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Cytotoxicity and fragmentation pattern of *Datura metel* L. leaves using ultra-performance liquid chromatography-mass spectroscopy

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ABSTRACT

This scientific investigation was conducted to evaluate the cytotoxicity and ascertain the pattern of fragmentation of *Datura metel* L. leaves using ultra-performance liquid chromatography-mass spectrometry. Phytochemical screening, isolation, and cytotoxic testing with the brine shrimp lethality test have been carried out on *D. metel* L. leaves extract. Preparative thin-layer chromatography with chloroform:n-hexane (1:1 *v/v*) eluent was used to carry out the compound isolation process. The octadecyl silica column was the stationary phase employed. The motion phase that was used was an isocratic elution system with a positive ion mode and acetonitrile:water (15:85 *v/v*) ratio. The retention time (tR) on the chromatogram was 3.20 minutes, and there was just one visible distinct peak. The results showed that the *Datura* leaves contain flavonoids, alkaloids, steroids, and saponins. The cytotoxic activity test showed a high cytotoxic potential of *Datura* leaf extract with an LC_{50} value of 46.1636 µg/ml. The result also showed that the isolate was a steroid that belongs to the Withanolide group, namely, Baimantuoluoline D. The isolate had a molecular weight of $[M^+]$ 504.0591 m/z with daughter fragments forming 477.2583 m/z $[M^+$- CH3-OH] and 301.1780 $[M^+-C_9H_{11}O_4]$. There are more than 600 activities of the Withanolide group that have been documented. However, the activity showed that Withanolide isolated from it has anti-microbial, anti-inflammatory, and cytotoxic potential. The phytochemical screening results showed that the *Datura* leaves contained alkaloids, flavonoids, steroids, and saponins. It is intended that a pharmaceutical formulation including *D. metel* essential oil as the active component will be created for further research.

INTRODUCTION

Datura (*Datura metel* L.) is a typical Asian plant commonly found throughout South Asia's tropics to Southeast Asia (Indonesia). This plant has an annual cycle, characterized by thorny fruit, 0.40–1 m high. The leaf size is ±15 cm, and the colors of the flower are either purple or white (Gaire and Subedi, 2013). The use of *Datura* as traditional medicine has been documented for centuries, and empirically, it is used as an asthma medication by utilizing its dried leaves. *Datura* is also used as an anti-bacterial, antiseptic, narcotic, and sedative (Carpa *et al*., 2017; Wu *et al*., 2020). *Datura metel*, which has a big population but has not been widely developed as an active ingredient in this therapy since the study of chemicals and fragmentation, has not been thoroughly applied. In the previous study, all components of the *Datura* plant were analyzed, but just phytochemical screening was done and no particular analysis of the active compounds was done. Antony *et al*. (2019) identified all parts of the *Datura* plant: the roots, stalks, leaves, flowers, fruits, and seeds, containing terpene and alkaloid compounds. The most alkaloid content is found in its roots and seeds. The Withanolide group, which is a steroid, is also a terpene group. The known alkaloid groups are atropine, hyoscyamine, scopolamine, and hyoscine. Besides, fatty substances and calcium oxalate were also found in the fruit (Antony *et al*.,

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2019; Carpa *et al*., 2017; Guo *et al*., 2018). *Datura* is classified as a toxic plant, with alkaloid compounds as the trigger factor. Several types of alkaloid compounds were found in hyoscyamine, atropine, and scopolamine (Carpa *et al*., 2017; Iranbakhsh *et al*., 2006). Identification of various secondary metabolites is needed to determine the specific effectiveness of *Datura* (*D. metel* L.). Identification can be done using ultra-performance liquid chromatography-mass spectroscopy (UPLC-MS) by looking at the specific fragmentation of *Datura* (*D. metel* L.). Several previous studies have tested the content of *D. metel* using high-performance liquid chromatography and gas chromatography-mass spectra (Hossain *et al*., 2013a, 2013b). This analysis was carried out to identify the phenolic content of *Datura* (*D. metel* L.).

Vadlapudi and Kaladhar (2012) researched *Datura* leaf extract. Methanol and n-hexane extracts at a concentration of 2 mg/ml formed inhibition zones of 6 and 8 mm, respectively, against *Staphylococcus aureus*. Yang *et al*. (2014) successfully isolated nine new isolates of the Withanolide group. Isolate 5,7-dimethyl 6-hydroxyl 3-amine sitosterol is isolated from *Datura* leaves, showing inhibition of *P. aeruginosa*, *B. subtilis*, *S. typhi*, *K. pneumonia*, *S. aureus,* and *P. mirabilis* (Okwu and Igara, 2009). One of them is the *Datura folisides,* a compound that shows a promising anti-inflammatory effect. Anti-inflammatory activity test used rat macrophages (murine) RAW 264.7 stimulated by Lipopolysaccharide. The study on the cytotoxic potential of *Datura* leaves has also been carried out by Bellila *et al*. (2011). The *Datura* leaf isolates of the Withanolide group, Withametelin, and 12α-hydroxydaturametelin B, showed cytotoxic effects on human lung carcinoma cells (A549) and human colorectal adenocarcinoma cells (DLD-1). Both isolates showed cytotoxicity to A549 and DLD-1 cells with IC_{50} values of 7 and 2.0 μM, respectively. Therefore, further research is needed to identify compounds and their cytotoxic activities using the brine shrimp lethality test (BSLT). The compound's toxicity potential can be determined based on the number of deaths of the tested animals.

Furthermore, the identification and isolation of secondary metabolites were carried out. The maceration stage was conducted by fractionation using the liquid-liquid partition. The isolation and purification used preparative thin-layer chromatography (TLC). The purification results obtained pure isolates, which were then identified using UPLC-MS. In this study, an analysis of the cytotoxicity and fragmentation pattern of *D. metel* L. leaves using is conducted. The UPLC-MS technique combines two methods in one analysis system. The isolate purity identification can use the UPLC chromatography system to detect compounds using mass spectroscopy by looking at the compound's fragmentation pattern and molecular weight (Jian, 2017; Yang *et al*., 2020). This research is expected to be continued with research on the formulation of active pharmaceutical ingredients using *Datura metel* essential oil.

