Antifilarial activity of caffeic acid phenethyl ester on *Brugia pahangi* *in vitro* and *in vivo*

Nazeh M. Al-Abd^{a,c}, Zurainee Mohamed Nor^a, Quazim O. Junaide^a , Marzida Mansor^b, M. S. Hasan^b and Mustafa Kassim^b

^aFaculty of Medicine, Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia; ^bFaculty of Medicine, Department of Anesthesiology, University of Malaya, Kuala Lumpur, Malaysia; ^cFaculty of Medicine and Health Science, Department of Para Clinic, University of Aden, Aden, Yemen

ABSTRACT

Lymphatic filariasis (LF) is a vector borne disease caused by parasitic worms such as *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, which are transmitted by mosquitoes. Current therapeutics to treat LF are mainly microfilaricidal, and lack activity against adult worms. This setback, poses a challenge for the control and elimination of filariasis. Thus, in this study the activities of caffeic acid phenethyl ester (CAPE) against the filarial worm *B. pahangi* and its bacterial endosymbiont, *Wolbachia* were evaluated. Different concentrations (2, 5, 10, 15, 20 µg/ml) of CAPE were used to assess its effects on motility, viability and microfilarial (mf) production of *B. pahangi in vitro*. Anti-*Wolbachia* activity of CAPE was measured in worms by quantification of *Wolbachia* *wsp* gene copy number using real-time polymerase chain reaction. Our findings show that CAPE was found to significantly reduce adult worm motility, viability, and mf release both *in vitro* and *in vivo*. 20 µg/ml of CAPE halts the release of mf *in vitro* by day 6 of post treatment. Also, the number of adult worms recovered *in vivo* were reduced significantly during and after treatment with 50 mg/kg of CAPE relative to control drugs, diethylcarbamazine and doxycycline. Real time PCR based on the *Wolbachia* *ftsZ* gene revealed a significant reduction in *Wolbachia* copy number upon treatment. Anti-*Wolbachia* and antifilarial properties of CAPE require further investigation as an alternative strategy to treat LF.

KEYWORDS

Lymphatic filariasis; *Wolbachia*; caffeic acid phenethyl ester; microfilaricidal; *Brugia pahangi*; antifilarial drugs

Introduction

Lymphatic filariasis (LF) is a disease caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, affecting approximately 150 million people worldwide [1]. The infection is responsible for a global morbidity of 6.5 million disabled lives per year. About 1.5 billion people live with the risk of infection in tropical and subtropical regions of Asia, Africa, South America, Central America, and Pacific Islands [1]. In the year 2015, 73 countries were considered to be endemic for LF, out of which 55 still require mass drug administration (MDA) [2].

There are several synthetic drugs that have been employed as antifilarial agents, such as ivermectin (IVM), diethylcarbamazine (DEC), and albendazole (ALB) but most of them are primarily microfilaricidal, which has little to no effect on adult worms [3]. However, current strategies to eliminate LF involve community-based single oral treatment with combination of DEC or IVM and ALB, repeated annually or bi-annually over many years in order to reduce transmission [4,5]. Recently, a triple-drug therapy of DEC (6 mg/kg), ALB (400 mg) and IVM (200 µg/kg) was proven to be safe and efficient in

treatment of Bancroftian filariasis [6]. It is noteworthy that both 2-drug and 3-drug regimen are associated with adverse events. Therefore, there is a need for an alternative therapy, through innovative and extensive phytomedicinal research, to produce potential tolerable antifilarial drugs [7].

The preliminary evaluation of antifilaria activity of *M. cajuputi* showed that methanol extracts from the flower of *M. cajuputi* exhibit potent antifilarial activity. In addition, it was confirmed that the chemical structure of a phenolic compound, caffeic acid phenethyl ester (CAPE), was the principle active compound in the extract. CAPE is a natural bioactive compound found in many plants and is also known as one of the main components of propolis extracts from honey bee [8]. Previous reports have shown that CAPE exhibits anti-carcinogenic, anti-inflammatory, antibacterial, antioxidant, antiviral, antifungal and immunomodulatory effects [9–15].

