

## PHYTOCHEMICAL ANALYSIS OF *Citrus maxima* AND ITS EFFECT ON MALE REPRODUCTIVE SYSTEM IN HIGH-FAT DIET INDUCED SPRAGUE DAWLEY RATS

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### Abstract

Present study was designed to evaluate phytochemical constituents, hypoglycemic effect and the juice effect of *Citrus maxima* on sperm quality. In phytochemical screening, the juices were tested for availability of reducing sugar, flavonoid, anthraquinone, tannins, terpenoid, saponin and alkaloid. In animal study, the rats were subjected to high fat diet for 6 weeks to induce obesity and hyperglycemic. The rats were then divided into 3 groups (n=6); control, HFD and CM groups. The results obtained demonstrated that *C. maxima* juice possessed reducing sugars, terpenoids, and saponins. Body weight in CM group increased significantly ( $p \leq 0.05$ ) throughout the treatment period. Sperm count was higher in the CM group as compared to HFD group, even though the differences were not significant. HFD group showed significant decrease ( $p \leq 0.05$ ) in progressive motility (PM) sperms, highest percent in non-progressive motility (NPM) and non motile (NM) sperms compared to control group. HFD and CM groups showed significant decreased in normal sperm compared to control group, where CM group showed better result compared to HFD group. HFD displayed significant decrease ( $p \leq 0.05$ ) in blood glucose level compared to control group. It was concluded that the adverse effects of HFD on male reproductive system can be reversed by treatment with *C. maxima* juices, where it also displayed antihyperglycemic and anti-obesity effects.

**Keywords:** *Citrus maxima*, Reproductive system, High-fat diet, Phytochemical analysis

### Introduction

High-fat diet (HFD) is a diet that contain large amount of fats, especially saturated (tropical oils or animal) fats (Segen's Medical Dictionary, 2011). It could cause an increased in body weight and will results in formation of oxidative stress that has been promoted by high body mass index and the accumulation of fat (Roushandeh et al., 2015). Oxidative stress can cause undesirable impact on reproductive system especially on fertility, as well as causes hyperinsulinemia that could lead to diabetes mellitus and alteration in hepatic lipids (Vijaimohan et al., 2006). HFD could also lead to obesity which is a multifactorial that also can gives negative effect on fertility by decreasing the sperm motility and quantity (Roushandeh et al., 2015).

*Citrus maxima*, also known as citrus fruits, have been recommended in traditional herbal medicines as a remedy for diabetes mellitus (Mangesh et al., 2014). The fruits juices of *C. maxima* are rich in pectin, which are able to reduce blood sugar and cholesterol. Study by Kundusen et al., (2011) also found that *C. maxima* possess hesperidin and naringin, a well-known hypoglycaemic agent that able to enhance erythrocyte antioxidant enzyme activities and lowers the plasma lipids in hypercholesterolemic subjects. It also has high content of

flavonoids, compound, which have potent antioxidant property (Oyedepot & Babarinde, 2013). Despite the widespread use of *C. maxima* as a folk medicine to cure metabolic disorder that include diabetes mellitus and obesity, the study on the effect of this plant on male reproductive system are still very limited. Therefore, this study was designed to investigate protective effect of *C. maxima* in the development of obesity and diabetes mellitus as well as their effect on male reproductive system in high fat diet induced rats.

## **Materials and Methods**

### **Plant Sample Collection and Extraction**

*C. maxima* fruits were obtained at local supermarket around Bandar Pusat Jengka, Pahang. The fruits were thoroughly washed with tap water, peeled and the seeds were removed. The fruits were also sliced before blended to obtained the juices.

### **Phytochemical Screening**

This test was carried out as described by (Arora & Kaur, 2013) with some modifications.

#### **Test for reducing sugars (Fehling's test)**

Aqueous ethanol extract (0.5g in 5ml water) was added to boiling Fehling's solution in a test tube. Presence of green suspension and red precipitate indicate a positive test.

#### **Test for flavonoids**

Extract was added to 5ml of dilute ammonia followed by 1ml of H<sub>2</sub>SO<sub>4</sub>. Yellow coloration that disappeared on standing indicates the presence of flavonoids.

