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Research Article

Phytochemical Composition and *In vitro* Anthelmintic activity of *Combretum micranthum* G. Don Leaf Extracts on *Haemonchus contortus* of Small Ruminants

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Abstract | The incidence of gastrointestinal parasitosis associated with resistance to conventional anthelmintics observed in small ruminants has led to the development of alternative strategies focusing on the use of medicinal plants. Thus, the present study was carried out to assay total phenolics and evaluate the *in vitro* anthelmintic efficacy of aqueous and hydroacetone extracts of *Combretum micranthum* leaves on the parasite *Haemonchus contortus*. Following phytochemical assay, five increasing concentrations (0.6; 1.2; 2.4; 4.8 and 9.6 mg/ml) of the two extracts were prepared and tested for egg hatching and larval development in the presence of positive (Albendazole) and negative (PBS) controls. Aqueous and hydroacetonic extracts of *C. micranthum* significantly inhibited ($p < 0.05$) parasite life stages comparatively to negative control (PBS). Similarly, concentrations of both plant extracts inhibited egg hatching and larval development of the parasite in a dose-dependent manner. The inhibitory concentration 50 (IC_{50}) for egg hatching ranged from 2.189 and 2.994 mg/ml for the hydroacetone and aqueous extracts respectively, and that for larval development was 1.970 for the hydroacetone extract and 2.761 mg/ml for the aqueous extract. The addition of polyvinylpolypyrrolidone (PVPP) to the extracts showed that the tannins present were responsible for the bioactivity observed in both plant extracts. These results show that plant extracts have an effect on *H. contortus* and could serve as an alternative to synthetic anthelmintics. However, *in vivo* anthelmintic and toxicity tests should be considered in order to confirm their use by the farming community.

Keywords | *Combretum micranthum*, anthelmintic activity, *Haemonchus contortus*, parasite, small ruminants, Burkina Faso.

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INTRODUCTION

Pathologies caused by gastrointestinal parasites remain a major health concern in small ruminant farming. Through their harmful effects on infested hosts, gastrointestinal strongyles reduce the productivity of grass-fed ruminants, with negative repercussions on the economic profitability of the whole farm for farmers. *Haemonchus*

contortus infection of small ruminants is one of the most common animal parasitoses (80%) in Burkina Faso (Belem et al., 2005). To deal with this, breeders usually use conventional anthelmintics. Unfortunately, in recent years these molecules have become ineffective against these helminths, mainly due to their misuse and the resistance developed by the parasites themselves towards synthetic anthelmintics (Hoste et al., 2018). To overcome this shortcoming, a new approach aimed at enhancing traditional recipes based on medicinal plants is being initiated for better management of small ruminant parasitosis. A veterinary ethnobotanical study was conducted in the Bwa community of Burkina Faso to identify traditional medicinal practices for the care of small ruminant pathologies (Tianhoun et al., 2023a). According to the farmers surveyed in our previous study, *Combretum micranthum* plant leaves have an anthelmintic effect on gastrointestinal nematodes of small ruminants. Other therapeutic uses in traditional medicine have been reported by various authors. For Gueyé (2019), *C. micranthum* (Kinkéliba) leaves are classically used for coughs, bronchitis, constipation, malaria, bilious hematuric fever and all hepatobiliary affections. Leaf decoction is also used to soothe colic, diarrhea and dysentery (Hama et al., 2019). To provide a scientific contribution to the Bwa community's assertion, the present study has been initiated by evaluating the *in vitro* anthelmintic activity of *C. micranthum* extract against *H. contortus*.

MATERIAL AND METHODS

Study framework

The study was carried out in the laboratory located at the Saria experimental station which has a northern Sudanian climate with a rainy season from June to October and a dry season from November to May. Average annual rainfall is 800 mm.

Plant material

Combretum micranthum leaves were collected in October 2021 in the urban commune of Dédougou at the Boucle du Mouhoun region of Burkina Faso. They were harvested, dried at room temperature, then ground to a powder before being stored in the laboratory, protected from dust and humidity.

Animal material

Faeces from two donor sheep previously artificially infested with *H. contortus* L_3 larvae were used to obtain parasite eggs for immediate *in vitro* biological testing in the laboratory.

