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Effect of combined *Kaempferia parviflora* extract and fenitrothion treatment on male reproductive parameters in a murine modelShawn Samson Tze Xian Ong¹, Phek Jin Kwong², Mun Yee Chan³, GimCheong Tan¹✉¹Department of Allied Health Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), 31900 Kampar, Perak, Malaysia²Department of Agricultural and Food Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), 31900 Kampar, Perak, Malaysia³Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), 31900 Kampar, Perak, Malaysia

ABSTRACT

Objective: To evaluate the antioxidant activity of *Kaempferia (K.) parviflora* rhizome extract and study its protective effect against male reproductive impairment induced by fenitrothion.

Methods: The phytochemicals and antioxidant properties of *K. parviflora* ethanolic extract were assessed. Twenty-four male mice aged 4-weeks-old were assigned into four groups. Groups 1 to 4 respectively received extra virgin olive oil (served as the vehicle control), 20 mg/kg body weight (bw) of fenitrothion, 50 mg/kg bw of *K. parviflora* ethanolic extract, and 50 mg/kg bw of *K. parviflora* ethanolic extract plus 20 mg/kg bw of fenitrothion. Upon completion of 28 days of continuous feeding treatment, the mice were subjected to mating evaluation and the assessment of epididymal sperm quality, lipid peroxidation, testosterone level and histological evaluation.

Results: Eight phytochemical compounds were detected in the ethanolic extract of *K. parviflora* via GC-MS. The extract exhibited a DPPH radical scavenging activity with an EC₅₀ value of (0.870±0.007) mg/mL. Mice fed with fenitrothion in group 2 displayed an increase in relative testis weight, lipid peroxidation levels and a significant reduction in all sperm parameters (sperm concentration, progressive motility, and viability), as well as serum testosterone levels compared to the control mice in group 1 ($P<0.05$). On the other hand, mice fed with *K. parviflora* ethanolic extract in group 3 showed a significant increase in serum testosterone levels compared to group 1. Mice co-administered with *K. parviflora* ethanolic extract and fenitrothion in group 4 exhibited a significant improvement in testosterone levels, sperm concentration, motility, and viability compared to the mice administered only with fenitrothion in group 2 ($P<0.05$). Histological analysis of testicular tissue from group 4 further revealed improved testicular morphology in comparison to fenitrothion-treated mice.

Conclusions: Eight bioactive compounds were identified in *K. parviflora* ethanolic extract and the extract exhibited antioxidative properties. Fenitrothion administration impairs testicular function, reducing sperm parameters and testosterone while increasing lipid peroxidation. However, *K. parviflora* ethanolic extract co-

administration alleviates oxidative stress, improves sperm parameters and testosterone levels, and enhances testicular morphology. These findings suggest that *K. parviflora* ethanolic extract has the therapeutic potential in mitigating male reproductive adverse effects induced by fenitrothion.

KEYWORDS: *Kaempferia parviflora*; Endocrine disruptor; Fenitrothion; Reproduction; Male fertility

Key Points

Question: What effects can be observed on male reproductive parameters after combining *Kaempferia parviflora* ethanolic extract and fenitrothion treatment in a murine model?

Findings: *Kaempferia parviflora* ethanolic extract exhibited a protective effect against fenitrothion-induced damage in male mice by significantly restoring sperm concentration, sperm motility, and testosterone levels. Additionally, the combined treatment of *Kaempferia parviflora* ethanolic extract and fenitrothion also improved testicular histomorphology in the mice.

Meaning: *Kaempferia parviflora* ethanolic extract can serve as a potential alternative naturopathic treatment for improving male's reproductive health.

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1. Introduction

Fenitrothion belongs to a group of organophosphate compounds, also called as sumithion, which is commonly used in agriculture as pest control[1]. Despite fenitrothion being classified as a moderately hazardous chemical, the usage of fenitrothion has been banned in some countries due to its damaging effect towards the environment[2]. Mishandling or extensive usage of fenitrothion can potentially lead to several health implications on human as fenitrothion can be absorbed *via* inhalation, ingestion and dermal routes[3]. Studies showed that extensive exposure to fenitrothion could lead to adverse effects on liver, kidney, lung, nervous system and male reproductive system[3,4]. Fenitrothion is known as an endocrine-disrupting chemical and an oxidative stressor, leading to the significant reduction of sperm count and testicular damage, as well as the modification of epigenetics by DNA methylation and histone modification[4–6]. Studies have shown that fenitrothion residues can be found in drinking water and harvested crops from farms that use fenitrothion as pesticides even at its lowest concentration (1.30 µg/L)[2,6]. Chronic consumption of contaminated water and crops raises concerns about its potential impact on human reproductive health. In order to counteract the reactive oxygen species (ROS) that present in the body which leads to oxidative stress, naturopathy such as herbs with high antioxidant activity could ameliorate the fertility problem due to oxidative stress[7]. *Kaempferia (K.) parviflora* or *Krachaidum*, a subtropical plant found abundantly in North-eastern Thailand and South-western Laos is known to contain antioxidative properties[8]. *K. parviflora* contains more than 11 types of methoxyflavones which exhibit anti-inflammation, anti-cancer and aphrodisiac properties[7–9]. Studies found that *K. parviflora* extract improves low testosterone level in streptozotocin-induced diabetic male rats, promoting higher chances of reaching ejaculation in sexual activity[10]. Even though *K. parviflora* has shown its capability of boosting sexual performance and improving male fertility[10,11], up to date there is no reported research that examine the protective effect of *K. parviflora* extract on male infertility induced by fenitrothion. Hence, this study aims to evaluate the potential of *K. parviflora* extract in ameliorating the male reproductive impairment induced by fenitrothion in murine model.

2. Methods

2.1. Collection and extraction of *K. parviflora*

Fresh rhizomes of *K. parviflora* used in this study were authenticated using the reference to the morphology described by Labrooy *et al*[12]. The fresh rhizomes were washed, peeled, sliced and air-dried[13]. The dried slices of *K. parviflora* were then ground into powder and the *K. parviflora* powder was extracted with 95% ethanol as described by Sudwan *et al* with slight modifications[11]. The

extract was evaporated to dryness using a rotary evaporator and the final extracted components were freeze-dried before use[11,13]. The yield of *K. parviflora* extract was recorded as dry weight basis[14]. Ethanolic extraction method was chosen in this study because it could dissolve both polar and non-polar bioactive compounds with higher extraction efficiency and it is relatively safer for human use compared to non-polar solvents[7,14].

