Decreased expression of caspase$_3$ in penis and prostate tissues of rat after the treatment with *buceng* (*Pimpinella alpina* Molk & *Euricoma longifolia* Jack)

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Abstrak

Latar belakang: *Buceng {kombinasi pasak bumi (Eurycoma longifolia Jack) dan purwoceng (Pimpinella alpine Molk)} telah terbukti meningkatkan kadar testosteron (Te) dan menurunkan apoptosis. Namun belum ada bukti apakah efek tersebut dimediasi oleh penurunan ekspresi caspase$_3$. Tujuan penelitian ini adalah untuk mempelajari apakah pemberian buceng dapat menurunkan ekspresi caspase$_3$ sel penis dan prostat pada tikus jantan* Sprague Dawley.

Metode: *Studi eksperimental dilakukan pada 24 tikus jantan galur* Sprague Dawley*, umur 90 hari dengan berat badan (BB) + 300 g, dibagi menjadi 4 kelompok secara acak masing-masing terdiri dari 6 ekor. Kelompok A, tikus dikastrasi dan diberi buceng 50 mg. Kelompok B, tikus tanpa dikastrasi, langsung dimatikan sebagai kontrol positif. Kelompok C, tikus dikastrasi dan diberi akuades 2 mL, sebagai kontrol negatif. Kelompok D, tikus dikastrasi dan diberi* mesterolone *6,75 mg yang dilarutkan dalam air. Analisis statistik yang digunakan untuk menguji perbedaan ekspresi caspase$_3$ adalah uji MANOVA, dilanjutkan dengan* Post Hoc.

Hasil: *Analisis MANOVA pada empat kelompok menunjukkan perbedaan ekspresi caspase$_3$ yang bermakna (p = 0,000). Analisis tes* Post Hoc *menunjukkan bahwa ekspresi caspase$_3$ penis dan prostat pada kelompok A (buceng) (33,56; 35,83) lebih rendah bermakna dibanding kelompok C (kontrol negatif) (54,33;60,07) dan kelompok D (*mesterolone*) (51,91;56,21), p = 0,000, dan lebih tinggi dibanding kelompok B (kontrol positif atau tikus normal) (29,40; 27,72), namun secara statistik tidak bermakna (p = 0,826).*

Kesimpulan: *Pemberian buceng 50 mg/hari selama 30 hari berturut-turut dapat menurunkan ekspresi caspase$_3$ pada sel penis dan prostat.* ***(Med J Indones. 2013;22:2-8)***

Abstract

Background: *Buceng* {combination of pasak bumi *(Eurycoma longifolia* Jack*)* and *purwoceng (Pimpinella alpine* Molk*)}* has been proven to increase testosterone (Te) level and decrease apoptosis. Unfortunately, there is no evidence whether these effects are mediated by the declining of caspase$_3$. Objective of this study was to evaluate whether *buceng* could decrease the expression of caspase$_3$ of penis and prostate cells in Sprague Dawley male rats.

Methods: Twenty four Sprague Dawley male rats weighing 300 g (90 days old) were randomly assigned into 4 groups of 6 male rats. Group A, rats were castrated and received *buceng* 50 mg. Group B, rats were not castrated, sacrifices as positive control. Group C, rats were castrated and given 2 mL aquadest as negative control. Group D, rats were castrated and got of 6.75 mg mesterolone, dissolved in 2 mL water. MANOVA statistical analysis was adopted to examine the difference expression of caspase$_3$ in all groups. The comparison of caspase$_3$ expression between two groups exhibiting difference values were evaluated by Post Hoc test.

Results: MANOVA revealed statistically significant differences in the expression of caspase$_3$ of penis and prostate tissues among the four groups. Post Hoct test also indicated that expression of caspase$_3$ in group A (*buceng*) (33.56; 35.83) was significantly lower compared to group C (negative control) (54.33; 60.07) and group D (mesterolone) (51.91;56.21), p = 0.000, and higher compared than group B or normal rats (29.40; 27.72), but statistically not significant (p = 0.826).

