Rat Sperm Proteomic Analysis: Effect of the Antifertility Agent *Centellaasiatica*L.

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ABSTRACT

Oral intake of *Centellaasiatica*L. has been reported to have an antifertility effecton sperm quality. In this study the effects of *C.asiatica*L. treatment in male rat sperm quality and sperm protein expression were analyzed. Thirty-two adult male Sprague-Dawley rats twelve weeks old were divided into four groups: control group; low dose group (100 mg/kg body weight); medium dose group (200 mg/kg body weight); and high dose group (300 mg/kg body weight). All treated groups were force-fed with an ethanolic extract of *C.asiatica*L. and the control group was force-fed with distilled water, respectively for 42 days. Rats were sacrificed on day 43. Cauda epididymal rat sperm from control and high dose treatment were subjected to two-dimensional gel electrophoresis for comparison of protein expression profiles. Results showed that caudaepididymal sperm count and motilityin treated groups showed a significant decrease compared to control. Medium and high dose treatments showed the most significant reduction ($p<0.05$).Proteomic study revealed significant changes in sperm protein expression between control (282 spots) versus high dose treated group (234 spots). The matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis of the selected spots exhibited five spots differentially expressed identified as alpha enolase, aldolase A, sorbitol dehydrogenase, glutaminesynthetase and lipocalin. Sorbitol dehydrogenase was present in the control group, but not in the treated group. This enzyme plays an important role in sperm motility and is associated with the maturation of the germinal epithelial layer of seminiferous tubules.The lipocalin protein also disappeared in the treated group. This transport protein plays a significant role in epididymal function. It was concludedthat *C. asiatica*L.have antifertility activities in rats sperm quality and protein expression.

Keywords:Centella asiatica *L., sperm quality,proteomic*

Introduction

The development of antifertility or contraceptive agents from medicinal plants which are safe and effective with minimum side effects is a trend in pharmacological research today (Qureshi et al. 2006). Many studies conducted successfully on various plants identified compounds which have antifertility properties, but most of them are metabolically toxic to human (Sarkar et al. 2000). Previous reports suggested that some compounds in plants are capable of interfering with the production of androgenichormones, can modify spermatogenesis, or are abortifacients, as well as having spermicidal activity (Qureshi et al. 2006).

Centella asiatica L. (Pegagan, local name) is one of the popular herbs used in folk medicine in Indonesia for its wound healing, antibacteria and antioxidant-rich properties. Some active compoundshavingmedicinal properties have been described such as madecassoside, asiaticoside (glycoside), madecassic acid and asiatic acid (terpene acid). Studies have revealed the efficacy of *C. asiatica*in wound healing, improving memory, and

anticancer activity (Inamdar et al. 1996; Jaganath & Ng, 2000),while the antifertility effect of *C. asiatica*has been reported in animals (Dutta & Basu, 1968).

Despite many studies of *C.asiatica*, details of theeffectson the male reproductive system have not been fully elucidated. Therefore, in this study we aimed to investigate the effects of *C. asiatica*on sperm count, sperm motility, and sperm protein expression.

Materials and methods

Plant material

Centellaasiatica L. was obtained from a traditional market in Serdang, Selangor. The specimen was then identified and confirmed by botanist of Herbarium Laboratory, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia. *C. asiatica*(5000 g) was washed and dried at 45°C in an oven (Memmert) for 32 h prior to milling to apowder. Sample extraction procedure as described earlier was followed (Houghton & Raman, 1998). A Soxhlet apparatus was used with 90% ethanol as solvent. The sample was then concentrated using rotary evaporator (BüchiRotavapor® R-200/205) to yield approximately 88 g of a dark brown semi solid mass.

Animals

Thirty-two proven fertile adult (age 12 weeks old and weighing 260-300 g) male rats of the Sprague-Dawley strain were obtained from UKM's Animal House. They were divided into four groups of eight animals each. Animals were housed separately in polycarbonate cages (595 mm X 380 mm X 200 mm, Techniplast 1354, Italy) with saw dust as bedding material. The animals' room environment wasmaintained at 12:12h light and dark cycle for each 24h period within temperature of about 25^{o}C. Standard water and rat food pellets (Barastock Rat and Mouse Pelleted Feed, Australia) were administered *ad libitum.* General health conditions of the animals were monitored throughout the experiment. All experiments were conducted following the guidelines of the National Institutes of Health for the care and use of laboratory animals. The study was approved by the Animal Ethics Committee of the Faculty of Medicine, UKM.

