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The Potentiality of *Cabomba aquatica DC not Aubletii* Ethanol Extract as Larvicide against *Aedes aegypti* Larvae

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Abstract : *Cabomba* genus is well known as one of invasive aquatic plants around the world. The genus has brought both of environmental problems and economical significance in many countries, particularly for its vast distribution as ornamental plants in aquariums. Indonesia used to be one of importers of this plant. Recently, Indonesia has successfully cultivated the plant and turned into one of exporter countries of the plant.
The most common species of *Cabomba* plants cultivated in Indonesia is *Cabomba aquatic DC not Aubletii*. Previous studies suggested that the plant had one form of defense mechanism to protect itself from disturbing organisms around it. Yet, its potentiality as larvicide producing toxic substance to prevent larvae growth (including *Aedes aegypti* larvae) remains unknown. Considering this phenomenon, this study is aimed to identify the effect of ethanol extract produced by *Cabomba aquatica DC not Aubletii* against *Aedes aegypti* larvae.
The whole body of *Cabomba aquatica DC not Aubletii* was using ethanol 96%. The method of this research used ethanol extract to get secondary metabolite compounds that were identified based on Thin Layer Chromatography (TLC). In order to examine the potentiality of *Cabomba* extract, treatment groups (each consists of 25 *Ae. aegypti* larvae) were treated using different concentrations of *C. aquatica* extract for 24 hours duration.
The result of this study showed that there were two groups of *Cabomba aquatica DC not Aubletii* ethanol extract secondary metabolite compounds. Meanwhile, according to statistical analysis that the treatment groups indicated ethanol extract of *Cabomba aquatica DC not Aubletii* was significantly effective against *Aedes aegypti* larvae.
Based on the result, it can be concluded that *Cabomba aquatica DC not Aubletii* was effective as larvicide against *Aedes aegypti* larvae. This potentiality was associated with secondary metabolite compounds of its ethanol extract.
Keywords : *Cabomba,* larvicide, secondary metabolites.

Introduction and Experimental

Introduction

One of the members of *Nymphaeales* order named *Cabomba* genus is widely known as one of invasive aquatic plants. The biomass has caused severe problems in many countries particularly on how much cost has

been spent by the countries to eradicate and control the population of the biomass. Therefore, under-controlled *Cabomba* population may function as water filter and perform mutual symbiosis with fish [1-11]. *Cabomba*, the plant which was native to American downstream could give bad impacts on aquatic environment, recreational water activities, public safety, and water quality. Therefore it also owns economic significance as ornamental plants in aquariums due to its convenient care and market high demand on the plant [1,3,6,11,12,15].

As other aquatic plants, *Cabomba* plays important roles in aquatic ecosystem, such as sheltering other organisms and becoming place where other organisms breed and foraging for foods. Other functions include improving oxygen level, maintaining soil surface stability (due to its specific root). Even in certain conditions *Cabomba* plants also functions to maintain water clarity level and producing buffer compounds to neutralize water chemical contents. Based on this function, *Cabomba* can also be used as indicator whether water has been polluted by heavy metals or not[1, 2, 10, 16].

However, in certain conditions when *Cabomba* mass is overpopulated, several problems may exist. Excessive population of *Cabomba* plants may disturb convenient uses of water environment, such as for recreational purposes and imbalance the food chains in the ecosystem. Non-native aquatic plant invasion (also called exotic water plant invasion) such as by *Cabomba* may potentially destruct aquatic ecosystem which becomes a great detriment for human beings, fish, and living organisms within the ecosystem [1, 2, 3, 4, 6, 7, 10, 11].

In many countries where *Cabomba* plants originated, the governments had taken efforts to restrict and control *Cabomba* populations. Over than a decade ago, due to aggressive growth of *Cabomba* population in water ecosystem in several regions (including South East Asia), strict supervisions to quarantine *Cabomba* plants were conducted [1, 17].

Cabomba plants adapt quite well to artificial aquatic ecosystems. According to previous reports, *Cabomba* plant was easily cultivated within tanks with rapid growth and beautiful appearance. The tempting appearance makes the plant favorite ornamental aquarium plant. When being cultivated in aquariums, the plant indicates rapid growth [12, 13, 14].