Conclusion: The treatment of 50 mg *buceng*/day for 30 consecutive days could decrease caspase$_3$ expression in penis and prostate cells. ***(Med J Indones. 2013;22:2-8)***

Keywords: *Apoptosis, buceng (Pimpinella alpine Molk – Eurycoma longifolia Jack), caspase$_3$*

Combination of Indonesia indigenous plants, *pasak bumi (Eurycoma longifolia* Jack*)* and *purwoceng (Pimpinella alpine* Molk*)* and so-called *buceng* has been proven to increase testosterone (Te) level and decrease of apoptosis.[1] Apoptosis is a biological mechanisme involved in aging process and cellular degeneration. Subsequently apoptosis will induce aging and the decline in physiological function of organs.[2] The decrease in testosterone concentration usually occur in adult males aged 40 years or above. The decline in testosterone level is associated with the increase in apoptosis of cell and tissues, in which Te receptors exist,[3,4] whereas caspase$_3$ is an enzyme playing role in the execution activity in the most cellular apoptosis.[5] Accordingly, the increase in Te level and followed by the decrease in apoptosis is attributed to the decrease in caspase$_3$ activity. Unfortunately, there is no evidence whether the increase in Te level and the decrease in apoptosis during *buceng* treatment is mediated by declining in caspase$_3$.

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The exploration of the effect of *buceng* to decrease apoptosis through the decrease in caspase$_3$ is very important in order to recognize the apoptosis molecular pathway, in which *buceng* has been utilized. To date, the pathway remains unclear, thus verification of the effect of *buceng* on caspase$_3$ down regulation is required. In addition, people will not be optimally encouraged to use *buceng* treatment when verification of the effect is unproved.

On the other hand, the Indonesian Ministry of Health recommend that every medicines should prove its efficacy besides the clarity of mechanism of action.[6] Thus, the scrutiny of the effects of *buceng* as Indonesian indigenous plant toward caspase$_3$ is required.

Various evidences indicated that high level of Te is able to decline tissue apoptosis.[2,4,7] On the contratry, decrease in Te level gives rise many organs, particularly penis and prostate, to undergo apoptosis as indicated by increase in caspase$_3$ expression and formation of apoptotic body.[1,8,9] Both penis and prostate organs are regulated by Te, thus they are very sensitive to Te declining or deprivation. Caspase is protease enzyme capable to cleave peptide chain at aspartic acid residue.[5] This cleavage will activate endonuclease and protease enzymes in cell nucleus, thus inducing degradation of nucleus and cytoskeletal protein. In other word, caspase$_3$ is considered to serve as an executor enzyme in cellular apoptosis. *Buceng*, is an extract of Indonesian indigenous plant that in previous studies has been proven to increase the level of Te, lutenizing hormone (LH), follicle stimulating hormone (FSH), and decrease in apoptosis in Sprague Dawley male rats.[1,10] The objective of the present study was to elucidate whether the effect of *buceng* is mediated by the decrease in expression of caspase$_3$ of penis and prostate cells in Sprague Dawley male rats.

METHODS

This study used 24 Sprague Dawley male rats weighing 300 g (90 days old), that were randomly assigned into 4 groups of 6 rats (determined by Federer formula). All rats were immediately caged individually in line with their groups each for one week to undergo acclimatization. All rats got their daily chow and tap water ad libitum. Group A, was castrated and given 50 mg of *buceng* extract by oral gavage. Group B, was not castrated and directly sacrifices as such of positive control. Group C, was castrated and given 2 mL aquadest as negative control. Group D, was castrated and given 6.75 mg mesterolone, dissolved in 2 mL water. All treatment was given orally at 7.00 am to all rats for 30 consecutive days except in group B.

Buceng extract has been obtained from soxhlet extraction method with 99% methanol as a solvent. The 50 mg/mL dosis was determined based on *Caropeboka* study in monkey 75 mg and then converted into rat dosis. Rats in group A, C, and D were sacrified after 30 days of treatment, penis and prostate tissues were taken for assesment of caspase$_3$ by immunohystochemistery method. For group B after acclimatization, rats were immediately sacrificed, penis and prostate tissues were taken as well for measurement of caspase$_3$ of referrence. Prior to measurement, penis and prostate tissues were freezed with nitrogen solution and kept in temperature of minus 20ºC.[11]

The experimental study was undertaken in Animal Experimental Development Unit of Veterinary School of Gajah Mada University Yogyakarta, whereas assessment of caspase$_3$ was conducted in Anatomic Pathology Laboratory of Sarjito Hospital/ School of Medicine Gajah Mada University.

Statistical analysis to examine the difference expression of caspase$_3$ in all groups was conducted with MANOVA test and comparison between two groups were evaluated by Post Hoct Tukey HSD. All statistical analyses were carried out using SPSS software version 10, The p value of < 0.05 was taken as the limit of statistical significance with confident interval 95%.