Despite the documentation of antiparasitic activity of propolis on *Trypanosoma cruzi* [16,17], *Trichomonas vaginalis* [18], *Leishmania amazonensis* [19,20] and *Giardia lamblia* [21,22], there are relatively few reports

focusing on its bioactive component, CAPE. However, a recent study by ES Abamor [23] shows that CAPE has significant inhibitory effects on *L. infantum* promastigotes and amastigotes. To our knowledge, antifilarial activity of CAPE has not been investigated until now.

Thus, the current study investigates the potential antifilarial activity of CAPE and its possible mechanism *in vitro* and *in vivo* by targeting *B. pahangi* adult worms. The results of this study provide a basis for developing more effective treatment strategies against filariasis.

Methodology and results

Animal model and infection

Mongolian gerbils (*Meriones unguiculatus*) were used as experimental animal models in this study. The gerbils were initially obtained from Institute of Medical Research, Malaysia and have been maintained in the department of Parasitology, Faculty of Medicine, University of Malaya.

Male gerbils were fed on a rodent diet and water *ad libitum* and housed in hygienic compartments with standard conditions of light (12 h light/12 h dark) and temperature (~28 °C). The filarial *B. pahangi* was initially obtained from an infected cat and maintained by cyclical passage between gerbils and *Aedes togoi* mosquitoes. Infection in gerbils was established by intraperitoneal inoculation of 150 infective L₃ larvae for *in vitro* studies, while about 50 infective L₃ larvae were subcutaneously inoculated for *in vivo* studies. All animals and experimental procedures included in the present study were duly approved by the Ethics Committee for Animal Care and Use, Faculty of Medicine, University of Malaya, Malaysia, with Ethics No. (PAR/21/11/2011/ZMN (R)). All animals received care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences [24].

in vitro study

B. pahangi adult worms were recovered from the peritoneal cavities of infected male gerbils at 4 to 6 months post-infective period. The gerbils were euthanized by administration of overdose of 50 mg/kg, ketamine (Troy Laboratories, Australia), and the peritoneal cavity was opened and washed with 10 ml of PBS to recover the worms. These worms were freed from any contaminants by washing 3–4 times in RPMI 1640 medium containing 20 mM HEPES, 100 µg/ml penicillin and 100 unit / mL streptomycin (Invitrogen, USA).

For the *in vitro* culture, about 25 pooled adult worms of *B. pahangi* (12 male and 13 female) were used per well in a 6-well flat-bottomed culture plate (TPP Techno Plastic Products), containing 5 mL complete media (CM). The worms were incubated with (2–20 µg/ml) of CAPE and 25 µg/ml of ivermectin separately in 5 mL of CM that comprised of RPMI-1640 supplemented with 25 mM

HEPES buffer, 2 mM glutamine, 100 U/mL streptomycin, 100 µg/mL penicillin, 0.25 µg/mL of amphotericin B and 10% foetal bovine serum. Control cultures (no CAPE) containing the same amount of CM with DMSO were also prepared. Cultures were maintained for 7 days at 37 °C in a humidified atmosphere of 5% CO₂. Microfilariae (mf) released by adult female worms into the culture medium were monitored daily. Mf were quantified 4 times in 10 µl of culture medium and results were expressed as % reduction in mf released relative to mf released by control worms. The culture medium was replaced with fresh medium of drugs on days 2, 4, 6, and terminated on day 7. Parasite viability was assessed quantitatively by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [25]. Motility and mortality of adult worms were monitored. All cultured experiments were carried out in triplicate. IC₅₀ (concentration at which the parasite motility was inhibited by 50%) and cytotoxic concentration (CC₅₀) were determined according to the methods of Lakshmi, et al. [26] and Sashidhara, et al. [27] respectively. Selectivity Index (SI) of the agents were computed by CC₅₀/IC₅₀.