Recent researches on *Cabomba* plants had been conducted. One of them was conducted by Markom, et al. (2009) on phytochemical contents of *Cabomba furcata (Red Cabomba)*. They found that *Cabomba furcata (Red Cabomba)* from Tasik Chini, Malaysia contained several substances, namely *saponin, alkaloids, and flavonoids*. The three compounds were previously known for its potentiality as larvicide against insect larvae, including mosquitoes and flies [18]. Morrison and Hay (2011) reported that lipid extract of *Cabomba caroliniana* might constrain microbial culture which were isolated from leaf surface. The research also showed that the plant was able to lower the appetite of its predator (i.e. aquatic snails) by secreting chemicals as defense mechanism. It also found out that aquatic snails which consumed the plants became more "unhealthy" compared to aquatic snails which had not consumed the plant or before consuming the plants although based on research conducted by United States Department of Agriculture (USDA) in general *Cabomba* plants did not have toxic properties on mammals and vertebrates [19, 20].

The aims of this study were to identify phytochemical compound groups of *Cabomba aquatica DC not Aubletii* cultivated at Gunung Bunder, Pamijahan District, Bogor, Indonesia and to examine the effects of *Cabomba aquatica DC not Aubletii* ethanol extracts on *Aedes aegypti* larvae.

Experimental

This study was conducted in the laboratory of *Hospital of Specialization of Infection* and in Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University Surabaya starting from September 2013 until May, 2015. This study applied observational design intended to identify the type of phytochemical compounds contained in *Cabomba aquatica DC not Aubletii* ethanol extract using Thin Layer Chromatography (TLC) to identify saponin, alkaloid, phenol, flavonoid, steroid, tannin, and terpenoid contents. This study also applied experimental design intended to examine the effect of *Cabomba aquatica DC not Aubletii* ethanol extract on *Aedes aegypti* larvae measured through post-test only control group referring to standards proposed by WHO (2005). The results were presented within 24 hours with a slight modification.

Cabomba plants used in this study were directly collected from farmers in Gunung Bunder, Pamijahan District, Bogor, Indonesia. The collected *Cabomba* plants were transported to Purwodadi Botanical Garden,

East Java, Indonesia to undergo taxonomical identification. The identification result categorized the plants as *Cabomba aquatica DC not Aubletii* which was synonymous with *Cabomba caroliniana A Gray* according to *The Atlas of Invasive Plants.*

The collected *Cabomba aquatica DC not Aubletii* were sliced and dried up naturally (without solar exposure). 7 grams of *Cabomba aquatica DC not Aubletii* powder were obtained from 100 grams of sliced Cabomba aquatica DC not Aubletii (wet weight). Other prominent characteristic of this plant was on its water contents within the powder. The measurement on water content was measured using gravimetric analysis. Gravimetric analysis was conducted in three sessions, namely at 105°C temperatures for 4 hours, 105°C temperatures for 2 hours, and 105°C temperatures for 2 hours continuously. The gravimetric analysis was conducted at Laboratory of Analytical Chemistry, Faculty of Sciences and Technology, Airlangga University, Surabaya.

Secondary metabolites or phytochemical compounds produced by *Cabomba aquatica DC not Aubletii* were identified by extracting *Cabomba aquatica DC not Aubletii* powder inside non-toxic solvent such as ethanol 96% as recommended. The process was conducted at Laboratory of Pharmacognosy and Phytochemical Department, Faculty of Pharmacy, Airlangga University, Surabaya, East Java, Indonesia. This process was conducted using maceration method namely by dipping the powder inside ethanol 96% solution for 24 hours three times. The extract was separated from its solvent by evaporating the extract inside vacuum rotary evaporator. 106 grams solid ethanol (dark green powder) were obtained from 750 grams Cabomba powder through this process.

Secondary metabolites of *Cabomba aquatica DC not Aubletii* ethanol extract (as crude extract) were identified through Thin Layer Chromatography (TLC) method. TLC was conducted to identify four common secondary metabolites produced by either terrestrial plants or aquatic plants, namely alkaloids, flavonoids, phenols. And terpenoid compounds. TLC examinations were conducted based on standard methods applied in Pharmacognosy and Phytochemical Department, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia.

Aedes aegypti larvae were isolated from collected mosquito eggs using ovitraps. The isolated larvae were grown until reaching 3rd or 4th instar larva stadium. The object of this study consisted of *Aedes aegypti* larvae at 3rd and 4th instar stadium. The larvae were sorted randomly into treatment containers consisting of 25 larvae per container.