MATERIALS AND METHODS

Extraction

In this study, extraction was performed by maceration using methanol 96% solvent (Dirks *et al*., 2016). The extraction stage was conducted by soaking 150 g of *Datura* (*D. metel* L.) leaf powder in 3,000 ml n-hexane. Maceration was carried out for 3 days and stirred every 12 hours. The maceration of the n-hexane extract was filtered. The filtrate obtained was concentrated using a rotary evaporator (IKA Germany) until it became a thick extract, and the remaining residue was evaporated in an oven at 45°C for 48 hours. The residue that was evaporated was then macerated again using 3,000 ml ethyl acetate. Maceration was carried out for 3 days and stirred every 12 hours. The result of the maceration of ethyl acetate extract was filtered. The obtained filtrate was concentrated using an evaporator until the extract became thick, and the remaining residue was evaporated in an oven at 45°C for 48 hours. The evaporated residue is macerated. The maceration of the methanol extract was filtered, and the filtrate obtained was concentrated using an evaporator. The concentrated thick extract was weighed afterward (Arivalagan *et al*., 2018; Dirks *et al*., 2016).

Phytochemical screening

Alkaloid identification

A total of 2 g of the extract was put into a test tube, dripped with HCl 2N, and then divided into several test tubes. Each tube was added with each reagent. If the extract forms a white or yellow precipitate after adding Mayer's positive reagent, the extract contains alkaloids. The extract contains alkaloids if an orange precipitate is formed after positive Dragendorff's reagent (De Silva *et al*., 2017).

Flavonoid identification

Several extracts were put into a test tube; then 10 ml of hot water was added, boiled for 5 minutes, and then filtered. From the filtrate obtained 5 ml was taken and added with magnesium powder and 1 ml of concentrated HCl was taken and added with amyl alcohol; shake the mixture till separation. If an orange, red, or yellow precipitate is formed, it is positive for containing flavonoids (De Silva *et al*., 2017).

Saponin identification

Several extracts were put into a test tube; then 10 ml of hot water was added and cooled; then they were shaken vigorously for 10 seconds. Being positive for saponins is indicated by forming foam as high as 1–10 cm for not less than 10 minutes, and with the addition of 1 drop of 1% HCl, the foam will be stable (Pandey and Tripathi, 2014).

Steroid and terpenoid identification

Several extracts were macerated with 10 ml ether for 2 hours and then filtered. From the filtrate obtained 5 ml was taken and then evaporated in a cup. To the residue obtained, 2 drops of anhydrous acetic acid and 1 drop of concentrated H_2SO_4 were added. When we see what is produced, red is positive for steroids, while purple is positive for terpenoids (Pandey and Tripathi, 2014).

Cytotoxic test

To determine the activity of *Datura* leaves, a cytotoxic test was carried out using the BSLT method. BSLT is one of the initial screening methods for testing cytotoxic compounds (Ramachandran *et al*., 2011). The first thing to do was to incubate the *Artemia salina* L. eggs in the *A. salina* L. egg-hatching vessel for 24 hours of hatching and within 48 hours. Preparation of

the standard solution in each extract is done by dissolving 100 mg of the extract in 100 ml of seawater to obtain a 1,000 ppm standard solution. The standard solution was diluted to a series concentration of 10, 20, 40, 80, and 160 ppm. Tests were carried out by inserting 10 larvae of *A. salina* L. in a test tube containing the extract. After 24 hours, the number of dead larvae was counted and a probit analysis was performed to determine cytotoxic activity (Ramachandran *et al*., 2011).

Separation of active compound *D. metel* L. leaves

The sample of *Datura* (*D. metel* L.) leaf extract was diluted with methanol for the dotting process. The mobile phase was prepared with a ratio of chloroform:n-hexane (1:1), methanol:ethyl acetate (3:2), and n-hexane:ethyl acetate (2:3). The sample was dotted on the TLC plate and put into the chamber. The purpose of this process is to find the best eluent composition that will be used to separate the compounds contained in the *Datura* leaf extract in the next TLC process (Gandjar and Rohman, 2012).

Separation by preparative TLC was performed using silica gel F254 with 20 × 20 cm. The plate was activated by heating it in an oven at 100°C for ± 30 minutes to remove any moisture on the plate. The concentrated extract was dissolved in the solvent; then 7 spots were dotted along the lower boundary line using a capillary tube. Then it was eluted using an eluent, which produced the best separation on analytical TLC. Elution was stopped after the developer solution reached the boundary line. The eluted plate was dried, and the stains were observed using a UV lamp with a wavelength of 254 and 366 nm (Ferey *et al*., 2017; Wang *et al*., 2012).

Isolate identification

A total of 10 mg of isolates were weighed using analytical scales. Then they were dissolved by adding 10 ml of methanol to obtain a concentration of 1,000 ppm, filtered, and then used as a standard solution. A solution with a concentration of 10 ppm was made by pipetting 50 µl, each of which was put into a 5 ml volumetric flask, and then methanol was added to the marked line. The standard solution was filtered and put into the vial liquid chromatography. Then it was injected into the UPLC-MS QTOF (Waters) system with a mobile phase flow rate of 200 µl/minute; then we viewed the fragmentation spectrum data on the mass spectroscopy data.

The stationary phase for the liquid chromatography mass spectrometer (LCMS) test was a C_{18} column, and the elution method was isocratic with an acetonitrile:water (15:85 *v*/*v*) solvent ratio. An injection volume of 5 microliters and a flow rate of 0.2 ml/minutes were used for the test.

RESULTS

Datura (*D. metel* L.) leaves were screened for phytochemicals. The results obtained are positive and contain alkaloids, flavonoids, steroids, and saponins.

