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Full Length Research Paper

The aqueous extract of *Codiaeum variegatum* and its fractions exhibit in vitro antiparasitic activity against *Giardia lamblia* and *Trichomonas vaginalis*

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Abstract

The leaf decoction of *Codiaeum variegatum* is used by Cameroonian local population in the treatment of intestinal infections. The present study was carried out to investigate the antiparasitic activity of the aqueous extract of *C. variegatum* and its fractions against axenic culture of *G. lamblia* and *T. vaginalis*. Trophozoites of *G. lamblia,* and *T. vaginalis* were incubated separately with different concentrations of leaf aqueous extract of *C. variegatum* fractions and sub-fractions for 24 and 48 hours. Metronidazole was used as the positive control. The viability of trophozoites determined by using the quantitative colorimetric [3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) technique. All the extract fractions and sub-fractions were active against *G. lamblia* and *T. vaginalis*. The sub-fraction SF9B showed the highest antiparasitic activity against *G. lamblia* and *T. vaginalis* after 48 hours, but remain lower compared to metronidazole. Sub-fraction SF9, and SF9B2 showed moderate antiparasitic activities. Methanol, ethyl acetate fractions and aqueous extract exhibited low antiparasitic activities. However, no significant difference was observed between the anti-trichomonal activity of the methanol fraction compared to that of the SF9B2 (89.35 ± 5.02µg/mL) sub-fraction of *C. variegatum* after 48h of incubation. The aqueous extract, methanol, fraction, ethyl acetate, and sub-fractions SF9, SF9B and SF9B2 isolated from *Codiaeum variegatum* exhibited giardicidal and antitrichomonal activities therefore supporting the medicinal usage of this plant against intestinal infections.

Key words: *Codiaeum variegatum, Giardia lamblia, Trichomonas vaginalis.*

INTRODUCTION

Background

Giardia lamblia (also known as *Giardia intestinalis* or *Giardia duodenalis*) is the most common intestinal parasite of humans identified worldwide (Adam, 2001). This flagellated protozoan parasite frequently Human disease, mediated by damage of the enterocytes, loss of the brush border of the epithelial cells of the intestine, shortening of microvilli and altered epithelial barrier function, comprises acute to aqueous diarrhoea, flatulence, steatorrhea, nausea, abdominal pain, vomiting and, as complications in case of chronic disease, malabsorption and weight loss (Vivancos et al., 2018; Loderstädt and Frickmann, 2021). Despite the fact that giardiasis has a global distribution, higher infection rates (20-60%) have been reported in developing countries, mainly among socially and economically deprived populations. In developed countries, infections rates are lower, and *G*. *lamblia* is often involved in numerous outbreaks that have been attributed to an inappropriate water treatment (Carvalho, et al., 2014). *G. lamblia* was included in the World Health Organization (WHO) Neglected Diseases Initiative in a group of diseases including pathogens that have a common link with poverty (Savioli et al., 2006). Giardiasis is a common cause of diarrhea in nursery and primary school (Thompson, 2000), especially in undernourished individuals which in turn gives rise to nutritional deficiencies leading to growth failure and cognitive impairment.