In this examination, there were five different concentrations of *Cabomba aquatica DC not Aubletii* ethanol extract given to the treatment groups, one groups was treated as negative control group by giving no larvicide substance and one positive control group given 1% *temephos* 1 ppm solved in 100 mL aquabidest.

The procedures of the examination were: seven sets of beaker glass with 120 mL volume were filled with five different concentrations of *Cabomba aquatica DC not Aubletii* ethanol extract, *temephos* and aquabidest, and aquabidest (without larvicide substances for negative control group); 25 x4x 3rd and 4th instar stadium *Aedes aegypti* larvae were pxixut into each beaker glass; after 24-hour treatment, the number and percentage of dead larvae were counted and recorded. This procedure was conducted in four repetitions.

Results and Discussion

Based on gravimetric analysis, water contents of *Cabomba aquatica DC not Aubletii* ethanol extract was 9.49%. This result indicated that *Cabomba aquatica DC not Aubletii* powder was at prescribed condition when being extracted indicated by its water content was lower than 10%.

Figure 1 indicated four black spots. RF values of these spots were to be measured.

Figure 1. Result of TLC Examination on Positive Polyphenol Compounds

The recapitulation of these RF values was presented on Table 1 below:

Table 1. RF Values and Spot Color of Polyphenol Compounds Identified in TLC Examination

No.	RF Value	Spot Color
1	0.38	Black
2	0.50	Black
3	0.63	Black
4	0.81	Black

Cabomba aquatica DC not Aubletii is one of aquatic plant species that is widely cultivated in Indonesia as aquatic ornamental plants in aquariums because of its beautiful appearance and low-cost treatment. Indonesian aquatic plant farmers tend to cultivate this species because of it is quite easy and fast to reproduce through vegetative mechanism in aquarium or other artificial aquatic ecosystem and for high demands of this species in either local and international markets.

The water content of *Cabomba aquatica DC not Aubletii* ethanol extract suggested that *Cabomba aquatica* powder was at prescribed condition when being extracted indicated by its water content (according to gravimetric examination) was below 10%. *Cabomba* powder used in this study was obtained from all parts of *Cabomba* plant because it was almost impossible to dissect and divide the plant into its parts, such as stem, leaves, flowers, and roots due to its minuscule size as recommended in extracting powder procedure. In this study, ethanol 96% was chosen as solvent in extraction process because of its high polarity and non-toxic characteristic so that it was widely used in extract preparation before being tested to experimentation animals.

According to the result of TLC examination on *Cabomba aquatica DC not Aubletii* ethanol extract, the most prominent secondary metabolite quantities were polyphenols and terpenoids. Both of these compounds were widely known for its medicinal function and as toxin for living cell, depending on its dosage and concentration.

In general, this study showed that new prospect and challenges related to the use of *Cabomba aquatica DC not Aubletii* as natural treatment agents besides its former functions as aquatic ornamental plants in aquariums and as mutual symbiotic agent in fish cultivation. Based on the result of examination on *Cabomba aquatica DC not Aubletii* ethanol extract secondary metabolite contents, it was showed that the main secondary metabolites came from polyphenol compound and terpenoid compound classes. Previous studies mentioned polyphenol compounds group as one of dominant secondary metabolites found on the whole parts of aquatic plants submerged in water. Results of TLC examination on *Cabomba aquatica DC* ethanol extract showed accordance to the theory. *Cabomba aquatica DC* was one of aquatic plants which all of its parts were submerged in water [11, 16, 21].

The results of Thin Layer Chromatography examination conducted on *Cabomba aquatica DC* ethanol extract was qualitative description. It could not describe percentage of each secondary metabolites compound furthermore differentiating phytochemical compounds. These variables could be analyzed by measuring RF values quantitatively. Therefore, there were four different polyphenol compounds found on *Cabomba aquatica DC* ethanol extract.

According to the previous studies, polyphenol compounds group was known as phytochemical compounds widely used for its medicinal purposes such as anti-cancer. Studies also found that polyphenol compound group as one of plants secondary metabolites which potentially functioned as insecticide and larvicide against insects larvae. Another function of the plant which has been demonstrated by previous study, was the potentiality to "sicken" the predators consuming the plant regularly. The main function of secondary metabolites was as medicinal herbs. Various studies also identified and found plant secondary metabolites, namely alkanes, alkenes, alkynes, aromatic simple compounds, lactona, essential oils and fatty acids, terpenes, alkaloids, steroids, isoflavonoids, pterocarpan, and lignin [11, 21, 23].