The main solution from the thick methanol extract of *Datura* leaves (*Datura stramonium*) was diluted to 5 concentrations, namely, 10, 20, 40, 80, and 160 ppm, to be used in concentration orientation tests. Negative controls were seawater and shrimp larvae without the extracts. *Datura* (*D. metel* L.) leaves were tested for cytotoxicity. From the test, we obtained linear results. After that, a concentration orientation test was carried out to obtain the percentage of larval mortality in the range of 10%–90%, the test concentrations were 160, 80, 40, 20, and 10 ppm. This experiment was performed in three repetitions (triple) to obtain more accurate data. Larvae that were 48 hours old were put into vials containing 10 different concentrations each. After 24 hours of extract addition, the larvae mortality was observed. Table 3 shows the results of the cytotoxic test of *Datura* leaf extract.

Table 4 shows the testing of isolates from *Datura* (*D. metel* L.) using LCMS. The result obtained is the presence of a Baimantuoluoline E component in *Datura* leaves.

Larval mortality is monitored 24 hours after the extract was administered. The above number is put into the equation $y = bx + a$, where y is the 50% probit value of the death percentage, X is the concentration log, and the antilog X is the lethal concentration (LC_{50}).

Figure 2 shows the outcomes of the TLC-based isolate purification procedure. Scraped off are the primary compound

Table 1. LCMS optimum condition.

LCMS optimum condition	
Elution system	Isocratic
Mobile phase	Acetonitrile:water (15:85 *v*/*v*)
Stationary phase	C_{18} (Acuity 2.1 × 50 mm, 1.7 µm)
Flow rate	0.2 ml/minute
Injection volume	5 µl
Operation mode	Ion positive (M^+)

Table 2. Phytochemical screening of *Datura metel* leaf extract.

Compounds	Reagents	Positive indicator	Results	Note
Alkaloid	Mayer	White precipitate	White precipitate	+
	Wagner	Chocolate brown	Chocolate brown	+
	Dragendorff	Orange precipitate	Orange precipitate	+
Flavonoid	H_2SO_4	Blackish green	Blackish green	+
Steroid	Liebermann Burchard	Green	Green	+
Saponin	Aquades	Foamy	Foamy	+

Table 3. Cytotoxicity study of *Datura metel* leaf extract.

C (ppm)	Death larvae			Total test larvae	% Mortality	Log C	Probit
	I	II	III				
10	2	2	1	30	16.6	1	4.01
20	3	2	3	30	26.6	1.30	4.36
40	6	5	5	30	53.3	1.60	5.08
80	6	7	6	30	63.3	1.90	5.33
160	10	8	7	30	83.3	2.20	5.95
Control	0	0	0	30	0	-	-

Table 4. LCMS results of *Datura metel* isolate.

No.	Compounds	Time retention (minute)	m/z [M⁺]	Formula	Ion fragment		
					m/z [M⁺]	m/z [M⁺]	m/z [M⁺]
1.	Baimantuoluoline E	3.25	504.5	$C_{28}H_{42}O_8$	504.0591	477.2583	301.1780

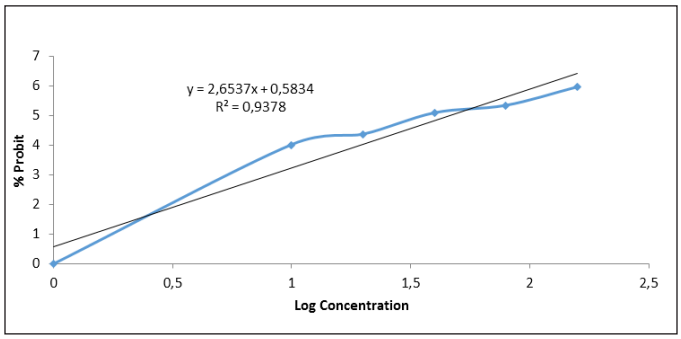

Figure 1. Linear regression between log concentration of *D. metel* leaf extract and the % probit of shrimp mortality.

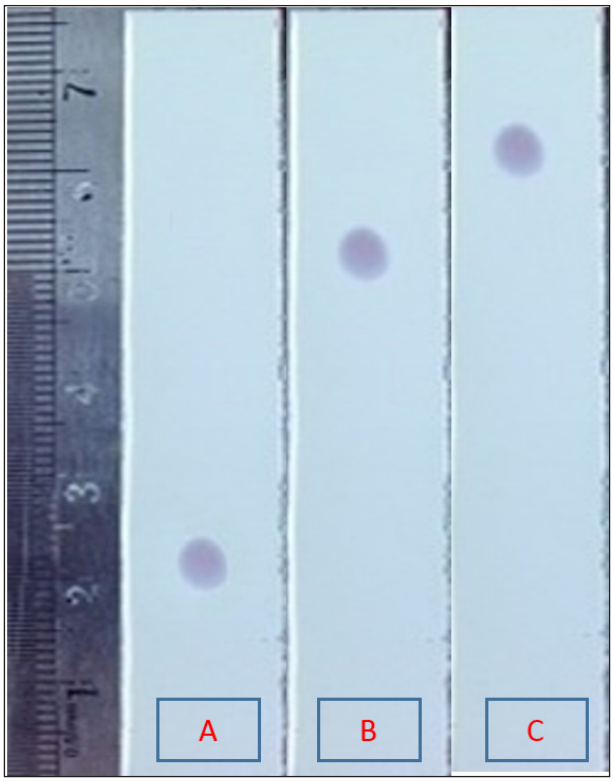

Figure 2. TLC results of *D. metel* isolate in different variations of mobile phase methanol:n-hexane. A: 4:1 *v/v*; B: 1:1 *v/v*; C: 1:4 *v/v*.

bands that have been projected to be present. To acquire the crystals of the isolate powder, the scraped powder was extracted with methanol, filtered, and evaporated.

Figure 3 shows one of the semi-polar elutions of the mobile phase of methanol:n-hexane (1:1 *v/v*) as a result of the isolate purity evaluation process.

Figure 4 shows the chromatograms of the isolates. The sharper the chromatogram peak gets the higher the indication that the compound only consists of one compound.

