EFFECT OF NITROGEN FERTILIZATION AND HARVESTING PERIOD ON PHYTOCHEMICAL CONTENT AND ANTIOXIDANT ACTIVITIES OF *Cymbopogon citratus*

WAN NUR AIN SYUKRIAH BINTI WAN MARZUKI RASHIDI

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ABSTRACT

Cymbopogon citratus is grown due to its economic benefits with high antioxidant activities. However, the biosynthesis of secondary metabolites and antioxidant activities of plants are often dependent on several factors such as the presence of nutrients and harvesting period. In this study, the effects of different nitrogen concentrations (0, 100, 200 kg/ha) and harvesting periods of 150, 180, 210, 240 and 270 days after transplant (DAT) on the yield, phytochemical content and antioxidant activities of *C. citratus* essential oil and ethanolic extract were evaluated. The experimental design used was a randomized complete block design (RCBD). The essential oil was extracted by hydrodistillation whereby ethanolic extract was obtained using the maceration technique. The evaluated phytochemical content comprises the total terpenoid content (TTC), total phenolic content (TPC) and total flavonoid content (TFC), while the antioxidant activities were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Correlations between the factors and responses were examined using Pearson's correlation and it was found that the highest yield of essential oil and citral content was from 150 DAT (200 kg/ha N), while highest ethanolic extract yield was obtained at 150 DAT (0 kg/ha N). Major compounds detected in essential oil were myrcene, neral, geranial, geranyl acetate, 5-epi-7-epi-α-eudesmol, selina-6-en-4-ol, hinesol, α-cadinol, juniper champor and farnesal (2E, 6E). Both extracts exhibited the highest DPPH scavenging and FRAP activities at 150 DAT and 210 DAT, respectively. As for ethanolic extract, TPC and TFC were the highest at 150 DAT, while TTC was the highest at two harvesting periods of 150 DAT and 180 DAT. Almost all dependent variables showed a strong correlation with harvesting period. Overall, 150 DAT was proven to be better in maintaining high yield, phytochemical content and DPPH activity while only FRAP of extracts showed the highest activity at 210 DAT. These findings showed that biological activity and phytochemical content in *C. citratus* vary in response to harvesting periods and nitrogen concentrations.

ABSTRAK

Cymbopogon citratus di tanam kerana nilai ekonominya dan juga mempunyai nilai aktiviti antioksida yang tinggi. Walaubagaimanapun, biosintesis metabolit sekunder dan aktiviti antioksida tumbuhan sering bergantung kepada faktor seperti kehadiran komposisi nutrien dan tempoh penuaian. Dalam kajian ini, kesan kepekatan kandungan nitrogen yang berbeza (0, 100, 200 kg/ha) dan tempoh penuaian pada 150, 180, 210, 240 dan 270 hari selepas pemindahan (DAT) pada hasil, kandungan fitokimia dan aktiviti antioksida minyak pati dan ekstrak etanol *C. citratus* dinilai. Reka bentuk kajian yang digunakan adalah Reka Bentuk Blok Lengkap Rawak (RCBD). Minyak pati diekstrak dengan kaedah hidrodistilasi manakala ekstrak etanol menggunakan maserasi. Kandungan fitokimia yang dinilai adalah kandungan sebatian terpenoid (TTC), kandungan sebatian fenolik (TPC) dan kandungan sebatian flavonoid (TFC). Manakala aktiviti antioksida dinilai menggunakan 2,2-diphenyl-1-picrylhydrazyl (DPPH) merangkap aktiviti radikal bebas dan pengurangan kuasa antioksida ferik (FRAP). Hubungan korelasi dianalisis dengan menggunakan korelasi Pearson. Hasil tertinggi minyak pati dan kandungan citral didapati pada 150 DAT (200 kg/ha N), sementara hasil ekstrak etanol paling tinggi diperoleh pada 150 DAT (0 kg/ha N). Sebatian utama yang dikesan dalam minyak pati ialah myrcene, neral, geranial, geranyl acetate, 5-epi-7-epi-α-eudesmol, selina-6-en-4-ol, hinesol, α-cadinol, juniper champor and farnesal (2E, 6E). Kedua-dua ekstrak mempunyai nilai DPPH dan FRAP tertinggi pada 150 DAT dan 210 DAT, masing-masing. Bagi ekstrak etanol, nilai TPC dan TFC tertinggi adalah pada 150 DAT, manakala TTC adalah tertinggi pada dua tempoh penuaian, 150 DAT dan 180 DAT. Hampir semua variable terikat menunjukkan hubungan korelasi yang kuat dengan masa penuaian. Secara keseluruhan, penuaian pada 150 DAT terbukti lebih baik dalam memberi hasil, kandungan fitokimia dan nilai DPPH yang tinggi dalam *C. citratus* manakala FRAP menunjukkan aktiviti yang tertinggi pada 210 DAT. Penemuan ini menunjukkan aktiviti biologi dan kandungan fitokimia dalam *C. citratus* berbeza sebagai tindak balas kepada tempoh penuaian dan kepekatan nitrogen.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	-	percentage
°C	-	degree celcius
µL	-	microliter
µM	-	micromolar
µm	-	micrometer
g	-	gram
kg/ha	-	kilogram per hectare
L	-	liter
m	-	meter
m/mol	-	millimol
m^2	-	meter square
mg	-	milligram
min	-	minutes
mL	-	milliliter
mm	-	millimeter
s	-	seconds
w/v	-	weight/volume basis
M	-	Molar
ANOVA	-	Analysis of variance
BHA	-	Butylated hydroxyanisole
BHT	-	Butylated hydroxytoluene
BVOC	-	Biogenic volatile organic compounds
DAT	-	Days after transplant
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
DW	-	Dry weight
FRAP	-	Ferric reducing antioxidant power
GAE	-	Gallic Acid Equivalent
GC-FID	-	Gass Chromatography-Flame Ionization Detector

