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PHYTOCHEMICAL PROFILING, ANTIOXIDANT ACTIVITIES AND ESSENTIAL OIL CONSTITUENTS OF *ANDROGRAPHIS PANICULATA*

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Abstact

Oxidative stress is involved in the pathogenesis of various diseases which lead to urgent need to investigate new, safe and effective source of antioxidant agents. This research proposed to investigate *in-vitro* and phytochemical constituent of the plant *Andrographis paniculata* using phytochemical analysis, GC/MS, DPPH, ABTS, FRAP and NO. Phytochemical analysis of *Andrographis paniculata* revealed the presence of tannins, total flavonoids, total phenol, total flavonols, and total proanthocyanidins. GC/MS analysis of essential oil of AP identified one major compound name benzenepropanoic acid elucited at 3.296 retention time and 0.74 area percentage. The ferric reducing potential of the extracts was concentration dependent and significantly different from that of rutin and vitamin E. The % inhibition of ABTS by the ethanol leaf extract of *Andrographis paniculata* was concentration dependent and compared favourably well with the rutin and vitamin E. In DPPH scavenging assays, the IC_{50} value of the ethanol leaf extract of *Andrographis paniculata* was < 0.025 mg/ml, while IC_{50} of rutin and Vitamin E were < 0.025 mg/ml and 0.08mg/ml. Nitric oxide IC_{50} for extract is 1.05mg/ml, Vitamin E is 1.2 mg/ml, and rutin is < 0.025 mg/ml. The present study showed high level of radical scavenging activity by ethanol leaf extract of *Andrographis paniculata* with higher antioxidant activities than Vitamin E but less than that of rutin. This show that *Andrographis paniculata* has antioxidant properties and the plant could be used in the prevention and treatment of diseases associated with oxidative stress.

Introduction

Most of the protective effects of plants on living cells have been attributed to their non-nutrient constituents such as carotenoids, flavonoids, isoflavonoids and phenolic acids (Badmus *et al.*, 2010). Different phytochemicals have been shown to possess a range of activities which help in protecting against lipid oxidation and by extension chronic conditions such as cancer, osteomyelitis or cardiovascular conditions (Masisi *et al.*, 2016; Ghosh *et al.*, 2016).

Antioxidants are substances that when present at low concentrations, compared to

those of the oxidisable substrate significantly delays or inhibits the oxidation of the substrate (Carocho and Ferreira, 2013). An important role of antioxidants is to suppress free radical-mediated oxidation by inhibiting the formation of free radicals by scavenging radicals. Radical scavenging action is dependent on both the reactivity and concentration of the antioxidant (Nimse and Pal, 2015).

Recently, there has been an increase interest in the therapeutic value of medicinal plants as antioxidants. Many forms of synthetic antioxidant products like butylated hydroxytoluene and tertiary butylated hydroxyquinone have been known to offer protection against oxidative stress, but they are often too costly and have been implicated in causing negative health effects such as radio-sensitization, increased toxicity of other chemicals, mutagenic activity, and tumor yield from chemical carcinogens (Mallawaarachchi *et al.*, 2015). Therefore, strong restrictions placed on their usage have prompted the need to search for naturally occurring antioxidants. Consequently, many plants have been investigated and found to contain varying amounts of antioxidant. Yet the need to find more information concerning the antioxidant potentials of plant species continues.

Andrographis paniculata Nees (Family: Acanthaceae) also known as 'Kalmegh' is part of Indian dietary component. It has been used in Indian and Chinese traditional medicine (Ojha *et al.*, 2012). This study is design to evaluate in-vitro antioxidant properties of *Andrographis paniculata* using FRAP, DPPH, ABTS and NO. Also to identify the phytocomponents of this plant and subjecting the plant leaves to Gas chromatography – Mass Spectrum analysis.

Mass spectrometry, coupled with chromatographic separations such as Gas chromatography (GC/MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for the analysis of non polar components and volatile essential oil, fatty acids, lipids (Sermakkani and Thangapandian, 2012).

Materials and methods

Plant collection and processing

The leaves of the plant *Andrographis paniculata* were collected from University of Ibadan Botanical Garden Ibadan, Ibadan North Local Government Area. The plants were identified and authenticated with voucher numbers: 2846 by herbarium curator, Department of Botany, University of Ibadan, Ibadan, Oyo State, Nigeria. The leaves of plant were cleaned with distilled water and air dried in a well ventilated shady room.

