# Antioxidant-rich *Clitoria ternatea* L. flower and its benefits in improving murine reproductive performance

Shu En GOH[1], Phek Jin KWONG[1,2,3] , Chong Lee NG[1], Wen Jie NG[1,3] , Kah Yaw EE[1,2]* 

## Abstract

*C. ternatea* is often used as a natural food colouring besides improving the aesthetic value of foods. It contains various antioxidants and bioactive compounds, which exert many functions, such as counteracting oxidative stress, anti-proliferative and anti-inflammatory activities. This study evaluated the antioxidant activity of *C. ternatea* flower extract and its protective effect on bisphenol A (BPA)-induced oxidative injury in female murine reproductive system. DPPH free radical scavenging activity ($EC_{50}$ 12.47 ± 2.96 mg/mL) and total phenolic content (4.59 ± 0.09 mg GAE/g) of *C. ternatea* flower extract were determined. Then, Institute Cancer Research (ICR) dams were administered with BPA and/or *C. ternatea* extract for six weeks to study on uterine histomorphology, percentage of pregnancy and offspring development. It was found that the uterus weight to body weight ratio from dam co-administered with BPA and *C. ternatea* was lower than dam treated with BPA alone, although no histopathological effect was found. However, the percentage of pregnancy and litter size from *C. ternatea*-fed group were both higher compared to BPA-fed dam. In conclusion, *C. ternatea* extract showed high antioxidant activity and exert potential protective effects against BPA on the reproduction performance in terms of improving the percentage of pregnancy and litter size.

**Keywords:** *C. ternatea*; edible flower; antioxidant; endocrine disrupting agent; bisphenol-A.

**Practical Application:** *In vivo* assessment on reproductive performance effect of oral administration of *C. ternatea* flower extract.

## 1 Introduction

Traditionally, flowers are often used as decorations or gifts because of their colourful appearance. However, some flowers such as chrysanthemum, daylily, lilac, mint, rose and violet can also be consumed and they are known as edible flowers. Even in ancient Chinese, Roman, Middle Eastern and Indian cultures, the edible flowers have been popularly consumed among the populations. Besides improving the aesthetic value and flavour of food, these flowers are known to contain many different nutrients and beneficial functional properties such as antioxidant and antimicrobial activities.

One of the well-known edible flowers in Asia is *C. ternatea* Linn. flower, commonly known as butterfly pea. It belongs to the Fabaceae family and its flower has been commonly used as a natural food colouring for its vivid deep blue colour in cooking. Furthermore, qualitative analysis of *C. ternatea* has found the presence of many bioactive compounds such as alkaloids, tannins, glycosides, resins, steroids, saponins, flavonoids and phenols (Manjula et al., 2013). The flower petals also contain a wide variety of polyphenols, the main polyphenol constituents found are anthocyanins (Pasukamonset et al., 2016). Study has also suggested that its flower possesses many health beneficial properties, such as tranquilizing effect, anti-inflammatory and antipyretic activities (Mukherjee et al., 2008). The water extract of butterfly pea flowers was reported to have anti-proliferative properties which inhibiting cancer cell lines (Neda et al., 2013). Furthermore, a recent study showed that butterfly pea flower

extracts helped in regulating biochemical indices of diabetes mellitus (Zingare et al., 2013).

In addition, the flowers of *C. ternatea* have diverse natural antioxidants that could counteract oxidative radicals (Youwei et al., 2008). As oxidative radicals are harmful to the body due to their reactivities toward biomolecules and associations with many diseases, such as intrauterine growth restriction, preeclampsia, endometriosis and polycystic ovary syndrome, *C. ternatea* flower, which is rich in a variety of natural antioxidants and bioactive compounds might thus provide protection against the oxidative damage and those reproductive diseases. Therefore, this study aimed to investigate the antioxidant activity of *C. ternatea* flower. The flower extract was then screened for its potential in ameliorating bisphenol-A (BPA)-induced adverse reproductive effects. Various reports have showed that BPA, which is an endocrine disrupting compound, could induce oxidative stress in the reproductive system (Fernández et al., 2010; Kim et al., 2001).