GC-MS	-	Gas Chromatography-Mass Spectrometry
IC_{50}	-	Concentration at 50% inhibition
LE	-	Linalool Equivalent
N	-	Nitrogen
QE	-	Quercetin Equivalent
RCBD	-	Randomized complete block design
TFC	-	Total flavonoid content
TPC	-	Total phenolic content
TPTZ	-	2,4,6-tripyridyl-S-triazine
UV-VIS	-	Ultraviolet-visible spectrophotometry

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Wan Nur Ain Syukriah Wan Marzuki Rashidi, Norhayati Muhammad, Nur Halimah Sairi, Nur Fazira Abdul Rahim, Balkis A Talip, Norazlin Abdullah and Mohd Fadzelly Abu Bakar (2019). Phytochemical analysis and *in-vitro* antiurolithiatic properties of selected Malaysian herbs. *Journal of Advanced Research in Fluid Mechanics and Thermal Sciences, 64*(1), 152-159. (SCOPUS). [Published].

CHAPTER 1

INTRODUCTION

1.1 Research background

Cymbopogon counts more than 635 genera and 900 species and *Cymbopogon citratus* (*C. citratus*) is one of the best-known species of the genus which is also known as 'serai makan' in Malaysia (Majewska *et al.*, 2019). It is a tall, perennial grass native to a warm temperate tropical region (Lawal *et al.*, 2017). The grass has a pleasant lemony aroma when cut due to a high amount of its main constituent called citral (Shahi *et al.*, 2005). It is commonly used in cooking and aromatherapy treatments such as massage oil and is often prepared as tea because of its fragrance. *C. citratus* essential oil is rich in terpene compound while ethanolic extract is rich in polar phenolic compound (Kusmardiyani, Alfianti, & Fidrianny, 2016; Rana, Das, & Blazqeuz, 2016). Both terpenes and phenol possess a wide array of bioactive compound benefits to human health. The use of *C. citratus* as essential oil and ethanolic extract is known for its multiple medicinal properties such as antioxidant, antimicrobial and anticancer activities from the presence of its phytochemical content (Anes *et al.*, 2017; Ekpenyong, Akpan, & Nyoh, 2015; Halabi & Sheikh, 2014; Trang *et al.*, 2020).

Based on the statistics from the Department of Agriculture (DOA), the total area of plantation for this crop increased every year, such as with 990 hectares in 2008, by which the increasing trend started from the year 2005 to 2008 (Abdullah, Sharu, & Ahamad, 2013). Nowadays, an estimated of 283 to 324 hectares of *C. citratus* cultivation in Beranang produces a daily yield of not less than five tonnes (Bernama, 2019 June 18). Therefore, developing a suitable strategy for plantation is seen as essential in meeting the growing market demand for these crops. The application of agricultural management is one way to overcome losses and maximize its yield.

