Afrizal Itam et al /J. Pharm. Sci. & Res. Vol. 10(3), 2018, 518-522

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Preliminary Phytochemical Screening, Total Phenolic Content, Antioxidant and Cytotoxic Activities of *Alstonia scholaris* R. Br Leaves and Stem Bark Extracts

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Abstract

Alstonia scholaris R.Br. (Family: Apocynaceae) is a plant that has been used by peoples as traditional medicine. Thus, the purpose of this study was the preliminary phytochemical screening to secondary metabolites of plant, and to determine of total phenolic content, antioxidant and cytotoxic properties of this plant extracts. Determination of total phenolic content carried out by the Folin-Ciocalteu method. Antioxidant activity has been determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method while to determine of cytotoxic activity has been used brine shrimp lethality test method. The results showed that all *A. scholaris* extracts either leaves or stem bark extracts have antioxidant activity against DPPH where IC_{50} of methanol, ethyl acetate and hexane extracts of leaves were 84.48, 237.29 and 295.40 µg/mL respectively, and IC_{50} of methanol, ethyl acetate and hexane extracts of stem bark were 89.36, 156.59 and 189.27 µg/mL, respectively. Total phenolic content of methanol, ethyl acetate and hexane extracts of leaves were 18.80, 5.33 and 2.63 µg of gallic acid equivalents (GAE)/10 mg dried extract, respectively, and total phenolic content of methanol, ethyl acetate and hexane extracts of stem bark were 13.04, 5.44 and 3.32 µg of gallic acid equivalents (GAE)/10 mg dried extract, respectively. Antioxidant activity of extracts are increased if their phenolic content is also increased with the regression equations y = -12.496x + 317.19 (R^2 = 0.9872) for leaves extract and y = -9.8981x + 217 (R^2 = 0.9862) for stem bark extract.

Keywords: *Alstonia scholaris*, Antioxidant, Brine shrimp lethality test, Phenolic, Phytochemistry,

1. Introduction

Secondary metabolites are the compounds which naturally occurring in plant or organism and having function as protective or preventive of diseases either for that plants or organisms. These compounds are not classified as nutrients, but they are also having bioactivities to human either for protective or treatment of diseases. The diseases that can be protected or treated by using these secondary metabolites among other are the disease which caused by occurring of oxidation on cell or human tissue such as cancer and other degenerative diseases. [1].

To prevent of occurring of oxidation can be used antioxidant. There are many secondary metabolites having antioxidant activity. One of them is flavonoids that also called phenolic or polyphenolic compounds. Function of flavonoids is as protectors to a wide variety of environmental stresses in plants, but in humans, function of flavonoids is as modifiers of biological response. Flavonoids have been proved to have antiallergenic, anti-inflammatory, antiviral, anti-carcinogenic, anti-aging, anti-diabetic, antimicrobial, antibacterial and antifungal activities [2, 3]. Medicinal plants serve as an important source of this secondary metabolites.

One example of plants that has been used by peoples as traditional medicine is *Alstonia scholaris* R.Br. (Family: Apocynaceae) which has been used among other for treatment to arthritis, impotence, wounds, earache, asthma, leucorrhoea, dog bite, fever, cancer, tumor, jaundice, hepatitis, malaria, skin diseases, diarrhea and others. [4]. The plant is a tree with of 10-50 m in high, and stem is stand up, woody, branching and dark green in color. Their leaves are single with lanceolate in shape with rounded edges, the base is tapered and the edges are flat. The leaves are 10-20 cm in long and 3-6 cm wide, the pinnate, the top surface is slippery, the length of the stalk is around 1 cm and the color is green. Local name of this plant is such as rite, pulai, pule (Indonesian), satiani, chattin, chatium (Bengali), white cheesewood, birrba, milkwood pine, milk wood, milky pine, black board tree, devil's tree, dita bark (English), chatian, satni, satwin, saitan-ki-jhad (Hindi), pulai, pulai linlin (Malay), alstonie (Germany) [4, 5, 6]. Phytochemical constituents of this plant is alkaloids among other echitamine, echitamine chloride, rhazine, nareline, pseudo akuammigi, scholarine, scholaricine, dihydrocondylocarpine, 19,20-Z-vallesamine and 19,20-E-vallesamine, flavonoids such as isookanin-7-o-alpha-lrhamnopyranoside, alstonoside, leucoanthocyanins. Beside that the plant containing phenolics, steroids, saponins and tannins [4].

