Pharmacology potency of thin layer chromatography steroid isolates of *Chlorella* sp. chloroform fraction

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Abstract. Al-Qur'an surah as Syu'ara verse 7 shows that Allah SWT grew many good and advantages plants, such as microalgae Chlorella sp. Chlorella sp. are contained some secondary metabolites, such as steroid compounds. Purpose of this research is to know toxicity levels and antioxidant activity steroid compound of chloroform and ethyl acetate fractions from hydrolyzed methanol extract of microalgae Chlorella sp. Cultivation is conducted on a laboratory scale using Sprouts Extract Medium (SEM) 4% and harvesting conducted every 10th day. Dry Chlorella sp. biomass was macerated using methanol solvent. The extract then hydrolyzed with 2 N of HCl and partitioned with chloroform, then the separation using Thin Layer Chromatography (TLC). Isolates conducted toxicity tests using Brine Shrimp Lethality Test (BSLT) and antioxidant activity test with the DPPH method. The toxicity test showed that TLC steroid isolates of chloroform fraction had LC_{50} values 8.796 (C1), 8.53 (C4) and 4.53 ppm (C5). The antioxidants test showed that TLC steroid isolates of chloroform fraction had had a value of EC_{50} is 2.26 1019 (C1) 163.10 ppm (C4) and 38.96 ppm (C5). Identification of steroid compounds with UV-VIS spectrophotometer has a maximum wavelength of 250.50, 270.5 and 281 nm. Identification with FTIR showed functional groups C=O (carbonyl), C-O (ether), -$C(CH_3)_2$ (geminal dimethyl), C=C and = CH (alkenes) attributed to steroid compounds.

1. Introduction

Microalgae *Chlorella* sp. is a low-level plant from green alga genus with a single cell of tiny microscopic which already widely studied. The green colour of the *Chlorella* sp. due to the dominant cell containing chlorophyll A and B, carotenoids and xantrofil. The advantages of microalgae *Chlorella* sp. of which can multiply quickly and easily in cultivation [1]. Microalgae life is not depending on the season, does not require a large place, and does not require a long time for harvesting [2]. Culture of *Chlorella* sp. in the laboratory scale cultivation is by the Sprouts Extract Medium (SEM). Sprouts is a common vegetable consumed, easily obtained, economical, does not produce a compound toxic effect, containing macro and microelements, vitamins, minerals, amino acids used as growth and development [3]. The results showed that SEM with a concentration of 4% is a culture medium that produces microalgae growth is very high compared to Seawater Medium (SWM) and Guillard Medium (GM) [4].

Results of previous studies have been conducted toxicity tests on the *Chlorella* sp. microalgae. The methanol extract of *Chlorella* sp. microalgae had LC_{50} values of 20.516 ppm [5], whereas LC_{50} ethyl acetate fraction is 43.3044 ppm. Results of previous studies stating *Chlorella* sp. have potential as antioxidants with EC_{50} values consecutively 18.610 ppm and 27.320 ppm [6]. The antioxidant activity

associated with the active compounds contained therein. Steroid compound in the ethyl acetate fraction of *Chlorella* sp. through phytochemical test and isolates identified using FTIR [7].

Steroids are one of the secondary metabolites of natural materials which have a variety of bioactivity. Generally used as an approach phytopharmacology. The pharmacological effects caused by steroids were different. The study says that the steroid discount cytotoxicity against cell myeloma (malignant tumour) [8] and can inhibit the action of the enzyme causes of prostate cancer [9]. Steroid compounds also have activity as an antioxidant [10].

Toxicity tests used in this study is the method of Brine Shrimp Lethality Test (BSLT) using larvae shrimp *Artemia salina* L and the antioxidant activity test using DPPH with various concentrations. Some of the advantages of the BSLT test which time quick test, the equipment used is simple and inexpensive, easy in treatment trials where one biological response observed was death, the number of organisms much that needs validation statistics with little samples [11].

Based on the exposure, it is necessary to isolate suspected steroid compounds have the highest activity in *Chlorella* sp. microalgae to know the level of toxicity and the resulting antioxidant activity. *Chlorella* sp. obtained from cultivation, extracted and hydrolyzed. Then, partitioned using chloroform. Steroid compounds then separated using TLC Preparative. The isolate was identified using FTIR and UV-Vis spectrophotometer.