Table 1. Body weight and caspase$_3$ expression in penis and prostate

Variables	Groups				p
	A	B	C	D	
	(n = 6)	(n = 6)	(n = 6)	(n = 6)	
BW (gr ± SD)	267.8 ± 25.21	268.7 ± 28.04	268,7 ± 28,54	266,4 ± 19,56	
Penis Caspase$_3$ (% ± SD)	33.56 ± 4.55	29.40 ± 4.54	54.33 ± 15.20	51.91 ± 15.08	< 0.01
Prostate Caspase$_3$ (% ± SD)	35.83 ± 10.41	27.72 ± 6.29	60.07 ± 22.43	56.21 ± 15.40	< 0.01

RESULTS

In the present study, 24 inbred Sprague Dawley male rats were used, thus the variability of genetic, age, and body weight can be neglected and therefore, the four groups are considered comparable. Caspase$_3$ expression both in penis and prostate is shown in table 1.

MANOVA test showed that the results were significantly different ($p < 0.05$). Post Hoc Tukey HSD also show that the expression of caspase$_3$ is significantly different in group A compared to group C and D, but not with group B (Table 2).

Table 2. The result of Post Hoc Tukey HSD test on caspase$_3$ penis and prostates

Variable		Group	p
Caspase$_3$	Penis	A vs B	0.826
		A vs C	< 0.001
		A vs D	< 0.001
		B vs C	< 0.001
		B vs D	< 0.001
		C vs D	0.953
	Prostate	A vs B	0.601
		A vs C	0.002
		A vs D	0.011
		B vs C	< 0.001
		B vs D	< 0.001
		C vs D	0.924

Difference expression of penis caspase$_3$

Light microscopy observation with 400 HF on the immune histochemistry staining of penis tissues is delineated in figure 1.

The mean of caspase$_3$ expression were calculated from all data collected from the observation of nucleus which show positive (brown) and the negative expression of caspase$_3$ (blue) in every 5 fields of observation. Table 1 indicates that the mean of caspase$_3$ positive expression in penis from group C was highest and followed by group D group A, and group B as the lowest.

Post Hoc Tukey HSD statistical analysis showed that the mean of positive expression of caspase$_3$ in group A was significantly lower compared to group C ($p < 0.001$), likewise when compared to group D. On the contrary, when compared to group B, the mean of positive expression of caspase$_3$ in group A was not significantly different ($p = 0.826$). In group C, the positive expression of caspase$_3$was higher compared to group D. However, this difference was statistically not significant ($p = 0.953$).

These results suggest that *buceng* treatment at the dose of 50 mg perday for 30 consecutive days in castrated Sprague Dawley male rats could decrease caspase$_3$ expression in penis cell. This effect was stronger compared to negative control and mesterolone group, but somehow equivalent to positive control or normal group.