#### **Test for saponins**

0.5g of extract was added to 5ml of distilled water in a test tube. Tube was shaken vigorously and present of persistent froth was observed. Three drops of olive oil were added into the froth and shaken firmly. Occurrence of emulsion indicate positive test.

#### **Test for tannins**

0.5g of extract was boiled with 10ml of distilled water in test tube and filtered. 0.1% aqueous iron chloride (FeCl<sub>3</sub>) was added to the filtrate and appearance of brownish green or blue black coloration showed a positive test.

#### **Test for terpenoids (Salkowski test)**

2ml chloroform was added in 0.5g of extract followed by careful addition of 3ml concentrated H<sub>2</sub>SO<sub>4</sub>. A layer of reddish brown coloration formed at interface layer indicates terpenoids presence.

#### **Test for anthraquinones**

0.5g of extract was heated with 10ml of H<sub>2</sub>SO<sub>4</sub> and filtered while hot. Filtrate was shaken with 5ml of chloroform. Layer of chloroform was pipetted into another tube and added with 1ml of diluted ammonia. Formation of pink/violet or red coloration indicates presence of anthraquinones.

#### **Test for alkaloids**

0.5g of extract was mixed with 10ml of 1% HCl, boiled and filtered. 2ml of dilute ammonia was added to 5ml of the filtrate. 5ml of chloroform was added to filtrate and shaken gently. Chloroform layer was extracted with 10ml of acetic acid. This solution was then separated

into two portions. One was added with Mayer reagent and another portion was mix with Draggendorff's reagent. Mayer reagent showed appearance of cream coloration, while Draggendorff's reagent showed presence of brown precipitate to indicate positive tests for alkaloids.

### Experimental design

The experiment was performed on 18 healthy adult male Sprague Dawley rats weighting between 150-200 g. The rats were fed with standard laboratory diets, given water *ad libitum* and maintained under laboratory conditions of temperature 22°C ( $\pm 3^\circ\text{C}$ ), with 12 h light and 12 h dark cycle. The selected rats were divided randomly into three groups. Each group consisted of six rats. The rats were grouped as listed in Table 1.

**Table 1** Groups of rat in the study

Groups	Treatment
Control	Normal control rats untreated (positive control)
High fat diet (HFD)	HFD control rats untreated (negative control)
<i>C. maxima</i> (CM)	HFD rats treated with <i>C. maxima</i> fruit juice (10 ml/kg).

### Induction of Obesity and Diabetes on Experimental Rats

Prior to the experiment, all rats were acclimatised for a week. CM group (HFD rats treated with *C. maxima* fruit juice) and HFD group (HFD rats untreated) were induced to obesity and diabetes for six weeks with HFD using sugar (30%) and white bread (50%). After six weeks, blood glucose level was measured using glucometer Accu-check Active, according to glucose oxidase method, by collecting the blood samples from rats tail.

### Body and Relative Organ Weight (ROW) Measurements

Initial and final body weights of the animals were recorded. At the end of treatment period (Day 31), all selected organs (lung, heart, liver, kidney, testes, and epididymis) were dissected out, freed from adherent tissues, weighed and recorded. ROW was calculated using the following formula:

$$\text{Relative organ weight (ROW)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on day of sacrifice (g)}} \times 100$$

### Sperm evaluations

Sperms from the cauda epididymis were extracted by cutting the organs and put into 2 ml of phosphate buffered saline (PBS). After 5 minutes incubation at 37 °C, the sperm morphology, motility, mortality and sperm count were obtained using the standard haemocytometric procedure, following the guidelines provided by World Health Organization (2010).

### Sperm morphology

A smear of semen were prepared on a slide and air dried, fixed and stained with eosin-negrosin. The slide were mounted with a coverslip and the slide brightfield optics at 1000 X magnification were examined. Approximately 200 spermatozoa per replicate were assessed for the percentage of normal forms or of normal and abnormal forms.

### Sperm motility

Sperm motility were determined immediately after liquefaction of the sample to minimize the

effects of dehydration of a sperm on motility. For each replicate, wet preparation were prepared, where the slide was examined with phase-contrast optics at 400 X magnification. Approximately 200 spermatozoa were assessed per replicate in different motile categories and graded as progressive motility (PM), non-progressive motility (NPM) or non-motile (NM).