The above data showed that *A. scholaris* has potential to be used as source of natural medicine to treat or manage of various diseases. The aim of study is to determine the antioxidant and cytotoxicity activities of the leaves and stem bark extracts of *A. scholaris*, and their correlation with total phenolic contents. Our expectation is *A. scholaris* extracts can be used as an antioxidant or to control of diseases that be caused by oxidative stress where these activities can inhibit or reduce or heal various diseases such as cancer

2. Materials And Methods

2.1. Chemicals and Reagents

Methanol, ethyl acetate and hexane for maceration were obtained from their techniques quality with distillation process. Folin-Ciocalteu, DPPH, and gallic acid were purchased from Sigma Chemical Company St. Louis MO, USA. Magnesium powder, hydrogen chloride, ferric chloride, acetic anhydride, Mayer reagent, sulphuric acid, chloroform, ammonia, sodium hydroxide, methanol p.a and sodium carbonate were purchased from Merck KGaA Darmstadt Germany.

2.2. Instruments

Filtrate from maceration was used rotary evaporator Heidolp WB 2000, antioxidant activity determination was used spectrometer UV/ VIS 1700 Series and the growth medium of brine shrimp was prepared with sea water in a small tank divided into two compartments.

2.3. Sample of *Alstonia scholaris*

A. scholaris leaves and stem bark were collected from West Sumatera, Indonesia. The leaves and stem bark were identified and the specimen was deposited in herbarium of Biology Department, Andalas University. Samples then were washed and dried at room temperature and furthermore samples were milled into powder.

2.4. Preliminary Phytochemical Screening

Preliminary qualitative screening of various phytochemicals based on Pawaskar, *et al.* (2017) [1] and Shah, *et al.* (2010) [7] methods with modification.

About 5 g of *A. scholaris* leaves and stem bark powders were macerated separately with 100 ml of methanol at the boiling point temperature for 5 minutes. The extracts were filtered and filtrate was concentrated by evaporation and the obtained residue were added with 5 mL of chloroform and 5 mL of water, and then the mixtures were shaken. After left for a few minutes, two layers would have formed. The aqueous layer (upper layer) was used to identification of flavonoids, phenolics and saponins, meanwhile the chloroform layer (below layer) was used to identification of triterpenoids and steroids.

a. Flavonoids Test
A volume of 1 mL aqueous layer was pipetted into test tube, and then to this solution was added magnesium powder and concentrated hydrogen chloride. Formation of red color indicates the presence of flavonoids.

b. Phenolics Test
A volume of 1 mL aqueous layer was pipetted into test tube, and then to this solution was added 3 drops of 1% ferric chloride solution. Formation of blue or green color indicates phenolics

c. Saponins Test
A volume of 2 mL aqueous layer was pipetted into test tube and then was shaken for a few minutes. Formation of frothing which is not loss after was added a few drops of hydrogen chloride, indicates the presence of saponins.

d. Triterpenoids and Steroids Tests
A volume of 1 ml of chloroform layer was pipetted into test tube, and was added 3 drops of acetic anhydride followed by 3 drops of concentrated sulphuric acid from side wall of test tube. Formation of violet to blue colored ring, indicates the presence of steroid moiety, while formation of reddish brown colored indicates the presence of triterpenoid moiety.

e. Alkaloids Test
About 1 g of *A. scholaris* leaves and stem bark powders were macerated separately with 10 ml of chloroform-ammonia 0.05 M for 10 minutes and were filtrated. 2 ml of filtrates were pipetted into test tube and followed by 2 ml of sulphuric acid 2 N, shaken and then was left for a few minutes. 1 mL of acidic layer (upper layer) was transferred to other test tube and was added with Mayer reagent. Presence of alkaloid was marked with formation of white colored precipitate

f. Coumarins Test
About 1 g of *A. scholaris* leaves and stem bark powders were macerated separately with 10 ml of methanol at boiling point temperature for 5 minutes and were filtrated. Filtrate were spotted on thin layer chromatography plate and then developed by ethyl acetate as mobile phase. Presence of coumarins was marked with blue colored fluorescence under ultra violet light 254 and 356 nm. This fluorescence will increase in intensity after spray with 1 % sodium hydroxide solution.