Trichomonas vaginalis parasitizes the urogenital human tract causing trichomoniasis, the most prevalent non-viral sexually transmitted disease worldwide, being responsible for 248 million new cases annually (WHO, 2012). After colonization, the parasite causes vaginitis, urethritis, and prostatitis (Petrin et al., 1998). Moreover, Trichomoniasis has been found to be associated with various health complications including pelvic inflammatory disease (PID), significant pregnancy complications, cervical cancer, prostatitis and infertility (Johnston and Dubbink, 2008). Significant pregnancy complications include pre-term labour, low birth weight and premature rupture of membranes (Glehn et al., 2017; Cornelius et al., 2012) Trichomonas vaginalis infection has also been associated with a high risk of acquisition and transmission of human immunodeficiency virus (HIV) (De Waaij et al., 2017) and pelvic inflammatory disease (Cherpes et al., 2006). Importantly, trichomoniaosis acts as a cofactor in human immunodeficiency virus (HIV) transmission and acquisition (Servillo et al., 2001; Van Der Pol et al., 2008). Since several decades, the most prescribed antigiardial and antitrichomonal drug is metronidazole, a 5-nitroimidazole with curing rates ranging between 80 to 95% (Ali and Nozaki, 2007; Thompson et al., 1993). Despite its efficiency, this treatment had undesirable side effects, and treatment failures are common with evidence of drug resistance (Shwebke et al., 2006; Dunn et al., 2010). Therefore, there is an increasing interest for the development of new antigiardial and antitrichomonal agents that can be effective against these parasites and less harmful to the patients. In this regards, natural products, especially medicinal plants, are known as a potent source of bioactivemolecules. *Codiaeum variegatum*(Euphorbiaceae) is a Cameroonian medicinal plant traditionally use in the treatment of intestinal infections. The leaf aqueous extract of this plant has been identified in our previous studies with a pronounced anti-amoebic activity among fifty-five medicinal plants collected from a survey within traditional healers in the Noun division (West region, Cameroon) (Moundipa et al., 2005). A bioassay-guided fractionation of this aqueous extract was carried out against trophozoites of *E. histolytica* in an axenic culture, and it was reported that several sub-fractions had significant higher anti-amoebic activity compared to the unfractionated aqueous extract (Mfotie et al., 2014). The anti-amoebic activity of the most potent sub-fraction (SF9B) was confirmed with the morphological characteristics of induced death in trophozoites through the destabilization of Gal/GalNAc lectin, an abundant parasite cell surface protein. Differential gene expression analysis using high-throughput RNA sequencing implies the potential mechanism of its anti-amoebic activity by targeting ceramide, a bioactive lipid involved in disturbance of biochemical processes within the cell membrane including differentiation, proliferation, cell growth arrest and apoptosis (Mfotie et al., 2014). An *in vivo* study aimed at evaluating the sub-chronic toxicity of this plant showed no significant adverse effect of *Codiaeum variegatum* with respect to the measured parameters (biochemical markers, weight, etc.) (Mfotie et al .2018). Knowing that the growth of *E. histolytica*, *G*. *lamblia* and*T. vaginalis* is inhibited by metronidazole, and regarding the mechanism of action of the subfraction SF9B which targets ceramide found in these three parasites, we hypothesized that the leaf aqueous extract of *C. variegatum*, and its fractions may also have inhibitory effect on *G*. *lamblia* and *T. vaginalis*. Therefore, the present study focused on the evaluation of their *in vitro* antiparasitic activities against axenic culture of *G. lamblia* and *T. vaginalis*.

MATERIAL AND METHODS

Preparation of plant extract and isolation of fractions

Leaves of *C. variegatum* were harvested, thoroughly

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washed with tap water, rinsed with distilled water, dried at room temperature and grinded into fine powder. The powder obtained was used to prepare the aqueous extract and the methanol, ethyl acetate fractions and different sub-fractions (SF9, SF9B, SF9B2) were isolated according to the protocol previously described (Mfotie et al., 2014).

In vitro culture of G. lamblia and T. vaginalis

The strain of *G. lamblia* (Portland1) and the symptomatic strain of *T. vaginalis* were cultivated on TYI-S-33 axenic medium (Carvalho et al., 2014). Trophozoites were harvested at the log-phase of growth after 72 hours post-inoculation, by chilling the tubes on an ice bath for 10 min. They were collected by centrifugation at 250 g for 15 min at 4°C. Pooled cells were suspended in sterile phosphate buffered saline (PBS; pH 7.2)andthe total number of trophozoites were counted microscopically with a haemocytometer on a Neubauercell-counterchamber. The cell number was adjusted with culture medium to prepare an inoculum of 10^5 parasites/mL.