Currently, the Malaysian Agricultural Research and Development Institute (MARDI) and The Department of Agriculture (DOA) recommended concentration of 70 kg/ha and 130 kg/ha of nitrogen for *C. citratus*, respectively (Syed Alwi *et al.*, 2005). However, no investigation has been done to uncover any effects of the different nitrogen concentrations towards *C. citratus* quality. Hence, nitrogen concentrations selected for this research is 0, 100 and 200 kg/ha based on the recommended nitrogen concentrations. Other than that, it is also proven that nitrogen and weather during cultivation are capable to affect the yield, phytochemical composition and antioxidant activity (Jardinetti *et al.*, 2016; Shehzad *et al.*, 2012; Zheljazkov *et al.*, 2011).

As such, it is necessary to understand how plants interacts in ecosystem and identify the management practices that allow them to maximize the use of growth resources in their environment. Research has uncovered that the availability of plant nutrients is an important factors in determining secondary metabolism synthesis within plants (Aires, Rosa, & Carvalho, 2006; Kopsell *et al.*, 2003; Stewart *et al.*, 2001). Nitrogen fertilizer is one of the most important growth factors in controlling the yield and quality of plants. Moreover, nitrogen also modulates the biosynthesis of secondary metabolites such as phenolic and terpenoid compounds (Aires *et al.*, 2006; Nurzyěska-Wierdak, 2013). The same goes for the harvesting periods in which every different plant species have their own optimum time to be harvested. Thus, the current study was conducted to investigate the effects of nitrogen concentrations and harvesting periods on *C. citratus* in terms of yield as well as the phytochemical content and antioxidant activities of essential oil and ethanolic extract.

1.2 Problem statement

The chemical composition of plants may vary according to several factors such as nitrogen concentrations and harvesting periods (Petropoulos *et al.*, 2020). The differences in environmental conditions could affect the active substances in the same plant species in terms of types, contents, and proportions of the chemical composition (Liu *et al.*, 2016). Other than that, active substances are the result of the interaction between plants and the environment, which is mediated mainly by the biosynthesis of secondary metabolites (Peñuelas & Llusià, 1997) and as a result, plants are able to tolerate environmental stresses (Sampaio, Edrada-Ebel, & Da Costa, 2016). However, it was found that the inhibition or synthesis of certain plant metabolites is related to the environment (Tiago *et al*., 2017). Hence, the influence upon the environment on plants such as nutrient limitation and growth conditions could suppress the production of chemical compounds in plants (Ncube, Finnie & Van Staden, 2012).

The harvesting periods of *C. citratus* have not been extensively studied although the data under this knowledge area is limited. For instance, lengthy harvesting periods could delay the supply and demand for *C. citratus* since farmers are not able to cope with the high commodity demand (Bernama, 2019 June 18). The lengthy harvesting periods can negatively impact the supply of the commodity and lead to supply shortages and higher prices for *C. citratus*. As recommended by the Malaysian Agricultural Research and Development Institute (MARDI), the current harvest takes place from 240 to 270 days after transplant (Abdullah, Herman, & Mohd, 2013). Since *C. citratus* is important in the industry, farmers must identify the proper harvesting periods for *C. citratus* so as to obtain high yield and quality but over shorter harvesting periods, especially since it is a common herb used in Malaysia.

No scientific studies have been conducted to establish the effect of nitrogen concentrations together with the harvesting period of *C. citratus*; hence, the mechanism behind these two conditions towards *C. citratus* remains unknown. Nitrogen is an essential nutrient for the life cycle of plants; however, the excessive use of this element may causes serious problems in agriculture as this can disrupt the production of many important plants. Other than that, the harvesting periods can also influence the biosynthesis and accumulation of chemical compounds (Adegbaju, Otunola, & Afolayan, 2020). Hence, this study will determine the ideal harvesting periods involving current farmers' practice and scientific recommendations.