## 2 Materials and methods

### 2.1 Materials

Flowers of *C. ternatea* Linn. were collected at Air Kuning, Perak, Malaysia. All reagents and chemicals were of analytical grade and purchased from Sigma Aldrich, USA; SIME Scientific,

Germany; Merck, Germany; Nacalai Tesque, Japan; HiMedia, India; and Bendosen, Malaysia, unless stated otherwise.

## 2.2 Aqueous extraction

Fresh flowers of *C. ternatea* were cleaned and then freeze dried using a ScanVac CoolSafe freeze dryer (LabogeneTM, Denmark). The freeze-dried flowers were ground into powder using an A11 basic analytical mill (IKA, Netherlands). Extraction of butterfly pea flower was carried out by stirring 5 g of freeze-dried material with 500 mL of distilled water at room temperature (25 °C) for 2 h. The crude extract was then centrifuged at 2000 rpm (1174 × g) for 10 min using a refrigerated Digtor 21 R tabletop centrifuge (Orto Alresa, Spain). The supernatant was collected and freeze dried. After drying, the freeze-dried sample was stored at -20 °C prior to antioxidant analysis and animal testing.

## 2.3 Determination of total phenolic content

Total phenolic content (TPC) in the crude extract was ascertained using the Folin-Ciocalteu method with modification (Saeed et al., 2012). Briefly, 250 μL of sample (1-2 mg/mL of dry extract in 80% (v/v) aqueous methanol) was mixed with 500 μL of 10% (v/v) Folin-Ciocalteu's phenol reagent and 2.5 mL of distilled water. The mixture was well-mixed and left in the dark at room temperature for 5-8 min. Then, 5 mL of 7% (w/v) $Na_2CO_3$ solution and 4.25 mL of distilled water were pipetted into the mixture. After that, the mixture was kept in dark condition and left for 2 h. The absorbance of the mixture was read at 750 nm. A standard curve was prepared using gallic acid solution (20-100 μg/mL in 80% (v/v) aqueous ethanol). TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter.

## 2.4 DPPH free radical scavenging assay

DPPH free radical scavenging assay was carried out according to a procedure described by Ee et al. (2019) with slight modification. DPPH radical solution was prepared freshly by mixing 10 mg of DPPH in 25 mL of 80% (v/v) aqueous methanol. An aliquot of 100 μL extract in various concentrations (0.1-100.0 mg/mL of dry extract in 80% (v/v) aqueous methanol) was mixed with 250 μL of DPPH free radical solution and 2 mL of 80% (v/v) aqueous methanol. The resulting solution was then agitated and left in the dark at room temperature for 20 min. After that, the absorbance (A) of the solution was measured at 515 nm. The control was assayed by replacing the extract with distilled water. The following Equation 1 was employed to calculate the percentage of inhibition for each extract:

$$\text{DPPH free radical scavenging activity} \left( \% \right) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (1)$$

## 2.5 Animal husbandry and treatments

In this study, Institute Cancer Research (ICR) strain mice (*Mus musculus*) were used. All the mice handling procedure and experimental design for the animal testing have been conducted as per the guideline set by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals) which were approved by the Scientific and Ethical Review Committee, Universiti Tunku Abdul Rahman. Female mice (n=32) at the age of 4 weeks old (20-25 g) and male mice (n=20) at the age of 8 weeks old (35-40 g) were housed in separated cages under the controlled environment of 20 ± 2 °C, relative humidity of 50 ± 15%, and 12 h:12 h light-dark cycle. Clean water and commercial mouse pellet feed (Gold Coin, Singapore) (500 g/cage/week) were provided *ad libitum* (5 g per mouse/ day) to all mice.

Female ICR mice were randomly assigned into four treatment groups (eight mice each). Treatment 1: vehicle control (VC) group, each mouse was administrated with 0.15 mL/kg body weight (BW) of olive oil, as this oil is used as a vehicle for BPA. Treatment 2: BPA group, each mouse was administrated with 5 mg/kg BW of BPA. The dosage of BPA used in this study was implemented from the research testing on the toxicity of BPA towards murine model by Tyl et al. (2002). Treatment 3: *C. ternatea* extract (CTE) group, each mouse was administrated with 100 mg/kg BW of CTE, this dosage was adopted from Iamsaard et al. (2014). Treatment 4: CTE + BPA group, each mouse was administrated with 100 mg/kg BW of CTE and followed by 5 mg/kg BW of BPA. All treatments were conducted daily for six consecutive weeks via intragastric route of administration (Ng et al., 2021).