Aqueous and hydroacetone extract preparation

Plant powder (100 g) were macerated under mechanical stirring with 1000 ml of distilled water (during 24 h) or hydroacetonic solution (during 72 h) 80: 20 v/v. After the filtering of extracts and vacuum concentration of the acetone phase, the supernatants were freeze-dried and then packaged at 4°C until the use.

Phytochemical testing

Phytochemical testing consisted of phenolic assay by determining total polyphenols and flavonoids contents using the method described by Meda et al. (2010); and condensed tannins content according the method of Agbangnan et al. (2012).

Total polyphenols assay: Total polyphenols were determined by mixing 125 µl of extract from each plant (at 0.1mg/ml in water) with 625 µl of Folin-ciolcalteu reagent (0.2 N). After 5 min incubation in the dark, 500 µl of sodium carbonate (Na2CO3, 75 g/L) were added to the mixture. The resulting solution was again incubated for 2 h in the dark, before total polyphenol levels were determined at 760 nm against a gallic acid calibration curve. The 125 µl distilled water + 625 µl Folin-Ciacalteu + 500 µl Na2CO3 mixture was treated like the samples and used as a blank. Each test was repeated 3 times and results expressed as mg (gallic acid equivalent) GAE/100 mg extract.

Total flavonoids assay: The total flavonoids contents were carried out using a homogenized mixture of 625 µl of plant solution of each plant (at 0.1 mg/ml in methanol) with 625 µl of aluminum trichloride (AlCl3) diluted to 2% in methanol. After 10 min incubation in the dark, flavonoid concentrations were measured at 415 nm, using a quercetin calibration curve. The test was repeated 3 times and the average calculated and expressed as mg (quercetin equivalent) QE/100 mg extract.

Condensed tannin assay: Three test tubes were prepared for each sample. Each tube received 0.5 ml of extract sample diluted to 1/100 followed by 1 ml of freshly prepared vanillin sulfate (1g in 100 ml of 70% sulfuric acid). Solutions were thoroughly mixed and left in a water bath (30°) for 15 minutes in the dark. The absorbances of each sample were read at 500 nm and the values expressed in mg (catechin equivalent) CE/100 mg extract.

In vitro anthelmintic tests

Five (05) increasing concentrations (0.6; 1.2; 2.4; 4.8; 9.6 mg/ml) of each extract were prepared by cascade dilution from 400 mg of each extract in 4 ml PBS (phosphate buffer saline) solution. The biological effects of the plant extracts were assessed using egg hatch inhibition and larval development tests.

Collecting and preparing *Haemonchus contortus* egg: Fresh faeces (sample of 200 g) from the two donor sheep

were collected directly from the rectum and placed in plastic bags before being processed in the laboratory in 1 liter of tap water. The mixture produced a relatively liquid suspension which was filtered through several sieves to remove coarse plant debris. The method described by Koffi Yao et al. (2018) was used to recover the eggs. To do this, the mixture was dispensed into 15-ml centrifuge tubes and then centrifuged for 10 minutes at 2 000 rpm. The supernatant obtained in the tubes was poured off, and 10 ml of saturated NaCl solution was added to the pellet at the bottom of the tube. This new suspension was also centrifuged at 1500 rpm for 10 minutes. The eggs in the upper meniscus of the tubes were collected in tubes and rinsed with distilled water. Egg concentration was estimated by counting the number of eggs in 10 µl of suspension on a microscope slide. A final concentration of 500 eggs/ml was obtained by concentrating the egg suspension with distilled water.

Egg hatch inhibition test: Firstly, approximately 100 eggs in 200 µl of egg suspension were pipetted into each well of two 24-well culture plates. Next, 200 µl of increasing concentrations of the aqueous and hydroacetone extracts of *C. micranthum* were added to give final concentrations of 0.6; 1.2; 2.4; 4.8 and 9.6 mg/ml in each well. Positive control (Albendazole 2 mg/ml) and negative control (PBS) were used, with 200 µl of egg suspension in each well for comparison. Four replicates were performed for each concentration of the two plant extracts and controls before the plates were incubated at 27°C for 48 h. After 48 h, two drops of formalin (10%) were added to each well of both plates to stop the assay. Percentage inhibition of egg hatching was calculated using the following formula from Coles et al. (1992):

$$Inhibition\ percentage\ (\%) = 100\ x\ (1 - \left(\frac{Pt}{Pc}\right))$$

Where 1 represents the total number of eggs, Pt the number of eggs hatched in the treated group and Pc the number of eggs in the negative control group.

