

RESEARCH ARTICLE

The Effect of Continuous Multistage Countercurrent Extraction (CMCE) Propolis Extract Administration on Leydig, and Sertoli Cells Counts, and Sperm Quality Induced With Monosodium Glutamate (MSG) (An Experimental Study on Male Wistar Rat Induced by Monosodium Glutamate)

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ABSTRACT

Introduction: ROS is the most common cause of male infertility. Monosodium glutamate (MSG) is a compound which produces ROS and decreases FSH and LH levels. Continuous Multistage Countercurrent Extraction (CMCE) propolis extract as an antioxidant is evidently capable of reducing ROS concentration. **Objective:** this research aims at evaluating the effect of CMCE propolis extract administration on Leydig cells and Sertoli cells counts and sperm quality of MSG-induced male wistar rats.

Methods: employing the Post Test Only Control Group Design, 18 rats were divided into 3 groups: control group, treated only with MSG (MS-G), and treatment groups, respectively treated with CMCE propolis extract at doses 8.3mg/day (CM8-G) and 10.8mg/day (CM10-G). CMCE propolis extract was given orally for 21 days. At the end of treatment, sperm and testis were taken to evaluate the Leydig cells and Sertoli cells counts and sperm quality. The sperm obtained from epididymis was analyzed using the WHO standards, while Leydig cells and Sertoli cells were counted from preparation using the HE stain method.

Results: the Post Hoc analysis result shows that the Leydig and Sertoli cells counts and sperm quality of CM8-G and CM10-G are significantly higher than those of MS-G, $p < 0.05$. The Leydig cells and Sertoli cells counts and sperm quality of CM10-G are significantly higher than those of CM8-G, $p < 0.05$.

Conclusion: CMCE propolis extract administration evidently increases the Leydig cells and Sertoli cells counts and sperm quality from the perspective of the count, motility and morphology of spermatozoa of MSG-induced male wistar rats.

Keywords: MSG, CMCE Propolis Extract, Leydig Cells Count, Sertoli Cells and Sperm Quality

ABSTRAK

Latar Belakang: ROS adalah salah satu faktor yang dapat menyebabkan pria menjadi infertil. Monosodium glutamate (MSG) adalah senyawa yang terbukti menghasilkan ROS, menurunkan kadar FSH, dan LH. Ekstrak Continuous Multistage Countercurrent Extraction (CMCE) propolis adalah antioksidan yang terbukti dapat menekan kadar ROS. **Tujuan:** penelitian ini bertujuan untuk mengevaluasi pengaruh pemberian ekstrak CMCE propolis terhadap jumlah sel leydig, sel sertoli, dan kualitas sperma pada tikus jantan wistar yang diinduksi MSG.

Metode: penelitian ini menggunakan metode eksperimental dengan rancangan Post Test Only Control Group Design. Subyek penelitian berjumlah 18 ekor tikus yang dibagi secara acak menjadi 3 kelompok: kelompok kontrol hanya mendapatkan MSG (MS-G), kelompok perlakuan dengan ekstrak CMCE propolis dosis 8.3mg/hari (CM8-G) dan 10.8mg/hari (CM10-G). Pemberian ekstrak CMCE propolis diberikan secara oral selama 21 hari. Di akhir perlakuan dilakukan pengambilan sperma dan testis. Sperma diambil dari epididymis untuk pemeriksaan kualitas sperma, sedangkan testis untuk pemeriksaan jumlah sel leydig sel sertoli. Analisis sperm dilakukan dengan menggunakan standar WHO, sedangkan testis dibuat preparat dengan menggunakan metode HE.

Hasil: hasil uji Post Hoc menunjukkan bahwa jumlah sel Leydig, Sertoli, dan kualitas sperma pada CM8-G dan CM10-G lebih tinggi bermakna dibanding MS-G, $p < 0.05$. Jumlah sel Leydig, Sertoli, dan kualitas sperma pada CM10-G lebih tinggi bermakna dibanding CM8-G, $p < 0.05$.

Kesimpulan: Pemberian ekstrak CMCE propolis mampu meningkatkan jumlah sel leydig, sel sertoli dan kualitas sperma yang dinilai dari jumlah, motilitas dan morfologi spermatozoa pada tikus wistar jantan yang diinduksi MSG.