## 2.6 Evaluation of the percentage of pregnancy and litter size

Five of the treated female mice from each treatment group were selected to mate with fertile male mice in a ratio of 1:1. The pairs were kept together in the same cage for five days and the numbers of pregnant female mice were recorded. The percentage of pregnancy (%) was calculated using the Equation 2 below:

$$\text{Percentage of pregnancy} \left( \% \right) = \frac{\text{Number of pregnant mice}}{\text{Total number of treated female mice}} \times 100\% \quad (2)$$

After the gestation period of 19-21 days, the total number of offspring (litter size) delivered were recorded. The pups were allowed to suckle for 28 days before recording the gender, anogenital distance and weight. The sex ratio for each treatment was calculated using the Equation 3 below:

$$\text{Sex ratio} = \frac{\text{Number of male offspring}}{\text{Total number offspring}} \quad (3)$$

## 2.7 Histological study

The remaining three treated female mice from each treatment group were euthanised and subjected to dissection. Subsequently, the entire uterus was biopsied and weighted for the analysis of toxicity (Nirogi et al. 2014). The uterus to body weight ratio was calculated using the Equation 4 below:

$$\text{Uterus weight to body weight ratio} = \frac{\text{Weight of uterus}}{\text{Body weight}} \quad (4)$$

The uterus was fixed with formalin and dehydrated in an increasing concentration of ethanol, then cleared in xylene and finally infiltrated and embedded with paraffin wax. Then, 5 μm

thickness of a section (Figure 1) was subjected to haematoxylin and eosin stain prior to microscopic observation of any morphologic abnormalities (Woo et al., 2018).

Leica ICC50 microscope and Leica Application Suite software (version 3.3.0) were used to analyse the images of stained tissues. Photomicrographs of the tissues were taken under magnification of 100×.

## 2.8 Statistical analysis

For total phenolic content and DPPH free radical scavenging assays, data were determined using linear regression or logarithmic regression in Microsoft Excel 2010. All the data obtained from dams before and after the treatment regimens were analysed using one-way analysis of variance (ANOVA) via SPSS statistical software version 18. Means comparison was also accomplished using Duncan's multiple range. The $p < 0.05$ was reported as significantly difference.

## 3 Results and discussion

### 3.1 Antioxidant activity of C. ternatea flower extract

In this study, CTE showed an $EC_{50}$ of $12.47 \pm 2.96$ mg/mL for DPPH scavenging activity, whereas the TPC in CTE was equivalent to $4.59 \pm 0.09$ mg GAE/ g extract. However, results were not in agreement with the DPPH scavenging activity of aqueous extract from C. ternatea flower reported in a previous study (Kamkaen & Wilkinson, 2009), where they reported that the aqueous extract of C. ternatea flower was showing lower $EC_{50}$ for DPPH scavenging activity (1 mg/mL) and lower total phenolic content (1.9 mg GAE/g extract). This could be possibly due to the geographical difference and other environmental factors where the C. ternatea was collected, as well as extraction methods and durations being applied, meanwhile the same study had also reported lower antioxidant properties in the ethanol extracts than other studies (Jadhav et al., 2013; Madhu, 2013). Despite dissimilarity of antioxidant value, many studies reported the potential protective effects of C. ternatea flower extracts against oxidative damage activities (Phrueksanan et al., 2014; Chayaratanasin et al., 2015; Chusak et al., 2018).

### 3.2 Effect of C. ternatea flower extract and BPA intake on the weights of maternal mice and their uterus, and histopathological evaluation

Generally, the mice from all the treatment groups showed increment in weight after treatment compared to their respective weight before treatment (Table 1). However, the average weight gain of dams from T2 ($6.00 \pm 1.73$ g) was significantly ($p < 0.05$) higher when comparing to dams from T1 ($3.14 \pm 2.19$ g). Result showed that the low dosage (5 mg/kg BW) of BPA feeding influenced the weight gain significantly. However, no statistical difference was found ($p \geq 0.05$) when comparing the weight gain of dams from control, T3 or T4 with T1 or T2, respectively. While for the mice that being fed in T3, the weight gain ($4.14 \pm 1.95$ g) was comparable to the control group ($4.86 \pm 1.57$ g), indicating the insignificant effect of the CTE on the growth of dams.