2.2. Determination of antioxidant activity of *K. parviflora* extract

Antioxidant activities of *K. parviflora* ethanolic extract was evaluated based on 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) assay as described by Les *et al* with slight modifications[15]. Triplicate measurements were performed for each concentration of *K. parviflora* ethanolic extract and the effective concentration required to inhibit 50% of DPPH free radicals (EC₅₀) was determined from the dose-response curve. Ascorbic acid was used as the positive control in this assay.

2.3. Determination of total phenolic and flavonoid contents of *K. parviflora* extract

Quantification of total phenolic content (TPC) was conducted based on Folin-Ciocalteu method described by McDonald *et al* with slight modifications[16]. Triplicate measurements were performed for each concentration of *K. parviflora* ethanolic extract and the TPC of the sample extract were determined as milligram of gallic acid equivalent per gram of *K. parviflora* extract dry weight (mg GAE/g dw).

In the meantime, the total flavonoid content of *K. parviflora* extract was determined based on aluminum chloride colorimetric protocol by Jimoh *et al* with slight modifications[17]. Triplicate measurements were performed for each concentration of *K. parviflora* extract and the total flavonoid content of the sample extracts were determined as milligram of quercetin equivalent per gram of *K. parviflora* extract dry weight (mg QE/g dw).

2.4. Gas chromatography–mass spectrometry (GC–MS)

Gas chromatography-mass spectrometry was performed to identify the compounds in the extract according to the protocol by Pitakpawasutthi *et al*[18]. BPX fuse silica column (30 mm × 0.25 mm, 0.25 µm) was used as the stationary phase. The oven temperature was raised from 60 °C to 240 °C at a constant rate of 3 °C /min. Helium was used as the carrier gas with 1 mL/min flow rate. Subsequently, one microliter of sample (one gram of *K. parviflora* ethanolic extract in 100 mL HPLC grade methanol) was injected *via* autoinjector with the split ratio of ten to one. Mass spectrometry was performed with the adjustment of electron impact positive mode at 70 eV. The eluted chemical constituents were analyzed and identified using NIST02 Mass Spectral library. The constituents were matched

with the MS library according to its mass spectra and retention time.

2.5. Mice husbandry and in vivo treatment of extract study

A total of 24 male and 36 female Institute of Cancer Research (ICR) strain mice, aged 4 weeks old [(30±5) g] were used in the *in vivo* study. They were all housed under a controlled environment [(23±3) °C], 12 hours light and 12 hours dark cycle. The mice were given access to pelleted mice chow from Gold Coin Feedmills Pte. Ltd and water *ad libitum*[19]. The mice were kept for a week in the controlled condition before the start of the experiment to allow the mice to acclimatize.

Twenty-four male mice were assigned randomly into four groups. Group 1 (the negative control group) received 5 mL/kg body weight (bw) of extra virgin olive oil twice, serving as the vehicle control[19]. Group 2 (the fenitrothion group) was administered with 20 mg/kg bw of fenitrothion, followed by 5 mL/kg bw of extra virgin olive oil[4]. Group 3 (the *K. parviflora* ethanolic extract group) received 50 mg/kg bw of *K. parviflora* ethanolic extract, followed by 5 mL/kg bw of extra virgin olive oil[20,21]. Group 4 (the test treatment group) was treated with 50 mg/kg bw of *K. parviflora* ethanolic extract, followed by 20 mg/kg bw of fenitrothion. All treatments were done *via* intragastric administration daily for 28 consecutive days.

After the 28 days treatment, three male mice from each treatment group were subjected to euthanization. The wet weight of the testis was recorded and the relative testis weight was calculated in percentage by dividing the testis wet weight with the body weight of mice[22]. The cauda epididymis was collected for sperm quality assessment, the testis was used for histomorphological studies while the blood was collected for the lipid peroxidation and testosterone level measurement.

2.6. Lipid peroxidation level measurement in blood serum

Lipid peroxidation activity was measured through the determination of serum malondialdehyde (MDA) levels using MDA ELISA kit (Elabscience, E-EL-0060). The procedures were conducted according to the manufacturer's protocol and the MDA levels for each treatment group were expressed in micromoles per liter (µmol/L).

2.7. Testosterone level measurement in blood serum

Testosterone level was measured *via* high performance liquid chromatography (HPLC) from Dikma® Technologies Inc. A HPLC grade testosterone standard with 98% purity was prepared in the concentration of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. Sample from each treatment group was prepared by taking ten microliter of blood serum mixed with 30% acetonitrile. The standard and sample were filtered before use. Diamonsil® C18 (2) column (150 mm × 4.6 mm, 5 µm) was used as the stationary phase. Mobile phase A (30 mM potassium phosphate dibasic, pH 8.0)

and mobile phase B (60 acetonitrile to 40 methanol) were prepared as the mobile phases of the chromatography. The mobile phase was set as gradient flow for 15 min (hold 40% mobile phase B for 0.6 min, change concentration of mobile phase B from 40% to 60% in 1.8 min, hold 60% mobile phase B for 2.2 min, change concentration of mobile phase B from 60% to 70% in 2.3 min, hold 70% mobile phase B for 2.8 min, change concentration of mobile phase B from 70% to 40% B in 1 min and equilibrate at 40% mobile phase B for 4 min). The column temperature was set at 30 °C while the wavelength of the spectrophotometer was set at 242 nm. All samples and standard were injected at the volume of ten microliter. A standard curve was plotted and the serum testosterone was calculated as shown below:

Serum testosterone (ng/mL) = (χ / 1 000) ÷ m × f × 1000
where

χ / 1 000 = sample peak area under curve

m = slope of standard curve

f = dilution factor of sample (f = 10)

2.8. Sperm collection and sperm quality assessment

The sperm suspension was obtained from cauda epididymis and mixed with 100 µL phosphate buffer saline (37 °C). The sperm parameters which include concentration, motility and viability were evaluated as per World Health Organization guidelines[23]. Sperm motility was graded according to the sperm motility patterns: rapid motility (grade A), slow motility (grade B), non-progressive motility (grade C) and immotile sperm (grade D)[23,24]. Sperm viability was assessed using eosin-nigrosin staining with a total of 200 spermatozoa counted per sample[24]. All sperm assessments were performed in triplicate. Sperm concentration and progressive sperm motility were determined as shown:

Sperm concentration = No. of sperm counted in a square box × 2 (dilution factor) × 10⁵ mL⁻¹

Progressive sperm motility (%) = [(grade A+grade B) ÷ 100] × 100%

2.9. Histological and morphological evaluation of testicular tissue

Testes harvested from the euthanized mice were used for histological assessment. Haematoxylin and eosin (H & E) staining was performed on sectioned testicular tissue using the ST Infinity™ H&E Staining System (Leica), following the manufacturer's guidelines. After staining, DPX mounting medium was placed onto the stained tissue and viewed at 400× magnification under Leica DM500 binocular microscope. Histological changes of the testicular tissue were recorded and evaluated using Johnsen's mean testicular biopsy score count[25].

