Research Article

Anti-Parasite Activity of Different Plant Leaf Extracts against Infective Stage Theront of *Ichthyophthirius multifiliis*

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Abstract | *Ichthyophthirius multifiliis* is a ciliate protozoa, that persists in freshwater fish, which affects aquaculture production. Its' control has adopted chemotherapeutics, and currently environmental friendly botanicals utilized against infective stage theronts. Two solvents, ethanol and chloroform solvents were used to extract five botanicals i.e., bitter kola (*Garcinia kola*), cashew leaf (*Anacardium occidentale*), bitter leaf (*Vernonia amygdalina*), lemon grass (*Cymbopogon citratus*) and ocimum leaf (*Ocimum gratissimum*) to investigate their efficacy against theronts for 24 hours. A 100 µl (480 theronts) were distributed into five groups; 0, 0.01, 0.03, 0.05 and 0.1 g/ml (A-E) (for all plant extracts used) in a 96 micro-wells chambers, with 3 sub-replicates of approximately 160 theronts. Mortality occurred in all groups except in the control group during the exposure period. 24-h theront mortality was highest in bitter leaf extracts and least in cashew leaf extract. Mortality was also dependent on extract concentration and extraction solvent. The two highest mortalities of 54.2% and 53.1% occurred at 0.1 g/ml ethanol and chloroform extracts of bitter leaf, respectively on 24-h exposure duration. The likelihood of a significant effect of duration of exposure on infective theront mortality resulting from the extracts was significant (Likelihood ratio [LR] χ^2 = 973.95, p < 0.0001). The lethal concentration (LC_{50}) estimates of bitter leaf ethanol and chloroform extracts were 0.137 g/ml (0.006 – 3.434) and 0.157 (0.004 – 6.099), respectively while the 95% confidence interval of the estimate overlapped with LC_{50} estimates of the other four extracts.

Keywords | Theronts, *Ichthyopthirius multifiliis*, Botanicals, Mortality, Aquaculture

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INTRODUCTION

Global food production has grown to 47% due to significant growth in aquaculture. Nigeria remains the most populous country in sub-Saharan Africa (approximately 200 million people); a staple food product such as fish has served as house hold diet in the recent times (FAO, 2017). The overall fish production in Nigeria per annum is approximately one million tons, of which 313,281 metric tons and 759,828 metric tons were from aquaculture and fisheries, respectively. With this, Nigeria ranks amongst the largest producers of fish in Africa, second to Egypt (FAO, 2017).

However, outbreaks of parasitic infections especially by an ectoparasite, and a single-celled organism, *Ichthyophthirius multifiliis* have also increased in aquaculture, causing serious economic losses. Nearly all the freshwater fish species are prone to infestation by these common ectoparasitic protozoa. It has been observed that the adult parasite (trophont) is protected by penetration in the host epithelium, current treatment plan target only the free swimming stages. Considering the life cycle of *I. multifiliis*, it is pertinent to know that infective theronts must be eliminated; having observed its' transient and shortened life span (48 h) nature in the absence of a viable host, fish. In the time past, fish farmers have adopted conventional chemotherapeutic

treatments such as; tannic acid (Alavian et al., 2018), acetic acid (Balta et al., 2008), ornidazole (Toksen and Nemli, 2010), metronidazole (Noga, 2010), potassium permanganate (Tieman and Godwin, 2001; Straus and Griffin, 2002; Balta et al., 2008; Noga, 2010), and sodium chloride (Shin et al., 2005) to control *I. multifiliis*. But the accumulation of chemical residues mostly persists in the body and tissues of the fish after treatment, thereby making the fish unacceptable to the consumers for consumption (Lusiastuti et al., 2017). To make up with these challenges, botanicals like *Moringa oleifera* (Ikele et al., 2020), *Polygonum cuspidatum* (Zhou et al., 2018), *Morus alba* root bark (Yao et al., 2010), *Cynachum paniculatum* (Ji-Hong et al., 2017), *Magnolia officinalis* (Yi et al., 2012), and *Sophora allopecuroides* (Yi et al., 2012) have been adopted as an environmental friendly, cheap, biodegradable residual-free alternative to chemicals against theronts.