Treatment

Animals were divided into four groups. The first three groups were administered with an ethanolic extract of *Centellaasiatica*at three different doses(Kumar & Gupta, 2002),i.e.a low dose (100 mg/kg), a medium dose (200 mg/kg), and a high dose (300 mg/kg), while distilled water was administered to the control group. All treatments were administered orally using a force feeding needle once a day from 10.00-11.00 am for 42 consecutive days.

Sperm count and motility assessment

After finishing 42 days of treatment, the following day,all rats were sacrificed under choloroform anesthesia. Sperm analysis was performed on the samples derived from the cauda epididymis. Sperm count was assessed using '*Improved Neubauer Haemocytometer'* based on previous reports (Comhaire & Mahmoud, 2006) and WHO manual (1999) with modification. Briefly, the cauda epididymis were minced using anatomical scissors, suspended in 15 ml of Biggers-Whitten-Whittingham (BWW)medium (Biggers et al. 1971; Kumar et al. 2007) prior to incubation in 5% CO_2incubator for 30 min at 37^{O}C to allow sperm swim-up. Sperm motility grade was determined usingWHO laboratory manual (1999). Statistical analysis was performed with SPSS 14.0 for windows software using one way ANOVA followed by LSD.

Proteomic Analysis of Rat Sperm

Cauda epididymal sperm obtained from high dose (300 mg/kg) and control groups were subjected to two dimensional gel electrophoresis for proteomic analysis. Protocols of the electrophoresis followed manual that has been previously described (Westermeier & Naven, 2002). Protein extraction from the cauda epididymal sperm in BWW mediumas described by Yunianto (2010).

Briefly, 10 ml of sperm were sucked from the surface of BWW medium and centrifuged (4000 rpm, 15 min, 4^oC). Sperm pellet yielded were extracted with 200 µl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% IPG buffer, 1mM PMSF) using ice in glass homogenizer and allowed to sit for 1h. After a final centrifugation (15,000 rpm, 20 min, 4^oC) the supernatant was removed, 60 mM dithiothreitol (DTT) was freshly added prior to frozen (-20^OC). Protein concentration was determined using the Bradford assay kit as previously reported (Bradford 1976, Rosenberg 1996).

A mini 2-dimensional electrophoresis system (*EttanIPGPhor Isoelectric Focusing,* GE Healthcare and Hoefer mini VE, Amersham Biosciences) was used. Isoelectric focusing was carried out in three steps (250 Vh, 500 Vh, and 8333 Vh, 50 µA per IPG strip, 20^OC, 8 h) (Lai 2006). After finishing the first dimensional electrophoresis, IPG strip was equilibrated in equilibration buffer (2% SDS, 50 mMTris-HCl pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 65 mM DTT for fisrt step and 0.135 M iodoacetamide for the next step) (Görg et al. 1987).Each equilibration step was run on the 3D rotator (Lab-line) for 15 min.The second dimensional electrophoresis was *Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis* (SDS-PAGE)using 12.5% acrylamide gels running at 100 V 20 mA (Lai, 2006). Protein molecular marker (Fermentas) was used in the process. Gels were stained with Coomassie Brilliant Blue R-250 (Biorad), scanned using*Image Scanner III* (GE Healthcare) and analyzed with the Imagemaster 2D Platinum ver.6.0 (GE Healthcare).

Image analysis of the gels was performed on three replicative gels of each group. The overlapping replicative gels were used to obtain a reference gel of each group to enable comparison between them. Quantification of the gel images was based on the mean relative volume (%vol) of the protein spots in each experimental group. According to the significant difference in relative volume ($P<.05$), the spots were cut out, digested and analyzed by *Matrix Assisted Laser Desorption Ionization-Time of Flight* (MALDI-TOF). The results were then identified using peptide mass profiling matched to the database (www.matrixscience.com).

Results

Sperm count and motility

Table 1. Sperm count (x 10^6) and sperm motility grade of control and treatment groups at 100 mg/kg, 200 mg/kg and 300 mg/kg.

Dose (mg/kg)	Sperm number (x 10^6)	Motility grade (a-d)
Control	32.91 ± 11.24	b
100	25.61 ± 1.96	b
200	23.34 ± 1.48*	c
300	21.83 ± 2.24*	c

* Significantly different compared to control ($p<0.05$).