Larval development inhibition test: The method described by Vernerova et al. (2009) was used. One hundred (100) µL of an *H. contortus* egg suspension was placed in each well of a 24-well plate and incubated for 24 h. After 24 h incubation, 50 µL of nutrient solution (Agar noble, DIFCO: 2%) and 100 µL of aqueous and hydroacetone extract concentrations were added to the larval solution to obtain final solutions of 0.6; 1.2; 2.4; 4.8 and 9.6 mg/ml before incubation at 27°C for 6 days. Negative (10% PBS) and positive (Albendazole 2 mg/ml) reference controls were set up. Each treatment was repeated four times. At the end of the six-day incubation period, the test was stopped by adding two drops of formalin (10%) to each well. The percentage of inhibition of larval development (IDL) was calculated as follows:

$$\text{IDL percentage} = \frac{\text{L3 larvae number}}{\text{L1 larvae number + L3}}\ \text{X 100}$$

Determining the role of tannins with PVPP

To confirm the role of tannins in anthelmintic effects, another incubation series was carried out for three (03) treatments: i) the negative control (PBS), ii) the maximum dose of the extract to be tested (9.6mg/ml) with polyvinyl-polypyrrolidone (PVPP) and a pre-incubation period of 3 h to bind the polyphenolic compounds (before exposure of eggs and larvae) and iii) the maximum incubation dose without PVPP (Makkar et al., 1995).

Statistical analyses

Collected data were entered and subjected to an analysis of variances to compare the means of the treatments applied, using the Tukey Kramer test at a statistical significance level of 0.05, with CoStat software (version 6.20.4) to assess the behavior effect of each plant extract. Beforehand, the data were log-transformed (log (x+1)) to obtain a normal distribution. The effect of concentrations was determined using the non-parametric Kruskall-Wallis test to assess the effect of plant extract concentrations. Inhibitory Concentration 50 (IC_{50}) was calculated for each extract using IBM SPSS software (version 20.0.0).

RESULTS

Phytochemical assay

The phenolic compound content of the extracts is shown in Table 1. The highest concentrations were obtained with the hydroacetonic extract: 43.7 mgGAE, 3.8 mgQE and 7.9 mgCE respectively, for total polyphenols, flavonoids and condensed tannins.

Table 1: Phenolic compound content of *C. micranthum* leaves.

Extract	Total polyphenols (mgGAE/100 mg extract)	Total flavonoids (mgQE/100 mg extract)	Condensed tannins (mgCE/100 mg extract)
Aqueous	12.2 ± 0.1	1.3 ± 0.4	0.9 ± 0.02
Hydroacetone	43.7 ± 0.3	3.8 ± 0.1	7.9 ± 0.6

GAE: gallic acid equivalent, QE: quercetin equivalent, CE: catechin equivalent

Inhibition of *Haemonchus contortus* egg hatching

The results of *H. contortus* egg hatch inhibition by plant

extracts are shown in Table 2 and Figure 1. The positive and negative controls recorded inhibition rates of 100% and 10%, respectively. The concentrations of the two plant extracts varied from 16.6 to 83.3% for the aqueous extract and from 33.3 to 79.1% for the hydroacetone extract.

Table 2: Percentage inhibition of *H. contortus* egg hatching by various concentrations of *C. micrantum* extracts and controls.

Concentration (mg/ml)	Extracts of *C. micrantum*	
	Aqueous extract	Hydroacetone extract
Albendazole	100 ± 0.0 dA	100 ± 0.0 eA
9.6	83.3 ± 2.3 cA	79.1 ± 3.8 dA
4.8	55.5 ± 3.3 bAB	66.6 ± 1.6 cAB
2.4	38.8 ± 1.7 bAB	50.0 ± 1.4 bAB
1.2	33.3 ± 1.9 bAB	44.4 ± 1.3 bAB
0.6	16.6 ± 1.9 aBC	33.3 ± 3.2 aAB
PBS	10.0 ± 0.0 aC	10.0 ± 0.0 aC
Probability	p < 0.05	p < 0.05

Lowercase letters (abcde) compare means between columns and uppercase letters (ABC) compare means between rows at the 5% threshold (p <0.05).