Therefore, current study tested various cheap, residual-free indigenous plants to ascertain their potency in the controlling/eliminating the free swimming stage, infective theronts of *I. multifiliis*.

MATERIALS AND METHODS

Plant Materials

Five kilograms of fresh leaves of *Ocimum gratissimum* (Ocimum leaf), *Anacardium occidentale* (Cashew leaf), *Garcinia kola* (Bitter kola), *Cymbopogon citratus* (lemon grass), and *Vernonia amygdalina* (bitter leaf) were collected from a nearby farm, in the University of Nigeria, Nsukka and were identified in the laboratory of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The fresh leaves were removed from the stalk, washed to remove debris and dust particles without squeezing. The leaves of the plants were dried under room temperature for two weeks and ground into fine powder, using a commercial blender and weighed, concentrated in both ethanol and chloroform, shaken vigorously and left for 24 hours. All the mixtures were filtered independently using a muslin cloth and evaporated to dryness. The percentage yields were obtained from the different plant extracts and calculated by dividing the weight of concentrated extract with the weight of dried-ground leaves, multiplied by 100. The extractants were stored in an airtight container until ready for use.

Phytochemicals

Phytonutrients such as alkaloids, flavonoids, saponins, tannins, terpenoids, and glycosides (Table 1) were analysed following the method described by Ajuru et al. (2017).

Experimental Fish and Parasite Isolation

Thirty (30) Ich infected juvenile *Clarias gariepinus* of average weight 97.24±13.26g and standard length 13.2±0.87 cm was obtained from Freedom fisheries, Nsukka, Enugu State, Nigeria. They were acclimatized and, fed twice daily at 5% body weight with commercial fish pellet feed and fecal matters were daily removed. The native strain parasite source infected *Clarias gariepinus* identified by the presence of visible white spots, and mature trophonts. The infected fish was anaesthetized with 150 g/ml tricaine methanesulfonate (MS-222, Sigma) and the skins gently scraped to dislodge the trophonts. Isolated parasites were transferred into beakers containing 500ml of dechlorinated tap water in order to remove organic matter for 4 h and were incubated in the dark chamber (slight modification) to allow for mitotic division which finally produced free swimming theronts (Figure 1) within 48 h. The 100 µl (480 theronts) were further distributed into five groups; 0, 0.01, 0.03, 0.05 and 0.1 g/ml (A-E) (for all plant extracts used) in a 96 micro-wells chambers, with a sub-replicates (3) of approximately 160 theronts were prepared for mortality test which lasted for 24 hours. Theront mortality (Figure 2) was observed and counted using olympus binocular microscope using 40X lens.