Motility grade according to WHO manual (1999).

a : rapid progressive motility; ≥ 25 µm/s at 37^OC and ≥ 20 µm/s at 20^OC

b : slow or sluggish progressive motility

c : non progressive motility; < 5 µm/s

d : immotility

Sperm count from treated and control groups are shown in Table1. The average rat sperm count was 32.91 x 106 ±11.24, 25.61 x 106 ± 1.96, 23.34 x 106 ± 1.48, 21.83 x 106± 2.24 for the control group, the low dose group, the medium dose group and the highdose group, respectively. Treatment with the high dose (300mg/kg) showed the most sperm count decrease as compared tolow dose (100 mg/kg) and medium dose (200 mg/kg). Thesefindings indicate that *Centella asiatica* treatment causednegative effect for sperm quality. Further LSD analysis also foundthat treatment in medium (200 mg/kg) and high dose (300mg/kg) gave the most significant value (p<0.05)in sperm count decrease as compared to the control.Sperm motility grade shown in Table 1 indicated that*Centella asiatica* treatment also produced an effect on the spermmotility grade, i.e., decrease from b (control and low dose)to c (medium and high dose). Analysis of both parameters,the sperm count and sperm motility grade, suggested a clearantifertility effectof the *Centella asiatica* treatment onmale rats.

Proteomic analysis

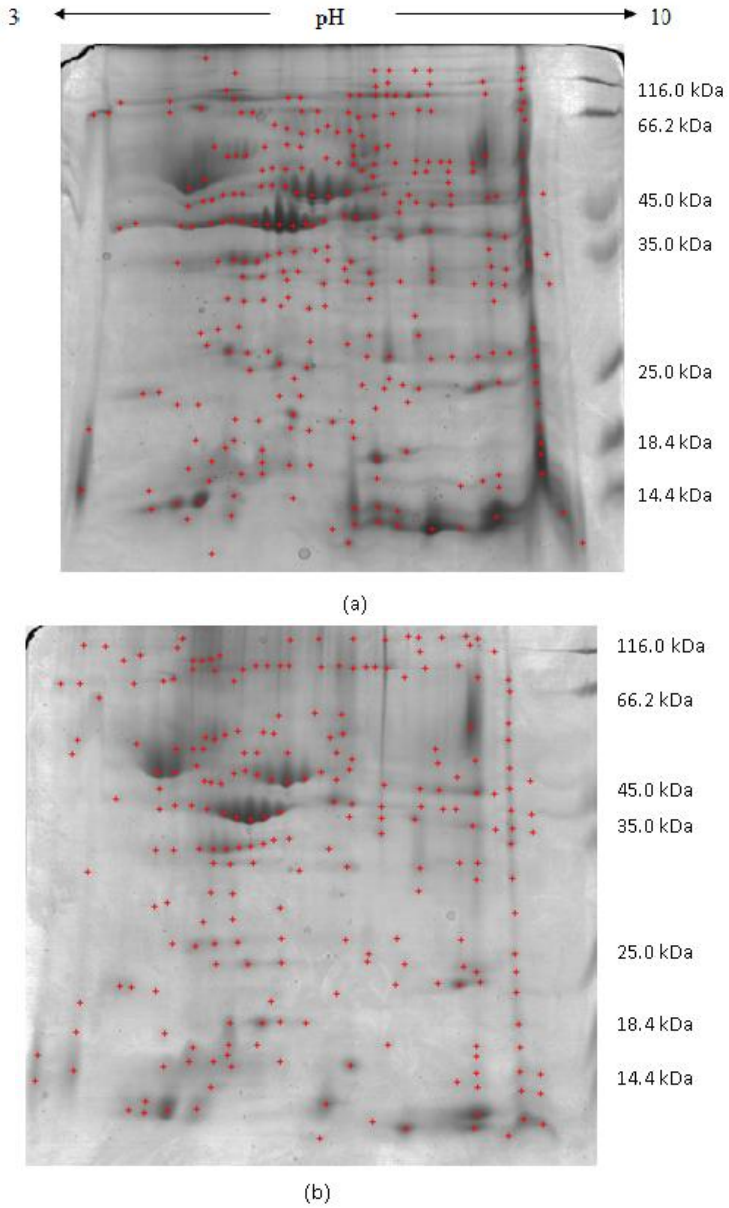

Fig 1. 2D SDS-PAGE gels (12.5%) of rat sperm using IPG strip pH 3-10, CBB stained. (a) control group (b) high dose group (300 mg/kg). Protein marker molecular weight (kDa) as shown on the right side of the gels