in vivo study

25 gerbils with patent infection (4 months post-infective larvae inoculation) showing progressive rise in mf were divided into five groups, each comprised of five animals. The first group received 10% dimethyl sulfoxide (DMSO) (no CAPE, vehicle control), the second group was given 50 mg/kg/day of doxycycline, the third group received 50 mg/kg/day of DEC, and the fourth and fifth groups received 20 and 50 mg/kg/day of CAPE, respectively. The injection was given daily intraperitoneally for 14 days. All substances were diluted in PBS and 10% DMSO. The efficacy of the drug on the filarial parasite was evaluated according to Misra-Bhattacharya, et al. [28]. A total of 50 µl of blood was taken from the tail vein of each animal (treated and control animals), and spread as three different thick smears which were air dried for 12–24 h. The smears were de-haemoglobinized with water and later stained with Geimsa stain. Blood was collected from each animal prior to the start of treatment at day 0 and at regular intervals of 15 days until day 120 post-treatment. The density of mf was assessed at each interval and the means were compared among the treated groups and control. After 120 days of post treatment, treated and untreated animals were sacrificed under terminal anaesthesia and heart, lungs, testes, peripheral lymph nodes and pleural cavity were removed and dissected carefully in PBS to recover the adult parasites. Assessment of macrofilaricidal/adulticidal efficacy of the extract was carried out by measuring percent reduction in adult worms recovered in the treated group relative to untreated animals as described previously [29].

Effect of CAPE on *Wolbachia* in vivo

The susceptibility of *Wolbachia* to CAPE was investigated based on the *wsp* gene copy number, and a method was developed for quantification of *Wolbachia*. Extraction of genomic DNA was performed using a QIAamp Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Depletion of *Wolbachia* was monitored by real-time polymerase chain reaction (qPCR). A primer pair was used to amplify a short (226 nucleotide) fragment of the *wsp* gene of *B. pahangi* *Wolbachia* i.e. Bp*wsp*-forward 5'TTGGTCTTGGTGTAGCATATA3', and Bp*wsp*-reverse 5'ACTTTTGTTCCTTATCCTCA3'. To verify the presence of a single amplification product, the primers were tested using a conventional PCR reaction performed on DNA extracted from *B. pahangi*. Reactions for PCR were carried out as follows: 3 min at 98 °C, 10 s at 98 °C, 15 s at 57 °C, and 30 s at 72 °C for 30 cycles. The amplification products were analysed by 1.7 % agarose gel electrophoresis. qPCR reaction mixtures of 1 µl of DNA template in a final volume of 25 µl were performed on a StepOnePlus System (Applied Biosystems, Germany). Each sample was tested in triplicate with biological replicate. PCR products of *wsp* (conventional PCR) were ligated into the pJET 1.2 vector (Promega, Madison, WI, USA) following the manufacturer's protocol. The cloned amplicon was confirmed and sequenced using automatic DNA sequencing followed by BLAST2 analysis (National Center for Biotechnology Information [NCBI (www.ncbi.nlm.nih.gov), Bethesda, MD, USA] with their corresponding sequences in GenBank. The concentration of purified plasmids that spanned the target regions for forward and reverse primers were measured by using a ND2000 NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). These measured plasmids were converted to copy numbers/µL according to the following formula (Equation (1)):

$$\text{DNA copy number} = \frac{[6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amount (g)}]}{[\text{DNA length (dp)} \times 660(\text{g/mol/dp})]}$$

Statistical analysis

Statistical data analyses were performed using statistical software, PRISM 6 (GraphPad, Software Inc., San Diego, USA). One-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons using Tukey's method were used to determine significant differences between the treatments in comparison with the control group. Statistical significance was established at alpha values less than 0.05 between the treated and their relevant controls groups.