2. Materials and methods

2.1. Material

Isolates *Chlorella sp*., Bean sprouts, shrimp larvae *Artemia salina* L., methanol pa, chloroform, ethyl acetate, n-hexane pa, 37% HCl, sodium bicarbonate, Lieberman-Burchard reagent (acetic acid anhydride, concentrated H_2SO_4, absolute ethanol), yeast, DMSO, DPPH, Ethanol and GF254 TLC plate.

2.2. Methods

2.2.1. Sample preparation of Chlorella sp. microalgae biomass

Chlorella sp. isolates 150 mL was inoculated into each 900 mL SEM 4% (36 mL extract of bean sprouts and 864 mL of distilled water) in Erlenmeyer and incubated for 10 days with photoperiodicity 14 hours of light and 10 hours of darkness [12], Harvesting biomass *Chlorella* sp. performed on the 10^{th} day. Biomass *Chlorella* sp. dried, then scraped and weighed as dry weight [13,14]. Analysis of Water Content Microalgae *Chlorella* sp. done by an empty cup and saucer containing 0.5 gram of sample is heated and weighed to obtain the weight of the cup is constant. The water content of biomass samples *Chlorella* sp. calculated using the formula (1) [15]:

$$Water\ Content = \frac{(b-c)}{b-a}\ x\ 100\ \% \quad (1)$$

Where a is the weight of the cup is empty, b is the weight of the cup + the sample before drying, and c is the weight of the cup + after the samples were dried.

2.2.2. Extraction microalgae Chlorella sp. with maceration

Chlorella sp. biomass as much as 30 g of dried and added to methanol 150 mL and put into Erlenmeyer cap, maceration for 24 hours and on the shaker at 120 rpm for 5 hours at room temperature. Then filtered using a Buchner funnel. The residue obtained macerated back up to 5 times the extraction process. The filtrate obtained, the solvent is evaporated using a rotary vacuum evaporator to form a concentrated extract of *Chlorella* sp. concentrated extract obtained is weighed and then calculated the yield of the resulting extract.

2.2.3. Hydrolysis and partition methanol extract microalgae Chlorella sp.

The methanol extract as much as 2.5 g inserted into the glass beaker, then hydrolyzed with 5 mL of 2 N HCl and stirred using a magnetic stirrer hot plate for 1 hour at room temperature [16]. The hydrolyzate

obtained is added with sodium bicarbonate until pH neutral. Then added 12.5 mL of solvent chloroform, then shaken and allowed to stand to form two layers, namely an organic layer and a water layer. Then each layer separated. The partition process performed 5 times. The organic layer is then concentrated by purged with N_2 gas. Concentrated extracts were then weighed and the yield is calculated.

2.2.4. Separation of steroid compounds by Preparative Thin Layer Chromatography (PTLC)

2.2.4.1 Separation of steroid compounds by Analytical Thin Layer Chromatography (ATLC)
Separation of steroid compounds with analytical TLC using GF254 silica gel plates which were activated in the oven at 100 °C for 30 minutes to remove water from the plates. Furthermore, each plate was given size of 1 x 10 cm. *Chlorella* sp. chloroform fractions which have been reconstituted with the solvent spotted at a distance of 1 cm from the bottom edge of the plate with the capillary tube, then drain and eluted using gradient eluent of n-hexane: ethyl acetate (4.75:0.25; 4.5:0.5; 4.25:0.25, 4:1; 3.75: 1.25 and 3.5: 1.5) [7]. The stain of separation results then observed under UV light at wavelengths of 254 and 366 nm.

2.2.4.2 Separation of steroid compounds by Preparative Thin Layer Chromatography (PTLC)
Samples were taken as the result of partition 10 mg and then diluted with 10 mL chloroform. Separation KLTP used steroids with silica gel plate F_{254} is activated by heating in an oven at a temperature of 100-105 °C for 30 minutes. Each plate with a size of 10×20 cm. *Chlorella* sp. chloroform fractions which have been reconstituted with the solvent spotted at a distance of 1 cm from the bottom edge of the plate with the capillary tube, then drain and eluted using the eluent n-hexane: ethyl acetate (3.75:1.25). The stain of separation results then observed under UV light at wavelengths of 254 and 366 nm.

2.2.5. Toxicity test steroid compounds against larvae of shrimp Artemia salina Leach

2.2.5.1 Hatching larvae shrimp Artemia salina Leach
A total of 250 mL of seawater included in the hatching container, put the eggs *Artemia salina* 2.5 mg then aerated. The eggs will hatch within ± 48 hours and ready to be used as a test target toxicity.