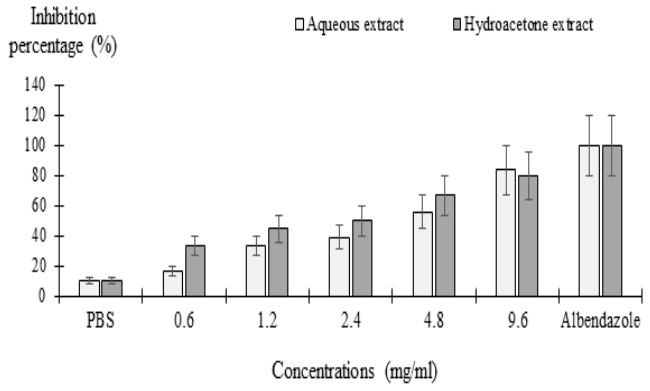

Figure 1: Dose-response profile for inhibition of hatching of *H. contortus* eggs by various concentrations of aqueous and hydroacetone extracts of *C. micranthum* during the study.

All concentrations of both plant extracts resulted in a significant increase (p < 0.05) in the rate of inhibition of egg hatching compared with the negative control.

Application of the Kruskall-Wallis test revealed a significant difference (p <0.05) in the percent inhibition of egg hatching with increasing concentrations of aqueous (dl = 4 ; p = 0.0037) and hydroacetone (dl = 4 ; p = 0.0037) extracts of *C. micranthum*.

Inhibitory concentration 50 (IC_{50}) values for the two extracts tested from the *C. micranthum* plant, determined by the analytical-probit method from the applied concentration results, are shown in Table 3. The value obtained for the aqueous extract is higher (2.994 mg/ml) than that for the hydroacetone extract (2.189 mg/ml).

Table 3: Values (mg/ml) of inhibitory concentrations 50 (IC_{50}) of *H. contortus* egg hatch.

Extracts of *C. micranthum*	IC_{50} (mg/ml)	LIC - LSC
Aqueous extract	2.994	1.654 - 7.420
Hydroacetone extract	2.189	1.004 - 3.404

LIC : lower confidence limit, LSC : upper confidence limit

Inhibition of *Haemonchus contortus* larval development

The inhibition of larval development by *C. micranthum* extracts are shown in Table 4 and Figure 2. The inhibition percentage of the PBS control was 5.0%, while the inhibition percentages of the extracts ranged from 21.1% to 84.2% for the aqueous extract and from 36.8% to 80.2% for the hydroacetone extract. Apart from the 0.6 mg/ml concentration of the aqueous extract, the inhibition percentage of the control was significantly (p < 0.05) lower compared to the percentages obtained with the hydroacetone extract concentrations and the other concentrations of the aqueous plant extract.

Table 4: Percentage inhibition of *H. contortus* larval development by various concentrations of *C. micranthum* leaf extracts and controls tested in the study.

Concentration (mg/ml)	*C. micranthum* leaf	
	Aqueous extract	Hydroacetone extract
Albendazole	94.7 ± 1.5 cA	94.7 ± 1.5 dA
9.6	84.2 ± 6.1 cA	80.2 ± 6.6 cdA
4.8	57.8 ± 17.1 bcAB	68.4 ± 12.1 cAB
2.4	42.1 ± 2.7 bAB	52.6 ± 10.5 bAB
1.2	36.8 ± 0.0 bAB	47.3 ± 12.1 bAB
0.6	21.1 ± 10.5 aB	36.8 ± 0.0 aAB
PBS	5.0 ± 4.1 aC	5.0 ± 4.1 aC
Probability	p < 0.05	p < 0.05

Lowercase letters (abcd) compare means between columns and uppercase letters (ABC) compare means between rows at the 5% threshold (p <0.05).

The Kruskall-Wallis test showed a significant difference (p < 0.05) in the percentage inhibition of larval development with increasing concentrations of the aqueous (P = 0.0063) and hydroacetone (P = 0.0057) extracts of *C. micranthum* tested.

The results of the inhibitory concentration 50 (IC_{50}) obtained from the two plant extracts as a result of probit analysis using the applied concentration results are shown in Table 5. The aqueous extract obtained a higher IC_{50} (2.761

mg/ml) than the hydroacetone extract (1.970 mg/ml).