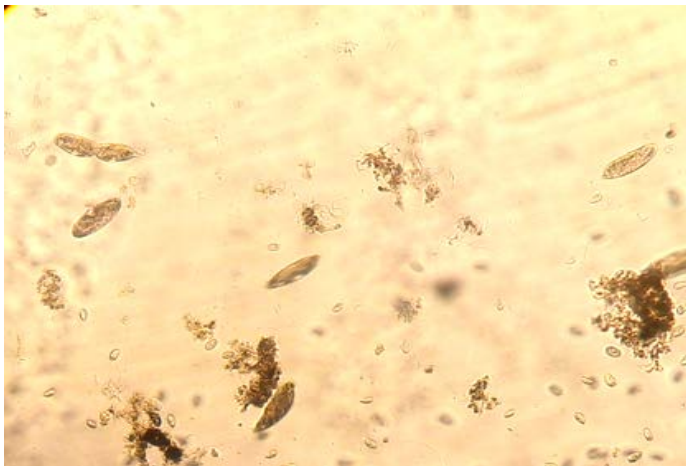

Figure 1: Free swimming theronts after 48 hours of culture

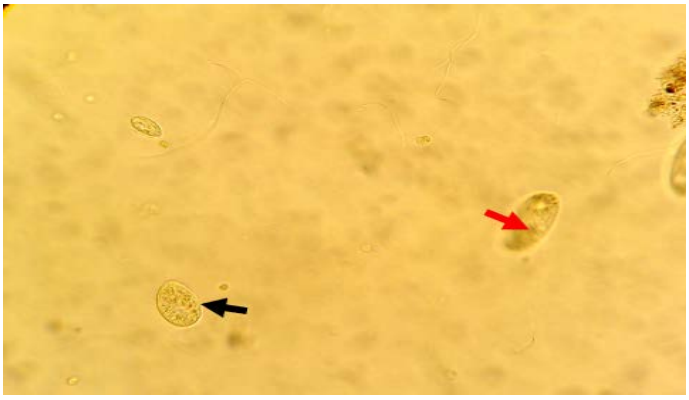

Figure 2: Microscopic representation of live (black arrow) showing fusiform to pyriform shape and intact cilia and dead theronts (red arrow) showing rounded body and deformed cilia after exposure to different botanicals for 24 hours.

Table 1: Qualitative phytonutrient of plant materials

Botanicals	Cashew Ethanol leaf extract	Ocimum ethanol leaf extract	Bitter kola ethanol Leaf extract	Lemon grass ethanol leaf extract	Bitter leaf ethanol leaf extract	Cashew Chloroform leaf extract	Ocimum Chloroform leaf extract	Bitter kola Chloroform Leaf extract	Lemon grass Chloroform leaf extract	Bitter leaf Chloroform extract
Alkaloid	+	++	++	+	+	+	+	++	++	+
Flavonoid	+	+	++	++++	+	-	+	+	-	+
Saponin	+	++	+	+	+	++	+	-	-	-
Tannin	+++	+	+++	+	+	-	-	+	++	+
Terpenoid	+	+	+	+	+++	-	+	+	-	-
Glycosides	+	-	+	-	+	+	+	++	+	+

Table 2: Cumulative mortality of *Ichthyophthirius multifiliis* theront on exposure to different extracts (initial theront load, 100 µL ~ 480 theront).

Extract	Solvent	Conc. (g/ml)	Mortality (%) at exposure duration (h)			
			0 h	6 h	12 h	24 h
Bitter leaf (*Vernonia amygdalina*)	Ethanol	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	45 (9.4)	153 (31.9)
		0.03	0 (0)	11 (2.3)	83 (17.3)	153 (31.9)
		0.05	0 (0)	0 (0)	24 (5.0)	160 (33.3)
		0.1	0 (0)	7 (1.5)	49 (10.2)	260 (54.2)
	Chloroform	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	21 (4.4)	153 (31.9)
		0.03	0 (0)	0 (0)	17 (3.5)	109 (22.7)
		0.05	0 (0)	0 (0)	15 (3.1)	164 (34.2)
		0.1	0 (0)	0 (0)	145 (30.2)	255 (53.1)
Lemon grass (*Cymbopogon citratus*)	Ethanol	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	35 (7.3)
		0.03	0 (0)	0 (0)	0 (0)	108 (22.5)
		0.05	0 (0)	5 (1.0)	6 (1.3)	128 (26.7)
		0.1	0 (0)	5 (1.0)	7 (1.5)	154 (32.1)
	Chloroform	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	28 (5.8)
		0.03	0 (0)	0 (0)	0 (0)	43 (9.0)
		0.05	0 (0)	0 (0)	0 (0)	72 (15.0)
		0.1	0 (0)	0 (0)	0 (0)	210 (43.8)
Bitter kola (*Garcinia kola*)	Ethanol	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	19 (4.0)
		0.03	0 (0)	0 (0)	27 (5.6)	101 (21.0)
		0.05	0 (0)	0 (0)	32 (6.7)	121 (25.2)
		0.1	0 (0)	19 (4.0)	33 (6.9)	135 (28.1)
	Chloroform	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	11 (2.3)	83 (17.3)
		0.03	0 (0)	0 (0)	15 (3.1)	94 (19.6)
		0.05	0 (0)	0 (0)	22 (4.6)	76 (15.8)