2.5. Extraction

Two hundred gram of the powdered leaves and stem bark were macerated separately with 200 mL of methanol, ethyl acetate and hexane in separated flasks at room temperature for overnight. The mixtures were filtrated, and filtrate were dried using a rotary evaporator and concentrated extracts were stored in freezer in airtight container.

2.6. Antioxidant Activity by DPPH Free Radical Scavenging Method

Determination of antioxidant activity of extracts by DPPH free radical scavenging based on Muslim *et. al* (2010) [8], Itam *et al.* (2015) [9] and Mani *et al.* (2017) [10] methods with some modifications. Ten mg of methanol, ethyl acetate and hexane extracts (leaves and stem bark extracts) were dissolved in methanol with total volume 10 mL in separated volumetric flask (stock solution 1,000 µg/mL). Various concentration of extracts were prepared from these stock solutions, where 6.25 to 400 µg/mL for leaves methanol extract and 150 to 400 µg/mL for ethyl acetate and hexane extracts, meanwhile 12.5 to 200 µg/mL for stem bark extracts ibn methanol. Then 0.1 mL of a DPPH solution in methanol (0.1 mM) was mixed with 0.1 mL of these extract solutions in separated tube. After incubation 30 minutes at room temperature in the dark, the absorbance was recorded at 517 nm using spectrofotometer UV-VIS 1700 Series. A control consists of 0.1 mL of methanol and 0.1 mL of 0.1 mM DPPH. Ascorbic acid was used as references compound. The free radical scavenging activity of the extracts was calculated (FRSA, %) using equation:

$$\text{FRSA } (\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{sample} is the sample solution absorbance, and A_{control} is the of the control solution absorbance. Inhibition concentration 50% (IC50) was calculated using regression equation from the calibration curve that obtained from the graph of extract concentrations (X-axis) plotted against radical scavenging percentage (Y-axis).

2.7. Cytotoxic Activity by the Brine Shrimp Lethality Method

Cytotoxic activities of extracts on brine shrimp was performed following Mudi *et al.* (2009) [11] and Meyer *et al.* (1982) [12] methods with some modifications. A small tank that divided into two compartments and containing sea water was used to growth medium of larvae. The shrimp eggs of *A. salina* were put to the covered compartment, while above the open side of the tank was placed a lamp to attract hatched shrimps through perforations in the partition wall. The shrimps mature as nauplii are ready for the assay after 48 hours. Ten mg of each extract (leaves and stem bark extracts) was dissolved in 10 mL of methanol (volume total), so that obtained 1,000 µg/mL of extract solution as stock solution. Each of stock solution with various volume were transferred using a micropipette into separate vials to obtain various concentration of extracts. These solutions were allowed to dry at room temperature, and then, residue was dissolved in 50 µL of DMSO and 2 mL of sea water. Furthermore, 10 of nauplii were introduced into each mixture and the volume of mixture was made up to 5 mL with sea water. The control is 50 µL of DMSO and the volume was made up to 5 mL with sea water. After 24 hours introducing the nauplii, the survival number at each dosage was counted and recorded. LC_{50} values of extract were determined using probit value and regression equation.

2.8. Determination of Total Phenolic Content

Total phenolic content of leaves and stem bark extracts were determined using Folin-Ciocalteu reagent method that based on Kosar *et al.* (2005) [13] and Sen *et al.* (2013) [14] with modification. Ten mg of methanol, ethyl acetate and hexane extracts of leaves and stem bark were dissolved in methanol using the different volumetric flask 10 mL. And then 0.5 mL of these each extract solutions was pipetted into test tubes that containing 1.0 mL of Folin-Ciocalteu reagent. After five minutes, 2.0 mL of sodium carbonate (7%, *w/v*) was added to this mixtures, and the volume of mixture was made up to 10 mL with water and the test tubes were shaken thoroughly. The mixtures were incubated for 2

hours at room temperature, and after that, absorbance of each mixture were measured at 760 nm using spectrometer UV/VIS Shimadzu Pharma Spec UV-1700. Gallic acid was used as standard to obtain a calibration curve, and phenolic content of extracts were expressed as gallic acid equivalent per 10 mg dried extracts.