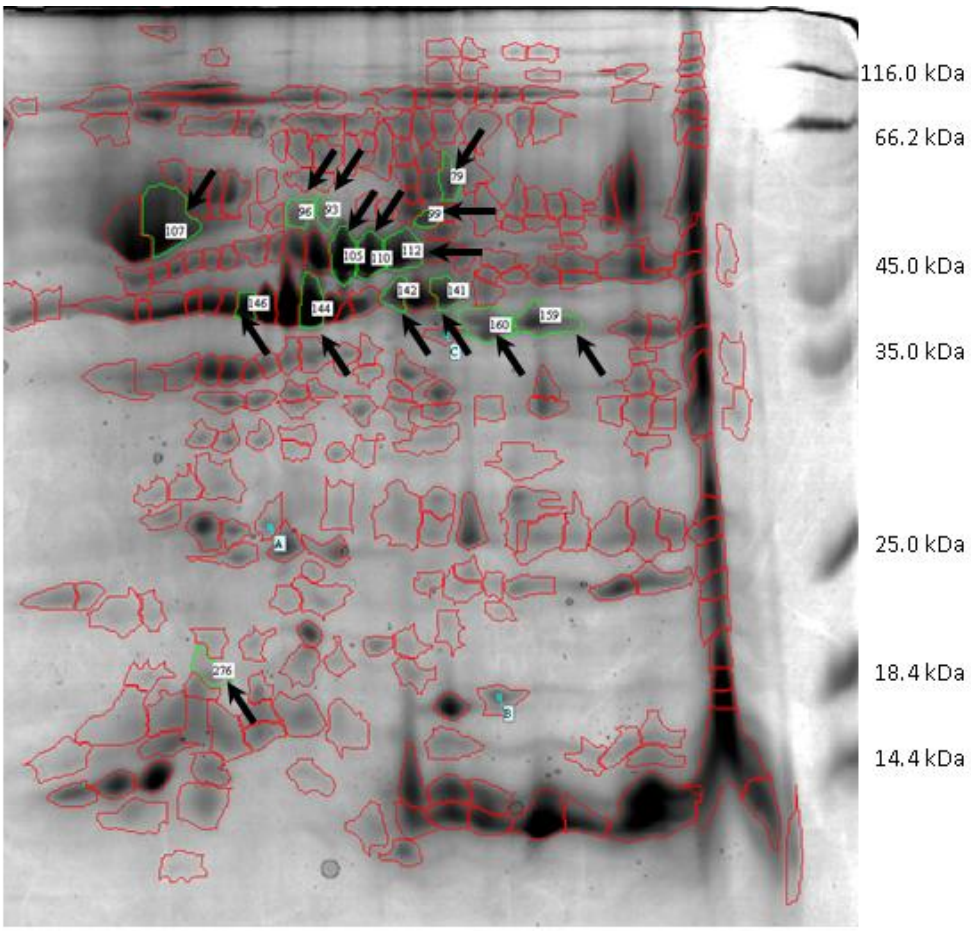

Fig. 2. 2D SDS-PAGE gel of control group showed position of reference spots. Spots appointed was the reference for quantification. Number showed reference identity for each spots identified.

Image analysis results depicted 282 versus 234 protein spots detected from control and treatment (high dose, 300 mg/kg) groups, respectively. Molecular weights (14.4 kDa-116 kDa) were as shown on the right side of the images (fig. 1). Quantification of mean relative volume (%vol) of both groups described 15 protein spots which were most significantly different ($p<.05$) in expression between the treatment as compared to the control group. Each spot was denoted by a reference identity number of protein spot in the control group (fig.2). The graphs shown in the images depicted mean relative volume's value. Nine protein spots were down-regulated (spot numbers: 1, 2, 3, 4, 6, 7, 8, 9 and 10) whereas three spots were up-regulated (spot numbers: 5, 14 and 15). Three protein spots which were present in the control groupdisappeared in the treatment group (spot numbers: 11, 12 and 13) (fig.3).