2.2.5.2 Toxicity test
Toxicity tests carried out on isolates suspected steroid compound, and then scraped and centrifuged. Separate the filtrate is taken from the lees, then evaporated the solvent. The resulting isolates were weighed and dissolved using a suitable solvent.

A stock solution was made by sampling isolate PTLC results as much as 2.9 mg dissolved in 5 mL of solvent to obtain a stock solution. Solvents are used according to the solubility properties of the sample. Steroid isolates from stock solution are made with three series of concentrations. Then put into each bottle vial which has been prepared. The solvent was evaporated to dryness. Yeast solution is prepared by dissolving 3 mg in 5 mL of seawater. A drop of baker's yeast solution and 50 mL of DMSO was added to the vial bottle containing isolates were then shaken until dissolved. 10 *Artemia salina* L larvae inserted in the bottle vial and signed by sea to a volume of 5 mL [17].

The controls are used are DMSO controls and the control of seawater. All bottles vial was placed under an incandescent bulb for 24 hours and observed the death of shrimp larvae. Then calculate the number of dead shrimp larvae.

2.2.6. Antioxidant activity test with DPPH

2.2.6.1 Antioxidant potential measurements in samples. Ethanol 95% was 4.5 mL pipette then added a solution of 0.2 mM DPPH, 1.5 mL, was added to cuvette until full. Furthermore, λ_{maks} solution sought and recorded measurement results λmaks for use at a later stage [18].

Samples isolate steroid made various concentrations (1, 2, 3, 4 and 5 ppm). Extract each pipette of 4.5 mL and 1.5 mL DPPH added 0.2 nM and then incubated at 37 °C for a period of stability. An absorbance measurement using spectrophotometry at a wavelength of 515.0 nm. Absorbance data obtained by each concentration of each extract calculated values per cent antioxidant activity using equation (2):

$$\text{Antioxidant Activity} = \frac{\text{AK}-\text{AS}}{\text{AK}} \times 100 \quad (2)$$

Where AK is the absorbance of the control and the AS are Absorbance sample. Controls used is 0.2 mM DPPH solution of 1.5 mL in 4.5 mL of 95% ethanol. Determination of EC_{50} values is done by obtaining a regression equation using the program "prism GraphPad software, Regression for analyzing dose-response data".

2.2.7. Identification of steroid compounds Microalgae Chlorella sp. With UV-VIS Spectrophotometer. Isolates of steroid from preparative TLC results were identified using UV-VIS spectrophotometer at a wavelength of 200-800 nm.

2.2.8. Identification of Compounds Steroids Microalgae Chlorella sp. with the FTIR spectrophotometer. Isolates of steroid preparative TLC results were identified using FTIR spectrophotometer by mixing the evaporated steroid isolates with 0.2 g KBr pellets.

3. Results and discussion

3.1. Sample preparation of Chlorella sp. microalgae biomass

The results showed that during the cultivation process from 0 to 10th days, microalgae discolouration on inoculum every day from yellowish green to dark green. This indicates an increase in cell density that indicates the utilization of the nutrients contained in SEM by *Chlorella* sp. cells [12]. Drying is not done using the oven or in direct sunlight, because it feared could damage secondary metabolites contained in *Chlorella* sp. microalgae which are not resistant to heat. Results of drying at room temperature are obtained *Chlorella* sp. biomass dry, greenish-black powder.

Analysis of water content used to determine the amount of moisture contained in *Chlorella* sp. microalgae. Samples with low water levels can affect the resistance of the sample during storage. The water content in the biomass of *Chlorella* sp. microalgae Dries gained by 10.25%. The process of extracting maximum water levels are required for the extraction process to run smoothly in the amount of 11% [19]. The water content greatly affects the extraction process. Methanol will hydrogen bond with the water contained in the sample so that the water content in the sample also extracted [20]. This effect on the yield of the extraction.

3.2. Extraction microalgae Chlorella sp. with maceration

Extraction of the active compound *Chlorella sp.* microalgae using maceration with methanol p.a. Steroid compound which is the active compound in the *Chlorella sp.* microalgae generally binds to glycoside which tend to be polar, so that the active compounds to be extracted into the solvent polarity which has a common trait with methanol. Results maceration, then concentrated by rotary vacuum evaporator. From 8.4 g of biomass was produced 2.0387 g of concentrated extract and the yield was 24.29%.