### Sperm count

Undiluted sperm suspension was placed on both sides of haemocytometer chamber. The samples were assessed within 10 minutes and counted for at least 200 spermatozoa per replicate under 400 X magnifications (World Health Organization, 2010). The concentration of spermatozoa per ml were calculated using this formula:

$$\begin{aligned} \text{mil/ml} &= (\text{Number of sperm (N)} \times \text{dilution factor}) / (\text{no. of square} \times \\ &\quad \text{depth}) \times 1000 \\ &= (N \times 20) / (4 \times 0.1) \times 1000 \\ &= N \times 50,000 \end{aligned}$$

### Statistical Analysis

All data were expressed as mean  $\pm$  standard error of mean (SEM) for each groups (six rats per group). Differences for both treated and control rats were analysed using One-way Analysis of Variance (ANOVA). A  $p$  value of 0.05 or less ( $p \leq 0.05$ ) was considered to be significant.

## Result and Discussion

### Phytochemical Screening

The results (Table 2) revealed the presence of reducing sugars, terpenoids and saponins. Flavonoids, anthraquinones, tannins and alkaloids were found to be absent.

**Table 2** Phytochemical screening of *C. maxima* juices

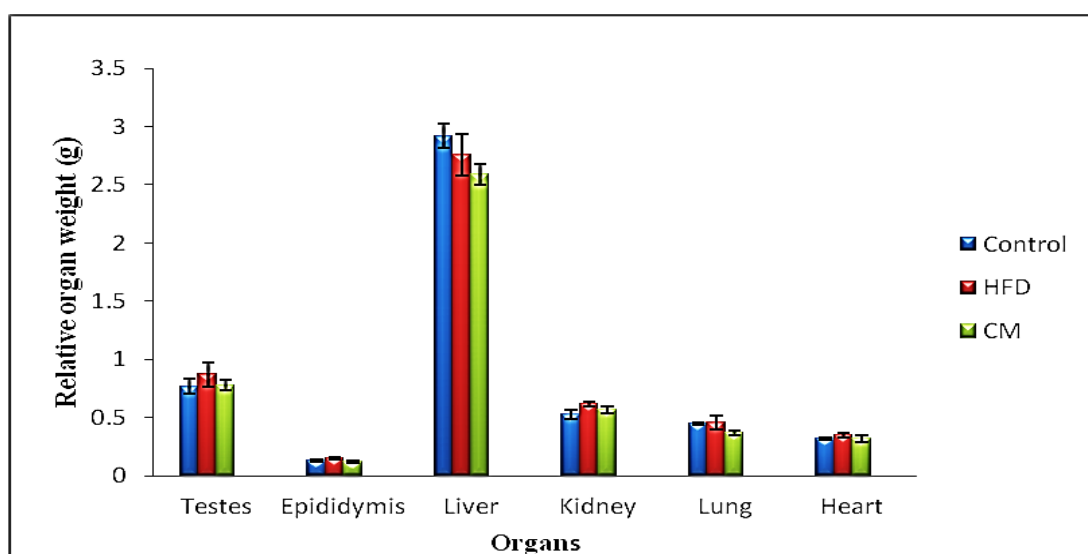
Phytochemical constituents	Detection in <i>C. maxima</i> juices
Reducing sugars	+
Flavonoids	-
Anthraquinones	-
Terpenoids	+
Saponins	+
Tannins	-
Alkaloids	-

<sup>+</sup> Indicates presence of compounds, while <sup>-</sup> indicates absence of compounds

Presence of these phytochemical compounds may have effects to blood glucose and body weight of the rats. According to Kumar, (2011) *C. maxima* contain high quantity of saponin that possesses haemolytic activity and cholesterol binding properties, where it might be responsible to cause body weight reduction in CM group. Besides that, study by Lv et al., (2015) also supported that citrus fruit and their peel have an ability to act as anti-obesity and anti-hyperglycemic towards HFD mice. Components that were believed to exert such effect were limonoid, nomilin, polymethoxyflavones and coumarin derivatives.