3. Results And Discussion

3.1. Preliminary Phytochemical Screening

Present, phytochemicals analysis of plants is gaining importance due to the very reason to know bioactivities of plants including antioxidant properties which are used for treatment of wide array of infectious and chronic diseases.

The results of preliminary qualitative screening of various phytochemicals from the leaves and steam bark of *A. scholaris* are expressed in Table 1 which showed that the phytochemicals that can be detected in this plant is flavonoids, phenolics, saponins, triterpenoids, steroids and alkaloid either in leaf or stem bark. Dey (2011) [4] has also reported the presence of alkaloids, flavonoids, phenolics, steroids, triterpenoids and saponins in *A. scholaris* plant. Therefore, this plant has ability as antioxidant because containing flavonoids, phenolics and alkaloids which having activity as antioxidant.

3.2. Extraction

The results of extraction that using maceration method to leaves and stem bark of *A. scholaris* with methanol, ethyl acetate and hexane solvents are presented in Table 2. Solubility of leaves and stem bark components in these solvents are following order: methanol > ethyl acetate > hexane. Solubility in hexane solvent is a half than that of ethyl acetate and methanol solvents for leaves sample, meanwhile for stem bark, solubility of its components in hexane solvent is a half than that of ethyl acetate solvent and less than a half compare to methanol solvent. It means, both of sample containing more the components that more polar properties. This suitable with previous report, that this plant containing alkaloids, flavonoids and phenolics, where these compounds are categorized the polar compound [4]. Therefore, to isolate of these compounds is used methanol or ethyl acetate as solvents

3.3. Antioxidant Activity of Extracts by DPPH Free Radical Scavenging Method

DPPH is a stable free radical that be caused happening delocalization of spare electron that occurring in the entire molecule. Donation of antioxidant proton that presence in sampel to DPPH radical reduces violet colour of DPPH to yellow coloured, 1,1-diphenyl-2-picrylhydrazyn [15, 16].

All extracts, either leaves extracts or stem bark extracts were able to reduce the stable radical DPPH to yellow-coloured. This show that all extracts have antioxidant activity. Free radical scavenging activity to DPPH of these extracts which using various concentration are shown in Fig.1 and Fig. 2, respectively and their inhibitory concentrations 50% (IC_{50}) are presented in Table 3. The Figure 1 and 2 show that antioxidant activity of extract increases with increasing of extract concentration. The order of antioxidant activity to DPPH is follow methanol > ethyl acetate > hexane extracts either leaves extracts or stem bark extract. This is also supported by IC_{50} value of extract where IC_{50} of methanol, ethyl acetate and hexane extracts of *A. scholaris* leaves are 84.48, 237.29 and 295.40 µg/mL respectively and IC_{50} of methanol, ethyl acetate and hexane extracts of *A. scholaris* stem bark are 89.36, 156.59 and 189.27 µg/mL, respectively. IC_{50} is a concentration of sample needed for reduce of 50% of DPPH. It means that the methanol extract of leaves and stem bark are categorized as strong antioxidant and ethyl acetate extract of leaves and stem bark and also hexane extract of stem bark are categorized as moderate antioxidant, meanwhile leaves hexane extract is categorized as weak antioxidant [17, 18]. However, antioxidant activity of this plant extracts is lower than ascorbic acid as reference where IC_{50} of ascorbic acid is 3.70 µg/mL.