Extraction

The air-dried leaves of *Andrographis paniculata* were grinded and the grinded powder was extracted in cold ethanol in a screw-capped flask and shaken at room temperature. The solvent was filtered, squeezed off and evaporated off under reduced pressure in a rotatory evaporator at 40°C to obtain semi-solid crude extract

which was store at 4°C. The stored ethanol extract of *Andrographis paniculata* (EEAP) was then used for the studies.

Chemicals used

The following chemicals were used for the various experiments: 1,1diphenyl-2 picrylhydrazyl (DPPH), 2,22 -azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), vanillin, aluminium chloride ($AlCl_3$), potassium acetate (CH3CO2K), ferric chloride (FeCl2), BHT, ascorbic acid, rutin, Folin-Ciocalteu reagent, sodium carbonate (Na2CO3), phosphate buffer, potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), glacial acetic acid (CH3COOH), sodium nitroprusside (Na2[Fe(CN)5NO]2H2O). They were purchased from Merck, Gauteng, South Africa. All other chemicals used were of analytical grade.

Determination of total phenols

The amount of phenol in the whole leaf extracts of *A. paniculata* was determined spectrophotometrically using the modified method of Wolfe *et al.*, (2003) with Folin Ciocalteu reagent. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water at a concentration of 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 s and left to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of gallic acid equivalent using the calibration curve: Y=14.885x, $R2$=0.9961, where x is the absorbance and Y is the tannic acid equivalent.

Estimation of flavonoids

Total flavonoid content was determined using the method of Ordonez *et al.* (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of the sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Plant extracts were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as mg/g of quercetin using the following equation based on the calibration curve: Y= 11.922x, $R2$= 0.9955, where x is the absorbance and Y is the quercetin equivalent.

Total flavonols

Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran (2007). The reaction mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/l) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h at 20°C. Total flavonol content was calculated as mg/g of quercetin equivalent from the calibration curve using the equation: Y= 13.128 x, $R2$ = 0.9990, where x is the absorbance and Y is the quercetin equivalent.

Determination of total proanthocyanidins

The determination of total pro-anthocyanidins was based on the procedure of Oyedemi *et al.* (2010). To 0.5 ml of

mg/ml of the extract solution was added 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500 nm. Total pro-anthocyanidins content was evaluated at a concentration of 0.1 mg/ml and expressed as catechin equivalent (mg/g) using the calibration curve equation: $Y = 0.5825x$, $R2 = 0.9277$, where x is the absorbance and Y is the catechin equivalent.

Determination of tannins

Tannin determination was done according to the method of AOAC (1990) with some modifications. To 0.20 g of the sample was added 20 ml of 50% methanol. This was shaken thoroughly and placed in a water bath at 80°C for 1 h to ensure uniform mixing. The extract was filtered into a 100-ml volumetric flask, followed by the addition of 20 ml of distilled water, 2.5 ml of Folin-Denis reagent and 10 ml of 17% aq. Na2CO3 and was thoroughly mixed. The mixture was made up to 100 ml with distilled water, mixed and allowed to stand for 20 min. The bluish-green color developed at the end of the reaction mixture of different concentrations ranges from 0 to 10 ppm. The absorbance of the tannic acid standard solutions as well as sample was measured after color development at 760 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent using the calibration curve: $Y = 154.45x - 0.0485$, $R2 = 0.9585$, where x is the absorbance and Y is the tannic acid equivalent.

Antioxidant assay

The antioxidant activities of the whole leaf extracts of *A. paniculata* were determined using DPPH, ABTS, reducing power and nitric oxide.

Determination of ferric reducing power of the extracts

The reducing power of the whole leave extract of *A. paniculata* was evaluated according to the method described by Aiyegoro and Okoh (2010). The mixture containing 2.5ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of K 3Fe(CN)6 (1% w/v) was added to 1.0 ml of the extracts and standards (0.025–0.5 mg/ml) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 ml of TCA (10% w/v), which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was then measured at 700 nm against blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

DPPH radical scavenging assay

The method of Liyana-Pathiana and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical. DPPH (1 ml, 0.135 mM) prepared in methanol was mixed with 1.0 ml of ethanol extract ranging from 0.025 to 0.5 mg/ml. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The

scavenging ability of the plant extract was calculated using the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control}) \times 100.}$$

where, Abs control=the absorbance of DPPH +methanol, and

Abs sample=the absorbance of DPPH radical +sample (sample or standard).