Results

Inhibitory effect of CAPE on viability and microfilariae production in vitro

The viability of worms were evaluated via MTT assay. The results obtained from the MTT assays show that the

worms are less viable upon exposure to CAPE, which is significant ($p < 0.05$) at 20 µg/mL compared to controls 7 days post-treatment (Figure 1). Reduction in mf release was quantified in terms of percentage reduction in formazan formation. CAPE treated groups experienced significant ($p < 0.05$) reduction in mf release compared to the control group within the duration of the study (7 days) and is dose-dependent (Figure 2). In this experiment, a stable release of mf was observed in the control (untreated) female worms, unlike the sharp reduction and complete cessation of mf release by day 6 in the CAPE (20 µg/mL) treated group. The standard drug DEC required higher concentration (LC₁₀₀: 485 µg/mL and LC₁₀₀: 390 µg/mL) to kill the adult worms and mf

Figure 1. Effects of CAPE on *Brugia pahangi* adult worm viability. Worms were cultured and incubated with different concentrations of CAPE (2–20 µg/ml), ivermectin (25 µg/ml), or left untreated (control) for 7 days. Viability was measured by MTT reduction assay through reduction in formazan production. Data were represented as mean ± SEM, $n = 25$.

Figure 2. Effects of CAPE on microfilariae production from *B. pahangi* worms in vitro. Results are shown as reduction in microfilariae released in CAPE- and ivermectin-treated groups compared with untreated control group. Data were represented as mean ± SEM, $n = 13$.

respectively, while the IC_{50} of CAPE for the adult worms was $3.9 \pm 0.6 \mu\text{g/mL}$ and that of mf was $7.5 \pm 1.1 \mu\text{g/mL}$ (Table 1).

Inhibitory effect of CAPE on microfilariae production and adult worms in vivo

CAPE administered at 20 mg/kg and 50 mg/kg were compared to DEC and doxycycline given at 50 mg/kg each in *in vivo* for 14 days. The effects of the treatments on mf production over a period of 120 days were represented graphically (Figure 3).

CAPE treatments were able to reduce the density of circulating mf during and after treatment. CAPE 50 reduced the density of circulating mf by over 60% during the first 14 days of treatment, and reduction in mf density continued over 100 days post treatment (pt) observation. Subsequently, there was further decline in circulating mf density until day 120, at which over 95% of mf (compared to day 0 and control) had been cleared. Reduction in mf density became significant ($P < 0.005$) from 60 days pt when compared to DEC 50 treatment. When compared to DOX 50 treatment, there was high significance ($P < 0.001$) from the beginning of treatment until the end of observation period (Figure 3). However, CAPE 20 shows less than 40% reduction in circulating

mf density during the treatment period. There was no significant difference ($P > 0.05$) in mf reduction between CAPE 20 and DOX 50, but there was a high significant difference ($P < 0.005$) in mf reduction between CAPE 20 and DEC 50 at days 15 and 30 (Figure 3). Similarly, both DEC 50 and DOX 50 show high levels of mf reduction as expected, but could not completely stop mf production (Figure 3).

Furthermore, the effect of CAPE against adult worms *in vivo*, as indicated by number of worms recovered after treatments and observation period, shows that CAPE 50 is most effective against adult worms (Table 2). The adulticidal efficacy (about 58%) of CAPE 50 was more highly significant ($P < 0.005$) than efficacy shown by other drugs (Table 2). There was no significant difference ($P > 0.05$) in the efficacy displayed by the trio, DEC, DOX and CAPE 20, against adult worms, but DEC 50 was the least efficient (Table 2).

Quantification of Wolbachia in *B. pahangi*, based on single copy WSP

Presence of the *Wolbachia* target gene insert in the cloned amplicon was confirmed by gel electrophoresis, as illustrated in the photomicrograph (Figure 4(a)). Copy numbers for the *wsp* gene of *Wolbachia* in all groups are

Table 1. In vitro activity of CAPE on adult worms and mf of *B. pahangi*. Values are mean $\pm$ SEM.

	Effect on adult worms		Effects on mf		$CC_{50}^{\dagger}$ ($\mu\text{g/mL}$)	$SI^{\ddagger}$ for adult
	LC_{100}^* ($\mu\text{g/mL}$)	$IC_{50}^{\wedge}$ ($\mu\text{g/mL}$)	LC_{100}^* ($\mu\text{g/mL}$)	$IC_{50}^{\wedge}$ ($\mu\text{g/mL}$)		
CAPE	15.4	3.9 ± 0.6	20.9	7.5 ± 1.1	162.8 ± 13.8	47.9 ± 7.3
IVERMECTIN	38.7	24.7 ± 2.4	58.6	28.5 ± 2.2	266.3 ± 15.1	10 ± 1.2
DEC	485	453.9 ± 22.3	390	287.6 ± 23.3	3109.1 ± 327.5	6.9 ± 0.8

Note: DEC: Diethylcarbamazine.