Kata Kunci: MSG, Ekstrak CMCE Propolis, Jumlah Sel leydig, Sel Sertoli, dan Kualitas Sperma

INTRODUCTION

Infertility is a problem in the reproduction system faced by men throughout the world. (Zegers-Hochschild

et al., 2009). Twenty four to forty two percent of male infertility is caused by reduced sperm quality related to increasing ROS level (Singh, 2013; Martin-hidalgo et

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al., 2019). Excessive ROS level in the body may cause oxidative stress and cause male infertility (Singh, 2013). Chronic use of Monosodium Glutamate (MSG) may trigger ROS production and reduce follicle stimulating hormone (FSH) and Luteinizing Hormone (LH) levels, followed with reduced level of testosterone (World Health Organization (WHO), 2010). Luteinizing Hormone (LH) produced by anterior hypophysis serves to stimulate Leydig cells in testis to produce testosterone. Meanwhile, FSH, which is also produced by anterior hypophysis, influences Sertoli cells to secrete growth factor and form androgen binding protein (ABP) serving to bind intratesticular testosterone produced by Leydig cells and triggering spermatogenesis (Sutikno, 2014). Therefore, decreased LH and FSH levels resulting from exposure to MSG may cause reduced sperm quality with mediation of reduced spermatogenesis.

Monosodium Glutamate is a compound often used to improve food deliciousness in Asia (Sharma, 2015). However, excessive use of MSG may endanger the body because of formation of free radicals and oxidative stress (Sharma, 2015). Free radicals and oxidative stress may occur to testis and various cells in the body including cells in hypothalamic, pituitary and gonadal axis. Interference in the axis causes reduced level of LH, FSH and testosterone hormones. Reduced LH level causes Leydig cells unable to produce testosterone, while reduced FSH level inhibits spermatogenesis (Martin-hidalgo et al., 2019). In addition, ROS may directly damage Leydig cells and Sertoli cells. (Mahidin, Maulana and Susiyadi, 2018). Therefore, it is necessary to administer an antioxidant to reduce or even prevent excessive ROS production resulting from exposure to MSG. One of the important antioxidants derived from the nature is propolis, one of bee's products.

Propolis is a compound containing Caffeic acid phenethyl ester (CAPE), constituting flavonoids which are able to donate its electron to neutralize free radicals, thus various cell damages resulting from oxidative stress may be prevented. Various studies show that propolis may reduce MDA level and improve sperm quality because of free radicals from exposure to smoke (Hoesada, Nasihun and Isradji, 2016). Another study shows that propolis at dose of 200mg/kgBW/day evidently reduces the degree of inflammation and MDA level of tested animal (Prasetyo, Suparyanti and Guntur, 2013). In addition, propolis extract at doses of 9, 18 and 27mg/day for 30 days is able to improve spermatozoa parameter which decreases because of exposure to MSG (Nasihun, Widayati and Anindiya Kusuma W, 2012). On the other hand, in cervical cancer cells (Hela cell line), propolis ethanol extract

administration improves apoptosis rate mediated by reduced Bcl2 expression and increased p21 expression in cervical cancer cell culture (Susanto, Maryono and Purwanto, 2017).

Propolis may be extracted using ethanol solvent or water solvent. Propolis used in this research is one extracted using the Continuous Multistage Countercurrent Extraction (CMCE) technology method, thus it may easily be absorbed and improve its bioavailability. The result of antioxidant level measurement in CMCE propolis using the Oxygen Radical Absorbance Capacity (ORAC) method shows its relatively high level (21.921 $\mu\text{mol}/\text{gram}$) (Fauzan and Bagus, 2016). Therefore, CMCE propolis is expected to be used more effectively. The objective of this research is to evaluate whether CMCE propolis administration may increase Leydig cells and Sertoli cells counts and sperm parameter quality after MSG administration.

METHODS

This experimental research employed a Post Test Only Control Group Design. In this research, 18 three-month old male Wistar rats with body weight 200-250gram were divided into 3 groups: control group, only administered with MSG at dose of 140mgBW/day; treatment group, induced with MSG at dose of 140mg/BW/day and CMCE propolis extract 8.3mg/day (CM8-G) for 21 days; and treatment group induced with MSG at dose of 140mg/BW/day and CMCE propolis extract at dose of 10.8mg/day for 21 days. During the research, meal and drinking water were provided to all groups ad libitum. At the end of research (day 22), the rats were terminated and their sperm was taken from epididymis and ductus deferens, and tested for spermatozoa quality (count, motility, morphology). The Leydig cells and Sertoli cells counts were obtained from preparation stained with Hematoxylin Eosin (HE). Leydig cells and Sertoli cells counts were calculated using a microscope with 400x magnification. The research was conducted upon approval from the Ethics Commission of the Faculty of Medicine, Unissula.