2.10. Mating and post-parturition assessment

Three male mice from each treatment group were mated with untreated fertile 8-week-old female ICR mice at the ratio of one

male to three females. Both male and female mice were housed together for 14 days. The female mice were placed into the respective male cage daily in the evening (6 p.m.) and they were left undisturbed at night until the next morning (6 a.m.) (Kuala Lumpur time, GMT+8)[26]. The presence of vaginal plug, indicating on successful mating, was checked daily in the morning at the end of cohabitation period before separating both male and female mice[27]. Female mice with positive vaginal plug were labelled as day 0 of gestation and monitored until parturition. The mating period of mice lasted for 14 days and the mating index was determined as shown as below[28]:

$$\text{Mating index} = \frac{\text{Female mice with vaginal plug presence}}{\text{Total cohabitated female mice in a cage}} \times 100\%$$

Post-parturition analyses namely litter size, survival rate and sex skewness of the offspring were recorded and evaluated.

2.11. Statistical analysis

SAS[®] OnDemand for Academics was used to perform data analyses. Firstly, Shapiro-Wilk test was done to determine the normality distribution of the data collected. If the data were significantly normal distributed (parametric data), one-way ANOVA was performed, followed by *post hoc* comparison using Fisher's least significant difference (LSD) test[19]. If the data were not normally distributed (non-parametric data), Kruskal-Wallis H was performed, followed by Dwass, Steel, Critchlow-Fligner (DSCF) *post hoc* test. One sample *t*-test was conducted on the determination of sex ratio skewness in offsprings of the study group where the hypothetical value was set at 0.5[27]. All triplicate data collected were expressed in mean $\pm$ standard deviation (mean $\pm$ SD). The significant differences of the study were stated when the *P*-value was less than 0.05.

2.12. Ethics statement

Mice husbandry and experiment procedures were followed according to the guidelines set by National Institute of Health which were then approved by UTAR Scientific and Ethical Review Committee (Approval number: U/SERC/254/2022; Approval date: 29 November 2022).

3. Results

3.1. Antioxidant and phytochemical assessments of *K. parviflora* ethanolic extract

The ethanolic extraction of *K. parviflora* resulted in an extract yield of (5.58 $\pm$ 1.85)%. Meanwhile, DPPH radical scavenging assay of the extract has exhibited an EC₅₀ value of (0.870 $\pm$ 0.007) mg/mL. The total phenolic and flavonoid contents of the extract were (60.28 $\pm$ 2.93) mg GAE/g dw and (77.78 $\pm$ 10.18 mg) QE/g dw, respectively.

A total of 8 peaks were detected by GC-MS in the rhizome of *K. parviflora* ethanolic extract (Supplementary Figure 1). According to NIST02 Mass Spectral library, the identified compounds in the ethanolic extract of *K. parviflora* included 7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one, 5-hydroxy-3,7-dimethoxyflavone, 2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one, 5-hydroxy-7,4'-dimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, 5-hydroxy-3,7,3'4'-tetramethoxyflavone, 5,7,4'-trimethoxyflavone and 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol), as presented in Supplementary Table 1.

3.2. Effect of co-administration of *K. parviflora* extract and fenitrothion on testis index and lipid peroxidation level

The effects of *K. parviflora* ethanolic extract as well as fenitrothion on the relative testis weight of mice were evaluated. Relative testis weight of mice after fenitrothion administration appeared higher compared to the control group [(0.360 $\pm$ 0.052)% and (0.310 $\pm$ 0.024)%, respectively]. Whereas *K. parviflora* ethanolic extract treatment group showed a reduction in the relative testis weight [(0.290 $\pm$ 0.005)%] compared to the control group. Interestingly, a slight reduction of the relative testis weight to body weight of mice was shown in the co-administration of *K. parviflora* ethanolic extract and fenitrothion [(0.32 $\pm$ 0.02)%] in comparison with the fenitrothion treatment group as shown in Figure 1A. However, no significant difference was found in the relative testis weight among all treatment groups (*P*>0.05).

Additionally, similar trend was also observed in the lipid peroxidation level assessment as shown in Figure 1B. An approximately two-fold increase in MDA level was observed in the fenitrothion treatment group [(41.30 $\pm$ 3.86) μ mol/L] compared with the control group [(18.36 $\pm$ 3.95) μ mol/L] and the *K. parviflora* ethanolic extract treatment group [(23.96 $\pm$ 2.29) μ mol/L]. Meanwhile, the *K. parviflora* ethanolic extract and fenitrothion co-administered treatment group showed a slight reduction of approximately 15% in MDA levels to (35.94 $\pm$ 2.51) μ mol/L compared to the fenitrothion treatment group, though this level remained significantly higher than that of both the control and *K. parviflora* ethanolic extract treatment groups.

3.3. Effect of co-administration of *K. parviflora* extract and fenitrothion on epididymal sperm count, sperm quality and testosterone level

Mice treated with fenitrothion showed a significant decrease (*P*<0.05) in the sperm concentration, progressive motility and viability compared to the control group. Meanwhile, significant decrease of sperm concentration (*P*<0.05) was also shown in the *K. parviflora* ethanolic extract treatment group in comparison with the control group, while a slight decrease (*P*>0.05) was observed in both sperm progressive motility and viability of the *K. parviflora* ethanolic extract treatment group compared to the control group. Furthermore,