*100% death of parasite in motility assay; $\wedge$ Concentration of the agent at which 50% inhibition was achieved in motility assay.

$\dagger$ Concentration at which 50% of cells death was achieved in motility assay.

$\ddagger$ Selectivity index = CC_{50}/IC_{50} .

Figure 3. In vivo effects of CAPE, DEC, and doxycycline on *Brugia pahangi* microfilaria. Gerbils were treated with CAPE (20 mg/kg and 50 mg/kg), DEC (50 mg/kg), and doxycycline (50 mg/kg). Controls were given 10% DMSO (vehicle), and treatments were administered for two weeks. Mf density was examined every 15 days for 120 days. Data were represented as mean density of mf $\pm$ S.E.M, $n = 5$. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$.

illustrated in Figure 4(b). Interestingly, the highest dose of CAPE induced the most significant reduction of WSP among all groups. In samples exposed to CAPE 50 and

Table 2. In vivo macrofilaricidal/adulticidal activity of CAPE against *B. pahangi*.

Dose (mg/kg) i.p. × 14d (N)	No. of live worms			% reduction in worms ^a
	Male	Female	Total	
Vehicle	8.6 ± 0.7	14.4 ± 1.2	21 ± 2.2	–
50 (5)	7.0 ± 1.1	8.4 ± 1.5	15.4 ± 2.5	22.9 ± 13.3
50 (5)	7.0 ± 1.0	10.8 ± 1.5	17.8 ± 2.4	9.0 ± 17.9
20 (5)	6.2 ± 0.6	7.6 ± 1.6	13.8 ± 2.1	29.2 ± 13.9
50 (5)	4.2 ± 0.7	4.0 ± 1.3	8.2 ± 1.9	58 ± 10.37**

Notes: DOX: Doxycycline; DEC: Diethylcarbamazine; CAPE: Caffeic acid phenethyl ester.

N = Number of animals.

Data were represented as mean ± SEM.

^aPercent reduction in worm recovery was calculated relative to the mean number of worms recovered from control gerbils.

** $P < 0.005$.

doxycycline, *wsp* expressions clearly show a high significant reduction ($P < 0.005$) of *wsp* copy number, relative to the control and standard drug, DEC, groups. The CAPE 20 treated group also shows significant ($P < 0.05$) reduction in *wsp* expression compared to the control group. However, there was no significant difference ($P > 0.05$) in the expression of *wsp* in the DEC treated group compared to the control group.

Discussion

Several reports have demonstrated that the presence of mf in the peripheral blood persists even after treatment with DEC, possibly due to the microfilaricidal activity of DEC having no influence on adult worms, which live for long periods with sustained production of mf [30]. Therefore, the development of a therapy that kills the adult worm is required. Current advancements and successes in the effort to control LF employing

Figure 4. (a) Gel electrophoresis of copy number showing confirmation of gene insertion; (b) The effect of CAPE on *wsp* copy number of *Wolbachia* in *B. pahangi* treated with CAPE (20 and 50 mg/kg), doxycycline (50 mg/kg), DEC (50 mg/kg) and the control (saline/kg). CAPE concentrations and doxycycline showed significant (* $P < 0.05$, ** $P < 0.005$) reduction in *wsp* copy number over the control and standard drug, DEC. Data were represented as mean ± SEM.

anti-*Wolbachia* remedies have offered a comprehensive therapy for LF [31]. In this context, natural products have been an attractive alternative and continue to make valuable contributions as alternative controls for the parasitic worms [3,7,32]. However, the importance of *Wolbachia* endosymbionts for the development, survival, and pathogenicity of adult lymphatic filarial parasites in the human host has given an alternative avenue for the eradication of the adult filarial worm.

