International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.6, pp 1817-1823, Oct-Nov 2014

Effect of Methanolic *Momordica charantia* seed extract and Depot medroxyprogesterone acetate (DMPA) to quantity and quality of rat sperm

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Abstract: To determine the effectiveness of the methanolic *Momordica charantia* seed extract dosage and depot medroxyprogesterone acetate (DMPA) to develop a potential male contraceptive. Methods: The extract of *M. charantia* seeds is done every day for 8 weeks of 50 mg/100 kg body weight of rats. DMPA dose is 0.625 mg/100 g BW of rat intramuscularly and given once on the first day of treatment after the first oral administration of *M. charantia* seed extract. There are 6 treatment groups (3 treatment groups were given the extract of *M. charantia* and 3 aquades control group) and 5 replicates of each group. Group (1) Administration of the extract of *M. charantia* seeds for 4 hours or 0 week, (2) seed extract of *M. charantia* every day for 4 weeks, (3) seed extract of *M. charantia* every day for 8 weeks, (4) administration of aquades (control 1) for 4 hours or 0 week, (5) administration of aquades (control 2) per day for 4 weeks, (6) administration of aquades (control 3) for 8 weeks. The combination of these two materials can accelerate the decline in the production of progesterone (precursor testosteron) and ultimately reduce the quantity and quality of rat sperm. The results obtained are decreased number of sperm, motility, normal morphology and viability of sperm after administration of the methanol extract of *Momordica charantia* seeds+DMPA at the weeks-4 and 8 ($p<0.05$). Conclusion: The methanol extract of *Momordica charantia* seeds (50mg/100g BW of rat) + DMPA (0.625 mg/100g BW of rat) is very good and effective for materials developed into a male contraceptive in the future
Keywords : *Momordica charantia* seed extract, DMPA, number of sperm, sperm motility, sperm morphology, sperm viability, germ cell apopotosis

Introduction

According to the Institute for Development of Economics and Finance (INDEF) estimates that the population of poor people in 2009 could reach 40 million people (16.82%). Conditions of this population increase of 5 million when compared to survey Central Statistics Agency (BPS) in March 2008, noted the poor as much as 34.96 million people (15.42%)[1]. The situation triggered by the global economic recession and the population is not comparable to Indonesia's economic growth. As the best solution is to optimize economic growth while reducing the rate of population growth that continues to grow. Reported a population of Indonesia in 2020-2025 an estimated 285 million people . As the main cause is the birth rate continued to increase until the period 2002-2010. If allowed to continue, it would have been a source of disaster for this nation . If there is no support for the growth of the national economy will be worse happened . One effective way is to improved the family planning (KB). Because of population growth is not matched by employment causes unemployment. Based on this, the target is no longer BKKBN" demographic" but also to prevent the unemployment rate of growth in reducing poverty directly or indirectly[2]. The latest report from the Ministry of State and the National Family Planning Coordinating Board in 1999 there were acceptors of family planning as much as 56.17% of the population 15-49 years. Approximately 97.86% of all acceptors are women and 2.14% of them were male. Male

acceptor is 43.33% and 56.68% vasectomy use condoms. This proves the participation of men as acceptors very little of women[3]. One of the main causes is the lack of available male contraceptive contraceptives when compared with women . Therefore, to encourage more men to become family planning acceptors it is necessary to develop a new male contraceptive[4].

Male contraceptive that is currently circulating more with vasectomy and condoms reported more disadvantages. Vasectomy can make people become sterile (infertile) throughout his life, but it could happen one plan change in the number of family planning. While the use of condoms would still complain about comfort and especially the leakage current usage. So it needs to be developed "hormonal contraception" is ideal for men and a goal some researchers world and WHO[20] (World Health Organization) for over a decade.

Plant extract intramuscularly or orally alone or in combination with progesterone male known to inhibit spermatogenesis (sperm formation process) to azoospermia[5]. There are reports that testosterone may lead to azoospermia is reversible, with no serious adverse events and significantly effective in Asian populations, so that testosterone seems to be the chemicals that give hope for both male fertility control[6].