Similar method and finding of study on BPA have been reported by Sakaue et al. (2001). The study suggested that the increase in body weight was related to the function of endocrine system. The hormones in endocrine system play a fundamental role in regulating the metabolism to ensure the intestinal digested compounds are being converted into energy. Furthermore, hormones are also responsible for storage of excess fuel and

**Table 1**. Weight difference of maternal mice (dams) and their uterus weight to body weight ratios in different dietary treatment groups.

| Treatment^ | Weight difference of dam (g)* | Uterus weight to body weight ratio* |
|---|---|---|
| Control# | $4.86 \pm 1.57^{ab}$ | ND |
| T1: Olive oil | $3.14 \pm 2.19^{a}$ | $0.00297 \pm 0.00077^{ab}$ |
| T2: BPA | $6.00 \pm 1.73^{b}$ | $0.00389 \pm 0.00094^{b}$ |
| T3: CTE | $4.14 \pm 1.95^{ab}$ | $0.00150 \pm 0.00103^{a}$ |
| T4: MIX_(CTE + BPA) | $4.86 \pm 1.46^{ab}$ | $0.00120 \pm 0.00081^{a}$ |

ND = not determined; T = treatment; BPA = bisphenol-A; CTE = C. ternatea flower extract; MIX = combination of CTE and BPA; ^Refer section 2.5 for feeding regimes; *Data are means of three determinations with standard deviations; #No additional supplement besides the normal mouse pellet; a,bData in column with the same superscripts are not significantly different while data with different superscripts are significant different (p < 0.05).
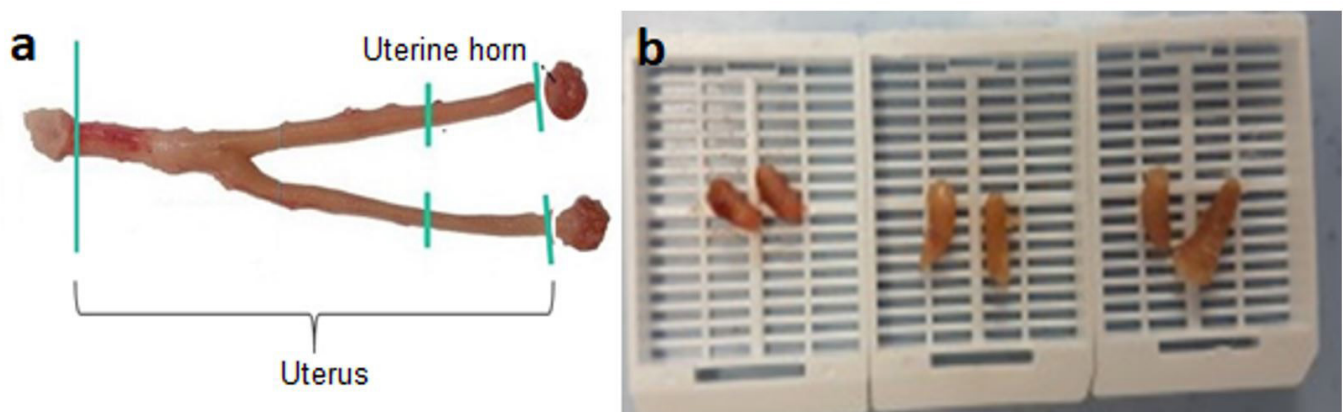


**Figure 1**. (a) Biopsied uterus; (b) placement of uterine horns on white cassette for tissue fixation.

mobilisation of fuel, and most notably for maintaining constant levels of blood glucose. However, BPA is known as one of the endocrine disrupting compounds and possesses the ability to mimic the action of endogenous oestrogen. These disruption activities will lead to an imbalance in metabolism and induction of diseases.

There are a number of genes that are likely to be involved in the control of the effect of BPA on adipocyte differentiation and the respective functions. Exposure to BPA during development or growing period could permanently alter the expression of gene in adipocytes, lipoprotein lipase activity and triacylglycerol accumulation. Also, it could even result in the presence of larger lipid droplets in the differentiated adipocytes (vom Saal et al., 2012). Interference in hormonal control of adipose tissue functions can therefore lead to inappropriate deposition of fat, higher weight gain and eventually it causes obesity.