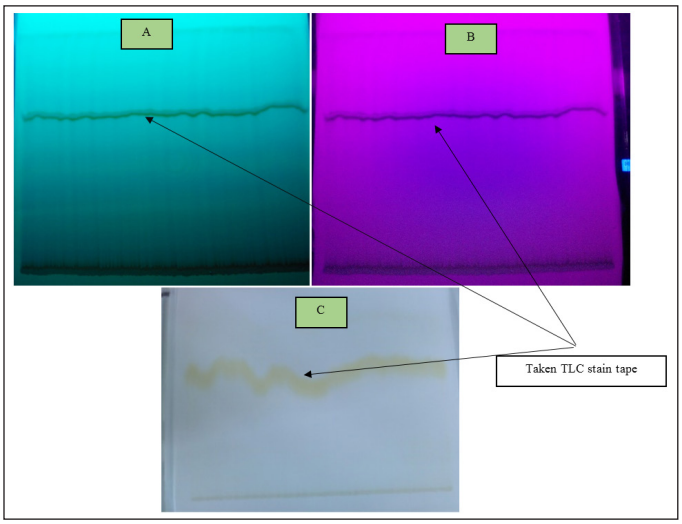

Figure 3. TLC results of *D. metel* with mobile phase chloroform:n-hexane (1:1 *v/v*). A: UV 254; B: UV 366; C: visible wavelength.

Figure 5 shows the results of the detection of isolates using a mass spectrometer. The mass spectrometer detector will identify the compound eluted from the LCMS column by ionizing it first and then measuring the mass ratio (m/z) and molecular fragments into small pieces. Furthermore, specific isolates were identified using a mass spectrometer to analyze these isolates' molecular ions and fragmentation patterns. The results of the detection of isolates using a mass spectrometer are shown in Figure 5.

Figure 6 shows the mass spectrum of *Datura* (*D. metel* L.) leaves. The molecular weight of the isolated substance was 504.0591 m/z, and it fragmented into smaller molecules with masses of 477.2583 m/z [M+ - CH_3-OH] and 301.1780 m/z [M+ -$C_9H_{11}O_4$]. More than 600 Withanolide-related actions have been reported.

DISCUSSION

Research has been carried out on *Datura* leaves. This research was preceded by an extraction process using 96% methanol. The extract obtained was then used for phytochemical screening tests. Furthermore, the extract's toxicity test was performed using the BSLT method to perceive its potential toxicity. The isolation process used preparative TLC using the appropriate eluent. The isolates obtained were then identified by the UPLC-MS technique.

Phytochemical screening

About 200 g of *Datura* leaf samples were extracted by the maceration method using 3,000 ml of methanol as a solvent (Carpa *et al*., 2017). Maceration was carried out for 6 × 24 hours. The macerated filtrate is evaporated using a rotary evaporator until a thick extract is obtained. The extract was obtained with a weight of 28.19 g and a yield of 14.09% from this stage. The physical

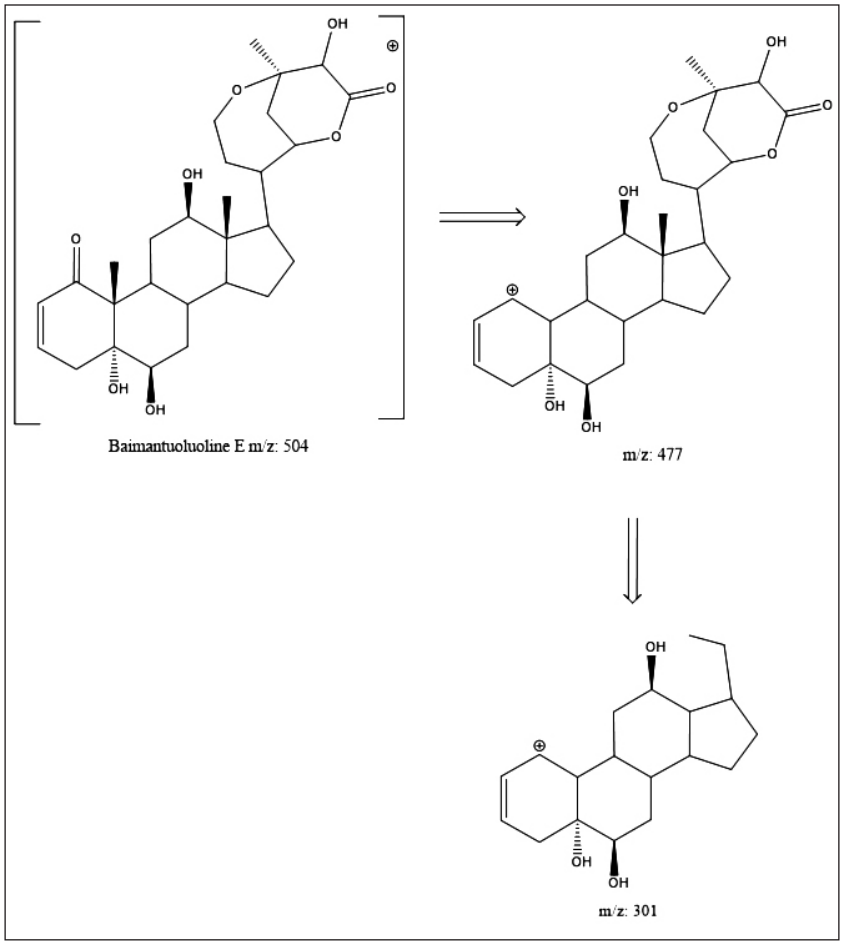

Figure 4. Fragmentation pattern of *D. metel* isolate (Xia *et al*., 2019; Yang *et al*., 2020).

properties of the extracts obtained are blackish-green, paste-like, and sticky.

The phytochemical compounds of the thick methanol extract produced from the extraction process were tested, which aims to determine the secondary metabolite compounds present in the sample. The phytochemical test in this study included identifying alkaloids, flavonoids, steroids, saponins, and tannins. Phytochemical test results can be seen in Table 2.

Based on the analysis conducted, samples of *Datura* leaves tested positive for containing flavonoids. It is indicated by the change in color to blackish green when added with the H_2SO_4 reagent. There is a change in color because flavonoids are phenolic compounds. Therefore, the color will change when an alkaline solution or ammonia is added (Hossain *et al*., 2013b, 2013b).