CMCE Propolis Extract

CMCE propolis extract is propolis specially extracted using Continuous Multistage Countercurrent Extraction method, thus any wax and resin are removed, leaving only beneficial materials such as flavonoids, flavon, polyphenols and phenolic acid ester which are easily absorbed.

Propolis Dose

Propolis extract dose used in this research was

Table 1. Average leydig cells count, sertoli cells count and spermatozoa count, motility and morphology.

Variables	Groups			P (anova)
	MS-G n=6 (X ±SD)	CM8-G n=6 (X ±SD)	CM10-G n=6 (X ±SD)	
Leydig Cell number (Σ)	3.53±0.242	4.46±0.242	5.53±0.206	0.000
Sertoli Cell Number (Σ)	18.73±3.1	25.03±4.5	31.43±6.5	0.002
Sperm Density (10 ⁶ /ml)	2.56±0.914	3.63±0.258	4.91±0.136	0.000
Motility (%)	41.33±1.211	53.83±1.472	65.33±1.633	0.000
Morphology (%)	54.50±2.174	65±1.414	76±2.191	0.000

the conversion from human dose of 300-600mg/day. The doses used for conversion were 450mg and 600mg. From these doses, the doses of conversion into those of rat with body weight 200gram were: 450mg x 0.018= 8.3mg/head/day and 600mg x 0.018= 10.8mg/head/day.

Testis Preparation Making

After testis tissue was taken, preparation was made in order from fixation with 10% buffer formalin, and thin slices then less than 4mm were made and stored in formalin. Paraffin block was made through dehydration with alcohol, clearing with xylene, and embedding with paraffin wax, slides of which were then made for observation under 400x magnification microscope.

Statistical analysis

The data were analyzed using one way Anova ($p < 0.05$), followed with post hoc LSD. The analysis result was deemed significant if $p < 0.05$.

RESULTS

This research was conducted in examining the effect of CMCE propolis extract administration on the increase in leydig cells and sertoli cells counts and sperm quality of MSG-induced male wistar rats for 21 days. The average Leydig cells and Sertoli cells counts are presented in table 1.

The research result shows that the highest Leydig cells and Sertoli cells counts and spermatozoa quality (count, motility and morphology) are with CM10-G group, followed with CM-G group, and the lowest is with MS-G group. The statistical Anova shows that Leydig cells and Sertoli cells counts and spermatozoa quality (count, motility and morphology) among the groups are significantly different, $p < 0.05$. In examining the difference between the two variables of the various

variables above, further analysis is needed as described below.

Leydig Cells Count

The Post Hoc analysis result shows that the Leydig cells counts of CM8-G and CM10-G groups are higher than that of MS-G group, $p < 0.05$. The Leydig cells count of CM10-G group is also significantly higher than that of CM8-G group, $p < 0.05$ (figure 1).

Sertoli Cells Count

The Post Hoc analysis result shows that the Sertoli cells counts of CM8-G and CM10-G groups are significantly higher than that of MS-G group, $p < 0.05$. The sertoli cells count of CM10-G group is also significantly higher than that of CM8-G group, $p < 0.05$ (figure 1).

Sperm Quality

Sperm quality is marked with concentration, motility and morphology of spermatozoa.

Spermatozoa Concentration

The Post Hoc analysis result shows that the spermatozoa concentrations of CM8-G and CM10-G groups are significantly higher than that of MS-G group, $p < 0.05$. The spermatozoa concentration of CM10-G group is also significantly higher than that of CM8-G group, $p < 0.05$ (figure 2).

Spermatozoa Motility

The Post Hoc analysis result shows that the percentages of spermatozoa motility of CM8-G and CM10-G groups are significantly higher than that of MS-G group, $p < 0.05$. The percentage of spermatozoa motility of CM10-G group is also significantly higher than that of CM8-G group, $p < 0.05$ (figure 2).