The present study shows that CAPE is very active against the adult worm in *in vitro* and *in vivo*. CAPE reduced worm motility and viability as assessed by the MTT reduction test. CAPE also reduced mf production more efficiently than the control, Ivermectin. Ivermectin was adopted as a control for *in vitro* studies due to previous studies indicating that it has stronger microfilaricidal and macrofilaricidal activity than DEC *in vitro* [33,34]. As well, DEC had been previously suggested to be ineffective against mf and adult worms *in vitro* [28]. The *in vitro* adulticidal activity of CAPE reported here is similar to other reports on newly a proposed class of antifilarial agents such as 3, 6- epoxy [1,5] dioxocines [35] and flavonoids like Naringenin and Hesperetin [26]. Similarly, the *in vitro* antifilarial activity of CAPE is similar to recent anti-parasitic reports of CAPE on *Leishmania infantum* promastigotes (51.0 ± 0.8 µg/ml) and amastigotes (19.0 ± 1.4 µg/ml) [23]; and *Gardia lamblia* trophozoites (63.1 ± 0.9 µg/ml) [22], although doses of CAPE reported in these studies are higher than those used in present study.

The antifilarial activity of CAPE *in vivo* shows that it is dose dependant, and 50 mg/kg of CAPE strongly reduced the circulating mf within the period of observation. Similarly, the adulticidal efficacy of CAPE was highly efficient at 50 mg/kg. The persistent reduction of mf after treatment with CAPE 50 can be attributed to its high adulticidal efficacy, which implies that there are less viable adult worms available to produce mf. Consequently, CAPE activity will aid in transmission blockage, as there will be less viable mf available for the mosquitoes vector while feeding. However, the drug of choice, DEC, showed strong microfilaricidal during the treatment period, as the mf continues to rise afterwards. It can be suggested that there are more viable worms still producing mf after treatment, as a result of DEC inability to kill the adult worms.

In the current study, a qPCR assay was used to evaluate the presence of *wsp* as an indicator of the presence of *Wolbachia* by determining the number of *wsp* transcripts present. By doing so, it was not possible to determine whether CAPE had a direct effect on *Wolbachia* or not. However, the treatment with CAPE 50 showed a decrease in number of *Wolbachia* based on the copy number, and presumably leads to a decrease in the number of worms recovered. This shows that the direct or indirect effect of CAPE 50 on *Wolbachia* might have led to the death of the worms and thus, reduced the amount of

worms recovered. Our results are similar to a previous study which suggested that removal of *Wolbachia* from *B. pahangi* leads to worm death and is also associated with fecundity [36]. Therefore, to determine the mechanism of action of CAPE, further studies must be conducted on *Wolbachia*-free filarial nematodes such as *Acanthocheilonema viteae*.

Conclusion

CAPE was demonstrated to reduce the motility and viability of both adult worms and mf *in vitro* and *in vivo*, as well as the copy number of *Wolbachia's* *wsp* gene *in vivo*. Thus, this work highlights for the first time that CAPE is a potential alternative to target LF. However, further investigation in both experimental models and humans to explore whether CAPE might be of use as an alternative antifilarial drug in humans is required.

Disclosure statement

The authors have declared that no conflict of interests exists, either financial or otherwise, from any company or other entities.

Funding

This study was funded by the Ministry of Higher Education, Malaysia [grant number FRGS FP011/2011A]; University Malaya Postgraduate Research Grant (PPP) [grant number PG085-2012B].