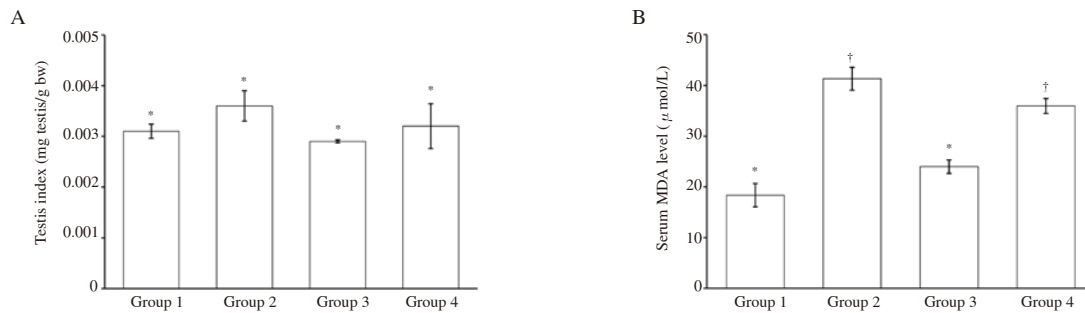


Figure 1. Relative testis weight and blood serum lipid peroxidation after treatment in mice. (A) relative testis weight and (B) blood serum malondialdehyde (MDA) concentrations in mice after different treatments ($n=3$). Group 1 (the negative control); Group 2 (fenitrothion); Group 3 (*Kaempferia parviflora* ethanolic extract); Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion). *†Means with different superscripts on top each bar are significantly different ($P<0.05$) using Kruskal-Wallis H test and DSCF *post hoc* test. *Means with same superscripts on top each bar are not significantly different ($P>0.05$) using Kruskal-Wallis H test and DSCF *post hoc* test.

Table 1. Sperm parameters and testosterone level analysis in mice of the experimental groups.

Parameters	Group 1	Group 2	Group 3	Group 4
Sperm concentration, $\times 10^6$ cells/mL	40.16 $\pm$ 1.02 [‡]	30.67 $\pm$ 0.26 [*]	33.91 $\pm$ 1.16 [†]	32.89 $\pm$ 1.28 [†]
Sperm progressive motility, %	79.00 $\pm$ 1.99 [‡]	33.00 $\pm$ 10.53 [*]	71.33 $\pm$ 3.79 [‡]	64.33 $\pm$ 3.79 [†]
Sperm viability, %	86.08 $\pm$ 1.40 [‡]	60.38 $\pm$ 5.65 [*]	76.48 $\pm$ 2.72 [‡]	73.44 $\pm$ 3.67 [†]
Serum testosterone, ng/mL	22.69 $\pm$ 2.74 [†]	4.50 $\pm$ 1.80 [*]	44.05 $\pm$ 5.13 [‡]	28.32 $\pm$ 3.81 [†]

Data are expressed as mean $\pm$ SD ($n=3$). *†‡Means within the same row with different superscripts differ significantly ($P<0.05$) by using LSD *post hoc* test. Group 1 (the negative control); Group 2 (fenitrothion); Group 3 (*Kaempferia parviflora* ethanolic extract); Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion).

Table 2. Mating index and mating period assessment of the experimental groups.

Parameters	Group 1	Group 2	Group 3	Group 4
Mating index, %	100.00 $\pm$ 0.00 ^{ns}	88.89 $\pm$ 19.24 ^{ns}	100.00 $\pm$ 0.00 ^{ns}	100.00 $\pm$ 0.00 ^{ns}
Mating period, days (14 days observation)	1.44 $\pm$ 0.31 [*]	11.13 $\pm$ 1.20 [‡]	1.33 $\pm$ 0.29 [*]	4.89 $\pm$ 0.45 [†]

Data are expressed as mean $\pm$ SD ($n=3$). *†‡Means within the same row with different superscripts are significantly different ($P<0.05$) by using LSD *post hoc* test. ^{ns}Means with same superscripts within a row are not significantly different ($P>0.05$). Group 1 (the negative control); Group 2 (fenitrothion); Group 3 (*Kaempferia parviflora* ethanolic extract); Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion).

the co-administration of *K. parviflora* ethanolic extract and fenitrothion treatment group showed a significant increase in sperm concentration, progressive motility and viability when compared to the fenitrothion treatment group.

Additionally, approximately one-fifth of the testosterone level was observed in the fenitrothion treatment group, while the *K. parviflora* ethanolic extract treatment group showed significant testosterone level that was about two-fold higher compared to the control group ($P<0.05$). Interestingly, an elevated testosterone level ($P<0.05$) of approximately seven-fold was observed in the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group compared to the fenitrothion treatment group (Table 1).

3.4. Histomorphological changes in testis of mice treated with *K. parviflora* extract and fenitrothion

Effects of *K. parviflora* ethanolic extract with or without fenitrothion on testis morphology were assessed by using histological evaluation (Figure 2) and Johnsen's mean testicular score count. A disorganized testicular cell with low visibility of spermatozoa at the lumen of seminiferous tubules was observed in the fenitrothion treatment group compared to the control group and the *K. parviflora* ethanolic extract treatment group. The result was similarly reflected in

Johnsen's mean testicular score count where the fenitrothion treatment group (7.15 $\pm$ 0.14) had a significantly lower score ($P<0.05$) compared to the control group (9.23 $\pm$ 0.07) and the *K. parviflora* ethanolic extract treatment group (9.30 $\pm$ 0.14). On the other hand, the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group showed an improvement of the structure of germinal epithelium with its abundance of spermatogonia, spermatocytes, spermatids and spermatozoans as compared to the fenitrothion treatment group. Similar result was obtained in Johnsen's mean testicular score count where the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group (8.77 $\pm$ 0.16) has significantly higher ($P<0.05$) testicular score compared to the fenitrothion treatment group.

3.5. Effect of co-administration of *K. parviflora* extract and fenitrothion on mating index, mating period, litter size, pups' survival rate and sex ratio skewness of pups

The data of mating assessment of the remaining live male mice in each treatment group as presented in Table 2. A lower mating index was observed in the fenitrothion treatment group while the remaining treatment groups have 100% mating success rate. However, no significant difference was found in the mating index among all treatment groups ($P>0.05$). Meanwhile, a significantly