Besides, the uterus to body weight ratio ($0.00389 \pm 0.00094$) of dam, which was fed with T2, was significantly ($p < 0.05$) higher compared to treatment group T4. Results showed that oral administration of CTE extract could prevent the BPA induced hyperplasia of uterus. However, no obvious histopathological change was observed upon examination of uterine morphology from all treatments (Figure 2). According to Papaconstantinou et al. (2000), ingestion of BPA can cause an increase in uterine weight on a dose-dependent manner. The uterotrophic actions of estrogenic substance result in an increase in uterine wet weight, which is an endpoint utilised in the standard uterotrophic assay (Reel et al., 1996). A similar study of Chandru et al. (2018) reported that oral administration of water based CTE at dosages up to 250 mg/kg BW on female rat did not change in the body weight significantly when comparing to control group. This suggested that the CTE are safe for oral consumption and could even be used to control weight gain after exposure to BPA, as demonstrated in this study.

### 3.3 Effect of *C. ternatea* flower extract and BPA intake on the success of establishing pregnancy and offspring development

The effects of CTE and BPA toward the percentage of pregnancy and litter size were also evaluated. The percentage of pregnancy of dams that were fed with BPA in T2 was the lowest (25%) among the other treatment groups (Figure 3). Similar observation was reported by Ng et al. (2021) showing that mice administered with BPA showed reduction in percentage of pregnancy compared to the control group. Result suggested that the BPA treatment on female mice could lead to lower fertility, indicating the adverse effects of BPA in the early processes of establishing pregnancy, such as the follicle development, ovulation or implantation of the embryos. It is noteworthy that the exposure of BPA has been associated with female fertility issues. Several studies have reported that BPA can disturb the oestrous cycle, scale down the primordial follicle pool, cause
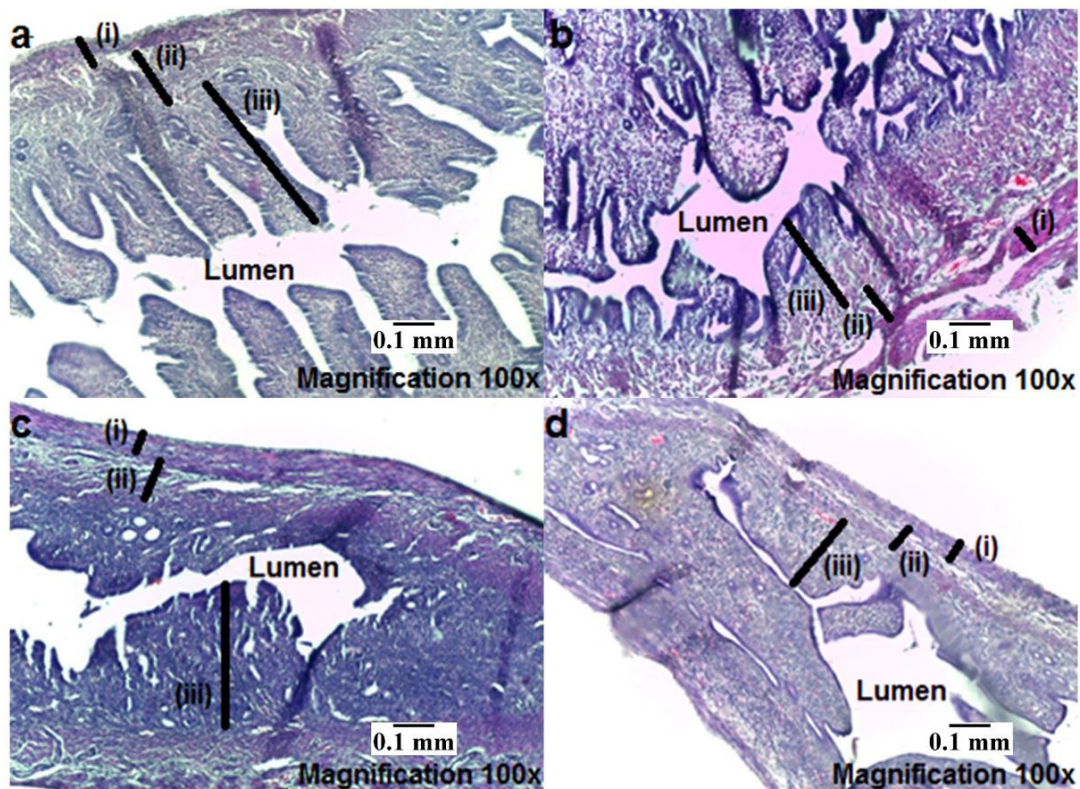