The effectivity of a plant extract as larvicide substance was influenced by many factors, such as plant species, parts of plant used, extraction method, and the type of solvent used in extraction process. Other factors which might affect the performance of plan extract as larvicide were plant's natural habitat or geographic origin, the numbers of diverse phytochemical compounds, and the maturity of plant itself [23].

In this study, the concentration of *Cabomba aquatica DC not Aubletii* ethanol extract was relatively high because the method applied produced crude extract therefore the phytochemical compounds resulted needed to be separated and processed further. The result also showed that at least there were four kinds of phytochemical compounds belonged to polyphenol group while six kinds of phytochemical compounds belonged to terpenoid groups. In this case, a quantitative examination needed to be conducted to determine Retention Factor (RF) Values of every stain appeared on TLC examination results.

Polyphenol compounds were found on almost all plants, particularly vascular plants. Related to the habitat of plants, polyphenol phytochemical compounds were often found on aquatic plants whose all (or half) of its parts was submerged in water [11, 21, 23]. Polyphenol compound was known for its potentiality as larvicide against mosquito larvae either by itself or along with other kinds of phytochemical compounds [11, 21, 24]. Secondary metabolites of polyphenol compounds that were known for its potentiality as larvicide against mosquito larvae were flavonoids, tannin, and lignin.

According to previous studies, the mechanism of polyphenol compounds group as larvicide was very fast and not very specific. The scope included proteins that could be associated with enzymes, receptors, molecular markers, ionic channels and structural proteins, nucleic acids, biological membranes, and other cellular and molecular components. Principally, although it included mechanism in broader sense, the mechanism affected physiological aspects of the target through various receptor sites which eventually caused nervous system abnormality/disorders [21, 26, 28].

Nervous system disorder could occur on the processes of forming, storing, releasing, binding, and retrieving neurotransmitters, on receptor activation and function, and on enzymatic disruption in signal transduction pathway. Other mechanism which had been studied was inhibition mechanism of acethylcholine inhibition by essential oils, Gamma Aminobutyric Acid (GABA) chloride channel gate by thymol, changing sodium and potassium ions through pyrethrin disruption, and cellular respiration inhibition by rotenone. Most of disruptions were also associated with calcium channel blocking by ryanodine, associated with cell membrane actions, related to octophamine reception by thymol, disruptions on hormonal balance, poisoning during mitosis process by azadirachtine, disruption on molecular basis of morphogenesis, and alterations in cholinergic behavior and memory system by essential oils [21, 27, 28].

Most of these mechanisms were affected by inhibiting the activation of *Acethylcoline Esterase (AChE). AChE* was the key enzyme responsible to stop nerve stimulation transmission through synapses. Based on observation, it was found that AChE was associated with resistance against organophosphate and carbamate. It was assumed that the resistance against insecticide by some insects were influenced by AChE activity [21, 27].

The results of RF value examination (as presented on Table 1) indicated the lowest RF value of polyphenol compounds was 0.38 while the highest value was 0.81.

The result of TLC examination using Silica Gel GF254 plate also identified the occurrence of terpenoid compounds. The mobile phase used on this examination was hexane : ethyl acetate (4 :1) and stains used was anisaldehyde compound –sulphuric acid.

Figure 2 showing the result of TLC examination indicating stains with six different colors: green, yellow, green, indigo/magenta, and purple when being observed under room light. This result suggested that *Cabomba aquatica DC* ethanol extract also contained secondary metabolites from terpenoid compound groups.

Figure 2. Result of TLC Examination on Positive Terpenoid Compounds

The results of RF value measurement on each colored stain (measured with mobile phase distance 8 cm) were presented on Table 2.

Table 2. Results of RF Values Measurement and Spot Colors of Terpenoid compounds Resulted from TLC Examination

No.	RF Value	Spot Color
1	0.19	Green
2	0.25	Yellow
3	0.38	Green
4	0.44	Green
5	0.50	Magenta
6	0.56	Purple

Figure 2 indicated the occurrence of six spots. Retention Factor (RF) of these spots were measured (and presented on Table 2). The results of RF value measurement indicated that *Cabomba aquatica DC not Aubletii* ethanol extract contained phytochemical compounds from terpenoid group with the highest RF value was 0.19 and the highest value was 0.56.