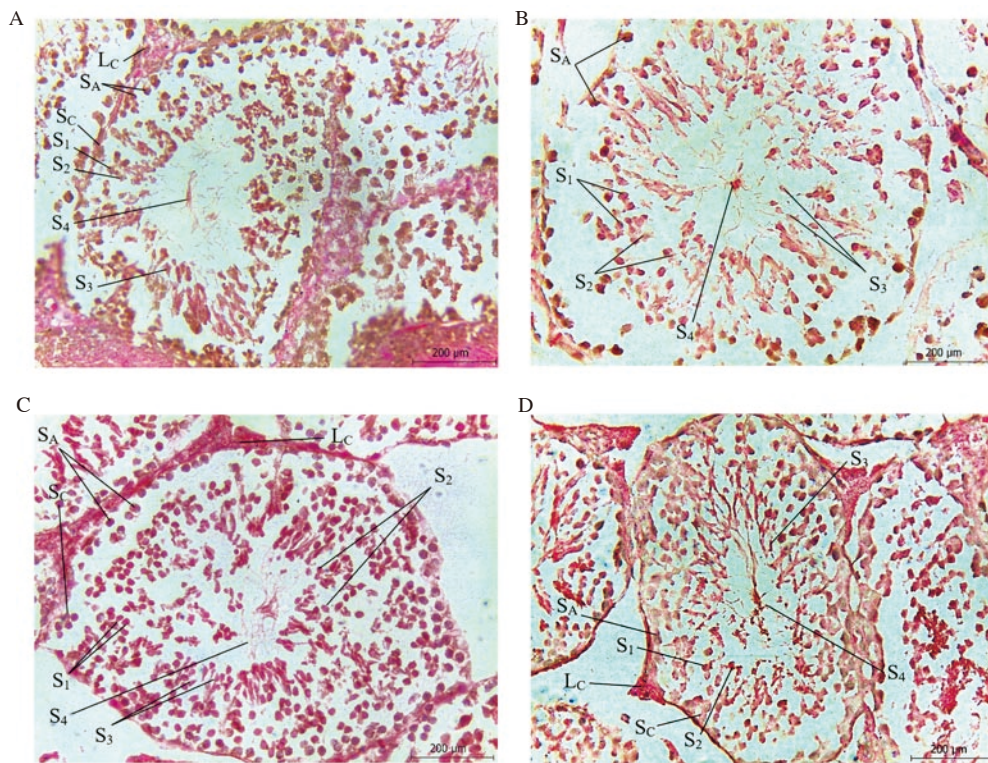


Figure 2. Histopathological images of seminiferous tubules in testis and spermatogenesis evaluation after treatments (magnification 400x; H & E staining). (A) Group 1 (the negative control); (B): Group 2 (fenitrothion); (C): Group 3 (*Kaempferia parviflora* ethanolic extract); (D) Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion). Lc: Leydig cell, Sc: Sertoli cell, SA: spermatogonium, S₁: primary spermatocyte, S₂: round spermatid, S₃: elongating spermatid, S₄: spermatozoan.

Table 3. Post-parturition analysis of experimental groups.

Parameters	Group 1	Group 2	Group 3	Group 4
Litter size per dam, <i>n</i>	12.20±0.62 ^{ns}	11.0±0.66 ^{ns}	11.10±0.45 ^{ns}	11.90±0.45 ^{ns}
Survival rate of pups, %	94.50±3.19 ^{ns}	89.90±3.41 ^{ns}	95.30±2.56 ^{ns}	94.40±2.42 ^{ns}

Data are expressed as mean±SD (*n*=3). ^{ns}Means with same superscripts within a row are not significantly different (*P*>0.05) by using LSD *post hoc* test. Group 1 (the negative control); Group 2 (fenitrothion); Group 3 (*Kaempferia parviflora* ethanolic extract); Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion).

(*P*<0.05) longer mating period was observed in the fenitrothion treatment group compared to the control group and the *K. parviflora* ethanolic extract treatment group. Interestingly, the mice that were co-administered with *K. parviflora* ethanolic extract and fenitrothion showed significantly shorter (*P*<0.05) mating period in comparison with the fenitrothion treatment group.

The mated female mice among all treatment groups were continuously observed until post-parturition and the parameters of post-parturition were evaluated (Table 3). An approximate 10% reduction in litter size per dam was observed in both fenitrothion and *K. parviflora* ethanolic extract treatment groups compared to the control group. Additionally, an 8% increase in litter size per dam was observed in the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group compared to the fenitrothion treatment group. The fenitrothion treatment group showed a 6% decrease in the survival rate of F1 progeny compared to the control group. The *K. parviflora* ethanolic extract treatment group had a slightly higher pup

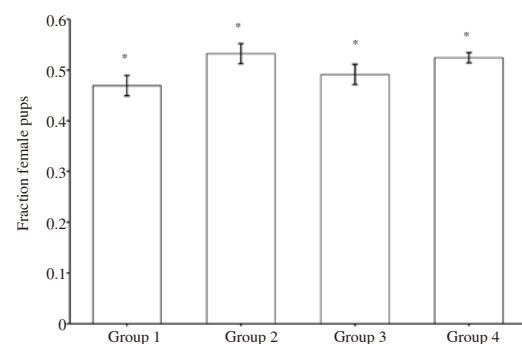


Figure 3. Fraction female pups' assessment in all treatment groups (*n*=9). Group 1 (the negative control); Group 2 (fenitrothion); Group 3 (*Kaempferia parviflora* ethanolic extract); Group 4 (*Kaempferia parviflora* ethanolic extract + fenitrothion). Means with same superscripts on top each bar are not significantly different from its hypothetical value (*P*>0.05).

survival rate than the control group, while the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group exhibited a 6% higher survival rate for F1 pups compared to the fenitrothion

treatment group. However, no significant differences were found in either litter size per dam or pup survival rates across all treatment groups ($P > 0.05$). Figure 3 shows the assessment of the female pup fraction among all treatment groups. A slightly higher female pup ratio was observed in the fenitrothion treatment group, followed by the combined treatment of *K. parviflora* ethanolic extract and fenitrothion group, the *K. parviflora* ethanolic extract treatment group and the control group. Notably, no significant difference was found in the mean fraction of female pups across all treatment groups when compared to the hypothetical value of 0.5 ($P > 0.05$).

4. Discussion

Antioxidant properties of a plant extract are commonly assessed based on the DPPH free radical scavenging activity, total phenolic content and total flavonoid content[28]. It was observed that the ethanolic extract of *K. parviflora* had a lower EC_{50} value for DPPH scavenging activity in the present study, implying that the ethanolic extract of *K. parviflora* possessed antioxidative properties[7]. In comparison with other studies, it can be observed that the present study showed relatively higher EC_{50} DPPH value in the ethanolic extract of *K. parviflora*, while the other extracts of *K. parviflora* showed similar EC_{50} values in DPPH scavenging activity[29,30]. This may be due to procedural differences, such as the duration of extraction of *K. parviflora*, which may have led to the differences observed in the present study[14,29,30]. Meanwhile, it was also noted that a higher phenolic content was observed in ethanolic extract of *K. parviflora*, whereas its flavonoid content was relatively low, as shown in the present findings. These significant findings were consistent with several studies on the evaluation of antioxidant content in different solvent extractions of *K. parviflora*[9,14,29]. It is well known that methoxyflavones, which are abundantly found in the rhizome of *K. parviflora* are non-polar and preferably soluble in less polar solvents[7,14]. However, other phytochemicals found in the rhizome of *K. parviflora* are highly polar and highly soluble in more polar solvent, contributing to the higher phenolic content in the ethanolic extraction of *K. parviflora*[31,32]. In conjunction with the reported findings, the ethanolic extract of *K. parviflora* exhibited the highest antioxidant activity compared to other solvent extractions of *K. parviflora*. Thus, ethanolic extract of *K. parviflora* was chosen for GC-MS analysis and *in vivo* studies.