In vitro antiparasitic assay

Trophozoites of *G. lamblia,* and *T. vaginalis* were incubated separately with different concentrations of *C. variegatum* aqueous extract, fractions and sub-fractions for 24 and 48 hours. Metronidazole (Sigma) was used as the positive control. The viability of trophozoite was examined on a microscope after addition of trypan blue (Sigma-Aldrich) (Bansal et al., 1987). This assay was also confirmed by using the quantitative colorimetric [3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)technique, as described by Ponce-Macotela et al., 2001. The percentages of inhibition were calculated according to the following formula:Inhibition percentage = $[(OD_{control} - OD_{test}) / OD_{control}] \times 100$ where OD is the optical density. The control represents 100% of viable parasites in the culture and the results were compared with the inhibition of metronidazole which is the reference drug against giardiasis and trichomoniaosis. The half maximal inhibitory concentration (IC50) was defined as the concentration of crude extract required to inhibit cell growth by 50%. The criteria used for defining the degree of activity of plant extracts in terms of inhibiting Giardia seemed to vary across tested products. Therefore, the following criteria previously proposed were used to classify the active fractions and sub-fractions: $IC_{50} \leq 100$ µg/mL as highly active; $100 < IC_{50} \leq 250$ µg/mL as active, $250 < IC_{50} \leq 500$ µg/mL as moderately active; $IC_{50} \geq 500$ µg/mL as inactive (Amaral et al., 2006).