### Body and Relative Organ Weight (ROW)

All relative organ weight of rats that includes testes, epididymis, liver, kidney, lung and heart were not significantly affected between all group (Figure 1). However, certain trend can be observed in HFD group rats, where all of organs, with an exception liver, were increased in weight as compared to control groups. Increased in these organs may be attributed by the increase of lipid deposition in adipose tissue around the organ (Fernandez et al., 2011). The slight decrease on weight of liver in both treated groups may be attributed by the effect of HFD to liver cell. The hepatocellular necrosis might occur due to the increasing degree of oxidative damage that induce increasing degree of hepatocyte swellings and apoptosis, leading to decrease of liver weight (Bais et al., 2014). Macroscopic examinations did not show any changes in organs colour of treated animals as compared with the control.

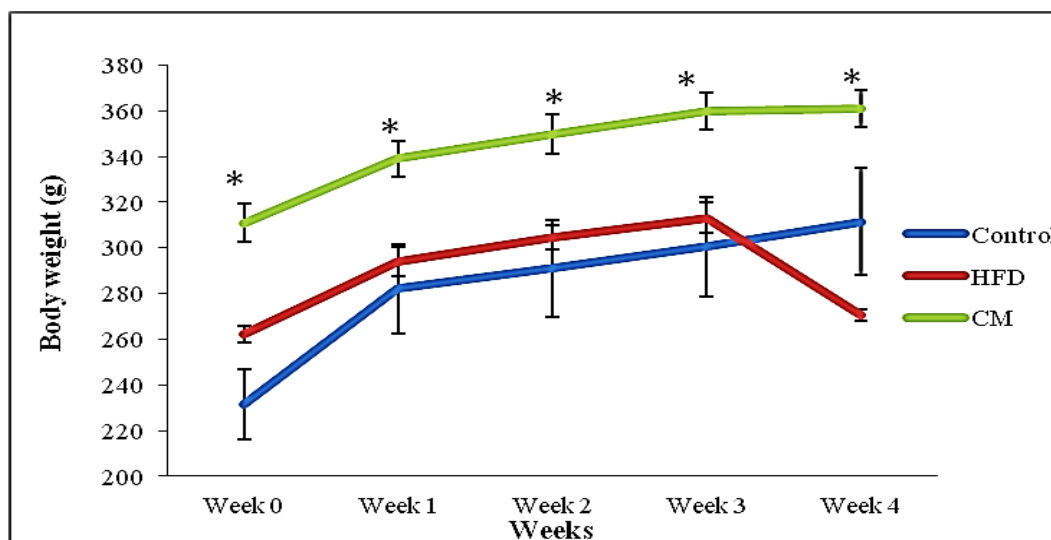


**Figure 1** Relative organ weight after treatment with *C. maxima*, HFD group and control group on Sprague Dawley rats.

Each value represents the mean  $\pm$  SEM (n=6)

All values were standardized to 100 g of body weight.

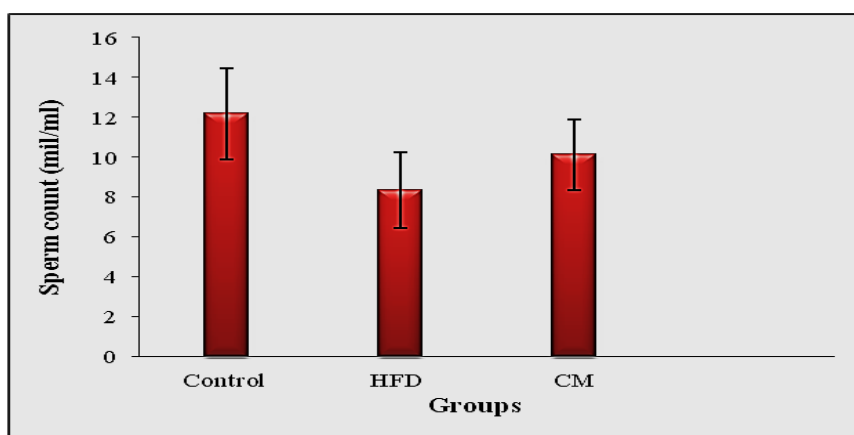
After pre-treatment to obesity (approximately 1 month), rats in CM group showed a significant increase of body weight compared to control and HFD groups. Among all the groups only HFD group showed a decrement of body weight during week 4. This was due to the facts that HFD rats were at risk for diabetes or having prediabetes after the induction of high fat diet (Hormone Health Network, 2013). The results for the body weight of the rats during treatment were presented in Figure 2.