GC-MS analysis of the ethanolic extract of *K. parviflora* detected 8 peaks in the chromatogram, with 5 of the compounds identified as methoxyflavone derivatives found in the rhizomes of *K. parviflora*[7]. Among the 5 detected compounds, 5,7,4'-trimethoxyflavone was also reported as one of the main constituents identified in the ethanolic extract of *K. parviflora* through GC-MS analysis in other studies[7,18]. Instead, 5-hydroxy-3,7-dimethoxyflavone was identified as the most abundant compound. The presence of this compound highlights

potential differences in the chemical composition of *K. parviflora* extracts, which could influence its biological activity. Earlier study reported that 5-hydroxy-3,7-dimethoxyflavone reduced the activity of glycogen synthase kinase-3 beta (GSK-3 β) enzymes that regulates glycogen metabolism and improved glycogen synthesis[7]. Meanwhile, the other identified methoxyflavones derivatives have been proposed in various studies to exhibit strong antioxidative, anti-inflammatory and anti-cancer properties[7,33]. In the meantime, the remaining 3 compounds detected in the present study have not been reported in any previous findings from phytochemical evaluations of *K. parviflora* extracts. One of the novel compounds identified, 2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one, belongs to the class of methoxyflavones and can also be referred to as 3'-hydroxy-3,7,4'-trimethoxyflavone, based on the chemical structure of methoxyflavones described by Chen *et al*[7]. This compound has not yet been reported for any potential bioactivity, but it is predicted to exhibit anti-allergic activity and inhibitory effects on acetylcholinesterase due to the presence of methoxy groups at positions 4' and 7 as proposed by findings in other studies[7,34]. 7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one, another novel compound identified, likely belongs to the class of methoxyisoflavones due to the presence of a methoxyphenylchromen-4-one skeleton. This structural feature suggests that the compound may possess anti-cancer properties, particularly due to methoxylation at the 4' position, similar to other isoflavone derivatives reported in various studies[35]. A notable finding of this study is the identification of a major compound, 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol), which accounts for approximately 22% of relative abundance. This compound belongs to the class of diarylmethane compounds and has not yet been explored for any bioactivity. Therefore, future study is required to focus on the isolation of this phytochemical from the rhizomes of *K. parviflora* using ethanolic extract and further investigate its potential bioactivity.

Fenitrothion-induced mice exhibited relatively higher testis weight compared to the control treatment group which was likely due to hydrocele where there is fluid accumulation in the inflamed seminiferous tubules that contributed to the increased testicular weight[4]. Fenitrothion induces oxidative damage in testicular cells by generating ROS and disrupting hormonal balance, which is essential for testicular fluid homeostasis[3,6]. Hence, it may lead to the development of hydrocele in the testis as observed in the present study. Meanwhile, significantly elevated serum MDA levels were detected in fenitrothion-induced mice compared to the control that indicates on the enhanced oxidative stress level. This result aligns with other studies in which elevated testicular weight and MDA levels were observed following fenitrothion administration in murine model[6]. Furthermore, exposure to organophosphates such as fenitrothion may increase oxidative stress level in spermatozoa, leading to spermatotoxicity in murine model[5,6]. In the meantime,

no significant changes in serum MDA level and relative testis weight were recorded in *K. parviflora* ethanolic extract treatment compared to the control group as safe dose was administered[21]. This is in line with the findings reported by Chivapat *et al* in which administration of *K. parviflora* ethanolic extract up to 2000 mg/kg body weight did not induce any observable adverse effect on male mice[36]. Interestingly, restorative effect of the relative testis weight to an extent similar to control was observed in the combined treatment of *K. parviflora* ethanolic extract and fenitrothion mice. This could be due to the antioxidative properties of *K. parviflora* ethanolic extract that scavenges ROS induced by fenitrothion, thus restoring testicular function and size in mice[6,14]. Simultaneously, a slight reduction in serum MDA level was recorded on the co-administration of *K. parviflora* ethanolic extract and fenitrothion. This finding highlights the antioxidative properties of *K. parviflora* ethanolic extract which may inhibit ROS activity by fenitrothion[7], thus leading to the reduction in further lipid peroxidation and the propagation of free radical chain reactions[7,9,37]. Similar effect was reported by Donia *et al* in which the study demonstrated a reduction in MDA levels in testicular tissue and improved antioxidant enzyme activity with the administration of green tea extract and vitamin C in fenitrothion-administered mice, suggesting their protective role as antioxidant against fenitrothion-induced oxidative damage[38]. However, further studies are needed to investigate the mechanisms between methoxyflavone derivatives in *K. parviflora* ethanolic extract and their effectiveness in scavenging ROS induced by fenitrothion.

Further, parameters such as sperm count, sperm motility, sperm viability and testosterone level were often used to determine male fertility in clinical study and animal model[23,24]. The treatment of fenitrothion alone in this study has shown significant adverse effect on male mice fertility, with the reduction all sperm parameters and testosterone level. This implied fenitrothion might have generated ROS in the testicular tissue which causes damages to spermatozoa and Leydig cells, leading to spermatotoxicity and significant reduction in testosterone level shown in the present study[37,39]. Interestingly, no significant changes were observed in any sperm parameters in the *K. parviflora* ethanolic extract treatment group, while a significant increase in serum testosterone levels was found compared to the control group. Other studies have reported similar findings using rat model, suggesting that the ethanolic extract of *K. parviflora* does not affect sperm count, motility and viability but possesses androgenic properties that may boost testosterone production[10,11]. Meanwhile, the administration of *K. parviflora* ethanolic extract in the present study significantly restored sperm concentration, motility, viability, and testosterone levels in fenitrothion-treated mice compared to mice receiving fenitrothion alone. These findings suggest on the role of antioxidant found in ethanolic extract of *K. parviflora* in scavenging ROS from fenitrothion, at the same time preventing the spermatozoa and

Leydig cells from further oxidative damages[6,7,14]. Similar findings were also reported in another study evaluating the role of quercetin in mitigating fenitrothion-induced spermatotoxicity in male rats[37]. Their results suggest that quercetin acts as a powerful antioxidant that scavenges and prevents further propagation of lipid alkoxyl and peroxy radicals potentially induced by fenitrothion[9,37,38]. Other relevant finding reported on the potential protective effects of palm oil tocotrienol-rich fraction in fenitrothion-administered male rats where they all possess antioxidative properties which prevents further free radical chain reaction in the spermatozoa[6,40]. These findings further support that *K. parviflora* ethanolic extract used in the present study which has been shown to possess antioxidant properties, exert protective effect on fenitrothion-induced spermatotoxicity and reduce testosterone levels in male mice.