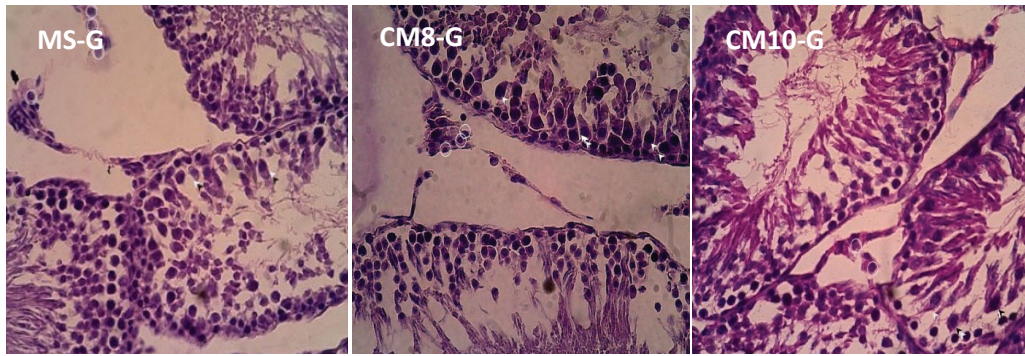


Figure 1. Leydig and Sertoli cells on Hematoxylin Eosin stain method identified by light microscope with 400x magnification in three groups. Post Hoc analysis: * p < 0.05

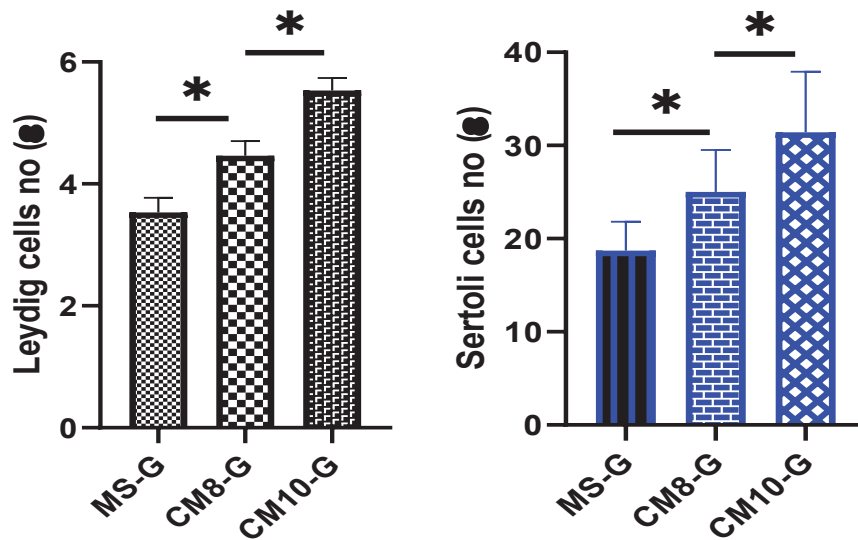


Figure 2. Post Hoc analysis on Leydig and Sertoli cells in three groups. *p < 0.05

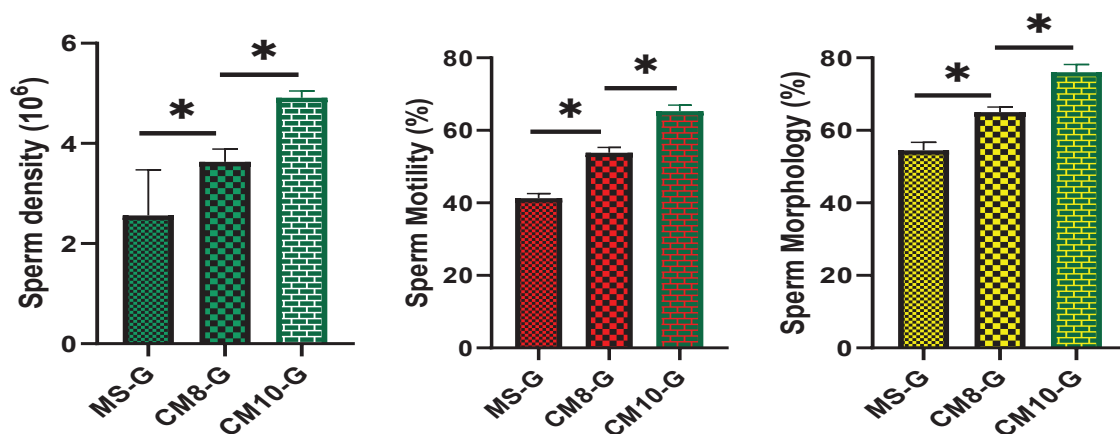


Figure 3. Post Hoc analysis on sperm quality in each group. Sperm density, Sperm motility, sperm morphology in three groups: *p<0.05

Spermatozoa Morphology

The Post Hoc analysis result shows that the percentages of normal spermatozoa morphology of CM8-G and CM10-G groups are significantly higher than that of MS-G group, $p < 0.05$. The percentage of spermatozoa morphology of CM10-G group is also

significantly higher than that of CM8-G group, $p < 0.05$ (figure 2).