**Figure 2** Body weight after treatment with *C. Maxima*, HFD group and control group on Sprague Dawley rats. Each value represents the mean  $\pm$  SEM (n=6),\* showed significant different at  $p \leq 0.05$  vs. control group

### Sperm Count, Motility and Morphology

The sperm count after treatment with *C. maxima* fruit juice were obtained where all the findings from the HFD group and CM group were comparable with those of the control group (Figure 3). Decreasing number of sperm in HFD and CM groups may be attributed by the effect of obesity that results from the HFD induction to the rats. Obesity will cause large amount of fat to accumulate in the scrotum that cause oxidative stress and production of reactive oxygen species (ROS), which will damage the sperm (Roushandeh et al., 2015). According to Fernandez et al., (2011), obesity will also increase the level of estradiol and decrease the testosterone level, which also will give negative effect to the production of sperm. Slight increment of the number of sperm in *C. maxima* treated rats showed protective effect of this fruit juice in improving the amount of sperm produced in testes.



**Figure 3.** Sperm count after treatment with *C. maxima* on Sprague Dawley rats. Each value represents the mean  $\pm$  SEM (n=6)

The results of sperm motility and morphology after treatment with *C. maxima* are displayed in Table 3. The progressive motility (PM) sperm for HFD group decreased significantly as

compared to control group by 63%. CM group shows slight decrement of 16% as compared to the control group although the value is not significant. The non-progressive motility (NPM) value for HFD and CM group shows slight increase with 31% and 27% respectively, as compared to control group, where the value is not significant. Non-motile (NM) sperm in HFD group showed the highest value (42.67%) followed by CM group with 69% and 6% increment respectively as compared to the control.

For sperm morphology, the values for normal sperms were significantly decrease for HFD and CM group compared to control group. It was observed that normal sperm count for HFD group showed lowest value with 57% decrement, followed by CM group with 53% decrement as compared to control group. Abnormal sperm count for HFD group and CM group also showed significant increase compared with control group. The highest value of abnormal sperm was observed in HFD group with 575% increment, followed by CM group with 530% increment as compared to control group. Figure 4 showed the sperm morphologies abnormalities observed during the experimental study.

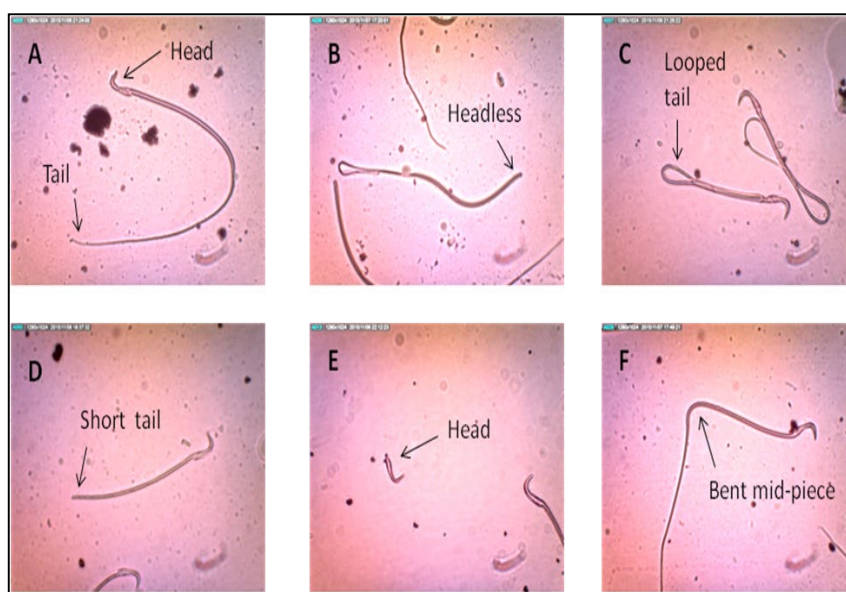
**Table 3** Sperm motility and morphology after treatment with *C. maxima* on Sprague Dawley rats