1.3 Research objectives

This study aims to examine the effects of different nitrogen concentrations and harvesting periods on the phytochemical content and antioxidant activities of *Cymbopogon citratus*. Meanwhile, the specific objectives are as follows:

i. To measure the yield and phytochemical content of *Cymbopogon citratus* essential oil and ethanolic extract under different nitrogen concentrations and harvesting periods.

ii. To evaluate antioxidant activities (*in-vitro*) of the *Cymbopogon citratus* essential oil and ethanolic extract under different nitrogen concentrations and harvesting periods.

iii. To correlate the yield, phytochemical content and antioxidant activities of the *Cymbopogon citratus* essential oil and ethanolic extract with nitrogen concentrations and harvesting periods.

1.4 Scope of the study

The scope of the study are as follows:

i. The harvesting periods applied in this study include 150, 180, 210, 240 and 270 days, while the nitrogen concentrations used were 0, 100 and 200 kg/ha.

ii. The treatment parameter (harvesting periods and nitrogen concentrations) was determined using randomized complete block design (RCBD) with factorial 3 × 5.

iii. The essential oil of *C. citratus* was extracted through hydrodistillation method.

iv. The ethanolic extract of *C. citratus* was obtained by using ethanol solvents through the maceration method.

v. Chemical compounds in *C. citratus* essential oil were identified using GC-MS and GC-FID.

vi. Phytochemical content was determined based on the Total Terpenoid Content (TTC), Total Phenolic Content (TPC), and Total Flavonoid Content (TFC).

vii. Antioxidant activities were determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays.

viii. The correlation of phytochemical content and antioxidant activities with harvesting periods and nitrogen concentrations was determined.

ix. The correlation between phytochemical content with antioxidant activities was determined.

1.5 Significance of the study

The growing demand for essential oils across the world was estimated at USD 6.63 billion and is projected to rise from 2018 to 2025, resulting in a large scale *C. citratus* plantation (Kumadoh & Ofori-kwakye, 2017; Lal *et al*., 2018). Products containing extract from *C. citratus* are essential in terms of aromatherapy, food, flavours, pharmaceuticals, disinfectants and insect repellent. Their importance is widely known and appreciated in plant chemotaxonomy. The use of herbs has also shown an increasing trend as the market findings indicates a significant increase in the sales of herbal supplements (Lindstrom *et al*., 2013). *C. citratus* is known to have numerous health benefits such as antioxidants, antimicrobial and anti-malarial activity (Lawal *et al*., 2017). In addition, the use of *C. citratus* as a preventive measure for numerous health problems are quite popular (Shah *et al*., 2011). Apart from that, herbs are said to be more affordable, give lesser side effects, easily assessable and improve overall health alternatives.

The investigation of harvesting periods and nitrogen concentrations are important in order to examine their influence on the phytochemical and antioxidant activities of *C. citratus*. If suitable harvesting periods and nitrogen fertilizer concentrations of *C. citratus* can be obtained, the highest quality of *C. citratus* extracts can be acquired. By identifying the most optimal parameter, it is possible to minimize costs, time and labour, most of which benefits such as improved bioactive properties from *C. citratus*. The results will also help in increasing their phytochemical content and antioxidant activities; hence, many of the benefits can be gained by manufacturing sectors as well as the consumer.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter presents the literature review related towards the research title. In this chapter, information on the yield, phytochemical and antioxidant activities of *C. citratus* essential oil and extracts from the previous studies are discussed, including the effects of nitrogen concentrations and harvesting periods with regard to these properties. The correlation effects and statistical analysis are also explained briefly.

2.2 *Cymbopogon citratus*

Cymbopogon citratus of the Poaceae family (formerly Gramineae) is locally known as lemongrass or 'serai makan'. This plant has been widely distributed due to its beneficial usage. Table 2.1 shows the taxonomic hierarchy of *C. citratus*. The genus *Cymbopogon* itself has about 180 species and it is usually easily recognizable by its long and thin grass and densely tufted root (Bertea & Maffei, 2009). It is perennial, robust, and up to 3 to 6 feet tall with thin long leaves (Abbas *et al.*, 2018). The *C. citratus* plants grows best in tropical or subtropical climates (Joshi, 2018).

