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Effects of Extracting Solvents on Total Phenolic Content, Total Flavonoid Content and Anti-Oxidant Activity of *Andrographis paniculata* from Kemaman, Malaysia.

Yahaya Najib Sani[1, 2*], Suleiman Danladi[1], Amirah Wan-Azemin[1], Mahadeva Rao US[1], Khamsah Suryati Mohd[3], and Saravanan Dharmaraj[1].

[1]Faculty of Medicine, Universiti Sultan Zainal Abidin, Medical Campus, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia.
[2]Bayero University, Kano, (B.U.K), P.M.B 3011, Kano, Nigeria.
[3]Department of Biotechnology, Faculty of Bioresources and Food Industry, University Sultan Zainal Abidin, Tembila Campus, 22200 Besut, Terengganu, Malaysia.

ABSTRACT

Andrographis paniculata Nee belonging to the family Acanthaceae, is common in Malaysia, Thailand and India. Traditionally, it has been used to treat many diseases including diabetes, upper respiratory tract infections and diarrhoea. The effect of extracting solvents (methanol, ethyl acetate, 50% ethanol/water and aqueous) on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of whole *Andrographis paniculata* was studied. TPC was measured using Folin-Cioucalteu method while TFC was determined using aluminium chloride method. Antioxidant activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The aqueous extract recorded the highest phenolic content (184.48 mg GAE/g), followed by ethanol:water (1:1 v/v), methanol and ethyl acetate (178.79, 58.78, 9.37 mg GAE/g) respectively. One way ANOVA showed that the TPC of aqueous and ethanol:water extracts did not differ significantly at $p<0.05$ among them but they differed from the other two extracts. Similarly, ethyl acetate extract recorded the highest TFC (0.44 mg QE/g) while methanol, ethanol:water and aqueous had similar average values for TFC at 0.43 mg QE/g. Consequently, the value for ethyl acetate extract was statistically different with the three others. Moreover, the antioxidant activity of ethanol:water indicated a good IC_{50} (93.30µg/ml) as compared to the standard (quercetin at 20 µg/ml) used in the experiment, while the other extracts have activity below 50%. The result of this study showed that TPC, TFC as well as antioxidant activity was influenced by extracting solvents and the choice of solvent for further study is suggested to be combination of ethanol:water. This is because it is similar statistically to aqueous in phenolic content and has highest antioxidant activity, which is beneficial for many diseases.

Keywords: *Andrographis paniculat,* polyphenols, solvents, antioxidant.

Corresponding author

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INTRODUCTION

Natural products especially that of plant origin are of great interest in the process of drug discovery, due to their large diversity in nature, permitting the identification of newer molecules of greater interest for the development of new therapeutic agents, as well as biochemical and molecular tools needed to clarify complex cellular and molecular mechanisms of action involved in most physiological and pathological processes. Furthermore, a growing world-wide interest in the use of phyto pharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, has been noted in recent years. It is believed that about 80% of world's population use plants as their primary source of medicinal agents. (Borris, 1996; Cordell, 1995; Martini et al., 2007).

Andrographis paniculata belongs to the genus Andrographis that is widely used for decades due to its known biological activities.

Figure 1: *Andrographis paniculata*

It belongs to the Family *Acanthaceae* comprising more than 40 different species. Biomedical literatures indicates the presence of medicinally important entalabdane Diterpenoids, noriridoides, xanthones, flavonoids and other miscellaneous compound that show an important pharmacological activities such as anti-diabetes, antidiarrheal, antibacterial, cardiovascular benefits, anti-inflammatory and hepato protective benefits. (Jarukamjorn et al., 2010; Mukherjee, Maiti, Mukherjee, & Houghton, 2006). Medicinal plants such as *Andrographis paniculata* Nees (Duraipandiyan, Ayyanar, & Ignacimuthu, 2006) have been reported to treat chronic as well as infectious diseases. In addition*, Andrographis paniculata* has been used as a medicinal herb for many centuries all over the world. It is widely and extensively used in Ayurveda, Unani and Siddha medicinal preparations as a remedy for various diseases. In Malaysia and other countries it is reported to have multiple clinical applications. It's an important cold property herb, used in fevers to remove toxins from the body. It is used to treat diabetics, upper respiratory tract infections, sore throat, fever, herpes, gastrointestinal tract infections, and some chronic diseases (Jayakumar, Hsieh, Lee, & Sheu, 2013).