ABTS radical scavenging activity

The method described by Adedapo *et al.* (2008) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 ml ABTS + solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min using a spectrophotometer. The percentage inhibition of ABTS+ by the extract was calculated from the following equation:

$$\% \text{ inhibition} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control}) \times 100.}$$

Nitric oxide scavenging activity

The modified method described by Oyedemi *et al.* (2010) was used to determine the nitric oxide radical scavenging activity of aqueous and other solvent extracts of *A. paniculata*. A volume of 2 ml of 10 mM of sodium nitroprusside prepared in 0.5 mM phosphate buffered saline (pH 7.4) was mixed with 0.5 ml of plant extracts, gallic acid and BHT individually at 0.025–0.5 mg/ml. The mixture was incubated at 25°C for 150 min. 0.5 ml of the incubated solution was mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic) acid at room temperature for 5 min with 1 ml of naphthylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min, followed by the measurement of the absorbance at 540 nm. The amount of nitric oxide radicals inhibited by the extract was calculated using the following equation:

$$\text{NO radical scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control}) \times 100,}$$

where Abs control= the absorbance of NO radicals + methanol, and
Abs sample = the absorbance of NO radical + extract or standard.

Hydro-distillation using Clevenger-type apparatus or extraction of volatile oils

About 100 g of the plant material was subjected to hydro-distillation using a Clevenger-type apparatus fitted with a condenser and connected to a heat-resistant 5-L round bottom flask. The plant material was heated in boiling water in the flask for about 3 hours, to produce a mixture of gases (oil vapor) which were conveyed with steam into the condenser, where they were cooled to below 30°C, producing two non-

mixing liquid phases: a lower hydrosol portion and the upper layer consisting of the essential oil. The condensed liquids were gravity fed into a separation funnel, where they were separated. The extracted oils were collected into small glass vials which were completely sealed before their analysis.

Gas chromatography-Mass spectrometry (GC-MS) analysis of volatile oils

The volatile oils were subjected to GC-MS analysis using an Agilent 7890 GC complex equipped with Agilent 5977A Mass selective detector system and a Zebron-5MS (cross-linked 5%- phenyl methyl polysiloxane) column (ZB-5MS 30 m X 0.25 mm X 0.25 µm). GC-grade helium was used as a carrier gas at a flow rate of 2 ml/min; splitless 1 µL injections were used. A needle with the samples (essential oils) was inserted directly into the inlet of the gas chromatograph. Injector temperature and ion source temperature were maintained at 280°C., while the initial oven temperature was 70°C. This was then ramped at 15°C/min to 120°C, then ramped at 10°C/min to 180°C and then ramped at 20°C/min to 270°C and finally held at this temperature for 3 min. The data obtained was gathered with ChemStation. Identification of the components of essential oils was accomplished by comparison of their retention times and mass spectra with those stored in NIST111 library.

Statistical analysis

All values were expressed as mean ± S.D. The test of significance between two groups was estimated by student's test. One- way ANOVA was performed using Graph Pad Prism version 4.00.

Results

Phytochemical constituents

Phytochemical analysis conducted on the ethanol leaf extract of *Andrographis paniculata* revealed the presence of tannins, total flavonoids, phenols, total flavonols, and total pro-anthocyanidins (table 1). The high contents of polyphenol observed in the ethanol leaf extract of *Andrographis paniculata* may be a contributing factor toward its antioxidant activity.

In vitro antioxidant activity of crude extract

The results of the antioxidant effects of ethanol leaf extract of *Andrographis paniculata* were as determined by the absorbance and scavenging percentage using FRAP, ABTS, DPPH, and NO inhibition.

Table I: Phytochemical analysis of ethanol solvent extracts of the leaf of *Andrographis paniculata*

Phytochemicals (mg/g)	Ethanol extract
Tannins	$1.33+0.14^{d}$
Total flavonoids	$6.71+1.82^{c}$
Total phenol	$13.77+1.22^{a}$
Total flavonols	$38.16+1.73^{c}$
Total proanthocyanidins	$46.35+8.58^{b}$

Data expressed as means +SD; n=3; Mean with the subscript; a=expressed as mg gallic acid/g of dry plant, b=expressed as mg catechin/g of dry plant materials, c=expressed as mg quercetin/g of dry plant materials, d=expressed as mg tannic acid/g of dry plant materials.