Table 2.1: The taxonomic hierarchy of *Cymbopogon citratus*

Class	Monocotyledonae
Order	Cyperales
Family	Poaceae
Genus	*Cymbopogon*
Species	*Cymbopogon citratus*

Previous studies have shown that *C. citratus* possesses antioxidant, antiamoebic, antimicrobial, antidiarrheal, antifungal, antiurolithiasis and antiinflammatory properties (Ahmed & Hassan, 2017; Manvitha & Bidya, 2014). Traditional practitioners often dry the leaves for making tea to relieve symptoms such as congestion, coughing, bladder disorders, and digestive problems (Lonkar *et al.*, 2013). Besides, the grasses have essential oil with high insect repellent activity (Soonwera & Phasomkusolsil, 2014). The fact that essential oil and its components are fast excreted and not accumulated in the organism strongly suggest that they can be successfully used as safe pharmaceutical medical drugs (Herman & Herman, 2015). In industry, *C. citratus* was extracted in the form of essential oil since citral, a major compound is a terpenoid which is easily extracted by hydrodistillation due to its volatile characteristics.

The plants are said to be originating from India; however, the plants are now widely naturalized in Indonesia, Malaysia, Myanmar, Nepal, and Thailand (Dama *et al*., 2011; Jamuna *et al*., 2017). The grass is cultivated in tropical and subtropical countries for the lemon-scented oil extracted from its leaves (Akhila, 2010). The production area of *C. citratus* cultivation in Malaysia is up to 1598 hectares and the total production is 13343 metric tonnes (Bernama, 2019 June 18). The first harvest can take place from 240 to 270 days after transplant as recommended by the Malaysian Agricultural Research and Development Institute (MARDI) (Abdullah, Herman & Mohd, 2013) and it can be harvested frequently during the active growing season, up to once every month. Furthermore, the number of harvests in a year depends on factors such as the temperature, rainfall, and the level of soil fertility (Joy *et al.,* 2006).

The plants grow in dense clumps and many cultivars from other countries have been acknowledged, which differ considerably in yield percentage and citral content (Rana *et al.*, 2016). Additionally, the same species of different localities may have different constituents and different species with different geographical distributions may have similar constituents in terms of essential oil. It is cultivated for 3 to 4 years and the crop is maintained through the vegetative propagation process by dividing the clumps (Desai, Parikh, & De, 2014). It was reported that the weed could threaten the normal growth of *C. citratus* and, hence, it should be avoided for the initial 60-75 days for better growth of *C. citratus* (Gawali & Meshram, 2019). The crop should be harvested from the second to the fourth year of planting to gain maximum herbage yield effect (Akhila, 2010).

2.3 Plantation of *Cymbopogon citratus*

The randomized complete block design (RCBD) is one of the most widely used experimental designs in *C. citratus* field studies (Bekele *et al*., 2019; Prins *et al*., 2010; Tajidin *et al*., 2012). The experimental units are grouped into blocks according to known or suspected variation which is isolated by the blocks (Bowers, 2012). Variation such as fertilizer and harvesting periods can be isolated by appropriate blocking (Ameen *et al.*, 2019). Within each block, the conditions are as homogeneous as possible; however, between blocks, large differences may exist (Dutilleul, 1993), resulting in relatively small gradients within each block so that the treatments may be compared under relatively homogeneous conditions. The treatments are assigned within the individual blocks at random with separate randomization for each block (Tajidin *et al.*, 2012). Figure 2.1 shows the *C. citratus* plantation by RCBD.