Moreover, *Andrographis paniculata* serve as potent stimulator of immune system by two approaches. The first was an antigen specific response; antibodies were made to counteract invading microbes and the second was none specific immune response; macrophages cells scavenged and destroyed invaders. Since *Andrographis paniculata* activated both responses, it may may be effective against scavengaing radicals and therefore, a potential anti-oxidant agent(Jarukamjorn & Nemoto, 2008).

Free radicals generated in the human body may increase the risk of chronic diseases such as cancer and cardiovascular diseases. These free radicals are usually produced through aerobic respiration. Although

the human body produces antioxidant enzymes to neutralize free radicals (Rafat, Philip, & Muniandy, 2010), a diet rich in edible antioxidants is recommended to assist the human body to protect itself from food borne free radicals. A variety of plant secondary metabolites have been reported to act as antioxidants and amongst them phenolic compounds form a major group. There are several reports on the contribution of phenolic compounds to the antioxidant potential of different plant species.(Cai et al., 2009).

Furthermore, free radicals can cause damage to cellular bio-molecules such as proteins, nucleic acid, ipids and carbohydrates, this may be a contributing factor in oxidative stress and several diseases. Antioxidants interfere and inactivates production of free radicals and play a key role to inactivate them(ISMAIL, MARJAN, & FOONG, 2004),(Halliwell, 1995). Compounds such as caretenoids, limonoids, tocopherols, ascorbates and polyphenols are found to be strong natural antioxidants agents. Nowadays, much interest is on the biological effects of phenols, since evidence shows that they provide protection against cancer, inflammation, and cardiovascular diseases. (Bajpai, Pande, Tewari, & Prakash, 2005; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Phillips et al., 2000).

Moreover, flavonoids are one of the polyphenolic compounds which are considered to have antioxidant activity, protecting the body system or cells against oxidation process. However, Hosu, Cristea, & Cimpoiu, 2014 reported the relevance of polyphenol compounds in determining the quality of red wines and therefore, flavonoid in abundance can influence the astringency, bitterness and colour of plant materials. Also numerous researches have shown that high concentration of flavonoids and phenolic compounds were significantly associated with reduced risk of cardiovascular diseases (CVD) through modulation of inflammation and improvement in vascular functions. Therefore, antioxidants especially polyphenols and flavonoids could have a protective effects against cancer, inflammation, diabetes, and infectious diseases of antioxidant agent on free radicals(Tharasena & Lawan, 2014). Numerous animal and human studies indicated that inflammation is a critical factor involved in the pathogenesis of liver(Wang et al., 2014). Therefore, flavonoids might be developed as a new natural drugs for the treatment of liver diseases by ameliorating hepatic inflammation.

Natural substances especially certain plants, are natural sources of phenols and may be used as an atioxidants(Kahkonen et al., 1999). However, this research studied the effects of extracting solvents on total phenolic content (TPC), total flavonoid content (TFC) and anti-oxidant activity of *Andrographis paniculata* from Kemaman, Malaysia In order to find out the potentials sources of solvent system for antioxidant activity that will be availble especially in commercial quantity locally.

MATERIALS AND MATHODS

Chemicals and standards

Quercetin, Folin-Ciocalteu, phenol reagent gallic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), sordium carbonate solution, methanol, ethanol were obtained from Merck (Germany). All other reagents are of analytical grade.

Plant materials

Andrographis paniculata was purchased from local herbal seller in Kuala Terengganu, Malaysia in September. The plant was identified, authentitecated by Norhaslinda Haron from Faculty of Agriculture and Animal sciences, Universiti Sultan Zainal Abidin, and deposited at the Herberium unit with voucher number 00266.

Extraction and preparation of plant extracts

The plant was washed with distilled water, dried at 40^{o}C and then grinded into powdered form (40mesh). The powdered plant was weighed using pre calibreted weighing balance and soaked in different solvents (methanol, ethylacetate, 50% ethanol/water and water) for 72 in a ratio of 1:10 respectively. The extracts were filtred using whatman® no 1 filter paper (whatman international maidstone) and evaporated to dryness using a rotary evaporator (Heidolph WB2000, Germany) at temperature 40^{o}C and a reduced pressure. Extracts were freez dried and kept at 4^{o}C for further use. Also stock solution of the plant extracts were prepered in 100% dimethylsulphoxide (DMSO) and kept at 4^{o}C for experiment.