ORCID

Quazim O. Junaid <http://orcid.org/0000-0002-1212-6706>

References

- [1] Brady M. Seventh meeting of the global alliance to eliminate lymphatic filariasis: reaching the vision by scaling up, scaling down, and reaching out. *Parasit Vectors*. 2014;7(1):1–19.
- [2] WHO. Weekly epidemiological record. Sep 23, 2016;39(91):441–460. Available from <http://apps.who.int/iris/bitstream/10665/250245/1/WER9139.pdf>
- [3] Sashidhara KV, Singh SP, Misra S, et al. Galactolipids from *Bauhinia racemosa* as a new class of antifilarial agents against human lymphatic filarial parasite, *Brugia malayi*. *Eur J Med Chem*. 2012;50:230–235.
- [4] Ottesen E, Duke B, Karam M, et al. Strategies and tools for the control/elimination of lymphatic filariasis. *Bull World Health Organ*. 1997;75(6):491–503.
- [5] Molyneux DH, Bradley M, Hoerauf A, et al. Mass drug treatment for lymphatic filariasis and onchocerciasis. *Trends Parasitol*. 2003;19(11):516–522.
- [6] Thomsen EK, Sanuku N, Baea M, et al. Efficacy, safety, and pharmacokinetics of co-administered diethylcarbamazine, albendazole, and ivermectin for the treatment of bancroftian filariasis. *Clin Infect Dis*. 2015 Oct 20;62(3):334–41.
- [7] Al-Abd NM, Nor ZM, Al-Adhroey AH, et al. Recent advances on the use of biochemical extracts as filaricidal agents. *Evid*