The result of Thin Layer Chromatography (TLC) examination indicated that *Cabomba aquatica DC not Aubletii* ethanol extract also contained terpenoids. Terpenoids was commonly found on both terrestrial and aquatic plants. Previous studies stated that terpenoids was a form of secondary metabolites which was widely known for its function as insecticide, including as larvicide against mosquito larvae [29, 30].

WHO (2005) described several characteristics of bio-larvicide. They are: environmentally friendly, bio-degradable, and having low toxicity on non-target organisms making bio-larvicide relatively safer for long-term application [31].

Terpenoids compound group was often found in combined forms along with other phytochemical compound such as combined with saponin forming terpenoid-saponin. Terpenoid-saponin also functioned as larvicide against mosquito larvae (including *Aedes aegypti*) [29, 30].

Phytochemical compound groups derived from plants or plant extract was commonly called secondary metabolite or secondary metabolite compounds. Basically, this compound was produced by plants as a response against threats and dangers existing in its environment. These threats and dangers might come from plants (as competitors) or from animals consuming the plants (consumers or predators) in the vicinity [19, 21].

Predators (or consumers) of the plants consisted of aquatic animals, crustaceans, and aquatic and land mammals whose habitat near aquatic ecosystem and whose source of nutrients included aquatic plants. This condition made the condition of aquatic plants became more vulnerable compared to other members of the ecosystem. As a response against this condition, aquatic plants conducted a form of defense mechanism namely by producing and secreting secondary metabolites to "repel" its predators [11, 19, 21].

Like polyphenols, terpenoids is included as aromatic compound, known for its potentiality as larvicide against mosquito larvae [23]. In general, the mechanism of terpenoids functioning as larvicide was similar to other phytochemical compound functionality as larvicide, namely by causing interference on nervous system of targeted insect which molecular processes have been explained earlier [23, 28].

The results of examination on *Cabomba aquatica DC Aubletii* potentiality as larvicide against collected 3rd and 4th instar *Aedes aegypti* larvae after 24-hour exposure to *Cabomba aquatica DC not Aubletii* ethanol extract was presented on Table 3 below:

Table 3. Average Percentage of Dead Larvae on Each Treatment Group

Treatment	Average Percentage of Mortality of Larvae
Negative control	0.00
1250 ppm	18.00
2500 ppm	52.00
5000 ppm	67.00
10000 ppm	84.00
20000 ppm	96.00
Positive control	100

Table 3 showed that the dead percentage of 3rd and 4th instar *Aedes aegypti* larvae of the five treatment groups which had been exposed to different dosages of *Cabomba aquatica DC not Aubletii* ethanol extract was found on 20000 ppm dosage (as the highest among treatment group). This value was much higher compared to negative control group yet this result was slightly lower than the value of positive control group which had been exposed to *temephos*).

The result of this examination was followed by statistical analysis in order to examine the significance of the results and to determine LC50 and LC90 values for 24-hour exposure with probit analysis test. The results of statistical analysis were obtained by comparing the average percentages of dead 3rd and 4th instar *Aedes aegypti* larvae. The comparison between 20000 ppm *Cabomba aquatica DC not Aubletii* ethanol extract dead larvae percentage and positive group of mortality larvae percentage indicated t-statistic value (p) 0.724 which was higher than significance margin (α) 0.05. This result indicated was no significant difference between the two treatments. The comparison between 20000 ppm *Cabomba aquatica* DC not Aubletii ethanol extract and negative control group indicated t-statistic value (p) 0.000 which is lower than significance margin (α) 0.05 indicating significant difference between treatment group and negative control group which was not exposed to ethanol extract at all. The comparison between 1250 ppm and 2500 ppm treatment groups indicated t-statistic value (p) 0.000 lower than significance margin (α) 0.05 indicating that there was significant difference between the two groups. The comparison among 5000 ppm, 10000 ppm, and 20000 ppm treatment groups indicating there were significant differences among the groups.

Meanwhile, LC50 and LC90 values for 24-hour exposure were 2405 ppm and 13500 ppm respectively. The results showed that the concentrations of *Cabomba aquatica DC not Aubletii* ethanol extract did affect *Aedes aegypti* 3rd and 4th instar larvae mortality rate. The higher concentration of *Cabomba aquatica DC not Aubletii* ethanol extract applied the higher *Aedes aegypti* 3rd and 4th instar larvae mortality rate.

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