3.3. Hydrolysis and partition of Chlorella sp. microalgae methanol extract

Hydrolysis is done to break bonds glycoside conducted by the addition of acid to be taken the steroid compound. Hydrolysis process carried out by the addition of 2 N HCl as an acid catalyst, then neutralized with sodium bicarbonate ($NaHCO_3$). This neutralization serves to stop the hydrolysis reaction is reversible.

The hydrolyzate obtained do partition using chloroform solvent. Concentrated extract partition results obtained greenish-black. The yield obtained from the partition using chloroform amounted to 32.80% (Table 1).

Table 1. The result of hydrolysis and partition.

Fraction	Weight of extract	Weight of fraction	% Yield
Chloroform	1.8564 g	0.5610 g	32.80

3.4. Separation of steroid compounds by Thin Layer Chromatography

3.4.1. Separation of steroid compounds by Analytical Thin Layer Chromatography (A-TLC). Illustration results of the separation patterns of eluent variations in A-TLC when observed under UV lamp are illustrated in Figure 1. Figure 1 shows that the separation patterns of 3.75:1.25 produce the much stains than the other eluent. So, the 3.75:1.25 eluent is used for further separation using P-TLC.

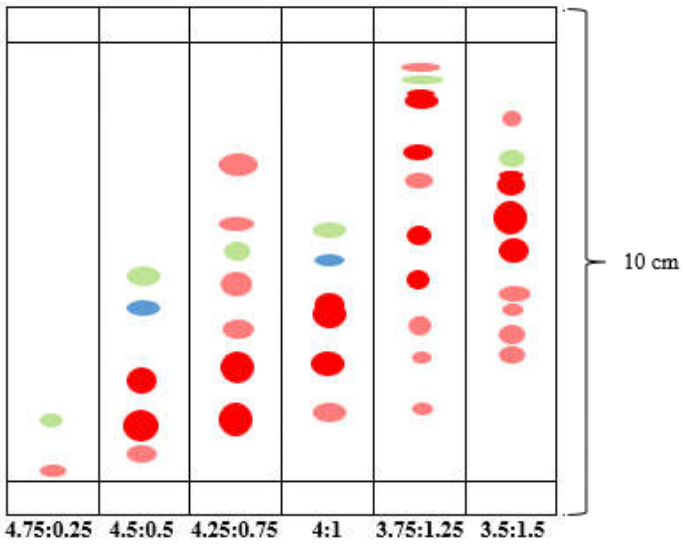

Figure 1. Illustration of A-TLC of *Chlorella* sp. chloroform fraction.

3.4.2. Separation of steroid compounds by Preparative Thin Layer Chromatography (P-TLC). Mobile phase for Preparative TLC which n-hexane: ethyl acetate (3.75:1.25) is the best eluent for separating steroids in Chlorella sp. microalgae. Identification of compounds separated on TLC plates using a stain that looked and value of Rf. The results were shown in Figure 2 and Table 2.

In Table 2, the 1st, 4th and 5th stains (C1, C4 and C5) with green, green and blue colour were alleged steroid compound with Rf 0.6869, 0.7670 and 0.9400. A bluish-green colour on a fern *Christella arida* showed a steroid compound [21]. Another study, that isolates the stem *bajakah tampala* steroid produces a bluish-green colour on the TLC plate [22]. Isolates obtained needle-shaped crystals.

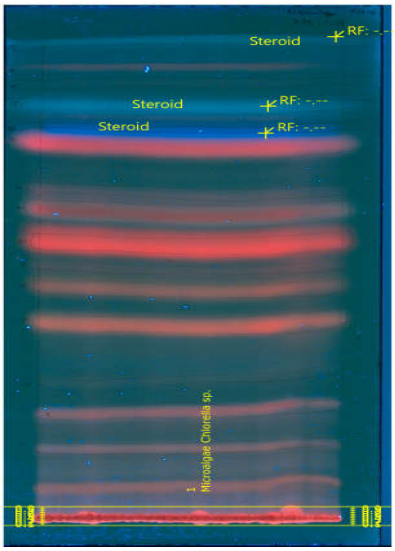

Figure 2. Result of P-TLC of *Chlorella* sp. chloroform fraction.

Table 2. TLC results of chloroform fraction of *Chlorella sp.* Microalgae.