Exposure to fenitrothion will induce the generation of ROS, leading to inflammatory damage in testicular tissue[3,4]. This damage is characterized by a decrease in the number of spermatogenic cells within the seminiferous tubules as well as the degeneration and disorganization of the spermatogenic epithelium. These findings are generally consistent with reported studies on fenitrothion-administered rats over 4 to 9 weeks of administration period[5,6]. The degeneration of germ cells observed in this study may result from apoptosis induced by ROS generation, which removes abnormal germ cells through the activation of caspases[3,6]. Other studies have also reported that fenitrothion can cross into testicular tissue *via* the bloodstream, reducing spermatogenesis by disrupting the maturation and differentiation of germ cells[4,5,37]. Meanwhile, no significant observation of degenerated, disrupted or disorganized germ cells was found in the seminiferous tubules of *K. parviflora* ethanolic extract-administered mice compared to the control group. This finding indicates that ethanolic extract of *K. parviflora* does not cause chronic testicular toxicity in mice. A study by Chivapat *et al* reported no testicular atrophy or orchitis in Wistar rats administered daily doses of 5, 50, and 500 mg/kg bw of ethanolic extract of *K. parviflora* for six months[36]. These findings strongly suggest that the ethanolic extract of *K. parviflora* does not exhibit testicular toxicity in murine models, which is consistent with the observations from the present histopathological study[7,21,36]. Furthermore, a restorative effect was observed in the testicular tissue of fenitrothion-administered mice with the co-administration of *K. parviflora* ethanolic extract, compared to the group administered with fenitrothion alone. Restorative signs such as the thickening of smooth muscle and slight congestion in the seminiferous tubules observed in the combined treatment of ethanolic extract of *K. parviflora* and fenitrothion group were also noted in another study involving the co-administration of quercetin and fenitrothion on Sprague-Dawley rats[37]. These findings may be attributed to the antioxidant properties of *K. parviflora* ethanolic extract which help ameliorate and restore fenitrothion-induced damage towards normal[6,7,37]. The present

findings are further supported by evidence that methoxyflavones from *K. parviflora* extract were found in the testes of rats within 1 to 2 hours after administration and were maintained for about 3 to 6 hours before being fully excreted[7]. Thus, the presence of methoxyflavones in the testis may scavenge free radicals generated by fenitrothion and further prevent ROS-induced testicular injury in a time- and dose-dependent manner[7,14,37].

Non-significant findings were observed in the mating index, litter size per dam and pups' survival rate in all treatment groups. This may be due to the limited sample size and the duration of treatment, which may not have been sufficient to demonstrate the significance of mating impairment as well as post-parturition effects in male mice[19]. However, a reduction in mating index, litter size per dam and survival rate of pups was observed in the fenitrothion-administered group and *K. parviflora* ethanolic extract-administered group compared to the control group, showing a similar pattern to findings reported by Yusoff *et al* and Sudwan *et al*[4,11]. Differences in dosage, exposure method and the possibility that the substances (*K. parviflora* ethanolic extract and fenitrothion) may affect in other conditions of the experiment model could be contributing factors to the present findings[4,11]. Interestingly, significantly longer mating period was observed in fenitrothion-treated male mice while a significantly reduced mating period was observed in mice co-administrated with the ethanolic extract of *K. parviflora* and fenitrothion. This may be due to the impact of fenitrothion causing lower testosterone level which reduced the sexual drive[4,5,37]. On the other hand, *K. parviflora* ethanolic extract enhances libido behaviour due to increase in androgen hormone[7,10]. Other relevant findings showed a significant decrease in mount and ejaculatory latencies in male rats administered with the ethanolic extract of *K. parviflora* for 4 weeks, while mount frequencies were significantly reduced in male rats administered with fenitrothion for 4 weeks[4,10,11]. These results may further support the current findings of the effectiveness of the *K. parviflora* ethanolic extract in reducing courtship time in fenitrothion-administered male mice.

There were no gross anomalies observed in the administered mice's offspring even though there were slight increase of litter size per dam and survival rate of pups in the combined treatment of the ethanolic extract of *K. parviflora* and fenitrothion parental mice compared to parental fenitrothion treatment group. These findings highlighted the potential occurrence of epigenetic modulation in sperm DNA which lead to defective sperm cell, thus reducing in successful fertilisation rate and the survival rate of pups[5,39,41]. The ethanolic extract of *K. parviflora* on the hand possessed antioxidative properties which may scavenge free radical and prevent spermatozoa from further oxidative damage caused by fenitrothion, thus potentially leading to higher successful fertilisation rate and more normal pups per dam as shown in the present findings[14,37]. A multigenerational study reported a significant decrease in Leydig cell count and observed several gross anomalies (such as short or absent tails and defective legs)

in the offspring, attributed to fenitrothion exposure in the parental male mice while there is no multigenerational study reported on the exposure of *K. parviflora* ethanolic extract on parental male mice[39]. The present study also showed no significant difference in the sex-skewness of offspring across the treatment groups compared to the hypothetical value. The current findings may suggest that fenitrothion or the ethanolic extract of *K. parviflora* does not directly influence sex differentiation in offspring. It was suggested that pesticides may not significantly affect sex skewness but may alter sex-specific behaviors in the offspring of male mice when they reached adulthood based on its endocrine disrupting properties[27,42]. However, there is no study reported on the sex-skewness or sex-specific trend in the birth of offspring influence by the administration of the ethanolic extract of *K. parviflora* or fenitrothion.