DISCUSSION

The research result shows that MSG administered at dose of 140mg/BW/day for 21 days evidently reduces

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leydig cells count, sertoli cells count and sperm quality. This is proven that the leydig cells count, Sertoli cells count and sperm parameter of the group administered with MSG are significantly lower than those of the other groups administered with propolis extract at dose of 8.3 or 10mg/day. This research result is in line with some previous researches that MSG administration may damage some organs such as kidney, liver, epididymis and prostate (Onyema, Farombi and Emerole, 2006; Onyema and Alisi, 2012; Hanipah et al., 2018). Such damage to various organs is related to oxidative stress caused by increased ROS level and increased lipid peroxidation marked with increased malondialdehyde (MDA) level, reduced oxidized glutathione (GSH) level and increased glutathione transferase (Onyema and Alisi, 2012; Hanipah et al., 2018).

Moreover, excessive use of MSG may also cause oxidative stress to testis cells, hypophysis and hypothalamus, which thus interferes in the performance of hypothalamic, pituitary and gonadal axis (Dong and Robbins, 2015). Consequently, gonadotropin releasing hormone (GnRH) secretion by hypothalamus decreases, followed with reduced FSH and LH levels secreted by anterior hypophysis. Decreased FSH level will be followed with decreased spermatogenesis, while decreased LH will be followed with reduced testosterone level produced by testis Leydig cells (Dong and Robbins, 2015). In addition, decreased FSH also causes Sertoli cells to fail to form androgen binding protein (ABP) as protein which transports testosterone to seminiferous tubules for spermatozoa maturation. Therefore, generally, excessive use of MSG may reduce sperm quality.

Furthermore, the research result also shows that CMCE propolis extract administration for 21 days, both at doses 8.3 and 10.8mg/day, evidently increases Leydig cells count, Sertoli cells count and sperm quality, covering density, motility and morphology of spermatozoa. This research result is in line with some previous researches that propolis administration may improve spermatozoa parameter (Capucho et al., 2012; Hoesada, Nasihun and Isradji, 2016). Sperm parameter improvement because of propolis administration is related to CAPE which serves as an antioxidant, thus it is able to neutralize ROS induced with MSG. Considering that CAPE is a strong antioxidant since it contains hydroxycinnamic acid and has group $\text{CH}_2=\text{CH}-\text{COOH}$, thus it has high electron donor capacity. In addition, CAPE is the most effective antioxidant flavonoid as a reactive species scavenger since after it has transferred atom H^+ flavonoid, it does not change to pro-oxidant (Bogdanov, 2013).

In general, the research result shows that CMCE

propolis administration at doses 8.3 and 10.8mg/day for 21 days to male wistar rats evidently effectively improves their reproductive function as marked with improved Leydig cells count, Sertoli cells count and sperm parameter. This research result is in line with another research stating that propolis administration at doses 9, 18 and 27mg/day for 30 evidently increases the concentration and percentage of normal spermatozoa morphology of male wistar rats induced with monosodium glutamate (Nasihun, Widayati and Anindiya Kusuma W, 2012). As previously hypothesized, MSG administration will damage hypothalamic, pituitary and testis organ axis and reduce sperm quality because of increased ROS. Therefore, improved sperm quality because of propolis administration is related to improved function of hypothalamic, pituitary and testis organ axis. Consequently, LH, FSH and testosterone hormones production will also improve (Capucho et al., 2012; Dong and Robbins, 2015). The statement is pursuant to previous study showing that CMCE propolis administration at doses 8.3 and 10.8mg/day evidently increases LH, FSH and testosterone level.

CONCLUSION

Administration of continuous multistage countercurrent extraction (CMCE) propolis extract increases leydig cells count, sertoli cells count and sperm quality of male wistar rats induced with MSG.