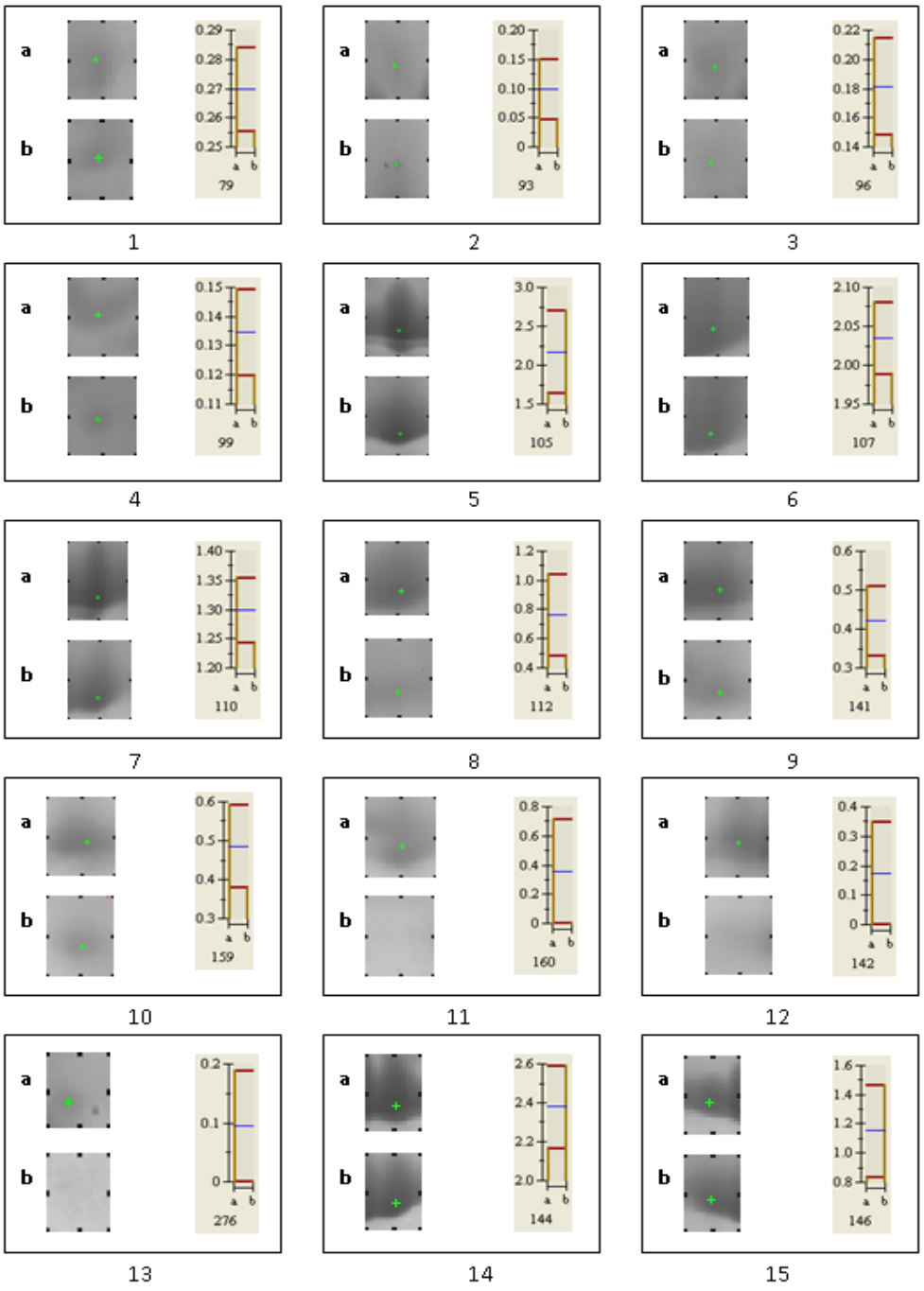

Fig. 3. Analysis result of the spots (a) control (b) treatment group showed different expression based on mean relative volume. The graph showed quantification value for each spots

Mass spectrometric analysis was performed on five protein spots (spot number 5, 10, 11, 12 and 13, correspond to reference identity spot numbers105, 159, 160, 142 and 276) selected based on the highest significance value (fig.3). Those spots met the expressional alteration criteria as reported previously (Kobayashi et al. 2009), i.e. spot number 5 was up-regulated, spot number 10 was down-regulated, whereas spot number 11, 12 and 13 disappeared. The *Probability Molecular Weight Search* (Mowse) result as described by Pappin et al. (1993) through the Mascot search engine (www.matrixscience.com) revealed spot number 5 corresponded to alfa enolase, spot number 10 corresponded to aldolase A,

whereas spot number 11, 12 and 13 corresponded to sorbitol dehydrogenase, glutamine synthetase and lipocalin respectively (table 2).

