head and (b) bent tail. B: Normal sperm morphology showed by the control group (1 000 × magnification); (a) hook-shaped head and (b) long-straight tail.

3.2. Sperm proteomic analysis

The analysis of 2D gel electrophoresis showed 234 protein spots were expressed in the treatment group while there were 282 protein spots in the control group (Figure 2). The analysis also showed that 48 protein spots were absent in the treatment group compared to the control group.

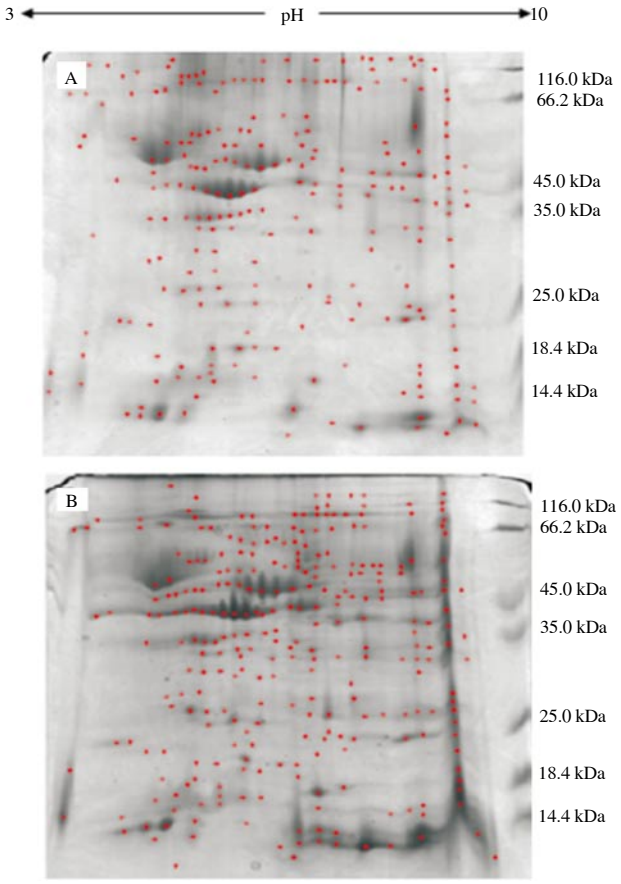

Figure 2. Position of 15 references protein spots identified in control group obtained from 2D gel electrophoresis.
The identified protein spots were appointed as references for quantification. A: Treatment group; B: Control group. The number of protein spots in the treatment group is shown to be less than the control group.

ImageMaster 2D Platinum Software (version 6.0) analysis showed 15 protein spots were significantly identified and the differences in protein expression between the control group and the treatment group (Figure 3 and 4). The identified protein spots were appointed as references for quantification. As shown in Table 1, the Mascott search engine managed to identify five protein in the treatment group; upregulated (spot 5), downregulated (spot 10), and disappeared (spot 11, 12 and 13).

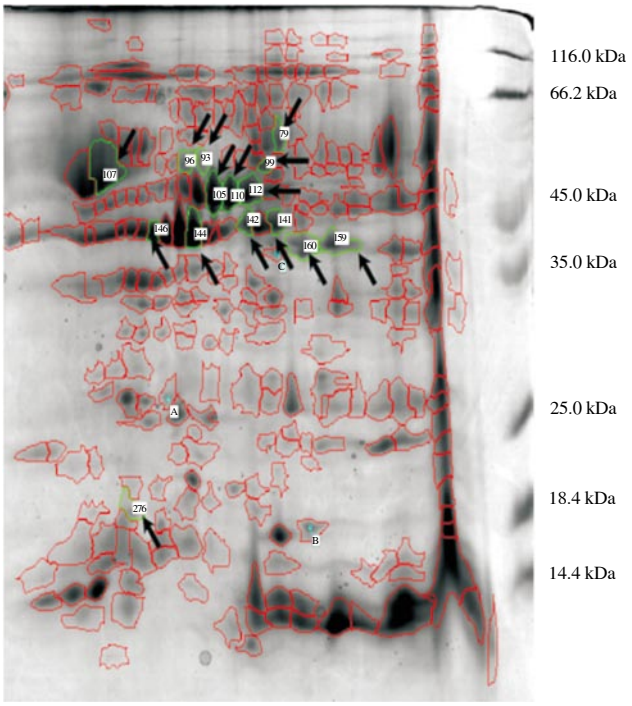

Figure 3. Position of 15 references protein spots identified in control group obtained from 2D gel electrophoresis.