The strong antioxidant activity on the DPPH radical is methanol extract, and then followed by ethyl acetate extract. Thus, in the methanol extract contain the components that have highest antioxidant activity than others. Methanol is a solvent which can dissolve the relative polar organic component. One of compound that can dissolve in methanol which has activity as antioxidant is phenolic or flavonoids. Even if these compounds has glycoside group, it would easier dissolve in relative polar solvent. There was a positive correlation between phenolic contents and its antioxidant activity [19, 20, 21, 22] and positive correlation between polyphenolic contents of *Agaricus blazei* and its antioxidant activity [23]. *A. scholaris* was also reported containing phenolic and flavonoids.

3.4. Cytotoxic Activity by the Brine Shrimp Lethality Method

Cytotoxic properties on *A. salina* larvae is conducted to various leaves and stem bark extracts of *A. scholaris*. The results are presented in Fig 3 and Fig. 4, respectively. These figures showed that increasing the extracts concentration the mortality of *A. salina* larvae is also increases. Cytotoxic properties of extract or sample is expressed with lethal concentration 50% (LC50) which is defined as the concentration that can kill a half of sample population of a specific test-animal. LC_{50} of these extracts are shown in Table 4 which express that the methanol and ethyl acetate extracts either leaves or stem bark has weak cytotoxic properties, meanwhile hexane extract both leaves and stem bark has no cytotoxic properties [24, 25]. Among these extracts, methanol extract is the extract that having highest cytotoxic activity than that of ethyl acetate and hexane extracts. This also has correlation to antioxidant activity which methanol extract has also highest antioxidant activity.

3.5. Determination of Total Phenolic Content

Total phenolic content of extracts was calculated based on gallic acid standard curve and the results are presented in Table 5. Total phenolic contents of extract either leaves extracts or stem bark extracts are following order: methanol > ethyl acetate > hexane extracts. This has correlation to solubility of component of leaves and stem bark in same solvents are also the follow same order.

Total phenolic content of this plant extract showed a concentration-response relationship on antioxidant activity to DPPH either leaves extracts or stem bark extracts where increase the phenolic content of extracts, antioxidant activity is also increase. In this case, antioxidant activity of extracts is expressed with IC_{50}. These correlations are shown in Fig. 5 and Fig. 6 which express relationship between total phenolic content and antioxidant activity (IC_{50}) of extract with regression equation $y = -12.496x + 317.19$ ($R^2 = 0.9872$) for leaves extracts and $y = -9.8981x + 217$ ($R^2 = 0.9862$) for stem bark extracts. Previous research, Sen S. *et al.* (2013) [14] also reported that there is correlation between phenolic content of extract with antioxidant activity to DPPH where increase in concentration is also an increase in scavenging capacity. Nyein *et al.* (2017) [26] also reported that the DPPH tests revealed there is a positive correlation between phenolic content and free radical scavenging activity where expressed that the IC_{50} is getting smaller with increasing total phenolic content. As known that the smaller the IC_{50}, it means higher the antioxidant activity.

Table 1. Results of Preliminary Qualitative Screening of various Phytochemicals from the leaf and steam bark of *A. scholaris* R. Br.

No	Phytochemicals	Leaf	Steam bark
1.	Flavonoids	+	+
2.	Phenolics	+	+
3.	Saponins	+	+
4.	Triterpenoids	+	+
5.	Steroids	+	+
6.	Alkaloids	+	+
7.	Coumarins	-	-

The sign of + denotes the presence of the compound meanwhile sign of - indicates absence of compound.

Table 2. The results of maceration to *A. sholaris* leaves and stem bark using methanol, ethyl acetate and hexane solvents

No	Sample	Solvent	Percentage (%)
1.	Leaves	Methanol	14.42
		Ethyl acetate	13.54
.		Hexane	7.22
2	Stem bark	Methanol	13.51
		Ethyl acetate	9.35
		Hexane	4.67

Table 3. IC_{50} values of various leaves and stem bark extracts of *A. scholaris* and ascorbic acid to DPPH

No	Sample	Extract	IC_{50} (µg/mL)
1.	Leaves	Methanol	84.48
		Ethyl acetate	237.29
.		Hexane	295.40
2	Stem bark	Methanol	89.36
		Ethyl acetate	156.59
		Hexane	189.27
3	Ascorbic acid		3.70