Datura leaf samples tested positive for alkaloid compounds. The deposits' formation indicates this in the three test tubes after the drops of Mayer, Wagner, and Dragendorff reagents. The reaction with Mayer's reagent formed a white precipitate, with Wagner's reagent, it formed a brown precipitate, and with Dragendorff's reagent, it formed a red-orange precipitate (De Silva *et al*., 2017). Based on the analysis carried out, the *Datura* leaves tested positive for containing steroid compounds. The color change can be seen after the Liebermann Burchard reagent addition, namely, green (Pandey and Tripathi, 2014; Tiwari *et al*., 2012). Based on the analysis, the *Datura* leaves tested positive for containing saponin compounds indicated by foam formation after the shaking process. The formation of foam indicates glycosides, which can form foam in the water (De Silva *et al*., 2017; Tiwari *et al*., 2012).

The same results were obtained by Dhawan and Gupta (2016), who carried out a phytochemical screening of *Datura* leaves. The results showed that the methanol extract of *Datura* leaves contains flavonoids, alkaloids, steroids, and saponins. The study also reported that the ethyl acetate and n-hexane extracts positively contained flavonoids, alkaloids, steroids, and saponins. Alabri *et al*. (2014) found that the methanol extract of *Datura* leaves contains alkaloids, flavonoids, and saponins but is negative in steroids. Many factors can influence these different results. Various factors cause secondary metabolite variability of the same species, including physiological variations, environmental conditions, geographical variations, and genetic and evolutionary factors (Figueiredo *et al*., 2008). Given that the samples obtained also show different geographic locawtions and environments, there are several studies that state that the influence of the environment, climate, and land affects the production of secondary metabolites in plants and in producing food reserves (Deduke *et al*., 2012; Sunic *et al*., 2021).

Secondary metabolites' content plays a role in providing antioxidant effects but through different biological mechanisms (Hossain and Nagooru, 2011; Suresh and Nagarajan, 2009). Most of

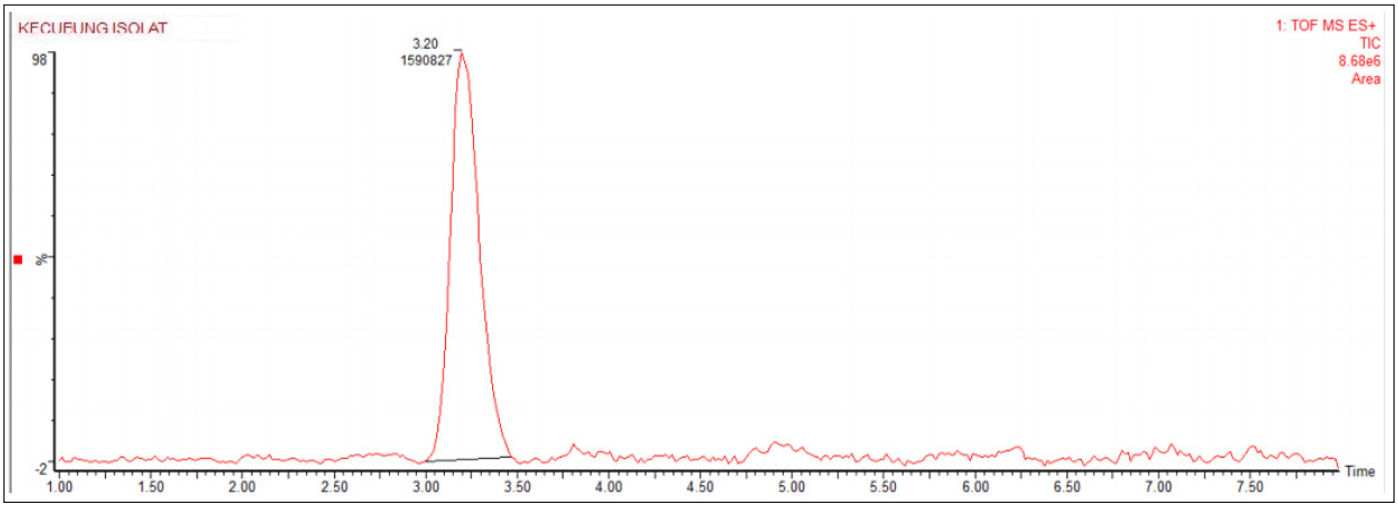

Figure 5. Peak chromatogram of *D. metel* isolate.