Another opinion stated that the achievement of azoospermia due to the hormone may occur through increased occurrence of apoptosis (programmed cell death) in spermatogenic cells[7]. As has been reported, that the suppression of spermatogenesis can occur in addition to the influence Depot Medoksiprogesteron Acetate (DMPA) through a negative feed-back mechanism, also through the mechanism of apoptosis. Progesterone is an earlier step prior to the formation of testosterone (T), which is an androgen which react directly with established ties to androgen receptors (RA)[8]. Androgen receptor family of nuclear receptors that react as ligand-responsive transcription factor[6]. In the testis, RA is located in Leydig cells, peritubular cells, and Sertoli cells[8].

Momordica caharantia containing the active substance cucurbitacin (momordikosida) which can be anti- mitotic[9]. Cucurbitasin classified as triterpene glycosides have the basic structure of cyclopentane perhidrofenantrena as also owned by steroids. Steroids can act as a reversible inhibitor of spermatogenesis and[10]. Testosterone freely diffuse through the plasma membrane and bind to RA form a complex that then interacts with androgen response elements in the promoters of target genes. Inducible transcription of target genes causing long term genomic effects[11] or inhibited depending on the factors associated with ligand - receptor complex is bound to the androgen response element[12]. In addition to the role of negative feedback from the shaft hipatalamus- pituitary-testes to decrease sperm in the testes, is also caused by a germ cell apoptosis mechanism that originated from testosterone deficiency intratesticular. Ultimately activates caspase 3 which is the executor factors (effectors) fragementation of DNA[13].

Increase in population in the period 2000-2005 is 1.34%, which can be a major problem for the development of the nation that should be reducing poverty. Needed improvement planning acceptors women and men that need development contraceptives. In this case, the man who became the target of developing a hormonal contraceptive that is effective, inexpensive, safe, does not decrease libido, revesibel and can be made public. This research it is necessary for the development of contraceptives by seeking local agricultural products (*Momordica charantia*)[14] as the main ingredient and combined with synthetic material patent (progesterone / Depot Medroxy Progesterone Acetate) [15] so that the effect is much better quality.

Methods

Animals

Thirty white male rats (body weight 150-200 g, 8-12 weeks) obtained from the Laboratory of Biological Science (Faculty of Matermatics and Natural Sciences) USU (University of Sumatra Utara) Medan, placed in a plastic cage 30x20x10 cm and covered wire. Basic cage coated rice husk 0.51 to 1 cm and replaced every 3 days. Room light 12 hours of light and 12 h dark, temperature and humidity in the room is left natural range. Feed (CP 551) of the plant Pockphan Tanjung Morawa and drinking (tap water) was supplied every day in *adlibitum*. Maintenance of experimental animals were placed in the laboratory of Biological Science USU Medan. The study had been began after approval Research Ethics Committee of the Faculty of Mathematics and Natural Sciences USU Medan.

Preparation of *M. charantia* seed extract and DMPA (Depot Medroxy Progesterone Aceteate)

M. charantia seed obtained from Rampah Sei Serdang in North Sumatra. Seeds obtained from the fruit of the sun dried, threshed to remove the seeds, then seeds are dried by heating sun or incubator 40^{o}C to constant

weight. Seeds then finely ground in a blender and filtered with a mesh-size of 40. Preparation of the extract was done by meseration uses 70% methanol. Methanol solvent separation is done by Rotary vacuum evaporator. The next extract is inserted into the oven 40°C until free of methanol (Indonesian Pharmacopoeia Edition IV[19]). *M. charantia* seed extract/DMPA preparation. Powder and extracted and examined their chemical ingredients in powder see below scanning electron microscope (SEM/Scanning Electron Microscope) brand desktop Phenom Phenom Pro X made in the Netherlands at the State University of Padang.

DMPA was manufactured and distributed by a pharmacy in Medan. The registered trade name for this product is Depo-Progestin. The compound was formulated as an aqueous suspension and packaged in 3 mL vials containing 150mg DMPA.