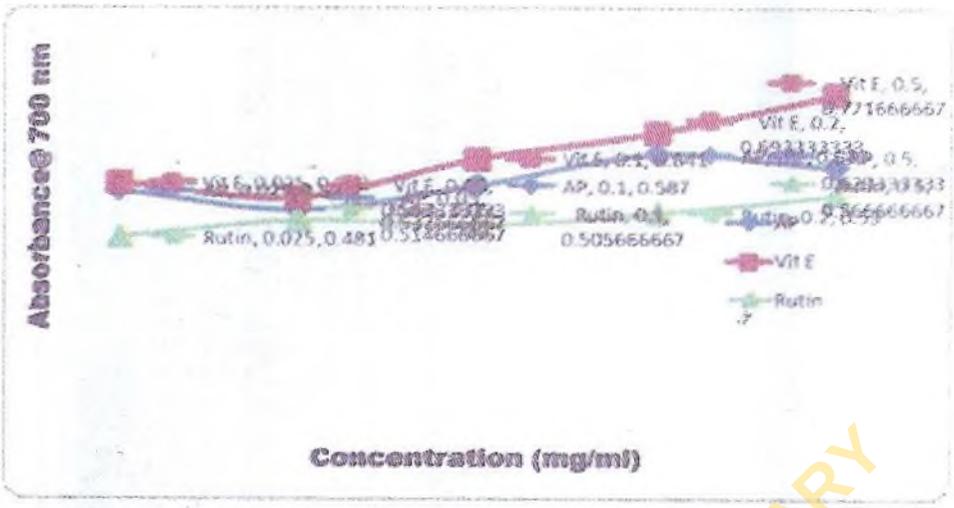

Figure 1: Reducing power of ethanol leaf extracts of *Andrographis paniculata*

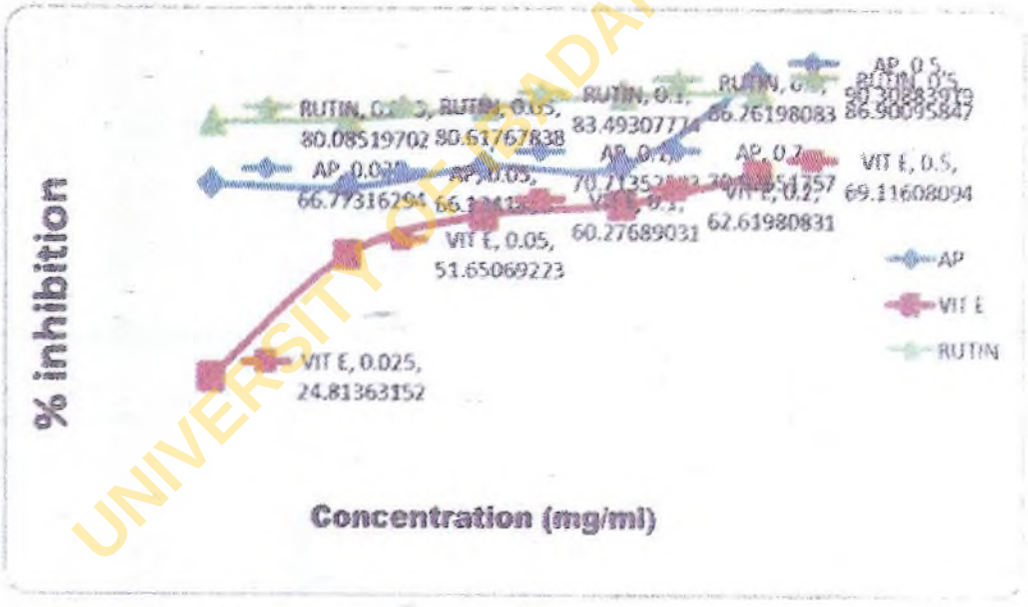

Figure 2: DPPH radical scavenging activity of ethanol leaf extracts of *Andrographis paniculata*