No.	Rf Value	Colour stains	Alleged Compound
1.	0.9400	Green	Steroid
2.	0.85.0	Red	-
3.	0.8056	Red	-
4.	0.7670	Green	Steroid
5.	0.6869	Blue	Steroid
6.	0.5100	Red	-
7.	0.4500	Red	-
8.	0.3400	Red	-
9.	0.6389	Red	-
10.	0.3167	Red	-
11.	0.2389	Brown	-
12.	0.1610	Red	-
13.	0.1278	Red	-

Table 3. LC_{50} values of each extract and isolates steroid microalgae *Chlorella sp.*

Sample	LC_{50} (ppm)
Methanol extracts	36.42
C1	8.795
C4	8.53
C5	4.53

3.5. Toxicity test of steroids compounds against larvae of shrimp Artemia salina Leach

Toxicity tests to extract methanol, and isolate steroids from chloroform fraction and ethyl acetate fraction. LC_{50} of each extract are listed in Table 3. Table 3 shows that steroid isolates had a lower LC_{50} value than methanol extract. The data explains that steroid compounds *Chlorella sp.* microalgae are more toxic than methanol extracts. A sample has a very toxic activity if the extract can kill 50% of test animals at concentrations less than 30 ppm [11]. The steroid compounds *Chlorella sp.* microalgae are had pharmacological potential as anticancer or antitumor.

3.6. Antioxidant activity test with DPPH

Measuring the antioxidant activity of the isolates results from TLC Preparative separation using the method of DPPH with variations of concentration. the concentration of DPPH control is 0.2 mM and measured at a wavelength of 515 nm. DPPH method based on measuring the absorbance of DPPH residual unreacted isolates with TLC Preparative separation results. DPPH radical reduction in the number marked by a colour change from purple to yellow. The colour change is due to the reaction between DPPH free radicals by hydrogen atoms from a steroid compound in TLC Preparative isolates to be 1,1diphenyl-2-picrylhydrazin. The colour change that occurs in this study are not entirely changed from purple to yellow, but only faded lilac.

The parameters used to determine the potential antioxidant. namely the per cent (%) antioxidant activity and EC_{50} values. EC_{50} is the concentration of the sample solution that will lead to a reduction of the activity of DPPH by 50%. The smaller the EC_{50} value, have higher the antioxidant activity [23]. Antioxidant activity of isolates TLC Preparative results was then analyzed using GraphPad software prism with non-linear regression equation Regression for analyzing dose-response data. EC_{50} values of isolates can be seen in Table 4. Table 4 shows that the C5 steroid isolates of microalgae *Chlorella* sp. have strong antioxidant potential. The antioxidant is very powerful if EC_{50} values of less than 50, said to be strong if EC_{50} values between 50-100, is said to be moderate if EC_{50} values between 100-150, and is said to be weak if EC_{50} values between 151-200 [4].

Table 4. E_{50} values of each extract and isolates steroid microalgae *Chlorella* sp.

Sample	EC_{50} (ppm)
C1	2.26×10^{19}
C4	163.10
C5	38.96

3.7. Identification of Compounds Steroids Microalgae Chlorella sp. with UV-VIS Spectrophotometer

Base on UV-Vis spectra, there are three peaks at wavelength of 250.50, 270.50 and 281 nm. According to Susilawati [24] the isolation and separation steroids compound in *Solanum torfum* leaves that result steroid with wavelength of 271 and 281 nm.

3.8. Identification of compounds steroids microalgae Chlorella sp. with the FTIR spectrophotometer

FTIR spectra of *Chlorella sp.* chloroform fraction steroid isolate are shown in Figure 3 and Figure 4. Analysis of FTIR spectrophotometer in Figure 3 shows the absorbtion in wave number 2957.6 cm^{-1}, 2926.0 and 2857.0 cm^{-1} presence of a methyl group (CH_3) and methylene (CH_2), C=O carbonyl in 1723.9 cm^{-1}. C=C absorption area in 1600.9 cm^{-1}. This suspicion is strengthened by the vibrations of C-H bend which indicates a geminal dimethyl groups are commonly found in the framework of steroid compounds. Vibration C-H bending contained in the absorption band 1459.3 and 1381.0 cm^{-1}. The peak in 1271.73 cm^{-1} indicates the presence of C-O vibration [25]. Absorption at wave number 855.0 cm^{-1} is the uptake of C-H alkene group with weak intensity.