REFERENCES

- Bogdanov, S. (2013) Propolis: Composition, Health, Medicine: A Review, Bee Product Science.
- Capucho, C. et al. (2012) 'Green Brazilian propolis effects on sperm count and epididymis morphology and oxidative stress', Food and Chemical Toxicology. Elsevier Ltd, 50(11), pp. 3956–3962. doi: 10.1016/j.fct.2012.08.027.
- Dong, H. V and Robbins, W. A. (2015) 'Ingestion of monosodium glutamate (MSG) in adult male rats reduces sperm count, testosterone, and disrupts testicular histology', Nutrition Bytes, 19(1), pp. 1–10.
- Fauzan, M. and Bagus, S. (2016) 'Administration of Propoelix® as an Adjuvant Therapy for Patients with HIV / AIDS in Sungailiat Hospital, Bangka', Medika, 3, pp. 128–138.
- Hanipah, E. N. A. et al. (2018) 'Monosodium Glutamate Induced Oxidative Stress in Accessory Reproductive Organs of Male Sprague-Dawley

- Rats', *Jurnal Sains Kesehatan Malaysia*, 10, pp. 67–73.
- Hoesada, I., Nasihun, T. and Isradji, I. (2016) 'The Effect of Propolis Extract on MDA Levels (Malondialdehyde) and Sperm Quality on Epididimis (Experimental Study on Wistar Strain Male Rats Exposed to Kretek Cigarettes)', *Sains medika*, 7(1), pp. 9–14.
- Mahidin, Maulana, A. M. and Susiyadi, S. (2018) 'Pengaruh Pemberian Ekstrak Etanol Daun Kemangi (*Ocimum basilicum* L) Terhadap Jumlah Sel Spermatogrnik Tikus Putih (*Rattus norvegicus*) Galur Wistar Jantan Yang Diinduksi Monosodium Glutamat', *Herb-Medicine Journal*, 1(1), pp. 19–30. doi: 10.30595/hmj.v1i1.2480.
- Martin-hidalgo, D. et al. (2019) 'Antioxidants and Male Fertility: from Molecular Studies to Clinical Evidence', *Antioxidants*, 8(89), pp. 1–21. doi: 10.3390/antiox8040089.
- Nasihun, T., Widayati, E. and Anindiya Kusuma W (2012) Proteksi Spermatozoa dengan Propolis dengan ROS Pada Tikus Jantan Galur Wistar Yang Diinduksi Oleh Monosodium Glutamat. Semarang: Bagian Andrology dan Biokimia Fakultas Kedokteran Universitas Islam Sultan Agung (UNISSULA).
- Onyema, O. O. and Alisi, C. S. (2012) 'Monosodium Glutamate Induces Oxidative Stress and Affects Glucose Metabolism in the Kidney of Rats', *International Journal of Biochemistry Research*, 2(1), pp. 1–11.
- Onyema, O. O., Farombi, E. O. and Emerole, G. O. (2006) 'Effect of vitamin E on monosodium glutamate induced hepatotoxicity and oxidative stress in rats', *Indian Journal of Biochemistry & Biophysics*, 43, pp. 20–24.
- Prasetyo, D. H., Suparyanti, E. L. and Guntur, A. H. (2013) 'Ekstrak Etanol Propolis Isolat Menurunkan Derajat Inflamasi dan Kadar Malondialdehid pada Serum Tikus Model Sepsis', *MKB*, 45(3), pp. 161–166.
- Sharma, A. (2015) 'Monosodium glutamate-induced oxidative kidney damage and possible mechanisms: a mini-review', *Journal of Biomedical Science*. *Journal of Biomedical Science*, p. 22:93. doi: 10.1186/s12929-015-0192-5.
- Singh, A. R. (2013) 'Spermicidal Activity and Antifertility Activity of Ethanolic Extract of *Withania somnifera* in Male Albino Rats', *International Journal of Pharmaceutical Science Review and Research*.
- Susanto, T. H., Maryono, S. and Purwanto, B. (2017) 'Pengaruh Ekstrak Propolis Terhadap Ekspresi Protein Bcl2, p21, dan Induksi Apoptosis Pada Sel Hela', *Biomedika*, 9(2), pp. 6–14.
- Sutikno, M. T. (2014) Pengaruh Ekstrak Etanol Propolis Terhadap Penurunan Jumlah Spermatid Mencit (*Mus Munculus*) yang dipapar Asap Rokok. Universitas Sebelas Maret.
- World Health Organization (WHO) (2010) WHO Laboratory Manual for Examination and Processing Human Semen. 5th edn. Geneva World Heal. Organ.
- Zegers-Hochschild, F. et al. (2009) 'The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology', *Human Reproduction*, 24(11), pp. 2683–2687. doi: 10.1093/humrep/dep343.