**Figure 2**. Photomicrographs of haematoxylin and eosin stained histological sections of uterus of mice in different dietary treatment groups (a) T1: Olive oil; (b) T2: BPA; (c) T3: CTE; and (d) T4: MIX$_{(CTE + BPA)}$. General histological of uterus structure (i) perimetrium; (ii) myometrium; (iii) endometrium.

premature ovary and disrupt steroidogenesis in a variety of animal models (Eichenlaub-Ritter et al., 2008; Rodríguez et al., 2010; Ziv-Gal et al., 2013). In mouse, the pool of ovarian primordial follicles is established since birth (Rodríguez et al., 2010). A study of Peretz et al. (2011) reported that exposure of BPA at 440 μM significantly decreased follicle growth, oestradiol, estrone, testosterone, androstenedione and progesterone levels throughout the 120 h of *in vitro* follicle culture system. However, all dams from the control group, T1 and T4 groups in this study were pregnant. Results suggested that oral administration of CTE extract might have ameliorated the BPA-induced adverse effects on the reproductive system of the dams. With reference to a similar study of Chandru et al. (2018), female mice treated with *C. ternatea* extract had oestradiol level higher than those present in control group. Bringing all together, the preliminary feeding tests using *C. ternatea* extract at 250 mg/kg BW have established safe and regular synthesis of sex hormones in female reproductive system.

In terms of the number of offspring (litter size), all the five different treatment groups gave birth to total of 146 pups. The only dam from the BPA group (T2), that was pregnant, gave birth to only four pups in total. Since there was only one replicate of litter size from the BPA group, the statistical analysis to compare the litter size for the treatment groups using One-Way ANOVA was conducted by excluding the BPA group. From the 146-litter size, 44 pups were from control group, 33 pups were from T1, 4 pups were from T2, 28 pups were from T3 and 37 pups were from T4. The average litter size from dams treated with T1 and T4 did not differ significantly ($p \geq 0.05$) compared to the control group which was without any treatment (Table 2). Results indicated that consumption of CTE had exerted protective effects against BPA during the pregnancy as the average litter size in the T4 group ($9.25 \pm 2.63$) was comparable to the control ($11.00 \pm 1.63$) and relatively higher than the T2 group.

In terms of the weight of offspring on 28 days old, the mean weight of male litter from dams of control, T1 and T4 groups was $17.00 \pm 2.94$ g, $22.25 \pm 3.40$ g and $27.00 \pm 2.00$ g, respectively, and T4 group was slightly higher than the mean weight of male litter from dam of T3 ($25.33 \pm 1.16$ g) group. On the other hand, T2 treated dam has three male pups with an average weight of 25 g and only one female at 18 g. Similarly, the weight of female litter from dams of T4 group ($23.50 \pm 2.65$ g) was significantly ($p < 0.05$) higher than the mean weight of female offspring from control group ($15.25 \pm 2.87$ g). There was no significant difference for litter from T1 and T3 groups ($19.00 \pm 1.83$ g and $22.67 \pm 1.53$ g, respectively). A similar study of Rubin et al. (2001) reported that the litters of BPA-treated female could have significant increase in body weight compared to those which were born in control dams. In addition, they reported that those rats exposed to low dose of BPA (1 mg/L) were heavier than those exposed to a higher dose (10 mg/L) of BPA. Future study on the physiological mechanism could clarify the effects of BPA on body weight.