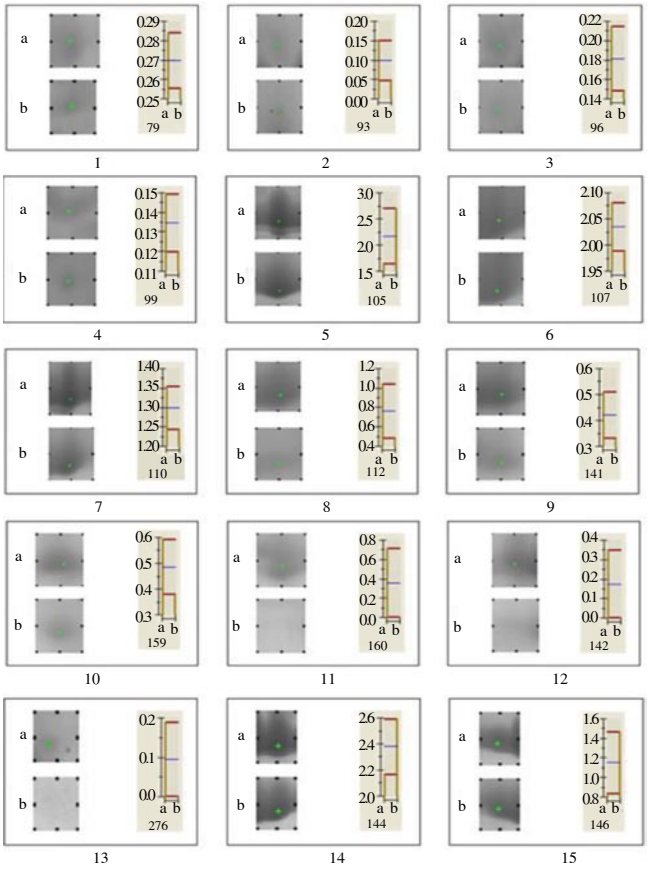

Figure 4. Analysis of 15 protein spots (numbered from 1 to 15).
a: control group; b: treatment group. Different protein expressions are shown based on mean relative volume. The graph on the right shows the quantification value for each spot.

Table 1
Mascot search engine results of selected protein spots.

Protein regulation	Spot No.	Score	Protein match	Mass (Da)	pI value
Up	5	785	Alpha enolase	54 346	5.81
Down	10	704	ALDOA	39 923	7.07
	11	440	Sorbitol dehydrogenase	38 780	7.14
Disappear	12	321	Glutamine synthetase	41 153	6.38
	13	356	Lipocalin	20 828	5.24

4. Discussion

This study shows that oral administration of 300 mg/kg *Centella asiatica* L. extract strongly influences the number of implantation sites and sperm protein. The sperm analysis showed a significant decrease in the sperm count [$(21.83\pm2.25)\times10^6$] of male rats treated with high dose of *Centella asiatica* L. extract compared to the control group[7]. This result is associated with the low number of implantation sites in the female rats that underwent fertility test with male rats treated with *Centella asiatica* L. extract. According to Dzuriaty[10], the number of progeny and percentage of infertile male mice treated with 300 mg/kg of *Centella asiatica* L. significantly decreased. This was also supported by Mas[11], who showed that the rate of in vivo fertilization in the treatment group (300 mg/kg of *Centella asiatica* L. extract) decreased. The study conducted by Heidari *et al.*[12] showed that *Centella asiatica* has toxicological effects to the reproductive system of male rats as the results shown the reduction of sperm quality, degeneration of seminiferous tubule and significantly decreased in serum testosterone, luteinizing hormone and follicle-stimulating hormone. Therefore, this study had proven that *Centella asiatica* L. extract possess antifertility properties due to the low sperm count reported[7] and low number of implantation sites.