Study Procedure

This study used two experimental material that methanol extract of *M. charantia* seeds + DMPA. The extract of *M. charantia* seeds is done every day for 8 weeks of 50 mg/100 kg body weight of rats. DMPA dose is 0.625 mg/100 g BW of rat intramuscularly and given once on the first day of treatment after the first oral administration of *M. charantia* seed extract. There are 6 treatment groups (3 treatment groups were given the extract of *M. charantia* and 3 aquades control group) and 5 replicates of each group. Group (1) Administration of the extract of *M. charantia* seeds for 4 hours or 0 week, (2) seed extract of *M. charantia* every day for 4 weeks, (3) seed extract of *M. charantia* every day for 8 weeks, (4) administration of aquades (control 1) for 4 hours or 0 week, (5) administration of aquades (control 2) per day for 4 weeks, (6) administration of aquades (control 3) for 8 weeks. Rats were sacrificed by dislocation of the neck and begins with annestesi at weeks 0, 4 and 8 left and right testis samples taken and fixed in Bouin solution for 24 hours and subsequent testicular histological preparations were made with standard histological methods for the observation of germ cell apoptosis. Cauda epidymis left and right were taken for examination of sperm samples in several parameters such as number of sperm, motility, morphology and viability of sperm.

Sperm number, motility of sperma, morphology and viability of sperm

Cauda epididymis from the testis removed and placed in 1 mL of physiological solution (0.95% NaCl) to allow sperm to swim so that it can be observed movement (motility). To calculate the sperm is placed in a Neubauer by using a pipette. Motility was determined by counting the number of sperma immotile and subtracting from the total number x 100%. Sperm morphology calculated by Giemsa staining of sperm with normal morphology until 100 sperm and multiplied by 100%. Sperm viability was determined by staining sperm with eosin Y and sperm viability was calculated by counting 100 sperm were viable (not colored/not absorb color) and then multiplied by 100%[16].

Germ cell Apoptosis

One testis from each rat was fixed in Bouin's fluid and paraffin embedded. Apoptotic cells were identified in tissue sections (5 mm) by ISEL using an in situ death detection kit, POD (Cat No. 11 684 817 001 – Roche). Briefly, sections were deparaffinized in xylene and rehydrated in a graded ethanol series (100%, 95%, 80%, and 70%), followed by phosphate-buffered saline. The sections were digested with buffer citrate for 30 min at 95 °C, washed with PBS, and then treated with PBS 4°C for 2 min. Sections were incubated with terminal deoxynucleotidyl transferase reaction mixture for 1.5 h at 37°C in a humidified atmosphere and the reaction stopped by immersion in buffer. Sections were then washed in PBS and incubated with peroxidase-conjugated anti-digoxygenin for 30 min at room temperature in a humid atmosphere. Following washing in PBS, sections were incubated with diaminobenzidene (DAB) substrate for 10 min at room temperature, washed in distilled water, dehydrated stepwise in ethanol (70%, 80%, 95%, and 100%) and xylene, and mounted with entelan. For quantification, slides were blinded and all tubules in two different sections of testis were counted for the absence or presence of 1 to 3 or > 3 TUNEL-positive germ cells. For every animal, at least 400 tubules were scored for TUNEL positivity.[15]

Statistical analysis

ANOVA bootstrapping, followed by *post hoc* test (software package, SPSS 20) was used to determine differences across time (0, 4, and 8 wk). Bootstrapping T test was employed to determine differences between two groups . Results are expressed as the mean ± SD. $P<0.05$ was considered to be significant.

Results and Discussion

Phytochemical testing botanicals and extracts of *M. charantia* seed

The data collection and test results phytochemical simplisia pare seed extract can be displayed in Table 1 below.

Table 1. Phytochemical test results botanicals and extracts of *M. chrantia* seeds

Compounds	Powder	Methanol Extracts
Phenolic	-	-
Flavonoids	+	+
Terpenoids	+	+

Ultrastructur of *M charantia* seed powder

To view and elaborate surface structure of pollen grains received pare with SEM results can be seen in Figure 1.