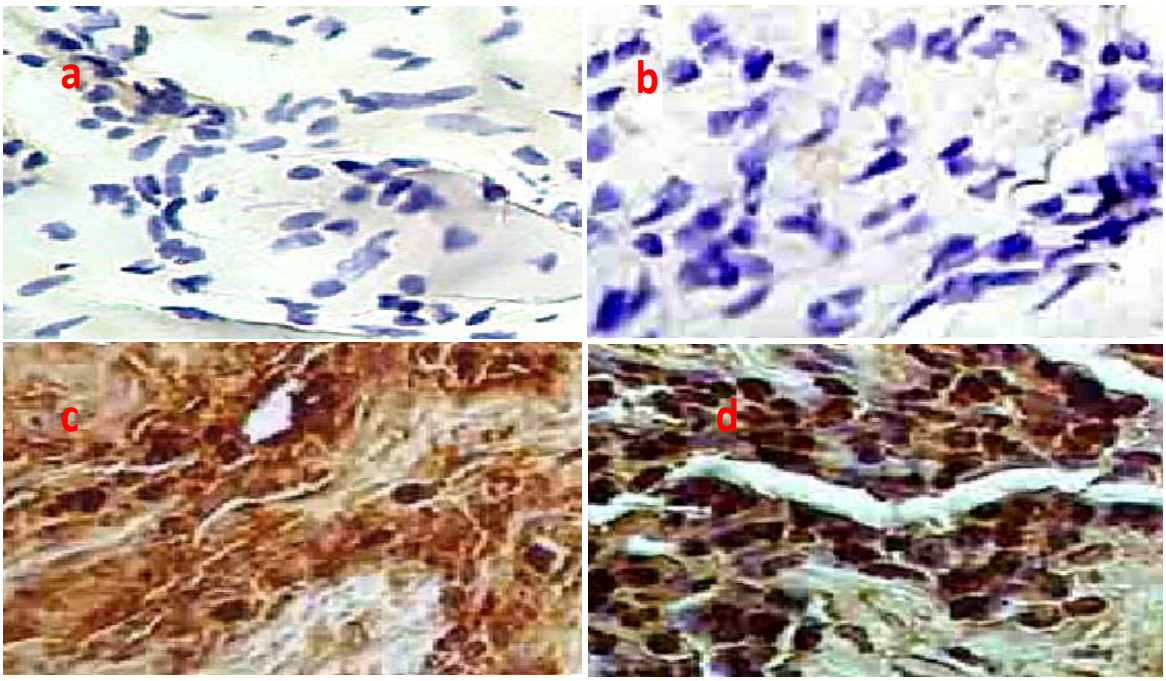

Figure 1. Penis caspase$_3$ expression in the castrated rats treated with buceng 50 mg (Group A), positive control non-castrated rats (Group B), negative control castrated rats (Group C), and castrated rats treated with mesterolone (Group D)

Difference expression of prostate caspase$_3$

Light microscopic observation with 400 HF on the immune histochemistry staining of prostate tissues preparation after 30 consecutive days of treatment with *buceng* 50 mg was shown in figure 2.

All data from this observation were calculated for the number of nucleus cells that indicate positive expression of caspase$_3$ (brown) and negative expression of caspase$_3$ (blue) from 5 fields observation each. Table 1 indicates that the mean of caspase$_3$ positive expression was highest in group C, followed by group D, A, and B.

Post Hoc analysis showed that caspase$_3$ expression in group A was significantly lower compared to group C ($p = 0.002$), likewise when compared to group D ($p = 0.011$). On the other hand, when compared to group B, the expression of caspase$_3$ in group A was slightly but not significantly, higher ($p = 0.601$). Caspase$_3$ expression in group C was also slightly higher than group D, however the difference was not statistically significant ($p = 0.924$) (Table 2).

DISCUSSION

The result of the present study indicated that positive expression of caspase$_3$ in *buceng* group was not significantly higher compared to positive control group. On contrary the expression of caspase$_3$ in *buceng* group was significantly lower compared to negative control and mesterolone group. The previous study of *buceng* in castrated and noncastrated male Sprague Dawley rats also indicated that *buceng* treatment could increase Te level and decrease apoptotic bodies in penis and prostate cells.[1] Considering caspase$_3$ is an apoptosis-executing enzyme, thus decreasing caspase$_3$ expression will consequently induce apoptosis. Taken together, the results of both the present and the previous studies indicated that *buceng* treatment possibly could inhibit apoptosis through the decrease in expression of caspase$_3$. However, the effect of *buceng* was slightly lower than positive control group or endogenous androgen. On the other hand, the potential effect of *buceng* on apoptosis was higher than that of castrated rats (low testosterone level), and mesterolone group.

Apoptosis is a process of programmed cell death caused by deprivation of growth factor or hormone, including testosterone.[7,8] Thus, when testosterone concentration is declining, the apoptosis process will be induced to start, followed by commitment of cells to death, and execution phase later on. Cytochrome C which is released from mitochondria regulates the commitment of cells to undergo suicide, whereas caspase$_3$ controls the execution phase of apoptosis.[4,9] *Buceng* is one of the herbs that has been traditionally utilized to enhance vitality and sexual potency.[12] The previous study has proven that *buceng* treatments was able to increase the testosterone and luteinizing hormon (LH) level, and spermatogenesis in male Sprague Dawley rats.[13,14] Furthermore, a number of evidence also indicated that *buceng* treatment could inhibit apoptosis in penis and prostate cells of rats marked by decrease