Statistical analysis

The effect of tested samples on the viability of *G. lamblia,* and *T. vaginalis* trophozoites, was analyzed by counting data following multiple comparisons using a factorial design layout. Each experiment was carried out in triplicate and data were submitted to the one-way analysis of variance (ANOVA) and the means were compared by Turkey's test. All analyses were conducted in SAS 9.2 for Windows (SAS Institute Inc., Cary, NC) and *p*-values less than 0.05 were considered as statistically significant.

The 50% efficient concentrations (EC_{50}) were calculated from concentration-response curves by using the non-linear regression analysis.

RESULTS

Table 1. Efficient concentrations (IC_{50})of the aqueous extract and fractions against trophozoites of *G. lambia* and *T. vaginalis* respectively.

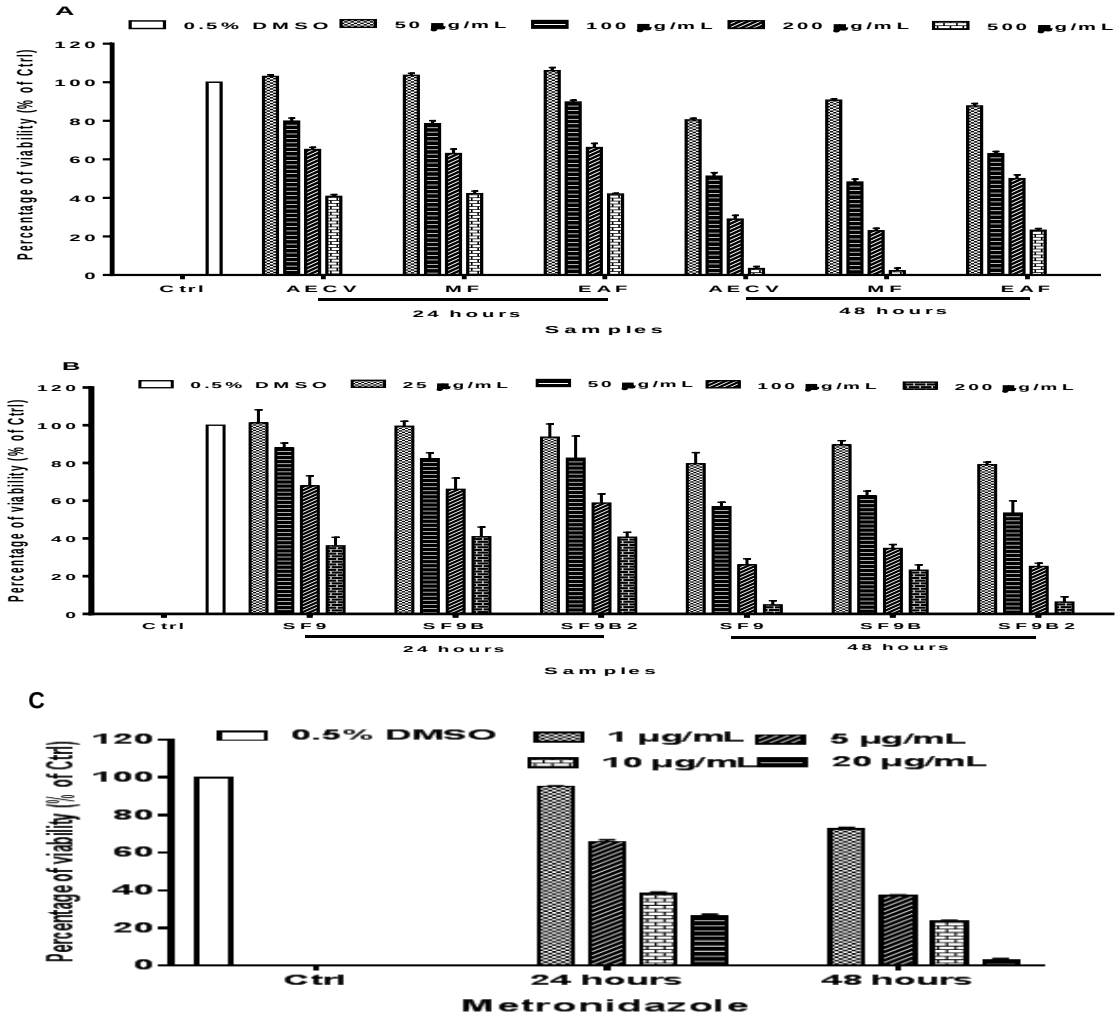
Tested Samples	EC_{50}(µg/mL)			
	giardicidal		Antitrichomonal	
	24 hours	48 hours	24 hours	48 hours
AECV	135.53 ± 7.79	82.19 ± 27.48	192.66 ± 4.12	118.90 ± 8.20
MF	74.43 ± 5.23	33.29 ± 5.99	161.25 ± 9.12	92.75 ± 3.61[a]
EAF	194.83 ± 43.85	123.38 ± 40.37	265.75 ± 43.35	180.08 ± 22.45
SF9	50.54 ± 1.66	19.40 ± 2.17	100.70 ± 8.90	56.61 ± 6.17
SF9B	33.48 ± 2.77	14.89 ± 1.37	75.24 ± 13.22	32.88 ± 6.79
SF9B2	72.47 ± 3.91	47.83 ± 6.34	137.36 ± 5.46	89.35 ± 5.02[a]
MTZ	8.96±0.02	2.98±0.46	7.667±0.799	3.205±0.551