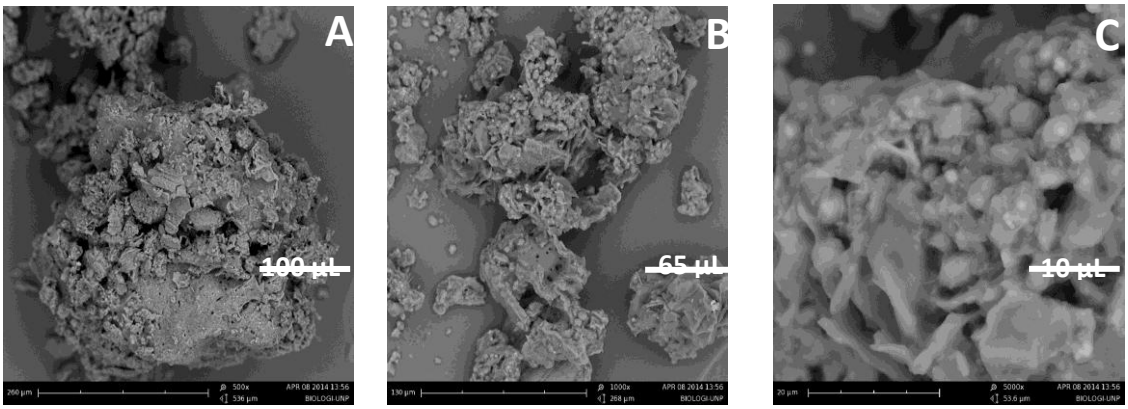

Figure 1. Results of Scanning Electron Microscope of the *M. charantia* seed powder (A) Magnification 500x, (B) Magnification 1000x, and (c) Magnification 5000X.

Sperm number, motility of sperm, morphology and viability of sperm

For more details, picture of the average number of sperm rats, motility of sperm, morphology and viability of sperm after administration of the methanol extract of *Momordica caharantia* seeds and DMPA can be seen in Figure 2.

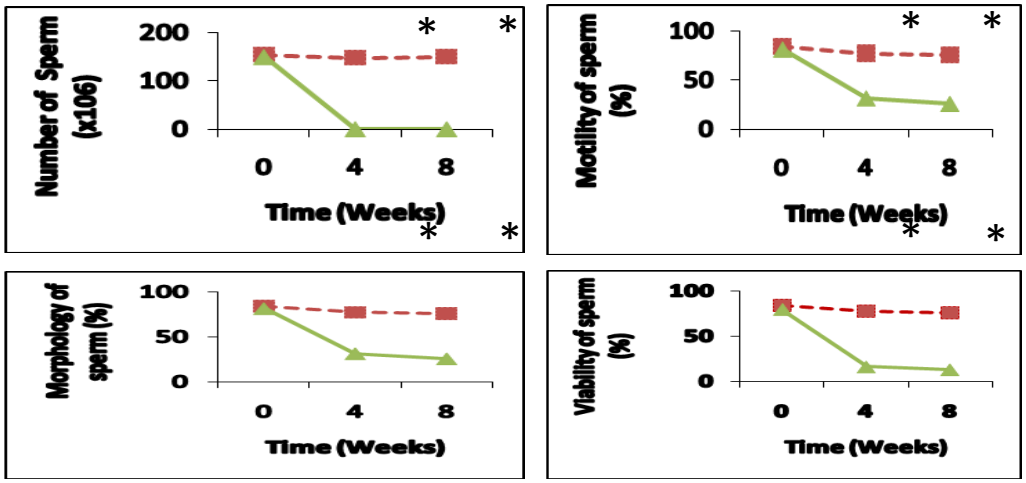

Figure 2. Line graphs the average number of sperm rats (A), motility of sperm (B), morphology (C) and viability of sperm (D) after administration of the methanol extract of *M. caharantia* seeds and DMPA for 8 weeks. *within groups different significantly (p<0.05),

Aquades M. charantia seed+DMPA

The results of the bootstrapping *t* test analysis between treatment and control at each weeks 4 and 8 were significantly different to the number of sperm, sperm motility, sperm morphology and sperm viability ($P<0.05$). This shows that there is a real effect of methanol extract of *M. charantia* seeds to the four parameters above.

Germ Cell Apoptosis

Figures 3 and 4 show the significant effects of the methanol extract of *M. charantia* seed germ cell apoptosis ($p<0.05$), especially at weeks 4 and 8.