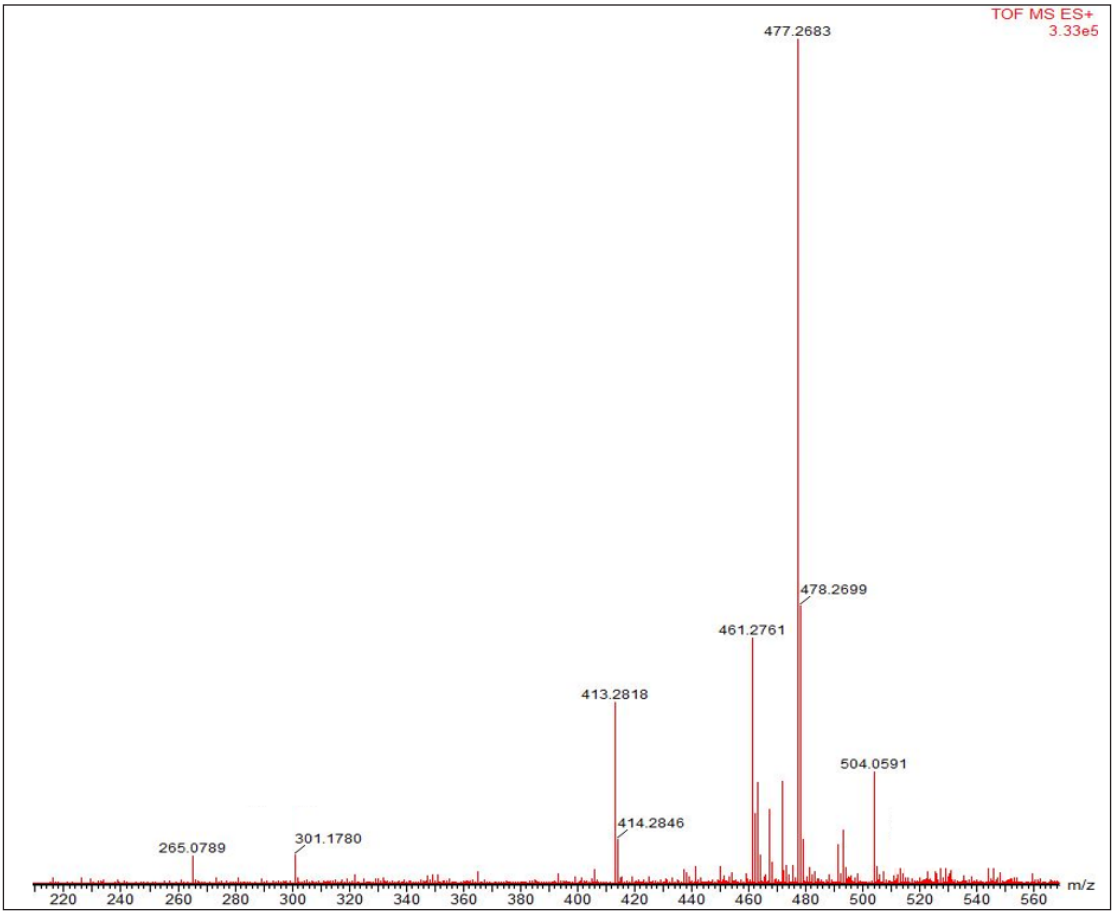

Figure 6. Mass spectrum of *D. metel isolate.*

the secondary metabolite components that have been isolated from *Datura* plants that show biological activity are extracts dissolved with polar solvents (Alabri *et al*., 2014; Gonzalez-Guevara *et al*., 2004). Several research results on the flavonoid group show high potential biological activity as antioxidant, anti-inflammatory, anti-microbial, anti-cancer, and anti-allergic reactions (Anyasor *et al*., 2010; Chao *et al*., 2002; Igbinosa *et al*., 2009; Thitilertdecha *et al*., 2008). Saponins are secondary metabolites that play a role in the plant defense system. Therefore, saponins show anti-microbial activity (Ayoola *et al*., 2008; Banso and Adeyemo, 2006). Phenolic compounds such as tannins and their derivatives are considered compounds that act as antioxidants or free radical scavengers (Akharaiyi, 2021; Sekar *et al*., 2012; Vadlapudi and Kaladhar, 2012).

BSLT cytotoxicity test

Cytotoxicity test was used to determine seawater and other larval mortality factors (Ramachandran *et al*., 2011). Thus, it can be ascertained that the addition of the extract only causes the death of larvae. The mortality results of *A. salina* Leach larvae in each vial were compared, including negative control vials. The total larvae used in each concentration with 3 repetitions of the experiment were 30 individuals. Therefore, the total number of larvae used in the entire experiment was 180. The percentage of mortality is obtained by multiplying the average mortality rate for larvae by 100%. The use of *A. salina* larvae in this BSLT test is because shrimp larvae have an affinity with mammals, such as having a DNA-dependent RNA polymerase as that of mammals (Bagheri *et al*., 2010).

The eggs of *A. salina* Leach used were shrimp larvae that were 48 hours old. At the age of 24 hours, the new larvae will enter the first instar phase, where the larvae cannot eat because their mouth and digestive tract have not been fully formed yet. Meanwhile, at the age of 36–48 hours after hatching, the larvae will metamorphose into a second instar, where the larvae will already have a perfect mouth and digestive system. Thus, the larval environment extract enters the larvae's bodies and causes death in the larvae (Chanda and Baravalia, 2011). Based on standard criteria, larvae are said to be dead if they do not move for 10 seconds of observation. Observation of larval mortality is carried out after 24 hours after giving the extract (Bagheri *et al*., 2010). The value above is entered into the straight-line equation $y = bx + a$, where the y value is the 50% probit value of the death percentage, X is the concentration log, and the antilog X is LC_{50} (Fig. 1).

The results of the toxicity test of the *Datura* leaf extract are shown in Figure 1. It can be concluded that the higher the concentration of an extract is, the higher the mortality rate of the larvae is. Calculate the LC_{50} using the straight-line equation $Y = 2.6537X + 0.5833$; then input the number 5 in the Y value to obtain LC_{50} 46.1636 µg/ml. An extract indicates a strong cytotoxic presence and is further analyzed if the LC_{50} value is <100 ppm. Calculation using this method produces LC_{50}, which is included in the toxic category. It indicates that *Datura* leaf extract has a very cytotoxic ability and should be further analyzed (Chanda and Baravalia, 2011; Ramachandran *et al*., 2011).

The biological response of plants does not come from one component but a mixture of various bioactive components from plants (Baravalia *et al*., 2012). The BSLT test results showed a correlation with the results of the phytochemical screening test. Phytochemical screening showed the presence of flavonoids, alkaloids, steroids, and saponins. Flavonoids and alkaloids are thought to form a complementary effect to kill shrimp larvae (Alabri *et al*., 2014; Subhadra *et al*., 2011). They work by acting as stomach poisoning. Therefore, when these compounds enter the larvae's bodies, their digestive organs will be disturbed. This compound will inhibit the taste receptors in the larvae's mouths. This results in the fact that the larvae fail to get a taste stimulus; thus they are unable to recognize their food, and as a result, the larvae die out of starvation (De Padua *et al*., 1999; Djali *et al*., Khairunnisa et al., 2018). Meanwhile, other bioactive compounds that cause shrimp larvae's death are steroids. Steroids and saponins in plants are toxic to insects, bacteria, and fungi and can be used as drugs to prohibit tumor cell growth in plants and animals (Nugrahaningsih *et al*., 2019; Rohmawati and Sutoyo, 2018).