Table 4. LC_{50} of various leaves and stem bark extracts of *A. scholaris* on *A. salina* larvae

No	Sample	Extract	LC_{50} (µg/mL)
1.	Leaves	Methanol	883.28
		Ethyl acetate	954.72
.		Hexane	2760.01
2	Stem bark	Methanol	680.07
		Ethyl acetate	834.67
		Hexane	2037.33

Table 5. Total phenolic content of various leaves and stem bark extracts of *A. scholaris*

No	Sample	Extract	Total phenolic content (µg GAE/10 mg dried extract)
1.	Leaves	Methanol	18.80
		Ethyl acetate	5.33
.		Hexane	2.63
2	Stem bark	Methanol	13.04
		Ethyl acetate	5.44
		Hexane	3.32

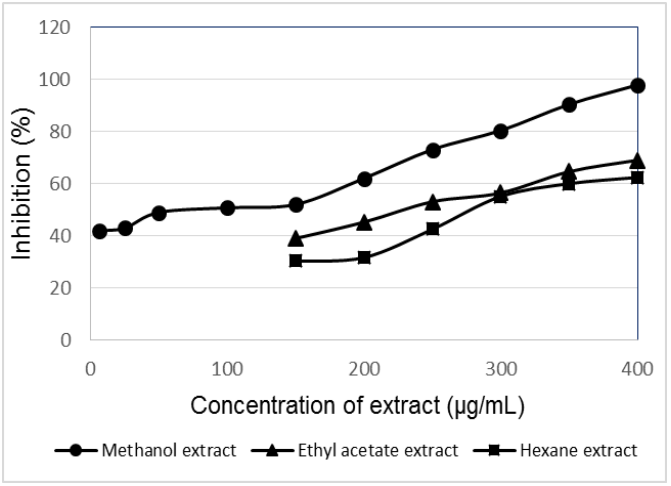

Fig. 1. Antioxidant activity of various concentration of *A. sholaris* leaves extracts to DPPH radical

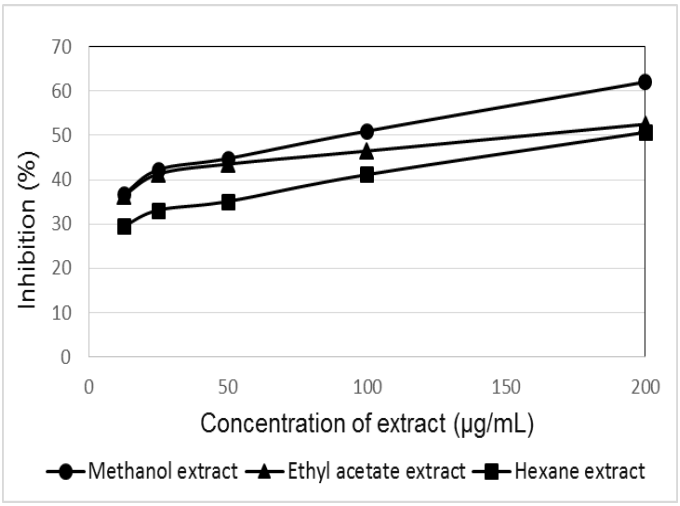

Fig. 2. Antioxidant activity of various concentration of *A. sholaris* stem bark extracts to DPPH radical

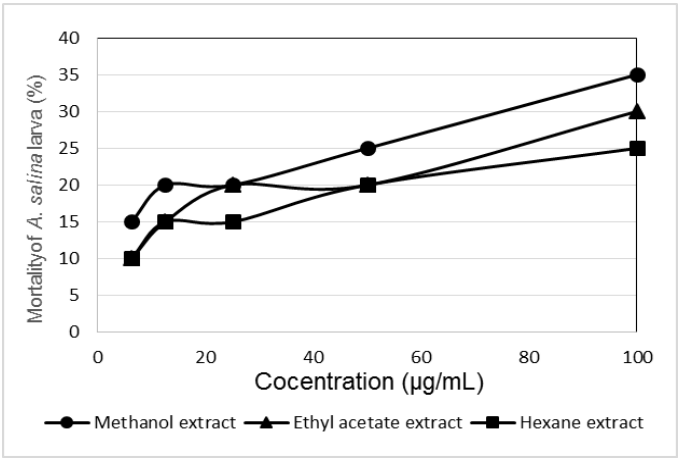

Fig. 3. Relationship graph between the concentrations of *A. scholaris* leaves extracts on mortality of *A. saline* larvae