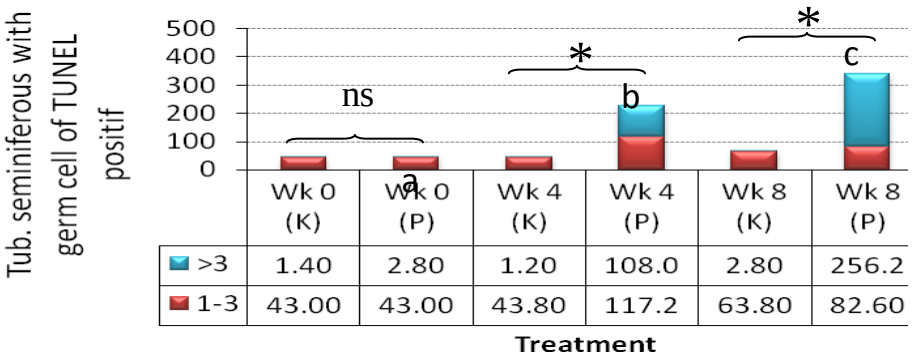

Figure 3 Histogram of germ cell apoptotis of rat after administration of the extract of *M. charantia* seeds+DMPA, >3 = number of seminiferous tubules with more than 3-positive germ cell apoptosis, 1-3 = number of seminiferous tubules which had 1-3 positive germ cell apoptosis, (K) Control, (P) extract of *M. charantia* and Treatment with administration (Week/Wk) = 0, 4, and 8, ts = $^{k,p}p>0.05$; *= $^{k,p}p<0.05$; $^{a,b}p<0.05$.

Discussion

Observed powder of *M. charantia* seed such as surface morphology, porosity, and surface. Morphology powder of *M. charantia* seed reported in Fig. 1A, 1B and 1C. Result of scanning revealed that the film is porous and smooth texture. The structure of *M. charantia* powder surface was uneven (Fig.1B). *M. charantia* powder surface showed randomly distributed micro space that looks like a crack. This structure particles indicates that *M. charantia* seed may have failed to crystallize (Fig.1C). Membrane surface characteristics play an important role in the ability to allow for methanol absorption. Cellulose fibers can increase the porosity of chitosan (Fig.1D). Bangyekan *et al*.,[17] *M. charantia* seed powder has a rough and uneven structure that does not cause the water molecules adsorbed. Morphology of *M. charantia* powder reinforced by reports SEM micrographs revealed that the surface structure of the *M. charantia* powder, *M. charantia*-reinforced has many similarities with amorphous *M. charantia* powder.

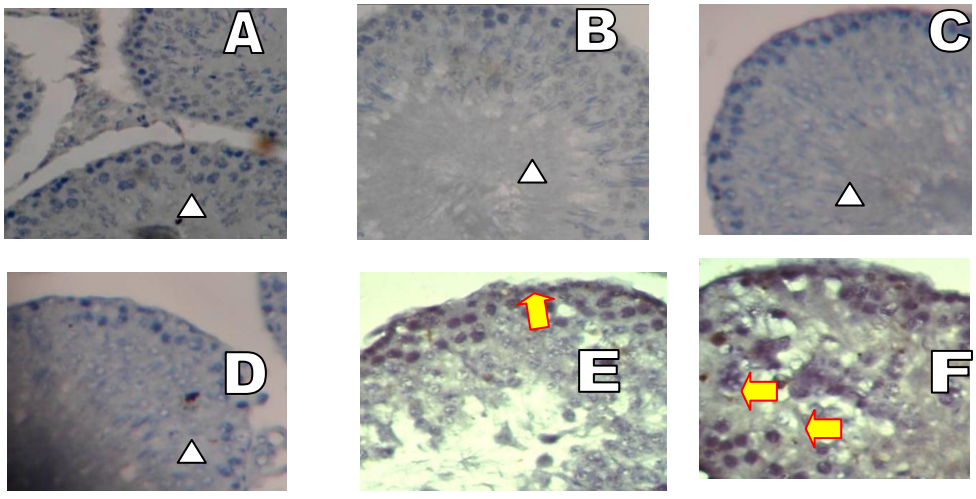

Figure 4. Germ cell apoptosis of rat after administration of the extract of *M. charantia* seeds+DMPA, (A) Control Week-0, (B) Control Week-4, (C) Control Week-8, (D) Treatment-A, (E) Treatment-B, and (F) Treatment-C, ➔ = germ cell apoptosis, △ = germ cells normal.