Determination of percentage yield of the extracts (%)

Gravimetrically using the dry weigt extracts (a) and the soaked sample materials (b) were used to determine the percentage yield using a formula as:

Percentage yield = (a/b) x 100

The yield for the extraction was calculated for each extracts and presented as a percentage yield (%).

Total phenolic content determination

TPC were measured with Folins reagent by the method of Blainski, Lopes, & De Mello, 2013 with some modification and expressed as gallic acid equivalent (GAE) on a dry weight basis. The extracts were diluted to 250µg/ml concentration. 1.25ml of Folin-Ciocalteu reagent was added to the mixture and incubated at room temperature for 3 minutes. Lastly 1.0ml of sodium carbonate (10%w/v)) was added and incubated at room temperature in a dark place for 30minutes. Absorbance values of the solutions were measured at 750nm spectrosphotmertrically.TPC was determined as gallic acid equivalent (GAE) based on Folin-Ciocalteau calibration curve using gallic acid (ranging from 2.5 to 160µg/ml) as the standard and expressed as mg gallic acid per gram of dry sample.

DPPH free radical sacavanging activity

Method of Musa, Abdullah, Kuswandi, & Hidayat, 2013 was adopted with little modification antioxidant activity (AOA) was assayed using free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and expressed as percentage of inhibition relative to the control. Base on the number of wells, 0.0041g/70ml of DPPH was weighed in a pre calibrated weighing balance and dissolved in 70ml methanol, wrapped and kept in a dark. 40µl of DMSO was added into 96 wells plate from B to H, 20µl of the samples were added into well A & B. Serial dilution was carried out in two-fold from well B to H making the concentration from A to G as 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 0 µg/ml respectively. 60µl of DMSO was then added to all wells after which 200µl of DPPH solution was added in all wells. The mixture was shaken vigorously and allowed to stanad at room temperature in a dark for 30minutes. The same procedure was reapeated for the standard sample (Quarcetin), while DMSO served as negative control in all wells (H). Each sample was assayed in triplicate. Decolouration due to reaction of antioxidants in samples with the stable free DPPH radical was measured spectrophotometerically using spectrophotometer (Elisa plate reader). Percentage inhibition was calculated and the concentration of the test sample to give 50% radical sacavenging acitivity was determined as IC_{50} values and compared wtih the standard sample (quercetin)

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

Total flavonoid content

Method of Marinova, Ribarova, & Atanassova, 2005 was adpoted with little modification to determine the TFC. 0.25ml of the diluted extracts of 250µg/ml concentration were mixed with solution containing 50µl of 1M aqueous potassiun acetate, 50µl of 10% (w/v) Alminium chloride and 2.15ml of 95% ethanol. The mixture was mixed, incubated at room temperature in a dark for 40 minutes; absorbance (*A*) was recorded spectrophometrically at 415nm wave lenth. The samples were assayed in triplicate. TFC was calculated using quercetin as standard and expressed as mg quercetin per gram of dry sample, using the calibration curve equation y = 216x – 23.294 and R^2 = 0.9934

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RESULTS AND DISCUSSION

Table 1: percentage yield of methanol, ethyl acetate, 50% ethanol/water and 1005 water extracts of *Andrographis paniculata*

Sample	Percentage yield (%)
Methanol	11.21 ± 2.22
Ethyl acetate	5.83 ± 1.37
Ethanol/water	19.60 ± 3.27
Water	4.53 ± 0.90
Mean ± SD	