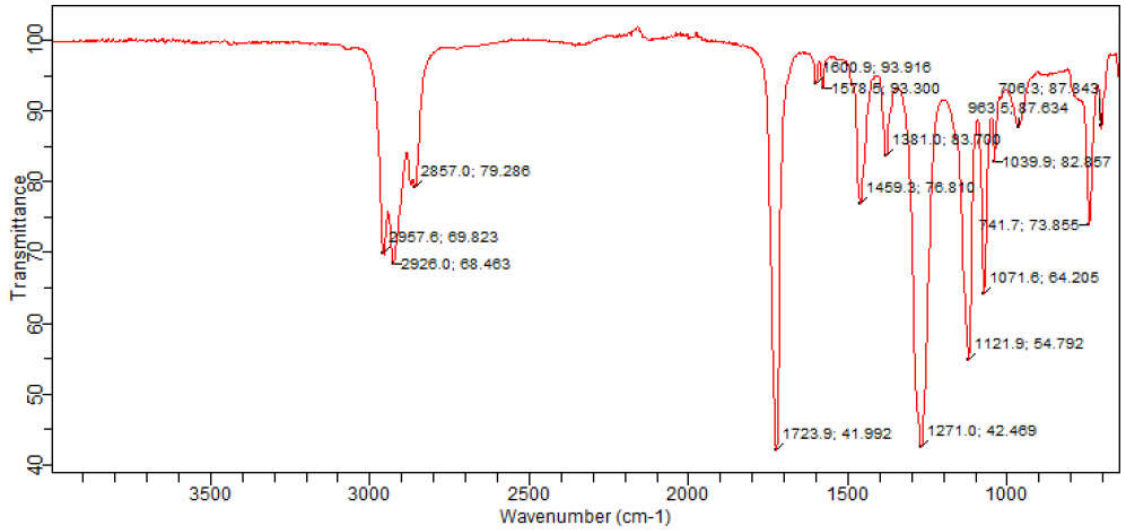

Figure 3. FTIR Spectra of C4 *Chlorella sp.* chloroform fraction steroid isolate.

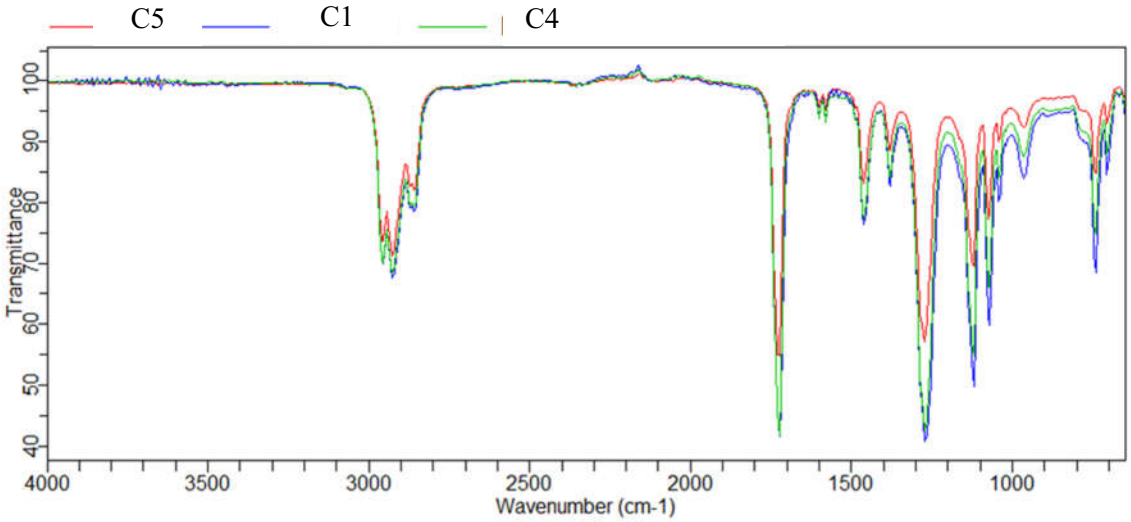

Figure 4. FTIR Spectra of C1 (blue), C4 (green) and C5 (red) *Chlorella sp.* steroid isolate.

4. Conclusion

Steroids isolate from TLC of *Chlorella sp.* Chloroform fractions have toxicity and antioxidant activity. LC_{50} value of TLC isolate of *Chlorella sp.* Chloroform steroid isolates (C1, C4 and C5) were 8.795, 8.53 and 4.53 ppm respectively. Antioxidant activity of *Chlorella sp.* Chloroform steroid isolates (C1, C4 and C5) were 2.26 10^{19}, 163.10 and 38.96 ppm. Based on phytochemicals test and Uv-Vis and FTIR identification showed that Chloroform fractions of *Chlorella sp.* contained steroids compound.

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