We obtain the number of sperm after treatment given *M. charantia* seed extract+DMPA was 0.52 and 0.23 million/mL at the 4 and 8 weeks respectively (Fig. 2A). The analysis also showed significant differences ($p<0.05$) between the control group and the treatment with aquades (solvent control) either at the week 4 and 8. This suggests a significant effect of methanol extract of *M. charantia* seeds+DMPA to number of rat sperm. It was caused by the presence of the active ingredient in the seeds cucurbitasin (*Momordica caharantia*) as antimitotic[9]. The nature of the process of spermatogenesis or cause cell division - cell spermatogonia into spermatocytes, spermatids and sperm to be blocked. So that the sperm produced are also being reduced. In addition to a combination of DMPA causes suppression of sperm more effectively through the hypothalamic - pituitary - testes. DMPA is that progesterone is the precursor of testosterone. Continuous increase in testosterone will cause the hypothalamus to inhibit the inhibitory activity in generating GnRH (or LHRH, FSHRH). So that the activity also inhibited the formation of pituitary FSH and LH. FSH and LH function in inducing the formation of sperm.

At the Fig. 2B it can be seen that a decrease in the percentage of sperm motility after treatment given *M. caharantia* seed extract + DMPA. Difference in the percentage of sperm motility mice after 4 weeks is the commission of 31.63%, and 8 weeks was 25.90%. The analysis also showed significant differences ($p<0.05$) between the awarding 0 to 4 weeks and 8 weeks. This suggests a significant effect of ethanol extract of the seeds of *Momordica caharantia* + DMPA rat sperm motility. Combination with DMPA causes suppression of sperm more effectively through the hypothalamic-pituitary-testes. Increased testosterone due to DMPA administration resulted in negative feed back so intratestikular decreased testosterone. Thereby disrupting the formation of sperm from and including the amount and quality of the mitochondrial membrane of sperma. Consequently be decreased sperm motility. Gu *et al*.,[17] stated that the provision of DMPA in combination with TU (testosterone undecanoate) has led decreased sperm motility.

Administration of *M. charantia* seed extract+DMPA was able to decrease in the percentage of normal sperm morphology. Decrease in the percentage of normal sperm morphology rat after 4 and 8 weeks were the commission of 25.48%, and 24.39% respectively (Fig. 2C). In accordance with research Ilyas (2007) which get reduced sperm normal morphology after the addition of TU and DMPA in rat. Intratesticular testosterone decreased was caused negative feedback therefore disruption of the process of formation of sperm (spermatogenesis) in the testis.

There was a real difference in sperm viability between the control group, aquades and methanol extracts of *M. charantia* seed groups at weeks 4 and 8 ($p<0,05$) (Fig. 2D). After the treatment given seed extract of *M. charantia* + DMPA rat sperm viability percentage after the commission of 4 weeks and 8 weeks of very low ie 15.83% respectively and 12.60%. This is consistent with research Ilyas *et al.*,[15,18] that the administration of TU + DMPA may reduce sperm viability as a result of the number of occurrences of germ cell apoptosis, especially in spermatid cells.

From Fig. 3 and 4 above have shown that *M. charantia* seed extract+DMPA increase the number of germ cell apoptosis that gametogenesis (sperm formation process) depressed. So that the sperm produced very little and eventually not be able to fertilize eggs (infertile). This is probably caused by the influence of terpenoids that are as trigger proaptoptosis proteins such as Bax, Bid, and p53. Increased expression of p53 protein proapoptosis cause program cell death or apoptosis. As a statements Liu *et al*.,[6] that, terpenoids derived from ginger plants can induce apotosis in endometrial cancer cells through activation of p53.

Conclusion

The use of Seed extract of *M. charantia* seed extract +DMPA hormones may be effective way to develop potential male contraceptives, and further studies on this topic should be carried out.

Acknowledgments

We are grateful to Hibah Bersaing team from DP2M Dikti Republic of Indonesia for providing funding for our research.

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