		0.1	0 (0)	0 (0)	19 (4.0)	90 (18.8)
Ocimum leaf (*Ocimum gratissimum*)	Ethanol	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	111 (23.1)
		0.03	0 (0)	0 (0)	0 (0)	175 (36.5)
		0.05	0 (0)	0 (0)	0 (0)	186 (38.5)
		0.1	0 (0)	0 (0)	0 (0)	217 (45.2)
	Chloroform	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	1 (0.2)	43 (9.0)
		0.03	0 (0)	0 (0)	2 (0.4)	78 (16.3)
		0.05	0 (0)	0 (0)	0 (0)	54 (11.3)
		0.1	0 (0)	0 (0)	2 (0.4)	89 (18.5)
Cashew leaf (*Anacardium occidentale*)	Ethanol	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	2 (0.4)
		0.03	0 (0)	0 (0)	16 (3.3)	27 (5.6)
		0.05	0 (0)	0 (0)	24 (5.0)	51 (10.6)
		0.1	0 (0)	0 (0)	72 (15.0)	181 (37.7)
	Chloroform	0.0	0 (0)	0 (0)	0 (0)	0 (0)
		0.01	0 (0)	0 (0)	0 (0)	14 (2.9)
		0.03	0 (0)	0 (0)	0 (0)	20 (4.2)
		0.05	0 (0)	0 (0)	17 (3.5)	37 (7.7)
		0.1	0 (0)	0 (0)	59 (12.3)	59 (27.7)

Table 3: Estimated lethal concentrations of different botanicals for theronts at 24 hours

Extract	Solvent	LCs	Estimated concentration (g/ml) at exposure duration		
			6 h	12 h	24 h
Bitter leaf (*Vernonia amygdalina*)	Ethanol	25	-	-	0.043 (0.007 – 0.253)
		50	-	-	0.137 (0.006 – 3.434)
		75	-	-	0.441 (0.001 – 138.28)
		99	-	-	7.768 (0 – 1897000)
	Chloroform	25	-	-	0.048 (0.007 – 0.316)
		50	-	-	0.157 (0.004 – 6.099)
		75	-	-	0.511 (0.0009 – 304.56)
		99	-	-	9.204 (0 – 6778221)
Lemon grass (*Cymbopogon citratus*)	Ethanol	25	-	-	0.078 (0.030 – 0.198)
		50	-	-	0.185 (0.031 – 1.103)
		75	-	-	0.442 (0.026 – 7.527)
		99	-	-	3.730 (0.015 – 952.07)
	Chloroform	25	-	-	0.060 (0.021 – 0.172)
		50	-	-	0.162 (0.022 – 1.20)
		75	-	-	0.439 (0.016 – 12.250)
		99	-	-	5.021 (0.006 – 4417.43)
Bitter kola (*Garcinia kola*)	Ethanol	25	-	0.223 (0.01 – 6.99)	0.084 (0.03 – 0.239)
		50	-	1.022 (0.004 – 120.83)	0.187 (0.026 – 1.321)
		75	-	4.302 (0.001 – 2183.78)	0.415 (0.019 – 8.886)
		99	-	132.615 (0.0001 – *)	2.942 (0.008 – 1086.4)
	Chloroform	25	-	-	0.117 (0.0036 – 3.818)

		50	-	-	0.568 (0.003 – 1136.14)
		75	-	-	2.758 (0 - *)
		99	-	-	132.58 (0 - *)
Ocimum leaf (*Ocimum gratissimum*)	Ethanol	25	-	-	0.011 (0.007 – 0.015)
		50	-	-	0.145 (0.102 – 0.254)
		75	-	-	3.611 (1.341 – 19.849)
		99	-	-	1058.2 (119.3 – 46424.9)
	Chloroform	25	-	-	0.1896 (0.003 – 11.009)
		50	-	-	0.8694 (0.0004 – 2147.8)
		75	-	-	3.988 (0 –491093)
		99	-	-	0 - *)
cashew leaf (*Anacardium occidentale*)	Ethanol	25	-	-	0.180 (0.049 – 0.653)
		50	-	-	0.387 (0.045 – 3.33)
		75	-	-	0.835 (0.04 – 17.55)
		99	-	-	5.50 (0.028 – 1068.6)
	Chloroform	25	-	-	0.364 (0.225 – 0.896)
		50	-	-	1.250 (0.581 – 5.43)
		75	-	-	4.292 (1.487 – 33.148)
		99	-	-	88.35 (14.79 – 2815.09)

*values over 2 000 000.