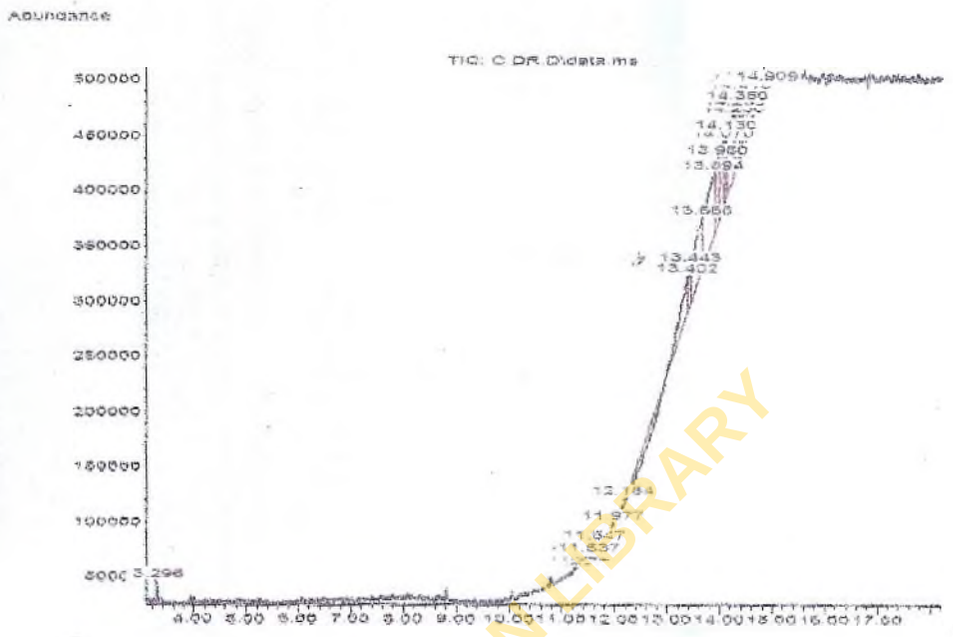

Figure 5: GC-MS chromatogram of essential oils of *Andrographis paniculata*

Ferric reducing antioxidant power (FRAP)

The potential of the plant extracts to reduce Fe3+ to Fe2+ by electron transfer is an indication of their antioxidant ability. Figure 1 shows the reducing power of the ethanol leaf extract of *Andrographis paniculata* in comparison with the standards (Vitamin E and Rutin). The ferric reducing activity of the extract was lower than that of vitamin E but higher than that of rutin the standard drugs used.

ABTS radical scavenging activity

Figure 2 shows the percentage inhibition of ABTS radical by ethanol leaf extract of *Andrographis paniculata*. The % inhibition of ABTS by the ethanol leaf extract of *Andrographis paniculata* was concentration dependent and compared favourably well with that of rutin and vitamin E used as standards in this study.

DPPH radical scavenging activity

Figure 3 shows the DPPH scavenging potential of the ethanol leaf extract of *Andrographis paniculata*. The IC_{50} value of ethanol leaf extract of *Andrographis paniculata* was <0.025 mg/ml, while that of Vitamin and rutin E are 0.08 mg/ml and <0.025 mg/ml respectfully.

Nitric oxide inhibition

activity (Sánchez-Moreno, 2002). In this study, the antioxidant ability of ethanol extracts of *Andrographis paniculata* was investigated, and *in vitro* radical scavenging activities were performed which showed promising results owing to the rich polyphenol profiles of *Andrographis paniculata*. Antioxidants activities were performed using DPPH radical scavenging activity, ABTS radical scavenging activity, NO radical scavenging activity and ferric reducing antioxidants power. The results showed that the extract significantly ($P< 0.05$) exhibited strong antioxidants activity compared to the standard (Rutin and Vitamin E) at the concentrations used.

The potential of the plant extracts to reduce Fe^{3+} to Fe^{2+} by electron transfer is an indication of their antioxidant ability (Gonçalves *et al*, 2013). The results of phytochemical of *Andrographis paniculata* revealed high content of polyphenolic compounds which are known to possess reducing ability and up-regulate antioxidant defenses.

DPPH activity of antioxidants may be due to their hydrogen-donating ability (Marín *et al*, 2016). The result shows that the extract had proton-donating potential and could act as free radical scavengers. Therefore, the extract could be used as therapeutic agent in free radical related diseases.

Nitric oxide is known as a mediator of some physiological process like inhibition of platelet aggregation and smooth muscle relaxation. Nitric oxide produce free radicals that act as a defense but excessive production of the free radicals could exacerbate inflammatory disease process (Jakubowska *et al*, 2016). The ability the extract of AP to significantly inhibit n oxide activity is a pointer to its free rad scavenging ability. The result from this st showed that Rutin exhibited the stron antioxidant activity, followed by that o extract thus indicating that this extrac stronger anti-oxidant activities than th vitamin E. These results support the u the plant therapeutically as we economically as antioxidant additiv nutritional supplements and should the be explored for novel antioxidant ager Wyk, 2008). The antiradical activity extracts could be related to the high c of polyphenols.

The GC-MS analysis of essent derived from *Andrographis pani* leaf by hydro distillation reveal presence of benzenepropanoic a *Andrographis panic* Benzenepropanoic acid is known t fatty acid content in the liver, plas also improves tissue insulin sensiti lahham *et al.*, 2010). It is also industries as fruits and feed a (MacFabe *et al.*, 2011).

Conclusion

This present study revea *Andrographis paniculata* posse antioxidant activities and this m in part to the phenolics prese extract. Therefore, *Andr paniculata* leaf is a potential go of natural antioxidant. This stud contributed to the validation of th potential of the extract of the *An paniculata* leaf.

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