AECV: Aqueous extract of *Codiaeum variegateaum*, MF: Methanol fraction was obtained after washing the AECV with methanol, EAF: Ethylacetate fraction was isolated from MF, SF9 was isolated from silica gel column fractionation of EAF, SF9B is the filtrate obtained from ethanol washing of SF9, and SF9B2 is the white crystal powder precipitated from SF9B, MTZ: Metronidazole. Data were obtained from three experiments and are presented as mean ± standard deviation (SD) compared to the control (DMSO)

Giardicidal activity of leaf aqueous extract of *C. variegatum* and its fractions

The Portland1 strain of *G. lamblia* was grown axenically on TYI-S-33 culture medium and incubated with different *C. variegateaum* aqueous crude extract, fractions (the methanol, ethyl acetate fractions) and sub-fractions (SF9, SF9B, SF9B2). Metronidazole (MTZ) was used as

Figure 1: Inhibitory effect of extract, fractions (A), sub-fractions (B) and MTZ (C) after 24 and 48 hours of treatment on trophozoites of *Giardia lambia*.

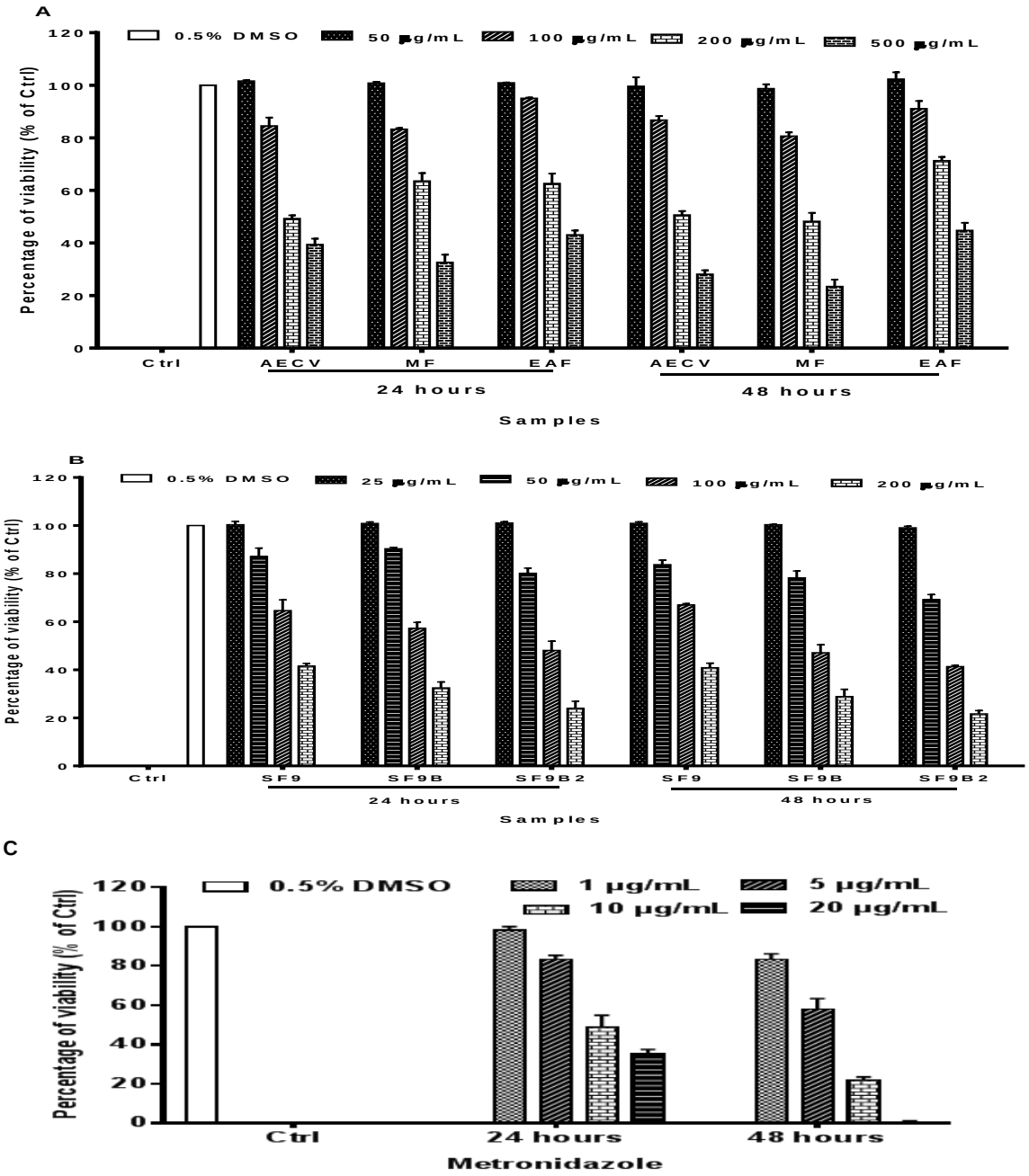


AECV: Aqueous extract of *Codiaeum variegateaum*, MF: Methanol fraction was obtained after washing the AECV with methanol, EAF: Ethylacetate fraction was isolated from MF, SF9 was isolated from silica gel column fractionation of EAF, SF9B is the filtrate obtained from ethanol washing of SF9, SF9B2 is the white crystal powder precipitated from SF9B, MTZ: Metronidazole. Data were obtained from three experiments and are presented as mean ± standard error of mean (SEM) compared to the control (0.5% DMSO).

reference drug. As result, extract, fractions and sub-fractions exhibited giardicidal activities in a concentration dependent manner. The period of incubation as well as fractionation process significantly influence *giardia* growth inhibition. In fact, the giardicidal effect increased progressively according to the fractionation step. By plotting the viability rate against concentration, IC_{50} of the extract fractions and sub-fractions were determined (Table 1). Despite the fact that none of them induced total mortality (0% viability) in the performed assay, we did not observe any stationary phase in Giardia parasites growth, (Figure 1).