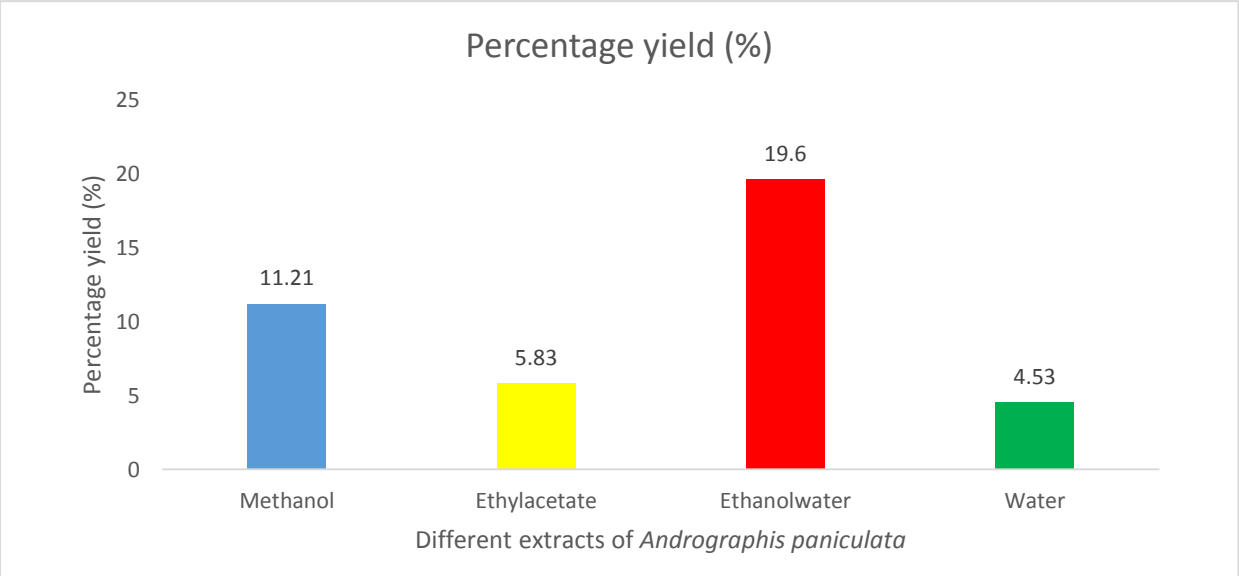

Figure 1: Percentage yield of different extracts of *Andrographis paniculata*

Table 2: Percentage inhibition rate of DPPH free scavenging radicals activity of different extracts of *Andrographis paniculata*

Sample	Percentage DPPH free Scavenging radical inhibition (%)
Methanol	46.60 ± 0.02
Ethyl acetate	25.70 ± 0.02
50% Ethanol/water	54.70 ± 0.02
Water	23.30 ± 0.02
Quercetin	84.70 ± 0.01

DPPH scavenging activity

Reaction between samples and stable free DPPH leads to donation of hydrogen atom by the sample and therefore, decolouration of the sample which was measured spectrophotometrically. Result indicates that 50% ethanol/water extracts has the highest inhibition rate (54.7%) (Table 2), while methanol extract, ethyl acetate and water extract indicates 46.6%, 25.7% and 23.3% respectively. However, all samples have significantly lower DPPH scavenging free radical activity compared to the standard (84.7%). Moreover, only 50% ethanol/water extract showed activity at IC_{50} (93.3µg/ml) compared with the standard IC_{50} (20µg/ml) (Table 3). The different extracts of *Andrographis paniculata* are not significantly different at the ($p>0.05$) indicating the homogeneity of the extracts.

Table 3: Minimum inhibitory concentration (IC_{50}) antioxidant activity of methanol, ethyl acetate, 50% ethanol/water and water extracts and standard Quercetin of *Andrographis paniculata* using DPPH free scavenging radical assay.

Extract	IC_{50} (µg/ml)
Methanol	-
Ethyl acetate	-
50% Ethanol/water	93.3
100% Water	-
Quercetin	20.0

Table 4: Total phenolic content of different extracts of *Andrographis paniculata*

Sample	Total phenolic content (mg of GAE/g of sample)
Methanol	58.78 ± 4.17^{b}
Ethyl acetate	9.37 ± 5.45^{a}
50% Ethanol/water	178.79 ± 21.72^{c}
Water	184.48 ± 12.57^{c}

This study investigates the TPC of *Andrographis paniculata* extracts (methanol, ethyl acetate, 50% ethanol/water and aqueous) (Table 4) were calculated using the standard curve of Gallic acid and presented as Gallic acid equivalents (GAE) per gram. The aqueous extract of *Andrographis paniculata* has shown to contain the highest TPC (184.48mg), followed by 50% ethanol/water, methanol and ethyl acetate (178.79, 58.78 and 9.37 mg) respectively. Ethyl acetate extract was found to have the lowest TPC. Previous research reported TPC of 75.86mg/g (Rafat et al., 2010). The mean phenolic content of different extracts was found to be significantly different ($p< 0.05$). Data expressed as mean ± SD (n=3 for each extract); means were compared by Bonferroni test. Extract with different lower case letters in the data (Table 4) are significantly different ($p<0.05$) while data with same identical lower case alphabets are have shown no significant difference ($p<0.05$).