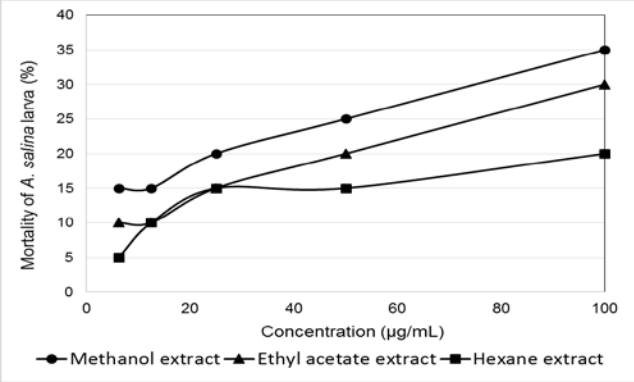

Fig. 4. Relationship graph between the concentrations of *A. scholaris* stem bark extracts on mortality of *A. saline* larvae

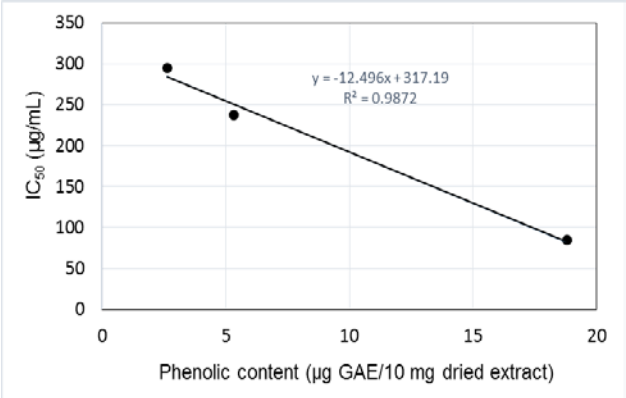

Fig. 5. Correlation curve between total phenolic contents of *A. scholaris* leaves extracts and their antioxidant activity (IC_{50})

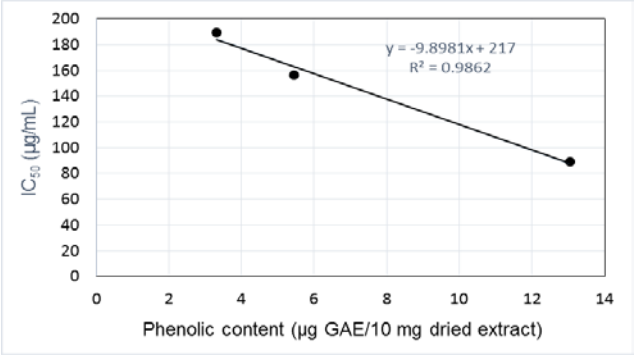

Fig. 6. Correlation curve between total phenolic contents of *A. scholaris* stem bark extracts and their antioxidant activity (IC_{50})

4. CONCLUSION

In conclusion, leaves and stem bark of *A. scholaris* contained secondary metabolites such as flavonoids, saponins, triterpenoids, steroids and alkaloid. Their extracts displayed free-radical-scavenging activity on DPPH radical and cytotoxic activity on *A. salina* larvae. Methanol extract of leaves and stem bark are categorized as strong antioxidant and ethyl acetate extract of leaves and stem bark and also hexane extract of stem bark are categorized as moderate antioxidant, meanwhile leaves hexane extract is categorized as weak antioxidant. Cytotoxic activity of methanol and ethyl acetate extracts either leaves or stem bark have weak cytotoxic properties, meanwhile hexane extract both leaves and stem bark have no cytotoxic properties. Antioxidant activities of extracts have positive correlation with their total phenolic contents, total phenolic concentration is increased, antioxidant activity is also increased, either leaves extracts or stem bark extracts.

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