Table 5: Values of inhibitory concentration 50 (IC_{50}) of larval development of *H. contortus* by two plant leaf extracts.

Extracts of *C. micranthum*	IC_{50} (mg/ml)	LIC – LSC
Aqueous extract	2.761	1.245 - 6.978
Hydroacetone extract	1.970	0.713 - 3.016

LIC: lower confidence limit, LSC: upper confidence limit

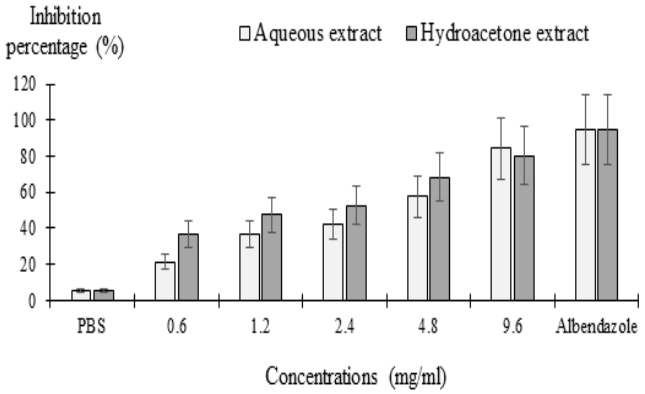

Figure 2: Dose-response profile for inhibition of larval development of *H. contortus* by concentrations of aqueous and hydroacetone extracts of *C. micranthum* compared with controls during the study.

Identifying the role of tannins

Inhibition of egg hatching: The plant extracts in contact with PVPP recorded inhibition percentages ranging from 5.5% for the aqueous extract to 9.7% for the hydroacetone extract (Table 6). In contrast, the two plant extracts not in contact with PVPP achieved egg hatch inhibition percentages of 72.2% for the hydroacetone extract and 88.8% for the aqueous extract.

Table 6: Percentage inhibition (%) of egg hatchability of the two *C. micranthum* extracts with and without polyvinylpolypyrrolidone (PVPP).

Extracts [9.6 mg/ml]	Inhibition percentages (%)		
	PVPP-free	PVPP- Added	PBS
Aqueous extract	88.8	5.5	10.0
Hydroacetone extract	72.2	9.7	

Inhibition of larval development: With the use of PVPP on larval development, the percentages of inhibition obtained were 10.5% for the aqueous extract and 14.5% for the hydroacetone extract. On the other hand, plant extracts that were not in contact with PVPP produced percentages of inhibition of larval development of 73.7% for the hydroacetone extract and 89.5% for the aqueous extract during the study (Table 7).

Table 7: Percentage inhibition (%) of larval development of the two *C. micranthum* extracts with and without polyvinylpolypyrrolidone (PVPP).

Extracts [9.6 mg/ml]	Inhibition percentages (%)		
	PVPP-free	PVPP- Added	PBS
Aqueous extract	89.5	10.5	10.0
Hydroacetone extract	73.7	14.5	

DISCUSSION

Due to the development of parasites resistant to synthetic anthelmintics, the use of new strategies focusing on the exploitation of plants rich in secondary metabolites of anthelmintic interest is proving imperative (Hoste et al., 2018; Degla et al., 2022). As part of this approach, the present study was carried out to scientifically validate the Bwa communities' assertions about the use of *C. micranthum* against gastrointestinal parasites in small ruminants in Burkina Faso. *In vitro* anthelmintic tests showed that different concentrations of the plant's aqueous and hydroacetonic extracts significantly inhibited egg hatching and larval development of the *H. contortus* parasite. In terms of egg hatching, the percentages of inhibition obtained ranged from 16.6 to 83.3%, and for inhibition of larval development, from 21.1 to 84.2%. These results also corroborate veterinary ethnobotanical surveys which have reported that the plant has therapeutic virtues against gastrointestinal parasites of small ruminants (Garba et al., 2019).