In this study, sperm proteomic analysis was done to determine the expression of protein which could play a role in male fertility. Mass spectrometric analysis was performed and 15 protein spots were identified. In the treatment group, two protein spots showed upregulated (spot 5 and 14), nine protein spots showed downregulated (spot 1, 2, 3, 4, 6, 7, 8, 9 and 10) and three protein spots showed disappeared (spot 11, 12 and 13). The probability molecular weight search (MOWSE) results obtained through Mascot search engine (www.matrixscience.com) revealed that the spot number 5 corresponded to alpha enolase, spot number 10 corresponded to aldolase A (ALDOA), whereas spot number 11, 12 and 13 corresponded to sorbitol dehydrogenase, glutamine synthetase and lipocalin respectively. The metabolic groups such as alpha enolase, ALDOA, and sorbitol dehydrogenase are the metabolic enzymes that involve in the glycolytic process to produce energy. Glutamine synthetase plays an important role in nitrogen metabolism while lipocalin acts as protein transporter.

Alpha-enolase (spot 5) or also known as enolase 1 (ENO 1) is a protein that has an alternative name which is 2-phospho-D-glycerate hydrolase. This type of protein exists in all mature tissues of mammals and acts as multifunctional glycolytic enzymes which involves in growth regulation, hypoxia tolerance, cellular stress, allergic reactions, and reproduction of organisms. ENO 1 also plays a role in immunoglobulin stimulation and activation of

plasminogen[13]. Subsequent studies have found that ENO 1 exists in rat testis and it is newly discovered that enolase, termed ENO 4 is also related to male reproduction and sperm activity[14].

ALDOA (spot 10) or known as fructose-biphosphate ALDOA is a protein included in the glycolytic enzyme group because it is involved in the glycolysis pathway during spermatogenesis. According to Krisfalusi *et al.*[15], a larger molecular weight of ALDOA band was identified in mouse sperm and fibrous sheaths isolated from human sperm[16]. Glutamine synthetase (spot 12) is an enzyme that synthesizes glutamine in various organs such as liver, lungs, muscles, and adipose tissues. Glutamine plays an important role in the transportation of nitrogen, carbon and energy between the organs. This protein is also involved in ammonia assimilation cycle, neurotransmitter metabolism and regulatory of epithelium cells proliferation[13].

A study conducted by Gebrie *et al.*[17] has structurally identified a group of lipocalin, which was epididymal retinoic acid-binding protein. This finding was supported by Srivastava *et al.*[18] who stated that lipocalin was also known as epididymal retinoic acid-binding protein[19]. Lipocalin has been associated with sperm in the epididymal fluid and it also has the ability to bind *cis*- and *trans*-retinoic acid. This concludes that lipocalin is involved in the retinoid carrier needed for epididymal function and sperm maturation. In this study, the analysed gels showed that lipocalin (spot 13) protein disappeared in treatment group compared to the control group. Therefore, it is suggested that epididymal function and sperm maturation did not occur due to the high dose of *Centella asiatica* L. extract. Besides, lipocalin and sorbitol dehydrogenase are the two main proteins involved in the spermatogenesis process. Hence, the inhibition of lipocalin enzyme due to the *Centella asiatica* L. extract could affect epididymal function and sperm maturation.

Sorbitol dehydrogenase (spot 11) is a dehydrogenase or reductase enzyme that converts sorbitol into fructose[20] via glycolytic pathway. The fructose is metabolized to ATP as energy source. This process usually occurs in the liver and seminal vesicle. Previous studies[21] reported that the energy source metabolized to ATP is important for sperm motility in the fertilization process. Sorbitol dehydrogenase activity occurs in the maturation of germinal epithelial layer of seminiferous tubule[22]. A reduction in the activity of sorbitol dehydrogenase could contribute to the inhibition of spermatogenesis and reduce sperm motility.

In conclusion, this study showed that the administration of 300 mg/kg of *Centella asiatica* L. extract could cause antifertility in rats. The sperm proteomic analysis showed at least five proteins including alpha enolase, ALDOA, sorbitol dehydrogenase, glutamine synthetase, and lipocalin, which play an important role in male fertility and could lead towards the development of contraceptive agent.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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