Statistical Analysis

Lethal concentration of the extracts against theronts of *Ichthyophthirius multifiliis* were computed using probit analysis. Theronts mortality (as count) was subjected to negative binomial regression with exposure duration, extract type, extraction solvent and extract concentration as fixed factors and mortality as dependent variable. This was followed by analysis of deviance. Data was analysed in R version 4.0.5 (R Core Team, 2021).

RESULTS

Cumulative mortality of theronts of *Ichthyophthirius multifiliis* on exposure to different plant extracts is summarized in Table 2. Mortality of theronts was dependent on duration of exposure; it increased with prolongation of exposure. Greater number of mortality occurred at 24 h compared to 12 h and 6 h. No mortality occurred at 0 h. Mortality occurred in all five extract types, bitter leaf, lemon grass, bitter kola, ocimum leaf and cashew leaf, and none occurred in the control for the exposure duration. 24-h theront mortality was highest in bitter leaf extracts and least in cashew leaf extract. Mortality was also dependent on extract concentration and extraction solvent. The two highest mortalities of 54.2% and 53.1% occurred at 0.1 g/ml ethanol and chloroform extracts of bitter leaf, respectively on 24-h exposure duration. The least 24-h theront mortality occurred at 0.01 g/ml cashew leaf extract. No mortality occurred within 6-h of exposure in ocimum leaf and cashew leaf treatment media.

From the negative binomial model, duration of exposure significantly affected mortality. A unit increase in mortality at 6-h of exposure was associated with a log count of mortality of 2.793 and 4.475, respectively at 12-h and 24-h, both being significant ($p < 0.0001$). Mortality increased on prolonged exposure. The effect of duration of exposure on infective theront mortality resulting from the extracts was significant (Likelihood ratio [LR] $\chi^2 = 973.95$, $p < 0.0001$). In 6-h, mortality was below 5% for each extract. No mortality occurred in chloroform extract at the 6-h duration. In ethanol extract, mortality occurred only in bitter leaf (0.05 and 0.1 g/ml), lemon grass (0.05 and 0.1 g/ml) and bitter kola (0.1 g/ml) (Figure 3). Extraction solvent affected mortality. The likelihood of a notable impact of exposure duration on infective theront mortality was least when compared to the other parameters investigated (i.e. extract type, exposure duration, and extract concentration), but it was however, significant ($\chi^2 = 27.69$, $p < 0.0001$). Extract prepared from chloroform was less effective compared to ethanol solvent extracts.

On 12-h exposure duration (Figure 4), the highest mortality occurred at 0.1 g/ml chloroform extracted bitter leaf extract (~ 30%). Mortality in all other extract in 12-h was below 20% (red broken line cut-off). Mortality of theronts by 0.03, 0.05 and 0.1 g/ml of bitter leaf, bitter kola and cashew leaf were usually significantly high compared to the control (Figure 2).