Similar results from other medicinal plants have been reported. Indeed, Koffi Yao et al. (2018) demonstrated the *in vitro* efficacy of hydromethanolic extracts of three plants (*Morus mesozygia*, *Ficus lutea* and *Albizia adianthifolia*) on *H. contortus* eggs and larvae in Côte d'Ivoire. In Burkina Faso, Tianhoun et al. (2020) also demonstrated the anthelmintic activity of aqueous and hydroacetone extracts of the *Cassia alata* plant on all three life stages (eggs, larvae and adult worms) of *H. contortus*. The efficacy of the extracts used was established in a dose-dependent manner, whereas a higher response was obtain with the highest concentration. This relative increase in effects is thought to be linked to the concentrations of active ingredients in the extracts tested (Hounzangbe-Adote, 2005; Ouédraogo, 2022).

In this study, the inhibitory concentration 50 (IC_{50}) of the hydroacetone and aqueous extracts of *C. micranthum* suggest that those for larval development (1.970 and 2.761 respectively) are relatively lower than those for egg hatching (2.189 and 2.994). This suggests that the aqueous and hydroacetone extracts of *C. micranthum* leaves are more active against larvae (larvicide) than eggs (ovicide). Consequently, the active chemical substances contained in the plant extracts would penetrate the larval cuticle more easily than the eggshell. They would do so by osmosis through

the circulatory system, hindering the development process of blastomeres and larvae and leading to death by starvation (Athanasiadou et al., 2001) or paralysis (Dobson et al., 1986). This finding runs counter to those of Ouédraogo (2022) with aqueous extracts of *Balanites aegyptiaca* on *H. contortus* and of Wabo-Poné et al. (2006) with the bark extract of *Canthium mannii* on *Ancylostoma caninum*.

The anthelmintic effects of *C. micranthum* extracts could be linked to the secondary metabolites it contains. Indeed, the anthelmintic properties of secondary metabolites such as tannins and flavonoids are reported by several authors (Hoste et al., 2009; Minaflinou et al., 2015; Koffi Ya et al., 2018; Maiga et al., 2020; Tianhoun et al., 2020). These metabolites would certainly act by inhibiting the oxidative phosphorylation of parasites (Vedha Hari et al., 2011) by binding to a protein that plays a protective role in the parasite's cuticle (Ongoka et al., 2012). This binding disrupts the integrity of the cuticle, leading to the death of the parasite (Athanasiadou et al., 2001; Vidyadhar et al., 2010). Condensed tannins also diffuse to the surface of egg and larval membranes to bind to free membrane proteins, thereby inhibiting egg hatching and inducing larval mortality (Brunet et al., 2007; Koffi Yao et al., 2018).

The use of PVPP to block the tannins contained in the extracts studied resulted in a significant drop in the rates of inhibition of egg hatching and larval development of *H. contortus*. This suggests that tannins are behind the efficacy of *C. micranthum* extracts on *H. contortus*. Study carried out by Mori et al. (2000) on *Caenorhabditis elegans*, a parasitic nematode, showed that tannins act on the parasite through their attachment to the cuticle of adults or the sheath of L_3 larvae, and alter nutritional and enzymatic processes. Also, addition of PVPP to aqueous and acetone extracts of *Acacia nilotica* and *Acacia raddiana*, showed that tannins were responsible for inhibiting egg hatching (*H. contortus*) and adult worm mortality (*Caenorhabditis elegans*), but not for inhibiting larval unsheathing (Zabré, 2018).

CONCLUSION

The results obtained show that extracts of the *C. micranthum* plant have anthelmintic activity on *H. contortus* eggs and larvae. This activity would justify its use as an alternative for the control of gastro-intestinal nematodes in small ruminants in rural Burkina Faso. However, further studies need to be carried out on *in vitro* efficacy on adult parasites and *in vivo* efficacy in small ruminants, with prior identification of safe measures for using the plant's leaves by rural livestock farmers.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interests regarding the publication of this article.

NOVELTY STATEMENT

This study is an assessment of ethnoveterinary practices in the management of internal parasitosis of small ruminants by local communities. It validates the anthelmintic efficacy of Combretum micranthum leaf extracts used by rural livestock farmers.

AUTHORS CONTRIBUTION

These authors have contributed to the study as followed. KA, MNTR and TDF initiated the study proposal and the methodology as part of TDF's thesis. TDF conducted experimentation and draft the manuscript. ZG, KB, THH, BAMG corrected the manuscript and provided their scientific reviews.

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