Table 5: Total flavonoid content of different extracts of *Andrographis paniculata*

Sample	Total flavonoid content (mg of QE/g of sample)
Methanol	0.43 ± 0.00^{a}
Ethyl acetate	0.44 ± 0.01^{b}
50% Ethanol/water	0.43 ± 0.00^{a}
100% Water	0.43 ± 0.00^{a}

Recent evidences suggest that oxidative stress is one of the primary factors in the development of degenerative diseases and normal process of aging. (Sen, Chakraborty, Sridhar, Reddy, & De, 2010; Shirwaikar, Patel, Kamariya, Parmar, & Khan, 2011)Reactive nitrogen species (RNS) and reactive oxygen species (ROS), are generated during normal physiological processes which are regulated by enzymatic and non-enzymatic antioxidant mechanisms. At low concentration they play a vital role in energy production, cell growth, phagocytosis, synthesis of biologically important compounds, but over production can lead to oxidative stress.(Sen & Chakraborty, 2011; Valko et al., 2007). Flavonoids have been found to have therapeutic application against different diseases caused by oxidative stress(Sen, De, Devanna, & Chakraborty, 2013). Ethyl acetate extracts demonstrate the highest total flavonoid content while methanol, 50% ethanol/water and aqueous extract have similar or all most the same TFC from *Andrographis paniculata* plant (Table 5). The mean flavonoid content of *Andrographis paniculata* extracts is significantly different ($p<0.005$). The data expressed as mean ± SD (n=3 for each extract); means were compared by Benferroni test ($p<0.05$). However, the mean total flavonoid content of different extracts (Methanol, 50% ethanol/water and aqueous extract) of *Andrographis paniculata* indicated by the same lower case identical alphabets show no significantly different ($p<0.05$), while the mean flavonoid content of ethyl acetate extract is significantly different from the other three extracts ($p<0.05$), indicated by lower case b alphabet

CONCLUSION

Physiologically phenolic compounds are very important to the body especially polyphenols. The antioxidant activities of the four extracts (methanol, ethyl acetate, 50% ethanol/water and aqueous extracts)

of *Andrographis paniculata* were evaluated and the result of 50% ethanol: water extracts was shown to have the highest antioxidant activity compared with the other extracts (methanol, ethyl acetate and aqueous extracts). The DPPH scavenging radical activity of 50% ethanol/water extract was shown to have the highest IC_{50} (93.3µg/ml) as compared with the standard. Although the other extracts have no activity at IC_{50}, ethyl acetate extract has the lowest amount of TPC while aqueous extracts contain the highest amount of TPC. This indicates that determination of total phenolic compounds and antioxidant may not be reliable using a single method.

TPC and percentage inhibition obtained from this study are comparable to the report made by Rafat et al., 2010 on ethanolic extract of *Andrographis paniculata*, with high TPC obtained using 50% ethanol/water compared with ethanol. However, the low DPPH scavenging radical activity may be due to several factors such as higher temperature and different solubility of the compounds in ethanol and aqueous. Different solvents used may contribute to the low antioxidant activity due to different solubility profile. Thus, solvent may play a vital role in activity of some compounds present in plants.

A lot of studies have shown that different antioxidant capacity exist between different varieties of the same plant. (Rafat et al., 2010) reported antioxidant activity of different parts of *Andrographis paniculata*. Also (Kedage, Tilak, Dixit, Devasagayam, & Mhatre, 2007) reported antioxidant activity of eleven varieties of grapes. (Henríquez et al., 2010) reported the antioxidant activity of different chile apple while (Yuri, Neira, Quilodran, Motomura, & Palomo, 2009) reported the antioxidant activity of the peel and flesh part of chile apple. Also (ISMAIL et al., 2004) reported antioxidant and total phenolic content of some common vegetables. However, this investigation on *Andrographis paniculata* is aims at providing information on the effect of solvent system in evaluating TPC, TFC and antioxidant activity using four different solvent on indigenous plant from Kemaman, Terengganu State in Malaysia.

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