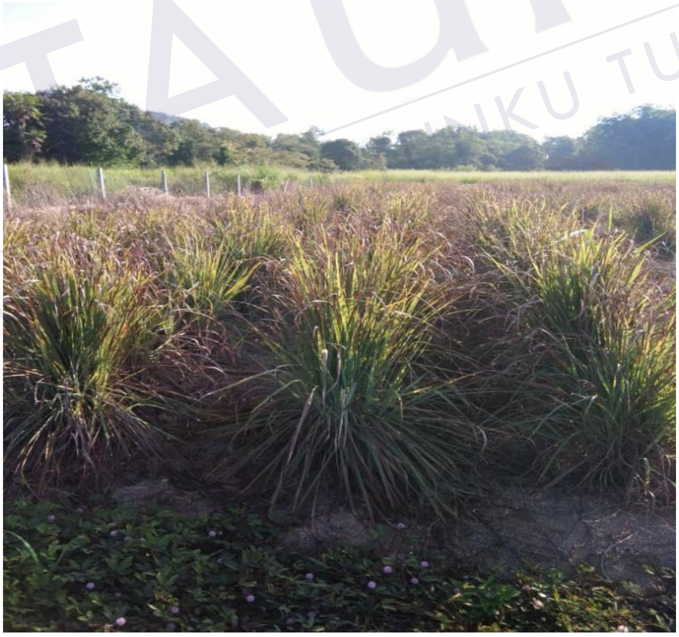

Figure 2.1: *Cymbopogon citratus* plantation at MARDI, Kluang

2.4 Essential oil

Hydrodistillation is a conventional technique in extracting essential oil from plants (Dhobi, Mandal & Hemalatha, 2009). In particular, there are three types of hydrodistillation that include water distillation, water-steam distillation and steam distillation (Vankar, 2004). This technique involves three main physicochemical processes: hydrodiffusion, hydrolysis, and decomposition by heat (Azmir *et al.*, 2013). Due to the influence of hot water and steam during the extraction, the essential oil from specialized secretory structures such as glandular trichomes, oil, or resin ducts is freed from the oil glands of the plant tissue (Kakaraparthi *et al.*, 2014). A direct hot steam with the plant parts during steam distillation leads to overheating of the plants, thus resulting in low-quality extracted oils (Sovová & Aleksovski, 2006). Hence, water distillation is more preferable since all surface area of the plants is immersed completely in the water. As a result, the surrounding water acts as a barrier to hinder the plants from overheating (Thakker, Parikh, & Desai, 2016).

2.5 Ethanolic extract

Solvent extraction such as macerations, decoctions, infusions, fluid extracts, tinctures or powdered extracts could be used to extract *C. citratus* (Handa *et al.*, 2008). Maceration involves soaking plant materials in a suitable solvent. Which will then be left at room temperature for the extraction to complete. The advantage of this method is that it does not involve high temperature, thus reducing the chances of loss of bioactive compounds. Moreover, scientists have discovered that highly polar solvents, such as ethanol, is highly effective in extracting phenolic compounds (Koffi *et al.*, 2010). The resulting extract that usually containing fragrance compounds, waxes, resins and dye compounds are called oleoresins (Nurzyěska-Wierdak, 2013). However, it is a time-consuming procedure with the use of a high volume of solvents. Another disadvantage includes the extract obtained through solvent-aided extraction containing traces of solvents that may contaminate foods (Ranitha *et al.*, 2014); hence, ethanol is preferable to recover polyphenols compared to other organic solvents because it is non–toxic and safe for human consumption (Do *et al.*, 2014).

2.6 Phytochemical

The medicinal prominence of plants lies in the chemical constituents present in them, which are termed phytochemicals or specifically known as secondary metabolites. The four major groups of secondary metabolites are terpene, phenols, glycosides and alkaloids. These secondary plant metabolites are classified according to their structure, which defines their specific functions in plant growth and development. In addition, continuous changes of abiotic and biotic stresses in the environment could also cause changes in the formation of these metabolites (Ewansiha *et al.,* 2012). Overall, this subtopic will go through specific active ingredients analyzed in this study, which include terpenoids, phenols, and flavonoids.

2.6.1 Terpenoids

Terpenoids are compounds that are made from isoprene; hence they are often referred to as isoprenoids. Table 2.2 shows the classes of terpenoids according to their isoprene unit. They are divided on the basis of their carbon skeleton (Grassmann, 2005). Different chemotypes of the *Cymbopogon* species contain varying major terpenoid compounds such as citral, geraniol, citronellol, piperitone and elemin (Avoseh *et al.*, 2015). Terpenoids in the *Cymbopogon* genus could be subcategorized into volatile and non-volatile types (Avoseh *et al.*, 2015). Many researchers have obtained positive terpenoid tests from *C. citratus* (Chowdury *et al*., 2015; Dama *et al*., 2011; Soares *et al*., 2013).

Table 2.2: Classes of terpenes found in *Cymbopogon citratus* (Vyshali, Saraswathi, & Mallavarapu, 2015).

Terpenoid	Number of C-atoms	Number of isoprene subunits
Monoterpene	10	2
Sesquiterpene	15	3

A study by Oluyemi, Ayodele & Oluyemi (2018) showed that the aqueous and methanol extract of *C. citratus* had a higher composition of terpenoids with the aqueous extract yielding 25.25 ± 0.08 ppm and the methanol extract yielding 41.03 ± 0.00 ppm. A study by Bouzenna *et al.* (2017) has proven that citral as the main aldehyde monoterpene in *C. citratus* demonstrated significant antioxidant activities.

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