BSLT is very sensitive because the *Artemia* larvae have fragile skin, making it easy for the solution to diffuse. Furthermore, the rapid growth of *Artemia* larvae resembles cancer cells' growth, making it easier for researchers to detect changes in biological responses (Mirzaei and Mirzaei, 2013; Thangapandi and Pushpanathan, 2014). Several previous studies have concluded that there is a direct relationship between toxic activity in BSLT and antiproliferative effects (Handayani *et al*., 2018; Sandrawati *et al*., 2019; Suzery and Cahyono, 2014). BSLT has a synergistic correlation with cytotoxic activity in some solid human tumors and pesticide activity. It has led to the discovery of a new class of natural pesticides and active antitumor agents (Chanda and Baravalia, 2011). Therefore, it might be suggested that the BSLT is an inexpensive, easy-to-master, and suitable preliminary test for predicting cytotoxic activity (McLaughlin *et al*., 1998).

Separation and identification of compounds

The fractionation of the compound components in the chloroform extract of *Datura* leaves began with determining the eluent (mobile phase) through TLC. The stationary phase used was the G60 F254 silica plate. Observations of the stains on the TLC plate were carried out using 254 and 366 nm UV lamps. The use of UV 254 lamps will cause fluorescent silica gel. It distinguishes it from silica gel, which binds to the compound (stain) and will appear blurry and differently clear (Fried, 2017). The search for the mobile phase (eluent) begins with a single eluent and then a combination of eluents to get the best separation shown through the separation stage between spots. TLC's mobile phase optimization is carried out until a suitable solvent is obtained based on a trial and error system (Coskun, 2016). The result is a combination of 2 solvents, namely, chloroform:n-hexane with the ratio of 1: 1 *v*/*v*. The combination of these solvents produces spots that are relatively far apart. Thus, it is used as an eluent in preparative TLC. From the calculation results, the Rf stain value is 0.61. The Rf value is still vulnerable to the recommended Rf value of 0.2–0.8 (Gandjar and Rohman, 2012).

The isolation process used TLC with a stationary phase of silica gel G60 F254 using a mobile phase of chlorophyll:n-hexane (1:1 *v*/*v*). The elution result consists of one dominant band and is separated from the other bands that are far apart to facilitate the scraping process. The results were observed under 254 and 366 nm UV lamps. The visible band at UV 254 nm is due to the interaction between UV rays and the plate's indicator, namely, silica gel F254 (Spangenberg *et al*., 2011). The plate will glow in UV light 254, while the stained area will cover the plate's light. Thus, the stain can be seen (Fried, 2017), whereas at a wavelength of UV 366, it will show the band's fluorosis and indicate the presence of a conjugated double bond (Ferey *et al*., 2017; Wang *et al*., 2012).

The results of the TLC scraped off were then refined using the same method. The isolates produced yellowish stains after being sprayed with cerium sulfate (Fig. 1). Cerium sulfate functions as a chelating agent. Thus, stains can appear in visible light (Harbone, 2001; Pandey and Tripathi, 2014; Wall, 2005). The main compound bands which have been predicted to be located are scraped off. The scraped powder was extracted with methanol, filtered, and then evaporated to obtain the isolate powder's crystals.

Figure 2 shows the results of the isolate purification process by performing the TLC.

To confirm the purity of the isolates, an evaluation was carried out using TLC (Coskun, 2016). The isolates obtained were eluted with three different types of eluents: polar, semi-polar, and non-polar. Figure 3 shows one of the semi-polar elutions of the mobile phase of methanol:n-hexane (1: 1 *v*/*v*) as a result of the isolate purity evaluation process.

The TLC technique was used to confirm the purity of the isolates, using eluent with 3 different variations, namely, polar, semi-polar, and non-polar with variations in the polar mobile phase of methanol:n-hexane (4: 1 *v*/*v*), semi-polar methanol:n-hexane (1: 1 *v*/*v*), and non-polar methanol:n-hexane (1: 4 *v*/*v*). The elution evaluation results of the isolates' purity showed that only one stain appeared in the three eluent concentrations. It indicates that the isolates obtained contain only one compound based on the TLC method (Coskun, 2016). Isolates are said to be pure should they are eluted using three variations of polar, semi-polar, and non-polar mobile phases, showing the consistency of only having one spot (Bajpai *et al*., 2016). The stains formed also showed that the polar eluent Rf was below 0.28, semi-polar Rf was in the middle of 0.61, and non-polar Rf was above 0.85. It occurs due to the eluent's influence, where the polar eluent will cause the isolate not to be eluted upward because hydrogen bonds are formed between the isolate and silanol on the TLC plate. When the eluent's polarity is reduced, the dimer interaction between the isolate and the TLC plate is reduced, causing the isolate to be more susceptible to Rf (Marston, 2011; Rossing and Chiaverina, 2000; Waksmundzka-Hajnos *et al*., 2008).

Wasnik *et al*. (2009) identified Withanolide using the TLC which documented the Rf. The approximate Withanolide was 0.61 with methanol:n-hexane (50:50%) as eluent. The spray reagent used to identify phytosterol groups in plants uses cerium sulfate. The presence of a steroid group from the alcohol group is indicated by a yellow to brown stain in visible light and a blue fluorescence on UV light 366 nm (Harbone, 2001; Sarker and Nahar, 2012). Based on the isolate chromatogram results of the spray, the cerium sulfate reagent shows a yellow-brown color. It indicates the presence of phytosterol compounds. The color that occurs is due to a substituted hydroxyl group on the steroid ring with a positively charged Ce (Harbone, 2001; Rossing and Chiaverina, 2000).

Isolate identification using LCMS

The initial step before carrying out the LCMS analysis is to optimize the instrument and its mobile phase. The purpose of optimizing the LCMS tool is to see the most suitable conditions for analyzing paracetamol in herbal samples. Based on the LCMS conditions' optimization results (Table 1), the optimal conditions for analyzing paracetamol are best obtained by using an isocratic elution system. According to Yu *et al*. (2016), in the isocratic elution system, the mobile phase used is regulated in a constant concentration that is pumped into the column. This study's type of column is the type of octadecyl silica column (ODS or C_{18}) with the silica gel component in it. This C_{18} column has a reversed-phase column and can produce the best separation with high levels of purity and accuracy (Jian, 2017).