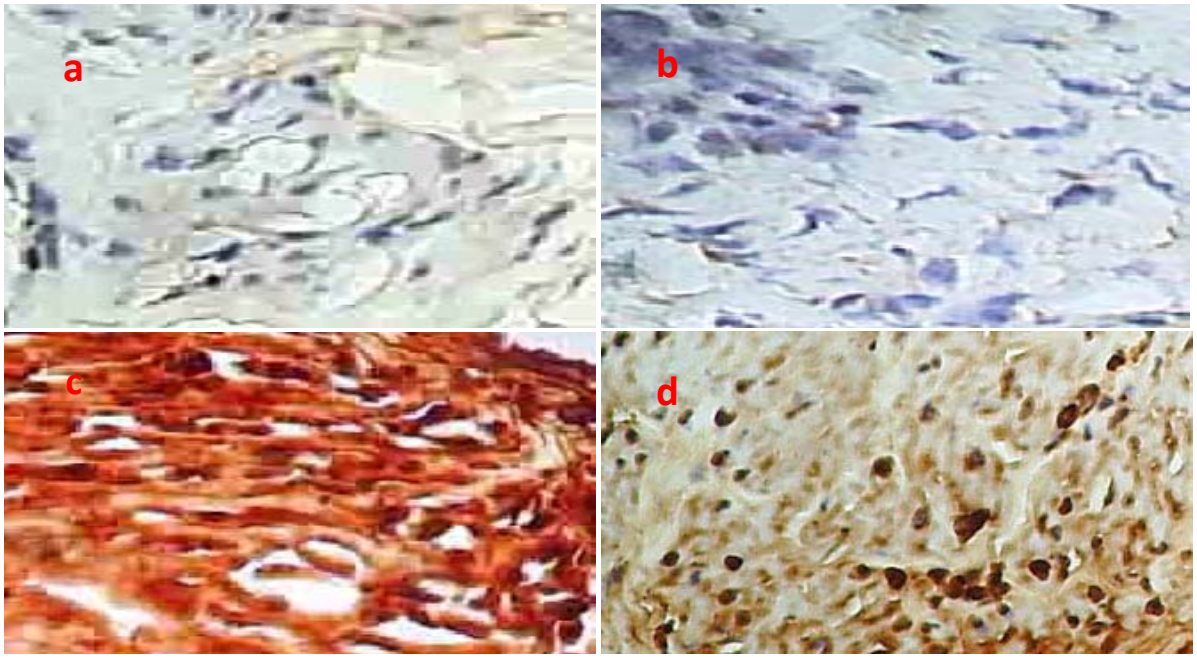

Figure 2. Prostate caspase$_3$ expression in the castrated rats treated with buceng 50 mg (Group A), positive control non-castrated rats (Group B), negative control castrated rats (Group C), and castrated rats treated with mesterolone (Group D)

in number of apoptosis bodies.[1] Various prior studies also indicated that testosterone could inhibit apoptosis in tubulus seminiferus and prostate cells through the suppression of tumor necrosing factor (TNF) and Fas mRNA. In addition, testosterone have been shown to affect the synthesis of Fas antigen, p53, Bax, and Bcl_2 expressed on androgen receptors.[3] With reference to the aforementioned testosterone and *buceng* effect, it can be predicted to inhibit apoptosis in penis and prostate cells by supression of $caspase_3$ expression. The results of the present study showed that treatment of *buceng* containing stigmasterol has proven to decrease $caspase_3$ expression and in-turn to decline the apoptosis process.

The expression of $caspase_3$ in *buceng* and positive control group were initially expected to be equal. However, this study showed that the expression of $caspase_3$ in *buceng* group was significantly lower compared to positive control group. The significant difference in $caspase_3$ expression is likely due to the inoptimal dose of *buceng,* and slow absorption rate of phytosterol contained in *buceng*.[15] So, the concentration of sterol in blood remain low leading to the low sterol in pheripheral tissues. It seems that in order to enhance the sterol conversion to testosterone, the dose of *buceng* treatment must be increased and extended. It is possible to do since high dose sterol, will not induce toxicity.[15] In addition, the endogenous testosterone is possibly more potent compared to natural testosterone (phyto-testoaterone). Further study is required to prove it.