We noticed the increase of activities with time for all the extract, fractions and sub-fractions. Methanol fraction, and SF9, SF9B, and SF9B2 sub-fractions were highly active against *G. lamblia*; whereas aqueous extract and ethyl acetate fractions were active after 24h. Among fractions, methanol fraction (MF) with an IC_{50} of 33.29 ± 5.99µg/mL exhibited the highest giardicidal activity compared to the ethyl acetate fraction (IC_{50} =123.38 ± 40.37µg/mL) after 48h of incubation. However, the ethyl acetate fraction (EAF) (IC_{50} =123.38 ± 40.37µg/mL) showed the giardicidal activity not significantly different from that of the aqueous extract (AECV) (IC_{50} =82.19 ± 27.48 µg/mL) after 48h of incubation. Among fractions and sub-fractions, SF9 and SF9B showed the highest giardicidal activity (IC_{50} =19.40 ± 2.17µg/mL and IC_{50} =14.89 ± 1.37µg/mL respectively) after 48 h and remained significantly lower compared to metronidazole (IC_{50} =2.98±0.46 µg/mL).

Figure 2. Inhibitory effect of extract, fractions (A) and sub-fractions (B) after 24 and 48 hours of treatment on trophozoites of *Trichomonas vaginalis.*



AECV: Aqueous extract of *Codiaeum variegateaum*, MF: Methanol fraction was obtained after washing the AECV with methanol, EAF: Ethylacetate fraction was isolated from MF, SF9 was isolated from silica gel column fractionation of EAF, SF9B is the filtrate obtained from ethanol washing of SF9, SF9B2 is the white crystal powder precipitated from SF9B , MTZ: Metronidazole. Data were obtained from three experiments and are presented as mean ± standard deviation (SD) compared to the control (0.5% DMSO).

Anti-trichomonal activity of *C. variegatum* extract and its fractions

The aqueous extract, fractions and sub-fractions of *C. variegatum* were also tested against the trophozoïtessymptomatic strain of *T. vaginalis*cultivated on TYI-S-33
axenic medium, and metronidazole (MTZ) was used as reference drug. *C. variegateaum* extract and its fractions showed anti-trichomonal activities which varied in a concentration-dependent manner and also influenced by the incubation time. The percentage of trichomonal viability was plotted against the concentration of tested samples (figure 2) which therefore allowed the determination of IC_{50} of the extract, fractions and sub-fractions (Table 1). All the extracts, fractions and sub-fractions exhibited anti-trichomonial activity. After 24h,

SF9B was highly active; SF9, SF9B2, methanol fraction and aqueous extract were active; ethyl acetate fraction was moderately active. The methanol fraction (MF) exhibited the highest anti-trichomonal activity among fractions with IC_{50}s of 161.25 ± 9.12 µg/mL and 92.75 ± 3.61µg/mL after 24h and 48h respectively. However, this activity of MF remains significantly lower than those of SF9 and SF9B sub-fractions with IC_{50}s of 100.70 ± 8.90 µg/mL and 75.24 ± 13.22 µg/mL after 24h; 56.61 ± 6.17µg/mL and 32.88 ± 6.79µg/mL after 48h of incubation respectively). However, no significant difference was observed between the anti-trichomonal activity of the methanol fraction compared to that of the SF9B2 (89.35 ± 5.02µg/mL) after 48h of incubation. Among fractions and sub-fractions of *C. variegatum*, the ethyl acetate fraction (EAF) exhibited the lowest anti trichomonal activity (265.75 ± 43.35µg/mL after 24h and 180.08 ± 22.45µg/mL after 48h respectively).