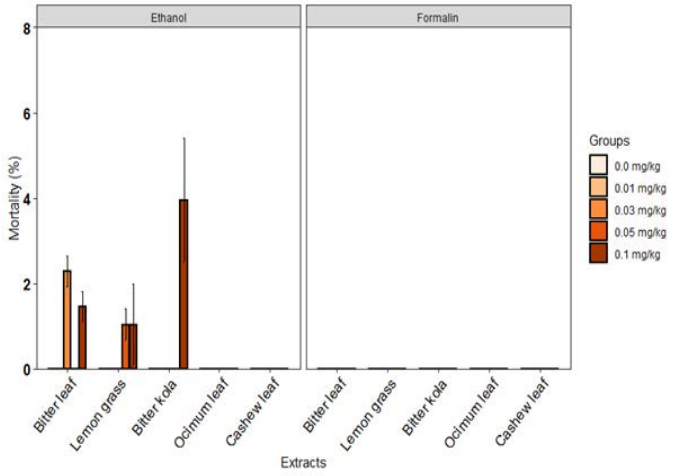

Figure 3: Mortality (%) of theronts in concentration of different plant extracts and extraction solvents at 6-h exposure duration.

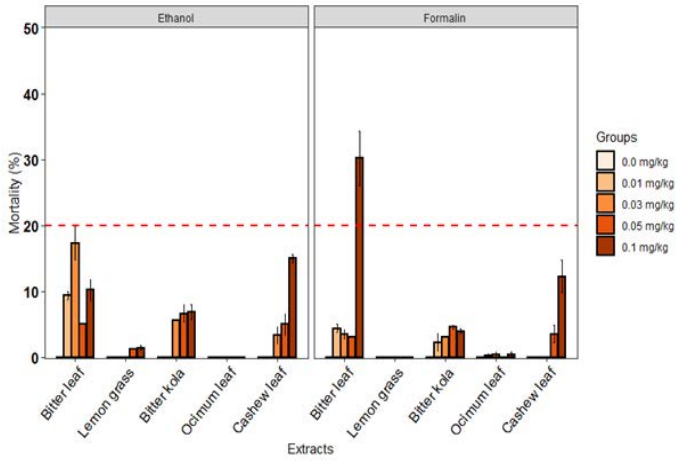

Figure 4: Mortality (%) of theronts in concentration of different plant extracts and extraction solvents at 12-h exposure duration.

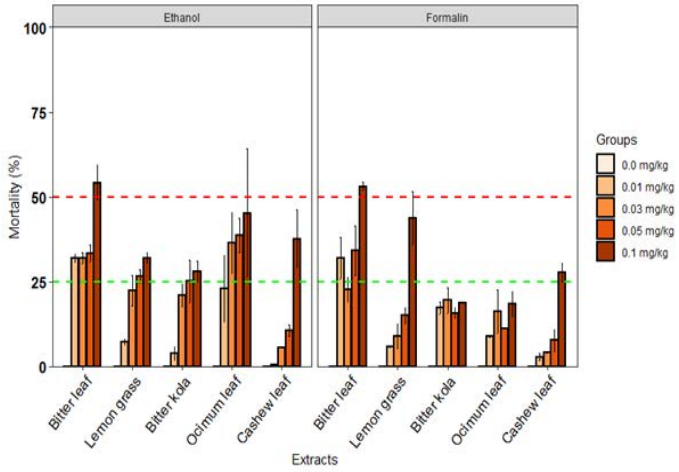

Figure 5: Mortality (%) of theronts in concentration of different plant extracts and extraction solvents at 24-h exposure duration.

At the end of 24-h exposure period, only 0.1 g/ml bitter leaf ethanol and chloroform extracts caused over 50% mortality of theronts (red dashed line cut-off). However, over 80% of the highest concentration of the extracts caused over 25% mortality in 24-h (Figure 5). Chloroform extracts of bitter kola and ocimum leaf were below 25% even at 0.1 g/ml on 24-h exposure. Bitter leaf ethanol and chloroform extracts performed better than other extracts as treatment for theront infection in fish *Clarias gariepinus*.

Estimated lethal concentrations of different botanicals for infective theronts

Lethal concentration estimates for the extracts were only possible for the 24-h exposure duration due to relatively low level mortality at other time intervals. The LC_{25}, LC_{50}, LC_{75} and LC_{99} of the extracts were presented in Table 3. The estimated median lethal concentration (LC_{50}) of bitter leaf ethanol and chloroform extracts were 0.137 g/ml (0.006 – 3.434) and 0.157 (0.004 – 6.099), respectively. The 95% confidence interval of the estimate overlapped with LC_{50} estimates of the other for extracts, namely; lemon grass, bitter kola, ocimum leaf, and cashew leaf.