In this analysis, an isolate chromatogram was obtained, which showed a sharp peak at the retention time (tR) of 3.20 minutes. According to Termopoli *et al*. (2019), the use of LCMS in the qualitative analysis was carried out by looking at the chromatogram peaks. The sharper chromatogram peak indicates that the compound only consists of one compound. The chromatograms of the isolates are shown in Figure 4.

Retention time (tR) is the time required for the analyte, which starts during the injection process until the column's separation process. The separation response will be sent in the form of a signal read by the detector. The small peaks formed around it indicate that impurities are present (Niessen and Correa, 2017). These impurities usually come from solvents or during the sample preparation process (Termopoli *et al*., 2019). However, the presence of impurities did not have a significant effect on the peaks of the isolates. It can be seen from the chromatogram peaks that are relatively far apart.

Table 4 shows the detected molecular weight and fragmentation patterns. The isolate is assumed to be a Baimantuoluoline D (m/z 504) compound, a steroid derivative from the Withanolide group (Xia *et al*., 2019). The Withanolide family has been detected in more than 600 different plants (Misico *et al*., 2011). Withanolide is formed from the Ergostane skeleton with side-chain modifications in the δ-lactone ring substituted on the C_{22} and C_{26} carbon chains (Guo *et al*., 2018). Withanolide derivatives have been known to have anti-inflammatory, antitumor, cytotoxic, and immunomodulatory activities (Wu *et al*., 2020; Xu *et al*., 2018). The isolate's molecular weight is known to be [M +] 504.0591 m/z, with a base peak of 477.2583 m/z. 477.2583 m/z [M + -CH_3-OH] and 301.1780 m/z [M + -$C_9H_{11}O_4$] daughter fragments were produced (Fig. 4). At the 477.2583 m/z fragments, there was a breakdown of hydroxy at Ring A of C_1 carbon and methyl at C_{19}. The termination of the hydroxy bonds is due to non-bonding bonds on the C_1 carbon; thus it is easier to be released. Meanwhile, the effect of Lewis acid on the proton of the neighboring hydroxy group (C_1) is to form hydrogen bonds, which result in the instability of C_{18} carbon. The formation of the 301.1780 m/z fragment is more due to the instability of the δ-lactone ring in the Ergostane framework due to epoxy in carbon substitution C22 C_{26} (Niessen and Correa, 2017; Yang *et al*., 2020).

The mass spectrum is an accurate, valid, and decisive identification because it can directly identify the structure of an unknown compound in a complex mixture even with a minimal concentration (Evard *et al*., 2016). Based on the mass spectra data obtained, the isolates analyzed showed 504, 427, and 301 m/z. This pattern is in line with that obtained by Yang *et al*. (2020), who confirmed that one of the secondary metabolites in *Datura* leaves is Baimantuoluoline D with a molecular weight of 504.5 m/z with ion fragments forming 504,477.301 m/z (Niessen and Correa, 2017; Yang *et al*., 2020). This fragmentation pattern confirms that the isolate is a Baimantuoluoline D.

The fragmentation pattern that is formed is also not abundant because the ionizer system used is electron spray ionization. The electron ionization (EI) system uses the Bombardment technique with a potential energy of 70 eV, which causes many fragments to be formed (Wei *et al*., 2019). In the ESI system using electrospraying technology, the isolate's molecular ion is obtained by evaporation, where the charged

liquid particles are reduced in size and the electric charge becomes closer together. The reduction in grain size due to evaporation continues to occur, to the point where the coulombic repulsive force overcomes and opposes the granules' cohesive force, resulting in the desolvation or breakdown of the solvent (Banerjee and Mazumdar, 2012; Schröder, 2012). The sample in the granules will be released/desorbed out in the form of $[M + H]^+$ or $[M-H]^-$. The formed fragments are not as many as in the EI system because the potential difference given is only 3–5 eV (Chen *et al*., 2011).

Research conducted by Yang *et al*. (2020) managed to find 85 Withanolide isolates. One of them is the *Datura mentaline* compound found in all parts of plants. *Datura foliside* compounds are found in all parts of the plant except in the seeds. Literature studies show the leaves' antifungal potential, especially related to the Withanolide content in *Datura* leaves (Chukunda *et al*., 2019; Dabur *et al*., 2004a, 2004b). Antifungal activity in *Datura* is found in leaves and fruit, while other parts are of very low activity. Roots are generally less active than leaves, fruit, and stems (Javaid and Saddique, 2012). Geographical differences significantly affect the existence of secondary metabolites in plant organs (Al-Snafi, 2017).

Apart from *Datura*, Withanolide is also found in the roots of *Withania somnifera*, which is a well-known ginseng from India (Misra *et al*., 2008; Trivedi *et al*., 2017). Several studies also show the Withanolide groups such as β-hydroxy-2,3-dihydro-withanolide F, Withanolide A, Withaferin A, Withanolide D, Ixocarpalactone A, Withanolide S, and Thiowithanolide (Chatterjee *et al*., 2010; Trivedi *et al*., 2017). Besides, it shows that isolates from *Withania somnifera* have various pharmacological activities including antioxidant, anti-cancer, immunomodulatory, hepatoprotective, neuroprotective, anti-inflammatory, anti-microbial, hypoglycemic effects (Budhiraja *et al*., 2000; Chen *et al*., 2011; Gorelick *et al*., 2015; Singh *et al*., 2010).

CONCLUSION

The phytochemical screening results showed that the *Datura* leaves contained alkaloids, flavonoids, steroids, and saponins. The cytotoxic activity of *Datura* leaf extract obtained LC_{50} 46.1636 µg/ml. The isolate obtained from *Datura* leaves is Baimantuoluoline D, a steroid base framework classified as a steroid derivative compound from the Withanolide group. This research helps to find a way to isolate the Withanolide group compounds, one of which is Baimantuoluoline D. Furthermore, activity tests are needed on Baimantuoluoline D isolates to develop medicinal compounds and to perceive their usefulness.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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The authors report no financial or any other conflicts of interest in this work.

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This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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