DISCUSSION

MTZ has long been used as the standard treatment of protozoan parasitic infections such as *E. histolytica*, *T. vaginalis* and *G. lamblia*. However, new alternative drugs obtained from natural sources are being investigated for the treatment of these infections due to the occurrence of MTZ-protozoan parasites resistant strains in addition to MTZ adverse effects (Gökmen et al., 2019; Rigo et al., 2017; Wassmann et al., 1999; Kapoor et al., 1999). Plant substances are being used as alternative sources for the treatment of various diseases, including parasitic diseases due to their useful therapeutic activities. Accordingly, several studies have been conducted to develop alternative drugs for the treatment of protozoan parasitic infections. Our previous studies intend to valorize the medicinal value of *Codiaeum variegatum* (var. *mollucanum*) in the treatment of intestinal amoebiasis. Therefore, the current investigation is realized in order to strengthen the local usage of this medicinal plant in the treatment of other protozoan parasitic infections such as *T. vaginalis* and *G. lamblia* infections. Several methods have been used to evaluate the cytotoxicity and viability of cells, including dye exclusion (trypan blue), colorimetric assays (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT)), fluorometric assays (alamarBlue assay and CFDA-AM assay), and luminometric assays (ATP assays) (Aslantürk et al., 2017). Previous studies have investigated plant extract activity against Giardia and Trichomonas by using dye exclusion and colorimetric assays to count viable cells (De Almeida et al., 2007; Wabo et al., 2011; Harris et al., 2000; Moon et al., 2006). In the present finding, the MTT assay was selected for the detection of viable Giardia and Trichomonas trophozoïtes grown on axenic media exposed to crude extracts, fractions and sub-fractions from *C. variegatum*. In fact, reduction of tetrazolium salt to formazan indicates an indirect evaluation of oxidative metabolism within the parasitic cells (Moon et al., 2006). In the present study, the sub-fractions of *C. variegatum* exhibited highly active antigiadial and antitrichomonal effect *in vitro* after 48 hours, fractions and aqueous extract showed active antiparasitic effect after 48 hours. Rayan et al., (2015) tested the activity of methanolic and aqueous *Terminalia ferdinandiana* fruit extracts against *G. duodenalis* and found the IC_{50} values of 704 µg/mL (inactive) and 143 µg/mL (active), respectively which is lower than those recorded in this study.Calzada et al., (2006) tested the *in vitro* activity of 26 plants against *Giardia lamblia* and found that Dorsteniacontrajerva, Senna villosa, and Rutachalepensis showed high activity against *Giardia* with IC_{50} values lower that 38 µg/mL. Compared with IC_{50} values reported in previous studies, high-to moderate activities of crude extracts, fractions and sub-fractions were observed in this study (Pinong et al., 2020). Furthermore, *Persea americana* has been reported as the most effective plant on *T. vaginalis* (Jimenez et al., 2013). In this study, the IC_{50} values of the chloroformic and ethanolic extracts of *P. Americana* seeds were obtained at concentration of 0.524 and 0.533 µg/ml, respectively, in comparison with IC_{50} at 0.037 µg/ml for metronidazole (Taran et al., 2006). The effect of *Persea americana* was significantly stronger than *C. variegatum*, although the efficacy of *Persea americana* was evaluated after 72 hours of incubation. The antitrichomonal effect ethanol and alkaloids extracts of *H. myrtifolium* was investigated, and it was found to be effective in the lysis of *T. vaginalis* trophozoite at the concentration of 400µg/mL and 150 µg/mL after 48 h respectively (Gökmenet al., 2019). The antitrichomonal activity of *C. variegatum* extract, fractions, and sub-fractions were higher than those of the *H. myrtifolium* ethanol extract as well as alkoid extract isolated from the same plant. Phytochemical screening of C. Variegatum extract showed the presence of several secondary metabolites including alkaloids (Ogunwenmo et al., 2007) which are known for their high antiparasitic activity (Gökmenet al., 2019: De Brum et al., 2011: Da silva et al., 2007).

CONCLUSION

The aqueous extract, methanol, fraction, ethyl acetate, sub fraction SF9, SF9B and SF9B2 of *Codiaeum variegatum* exhibited giardicidal and antitrichomonal activities. Although these plant extract, fractions and sub-fractions showed significant activity against *G. lamblia* and *T. vaginalisin vitro*, further *in vivo* studies are required to validate these results before its potential application in humans

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Statement of ethics

This is an *in vitro* study that does not involves human.

Conflict of Interest Statement

The authors have no conflict of interest to declare

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Author Contributions

SN Pechangou, and Kapil Goyal carried out all the *in vitro* experiment reported in this manuscript. SN Pechangou, EM Njoya, FN Njayou, PF Moundipa and R Sehgal designed the study. All authors read and approved the final manuscript.

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