Androgen, especially testosterone has several influences in various cells which have Te receptor including acessory glands, heart, bone, brain, other than penis and prostate. Consequently, people who have low level of Te will undergo these aforementioned organs degeneration and decline in physiological function, leading to appearance of symptomps and signs and these called andropause. Mostly men who undergo andropause take Te as hormone replacement therapy.[16] Mesterolone is one of the testosterone synthetic that have been used as a hormone replacement therapy in andropaused man. Mesterolone is favorably taken orally and immediately absorbed without being metabolized or inactivated by liver, thus the serum concentration of Te increase in line with the doses of treatment.[17,18] In the present study, the maximal dose that can be administered to the rats was 6.75 mg, higher dose could induce intoxication of rats indicated by weakness and sluggish. Subsequently, increasing the dose is impossible. The effects of intoxication are reversible after three days of treatment discontinuation. In addition, since mesterolone is the derivative of testosterone with alkylation of A, B, or C rings from steroid frame of cyclopentanoperhydrofenantren, this makes Te will be protected from liver metabolism.

The impresive results of the present study is that the $caspase_3$ expression was found to be significantly lower in group of *buceng* treated compared to that of mesterolone group. This result possibly related to high level of Te due to *buceng* treatment. Evidence from the previous study indicated that Te concentration after dosing of *buceng* was significantly higher compared to mesterolone treatment. The difference was seem to be caused by aglicon steroid conversion contained in *buceng* to Te mediated by enzymes of 17 α hydroksilase, 3 β hydroksisteroid dehydrogenase, and 17 β hydroksisteroid dehydrogenase in pheripheral tissues.[20,21] Aglicon steroid or sapogenin is stigma sterol and phytosterol which is bound with an olygosacharide or monosacharide such as glucose, silose, ramnose etc that easily dissolved in water (hydrophylic) and not toxic if taken orally. Hence, the sterol concentration rapidly increase and convert to Te.[22] This result can also be attributed to the methyl group (CH_3) in mesterolone, a derivative of Te undergoing alkylation at A, B, or C ring from the steroid nuclear.[17-19] This alkylation will inhibit apoptosis which mediated by covalent bound between two or more DNA bases, consequently will induce methyl-guanin and benzopyren bound formation, followed by formation of benzopyren-diol-epoxide-guanine bound at DNA and provoke mutation and then proliferation. Other evidence showed that alkyl group has an important role in generating cellular mytosis postulated via p53 inactivation, as such of checkpoint control of cell cycle.[23,24] Inactivation of p53 is caused by covalent bound at DNA base formed by alkyl group, thus provoke mutation at sequence DNA specific binding domain containing serin residue and therefore, unable to bind mdm_2. This condition make mdm_2 as a protein anti p53 will be freed and activated leading to inhibition of p53 activity. Controling of p53 by mdm_2 is not at DNA level, otherwise in protein level.[25] Decrease in p53 activity is associated with the dissapearance of the function of checkpoint control potency. Subsequently, the cell cycle does not stop in G1 phase (G1 arrest) in order to perform either reparation or apoptosis, instead, continously cleave leading to the change to malignant, eventhough the expression of $caspase_3$ increase.[23,24]

Compared to protodioscin (*Tribulus terestis* L) containing saponin steroid of furostanol, an natural androgen that have been launching to the markets, *buceng* have some more benefits because the furostanol point of action is only on stimulation of LH secretion of anterior hypophysis, without affecting on FSH secretion.[26] Whereas *buceng* is able to increase both

LH and FSH secretion.[13] The increase in LH secretion causes the increase in Te secretion by Leydig cell, meanwhile the increase in FSH secretion induces the incerase in number of LH receptor on Leydig cells resulting in more optimal Te secretion.[22]

Both chemical structures of furostanol (protodioscin) and sitosterol (*buceng*) have similarities which laid on OH clusters bound to the third of carbon atom of cyclopentanoprehidrofenantren nucleus. The cluster of OH cause formation of glycoside bound between sitosterols (aglycon) with olygosaccharide (hexose or pentose). The formation of glycoside bound will change the physical nature of sterol from nonpolar to polar.[22] Considering the similarity in the effects and chemical structures of both furostanol and sitosterol of *buceng*, it could be postulated that sitosterol of *buceng* contained in furostanol family is potentially capable to increase the DHEA concentration.

The present study indicated that *Buceng* treatment (phytoandrogen) is able to decrease the caspase$_3$ expression which seems likely attributed to increase in Te level. However, compared to endogenous androgen, *buceng* treatment is more inferior. In addition, *buceng* treatment is relatively safer than mesterolone since it has no alkyl group, so have no potential to provoke malignancy. This hypothesis needs further confirmary study.

The conclusion of the present study was that the treatment of *buceng* with 50 mg dose during 30 consecutive days is capable to decrease caspase$_3$ expression in penis and prostate.

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