The limitation of this study lies in the low sample size used, which may have affected the accuracy of certain reproductive parameters, such as mating success rate, litter size and pups' survival rate. The small number of mice used in the study may also contribute to the lack of statistically significant differences in these outcomes. Hence, future studies with a larger sample size are recommended to enhance the reliability and robustness of the findings on the effects of *K. parviflora* ethanolic extract and fenitrothion treatment.

In conclusion, there were eight bioactive compounds were identified in *K. parviflora* ethanolic extract in this study and the extract exhibited antioxidative properties. In addition, *K. parviflora* ethanolic extract at the dose of 50 mg/kg bw has shown protective effect on the reproductive impairment of male mice after exposing to fenitrothion (20 mg/kg bw) by the improvement of sperm count, sperm motility, sperm viability and testosterone level as well as the reduction of abnormal sperm morphology. The *K. parviflora* ethanolic extract was found to reduce the alterations and improve the testicular histological changes caused by fenitrothion exposure. The study highlights the protective effect of the *K. parviflora* ethanolic extract against fenitrothion-induced male reproductive damage. However, future studies are still necessary to evaluate the ameliorative effect of *K. parviflora* ethanolic extract at a longer duration of exposure, higher dosage of both *K. parviflora* extract and fenitrothion to have better evaluation on male aphrodisiac effect. The specific mechanism of action of the bioactive compounds in the *K. parviflora* ethanolic extract that exert the protective effect on male infertility needs to be studied and established. This might also be helpful in understanding the deleterious effects of residual fenitrothion (from agricultural use) on humans, as it contributes to male infertility—a global issue in the 21st century.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

Shawn Samson Tze Xian Ong conducted the experiment, analyzed the acquired data and prepared the manuscript. GimCheong Tan was the principal investigator of the grant while Phek Jin Kwong and Mun Yee Chan were the co-investigators of the grant. All investigators were involved in designing the study, analyzing all the acquired data and advised on the evaluation of result record. All authors read and approved the final manuscript.

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Effect of combined *Kaempferia parviflora* extract and fenitrothion treatment on male reproductive parameters in a murine model

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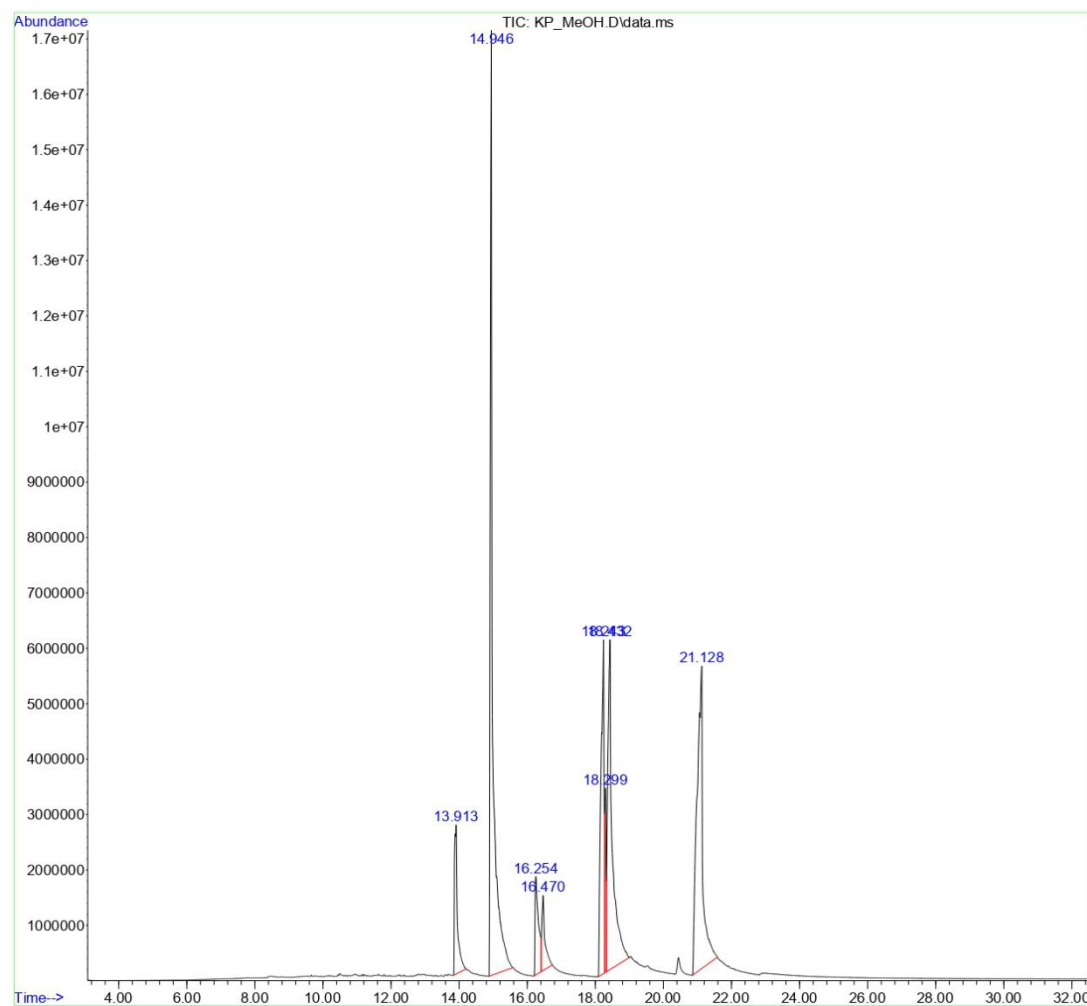
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Supplementary Materials

Supplementary Table 1. List of bioactive compounds present in ethanolic extract of *Kaempferia parviflora* detected by gas chromatography-mass spectrometry (GC-MS).

Retention time (min)	Compound detected	Peak abundancy (%)
13.910	7-Hydroxy-3-methoxy-2- <i>p</i> -methoxyphenyl-4H-chromen-4-one	5.48
14.945	5-hydroxy-3,7-dimethoxyflavone	29.94
16.257	2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one	4.18
16.472	5-hydroxy-7,4'-dimethoxyflavone	3.00
18.242	3,5,7,4'-tetramethoxyflavone	12.30
18.298	5-hydroxy-3,7,3'4'-tetramethoxyflavone	3.18
18.430	5,7,4'-trimethoxyflavone	19.53
21.130	2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol)	22.39



Supplementary Figure 1. Peaks are detected by gas chromatography-mass spectrometry (GC-MS) in the rhizome of *Kaempferia parviflora* ethanolic extract.