DISCUSSION

Efforts towards identifying suitable, economical, residual-free plant-derived compounds, as a better replacement of chemical strategies for the effective control of *I. multifiliis* has been increased at the recent times (Ikele et al., 2020). There is still paucity of literature on the efficacy of cashew leaf, *ocimum* leaf, bitter leaf, lemon grass and bitter kola leaf as a potential parasiticide in controlling theronts. Results of previous studies evaluating plant extracts for their anti-ich efficacy have suggested that crude extracts from some plant have compounds with significant effect against *I. multifiliis* (Buchmann et al., 2003, Yao et al.,, 2010, Zhang et al., 2013).

The exposure duration for killing theronts was adopted for evaluating a potential parasiticide and was used to evaluate anti-ich efficacy in the current study. Antiprotozoal activity screening of ethanolic and formalin extract of lemongrass, bitter leaf, scent leaf, bitter kola leaf and cashew leaf exhibited a good activity against *I. mulifiliis* theronts within 24hours exposure.

Ling et al. (2013) reported high efficacy of 1.25mg/l methanol extract of *Psoralea Corylifolia* in eradicating 100% infective theronts within 4h of exposure. Camacho et al (2010) reported short exposure to high dose of bronopol (20, 50 and 100mg/l) appeared to have marked effect on the survival of tormont, cysts, and theront stages of *I. multifiliis*.

Yi et al. (2012) reported that methanol extract of *Mag-*

nolia officinalis and *Sophora alopecuroides* displayed a high antiprotozoal activity against theronts within 4h and LC_{50} values estimated to be 2.45 and 3.43mg/l. There is little information available about the plants used in our study for the control of theronts. But further reports on the efficacy of herbal extract in controlling/eradicating the *I. multifiliis* infective stage theronts has been reported by (Zhang et al.,, 2013; Ikele et al., 2020). Likewise, Ekanem et al. (2004) reported eradication of ich parasite with 200mg/l *Mucus Pruriens* within 96 hours. There is concerted effort to utilize environmental friendly botanicals as a replacement agent against chemotherapeutics in controlling Infective stage theront Infection.

Test was conducted to evaluate the activities of ethanol and chloroform extract of these plant materials mentioned above against *I. multifiliis* theront. The results showed that theront susceptibility is dependent on the duration length of exposure, concentration and extract solvent. Similar results were also reported in previous studies; for example, garlic extract killed theronts at 62.5 mg l–1 within 15 h and (Buchmann et al., 2003). Pentagalloylglucose caused 100% mortality of theronts at 2.5mg l–1 in 4hrs exposure (Zhang et al., 2013).

CONCLUSION

Icthyophthirius multifiliis is a ciliate parasitic protozoa, commonly encountered in most cultured freshwater fish species. The infective stage theronts is the most difficult to control, due to its' survival rate, and quick penetration to the basal layer of the fish skin within few minutes. Our present findings offered a range of treatment method options for use in commercial farms systems not only for the control of Ich infestations but also other fresh water and marine protozoan's diseases. There is need for proper licensing of environmental friendly non-chemotherapeutics to control parasite infection in food fish farms. In addition, the most effective management strategy to use against Ich, however, may be site/farm specific and require a combination of the different treatments considered in the current study. Plant materials used in the current study has the potential of controlling the free living infective stage parasite, theronts within a short interval.

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CONFLICT OF INTEREST

No conflict of interest among the authors

NOVELTY STATEMENT

The research work currently evaluated potential of *botanicals* against *I. multifillis*. *O. gratissimum* has seldomly be used to control protozoa ich parasite.

AUTHORS CONTRIBUTIONS

Ikele, Chika Bright: designed the experiment, supervised the research and proof read the manuscript. Ikele, Chioma Faith: conducted the phytochemical screenings of the plant material. Uju Venita: conducted the experiment under supervision.

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