Sperm motility	Control (%)	HFD (%)	CM (%)
PM	43.08±1.47 <sup>a</sup>	16.00±5.28 <sup>b</sup>	36.00±2.98 <sup>a</sup>
NPM	31.67±1.68 <sup>a</sup>	41.33±8.76 <sup>a</sup>	40.25±3.53 <sup>a</sup>
NM	25.25±1.99 <sup>a</sup>	42.67±10.43 <sup>a</sup>	23.75±1.89 <sup>a</sup>
Normal	90.98±0.98 <sup>a</sup>	39.10±2.25 <sup>b</sup>	43.21±1.33 <sup>b</sup>
Abnormal	9.02 ±0.98 <sup>b</sup>	60.90±2.25 <sup>a</sup>	56.79±1.33 <sup>a</sup>

Each value represents the mean ± SEM (n=6)

Superscripts <sup>a, b</sup> within rows showed significant difference at  $p \leq 0.05$

The highest number of NM sperm, the lowest number of PM and normal sperm as compared to the control are shown by HFD group, followed by CM group. This indicate that treatment with *C. maxima* in CM group somehow helps to minimize the effects of obesity to the rats as compared to the untreated obesity rats in HFD group. It might be due to the component of *C. maxima* juice, which is pectin that according to [6] possess blood sugar and cholesterol lowering properties. They also stated that *C. maxima* fruit juice contains flavonoids which have antioxidant property. Antioxidant will protect sperm from ROS that cause lipid peroxidation and causes decrease in sperm quality (Roushandeh et al., 2015).



**Figure 4** Sperm abnormalities. (A) Normal sperms, (B) Sperms with headless tail, (C) Sperm with looped tail, (D) Sperm with short tail, (E) Sperm with tailless head and (F) Sperm with bent mid-piece

Previous studies proved that obesity caused the decrease in sperm parameter, as shown by HFD group, which results in poor quality of sperm, increased DNA fragmentation index and also decrease normal motility of sperm cells. There are also reports that obese mice have lower fertilization rate, sperm motility and increase in sperm intracellular ROS and DNA damage Fernandez et al., (2011).

### Fasting Blood Glucose

Analysis of blood glucose after treatment was presented in Table 4. Decrement of blood glucose for HFD group was the lowest as compared to control and CM groups with 1.42 mmol/L reduction.

**Table 4** Fasting blood glucose after treatment with *C. maxima* on Sprague Dawley rats

	Fasting blood glucose (mmol/L)		
	Before	After	Changes
Control	4.93	4.78	- 0.15 ± 0.16 <sup>b</sup>
HFD	5.10	3.68	- 1.42 ± 0.10 <sup>a</sup>
CM	5.08	4.70	- 0.38 ± 0.18 <sup>b</sup>

Each value represents the mean ± SEM (n=6)

Superscripts <sup>a,b</sup> within rows showed significant difference at  $p \leq 0.05$

Blood glucose for all the groups were within normal range when the treatment were started. After the high fat diet was introduced, approximately for 1 month, the blood glucose for HFD group fall to the range that indicates the insulin abnormalities production which cause hypoglycemia (3.68 mmol/L). Reactive hypoglycemia is one type of non-diabetic hypoglycemia and the possible causes of this condition might be due to the high amount of insulin being produced by pancreas. Secretion of insulin continue even after the glucose from meal has been digested, resulting in drops of of blood glucose level. Reactive hypoglycemic are more common in overweight or obese individual (Global Diabetes



Community ,2015). The other causes of reactive hypoglycemic according to Hormone Health Network, (2013) is being at risk for diabetes or having prediabetes. This situation can lead to failure to produce the correct amount of insulin. One of the symptoms as stated in Endocrineweb, (2015) is losing weight and this symptom was shown by the results of the decrement of body weight at week 4 of rats in HFD group (Figure 2).

### **Conclusion**

The results had demonstrated that HFD causes changes in certain parameters tested and *C. maxima* do have the antioxidant properties to counterattack the effect of HFD, even though the result were not significant. It is recommended to increase the dosage of *C. maxima* fruit juice and prolong the study for more than 1 month for future studies. This finding therefore establishes the scientific evidence for the usage of *C. maxima* on reducing blood glucose level and as an alternative on body weight management.

### **Conflict of interest**

The author(s) declare(s) that there is no conflict of interest.

### **Acknowledgement**

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