The percentage of male litter from T3 group ($78.33 \pm 0.12\%$) was significantly ($p < 0.05$) higher than the percentage of male litter from other treatment groups (Figure 4). T3 group had the highest percentage in male litter, while T4 group showed the highest

percentage of female litter. Results showed that when maternal mice were supplemented with T3, they had higher chance to produce more male offspring. However, the treatment of T2 was not included into the analysis because only one out of four dams was successfully pregnant. Evaluation on the sex ratio of offspring in this study shows that, maternal feeding with T3 resulted in significantly higher percentage of male offspring compared to other treatment groups. A study of McGraw et al. (2005) reported
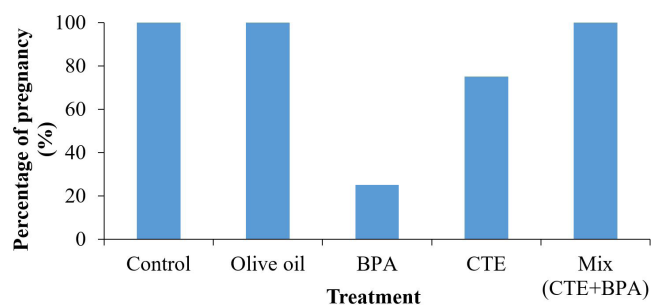


**Figure 3**. Percentage of pregnancy (%) of maternal mice in different dietary treatment groups.
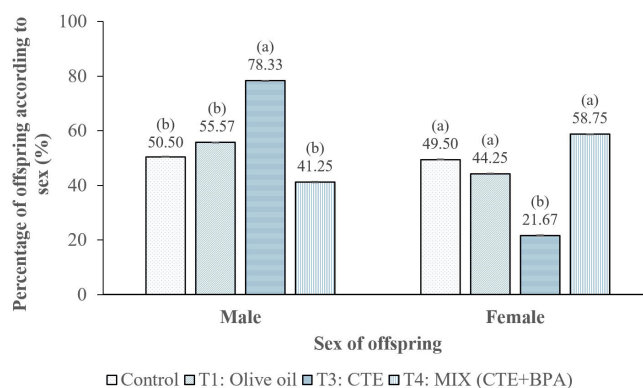


**Figure 4**. Percentage of male and female offspring derived from dams fed with different dietary treatments. [a,b]Data with different superscripts within the same sex group are significantly different ($p < 0.05$).

**Table 2**. Average number of offspring delivered and their average weights in different dietary treatment groups.

| Treatment[^] | Average number of offspring* | Average weight of offspring (g)* | |
|---|---|---|---|
| | | Male | Female |
| Control[#] | $11.00 \pm 1.63$[a] | $17.00 \pm 2.94$[a] | $15.25 \pm 2.87$[a] |
| T1: Olive oil | $8.25 \pm 1.71$[a] | $22.25 \pm 3.40$[b] | $19.00 \pm 1.83$[ab] |
| T3: CTE | $7.00 \pm 4.69$[a] | $25.33 \pm 1.16$[bc] | $22.67 \pm 1.53$[bc] |
| T4: MIX (CTE + BPA) | $9.25 \pm 2.63$[a] | $27.00 \pm 2.00$[c] | $23.50 \pm 2.65$[c] |

T = treatment; BPA = bisphenol-A; CTE = *C. ternatea* flower extract; MIX = combination of CTE and BPA; ^Refer section 2.5 for feeding regimes; *Data are means of three determinations with standard deviations; #No additional supplement besides the normal mouse pellet; a-cData in column with the same superscripts are not significantly different while data with different superscripts are significant different (p < 0.05).

that the maternal dietary with rich carotenoids, which is also known as a source of antioxidant, increased the percentage of male offspring than the female. Similar outcomes were observed in this study, where feeding the dam with CTE, which is rich in antioxidant, resulted in more male offspring. However, the mechanism behind the antioxidant effect on sex development still need to be explored in future research.

In conclusion, the finding of this study showed that oral administration of CTE does not exert negative effect on the maternal body weight. Promising protective effect of CTE against excessive enlargement of the uterus weight, that is induced by BPA, was demonstrated. In terms of fertility performance, BPA significantly reduced the fertility performance as the percentage of pregnancy and litter size were low, even though the histology of the uterus appeared to be normal. However, oral administration of CTE shows protective effect against BPA by improving the reproductive performance, including lower the uterus weight over body weight ratio, increasing percentage of pregnancy and litter size.

## Abbreviations

BPA: Bisphenol A. CTE: *C. ternatea* flower extract. GAE: Gallic acid equivalent. ICR: Institute Cancer Research. TPC: Total phenolic content.

## Ethical approval

This study was approved by the UTAR Science and Ethical Review Committee (SERC) with approval number: U/SERC/238/2019.

## Conflict of interest

The authors declare no conflict of interest.

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