- Based Complement Alternat Med. Nov 5, 2013;2013:13 pages. <http://dx.doi.org/10.1155/2013/986573>.
- [8] Bankova V. Chemical diversity of propolis makes it a valuable source of new biologically active compounds. *J ApiProd ApiMed Sci*. 2009;1(2):23–28.
 - [9] Murtaza G, Karim S, Akram MR, et al. Caffeic acid phenethyl ester and therapeutic potentials. *Biomed Res Int* 2014 May 29;2014:9 pages. <http://dx.doi.org/10.1155/2014/145342>.
 - [10] Ozturk G, Ginis Z, Akyol S, et al. The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers. *Eur Rev Med Pharmacol Sci*. 2012;16(15):2064–2068.
 - [11] Park JH, Lee JK, Kim HS, et al. Immunomodulatory effect of caffeic acid phenethyl ester in Balb/c mice. *Int Immunopharmacol*. 2004;4(3):429–436.
 - [12] Alici O, Kavakli HS, Koca C, et al. Value of caffeic acid phenethyl ester pretreatment in experimental sepsis model in rats. *Mediators Inflamm*. April 8, 2015;2015:6 pages. <http://dx.doi.org/10.1155/2015/810948>.
 - [13] Tolba MF, Azab SS, Khalifa AE, et al. Caffeic acid phenethyl ester, a promising component of propolis with a plethora of biological activities: a review on its anti-inflammatory, neuroprotective, hepatoprotective, and cardioprotective effects. *IUBMB Life*. 2013;65(8):699–709.
 - [14] Velazquez C, Navarro M, Acosta A, et al. Antibacterial and free-radical scavenging activities of Sonoran propolis. *J Appl Microbiol*. 2007;103(5):1747–1756.
 - [15] Meyuhas S, Assali M, Huleihil M, et al. Antimicrobial activities of caffeic acid phenethyl ester. *J Mol Biochem*. 2015;19(4(2)): 21–31.
 - [16] da Silva Cunha IB, Salomão K, Shimizu M, et al. Antitrypanosomal activity of Brazilian propolis from *Apis mellifera*. *Chem Pharm Bull (Tokyo)*. 2004;52(5):602–604.
 - [17] Salomão K, de Souza EM, Henriques-Pons A. Brazilian green propolis: effects *in vitro* and *in vivo* on *Trypanosoma cruzi*. *Evid Based Complement Alternat Med*. 2011 Feb 13;2011:11 pages. <http://dx.doi.org/10.1093/ecam/nep014>.
 - [18] Monzote FL, Sarriego RI, García PM, et al. Activity of Cuban propolis extracts on *Leishmania amazonensis* and *Trichomonas vaginalis*. *Nat Prod Commun*. 2011;6(7):973–976.
 - [19] Ferreira FM, Castro RA, Batista MA, et al. Association of water extract of green propolis and liposomal meglumine antimoniate in the treatment of experimental visceral leishmaniasis. *Parasitol Res*. 2014;113(2):533–543.
 - [20] Santana LC, Carneiro SMP, Caland-Neto LB, et al. Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of *Leishmania amazonensis*. *Nat Prod Res*. 2014;28(5):340–343.
 - [21] Freitas S, Shinohara L, Sforzin J, et al. In vitro effects of propolis on *Giardia duodenalis* trophozoites. *Phytomedicine*. 2006;13(3):170–175.
 - [22] Alday-Provencio S, Díaz G, Rascon L, et al. Sonoran propolis and some of its chemical constituents inhibit *in vitro* growth of *giardia lamblia* trophozoites. *Planta Med*. 2015;81(9):742–747.
 - [23] Abamor ES. Antileishmanial activities of caffeic acid phenethyl ester loaded PLGA nanoparticles against *Leishmania infantum* promastigotes and amastigotes *in vitro*. *Asian Pac J Trop Med*. 2017;10(1):25–34.
 - [24] Barthold SW, Bayne K, Davis M. Guide for the care and use of laboratory animals. Washington (DC): National Academy Press; 2011.
 - [25] Rao R, Weil GJ. In vitro effects of antibiotics on *Brugia malayi* worm survival and reproduction. *J Parasitol*. 2002;88(3):605–611.
 - [26] Lakshmi V, Joseph S, Srivastava S, et al. Antifilarial activity *in vitro* and *in vivo* of some flavonoids tested against *Brugia malayi*. *Acta Trop*. 2010;116(2):127–133.
 - [27] Sashidhara KV, Rao KB, Kushwaha V, et al. Synthesis and antifilarial activity of chalcone–thiazole derivatives against a human lymphatic filarial parasite, *Brugia malayi*. *Eur J Med Chem*. 2014;81:473–480.
 - [28] Misra-Bhattacharya S, Katiyar D, Bajpai P, et al. 4-Methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one: a novel DNA topoisomerase II inhibitor with adulticidal and embryostatic activity against sub-periodic *Brugia malayi*. *Parasitol Res*. 2004;92(3):177–182.
 - [29] Mathew N, Misra-Bhattacharya S, Perumal V, et al. Antifilarial lead molecules isolated from *Trachyspermum ammi*. *Molecules*. 2008;13(9):2156–2168.
 - [30] Johnston KL, Wu B, Guimarães A, et al. Lipoprotein biosynthesis as a target for anti-Wolbachia treatment of filarial nematodes. *Parasit Vectors*. 2010;3(1):99. <https://doi.org/10.1186/1756-3305-3-99>
 - [31] Pathak M, Verma M, Srivastava M, et al. Wolbachia endosymbiont of *Brugia malayi* elicits a T helper type 17-mediated pro-inflammatory immune response through Wolbachia surface protein. *Immunology*. 2015;144(2):231–244.
 - [32] Kumar K, Kumar M, Verma M, et al. Anti-wolbachia: a herbal approach to treatment of Filariasis. *Spatula DD-Peer Rev J Complementary Med Drug Discov*. 2014;4(2):101–108.
 - [33] Rao RU, Huang Y, Fischer K, et al. *Brugia malayi*: effects of nitazoxanide and tizoxanide on adult worms and microfilariae of filarial nematodes. *Exp Parasitol*. 2009;121(1):38–45.
 - [34] Misra S, Verma M, Mishra SK, et al. Gedunin and photogedunin of *Xylocarpus granatum* possess antifilarial activity against human lymphatic filarial parasite *Brugia malayi* in experimental rodent host. *Parasitol Res*. 2011;109(5):1351–1360.
 - [35] Sashidhara KV, Kumar A, Rao KB, et al. In vitro and *in vivo* antifilarial activity evaluation of 3, 6-epoxy [1, 5] dioxocenes: a new class of antifilarial agents. *Bioorg Med Chem Lett*. 2012;22(4):1527–1532.
 - [36] Chirgwin SR, Coleman SU, Porthouse KH, et al. Removal of Wolbachia from *Brugia pahangi* is closely linked to worm death and fecundity but does not result in altered lymphatic lesion formation in Mongolian gerbils (*Meriones unguiculatus*). *Infect Immun*. 2003;71(12):6986–6994.