Table 2. Mascot search engine result

Spots	Score	Protein Match	Mass (Da)	pI value
5	785	Alfa enolase	54346	5.81
10	704	Aldolase A	39923	7.07
11	440	Sorbitol dehidrogenase	38780	7.14
12	321	Glutamin sintetase	41153	6.38
13	356	Lipokalin	20828	5.24

Discussion

This study was designed to investigate the antifertility effect of *Centella asiatica* on the reproductive system of male Sprague-Dawley rats. The *C. asiatica*was chosen for this study because of its medicinal properties and it's wide use by people invarious parts of the world. Results of the present study revealed the significantdecrease in sperm count of the treated groups as comparedto the control group in a dose dependent manner. There werealso a decrease in the sperm motility grade. These findings underline the antifertilityeffect of *Centella asiatica* on the male rat's reproductivesystem.

To further elucidate the antifertility effect of this plant on the sperm quality at a molecular level, proteomic analysiswas implemented. Proteomic technology is an attempt to better comprehend many biological processes in detail, including spermatogenesis. Studies based on proteomics have revealed some proteins which play a significant role in spermatogenesis (Huo et al. 2004; Zhu et al. 2006).

Mass spectrometric analysis was the key to translate the two-dimensional separation result with ahigh level of sensitivity and specificity. This method enabled a complete protein study from the proteome analysis to the structural and protein interactions (Roepstorff 1997). Analysis and identification of the proteins resulting from the Mascot search engine described four proteins belonging to the metabolic enzyme group and one transport protein. The Alfa enolase,aldolase A and sorbitol dehydrogenaseare metabolic enzymes which are involved in glycolitic processes to produce energy. Glutamine synthetase was an enzyme involved in nitrogen metabolism, whereas lipocalin was a transport protein. Two proteins that were closely associated with the spermatogenesis were lipocalin and sorbitol dehydrogenase.

One group of lipocalin that has been structurally identified is Epididymal retinoic acid-binding proteinas reported by Flower et al. (2000). Uniprot protein database (2009) described lipocalin in this study, which is also known as Epididymal-specific lipocalin-5orEpididymalretinoic acid-binding protein. Lipocalin was associated with sperm in the epididymal fluid and had the ability to bind *cis*- and *trans*-retinoic acid. It was concluded that lipocalin is involved in the retinoid carrier needed for epididymal function or sperm maturation. The image analyzed gels showedthat lipocalin expression disappeared in the treatment group. Therefore, it was suggested that epididymal function and sperm maturation did not occur optimally due to *C. asiatica*treatment.

Sorbitol dehydrogenase is a dehydrogenase/reductase enzyme whose function is to convert sorbitol into fructose(El-Kabbani et al. 2004). This process usually occurs in the liver and seminal vesicle. Previous studies (Cao et al. 2009) reported that the energy source which was metabolized into ATP was important for sperm motility. After sorbitol is converted into fructose, through the glycolytic pathway, fructose is then metabolized into ATP. The study suggested that sorbitol was used as an alternative energy source for sperm motility. Sorbitol

dehydrogenase activity was also associated with the maturation of germinal epithelial layer of seminiferous tubule(Pant et al. 1995, 2004).A previous report (Srivastava et al. 1990) showed that seminiferous tubule damage was associated with decreased activity of sorbitol dehydrogenase.

A proteomic study underlined the antifertility effect of *C. asiatica* at a molecular level. Inhibition of lipocalin enzyme due to *C. asiatica* treatment affected epididymal function and sperm maturation. The reduction activity of sorbitol dehydrogenase contributed to the inhibition of spermatogenesis and a decrease of sperm motility grade. It is suggested that lipocalin and sorbitol dehydrogenase could serve as testicular toxicity biomarkers.

Conclusion

Overall, the results of this study suggest that the ethanolic extract of *C. asiatica*isan appropriate contraceptive agent in male rats. The chemical constituents of this plant have the ability to decrease fertility and affect sperm quality. The isolation, identification and characterization of the bioactive compound(s) of *C. asiatica* that exhibited antifertility activity are under further study.

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