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Research Article

In Vitro Antidiabetic, Anti-Inflammatory Effects of Methanolic Extract and Isolated Compound from *Andrographis paniculata* (Burm. F) Wall. Ex Nees from Kemaman, Malaysia

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ABSTRACT

Objective: This study investigated the activity of the extracts and the isolated compound on its potential *in vitro* antidiabetic, anti-inflammatory and potential anti-cancer effect, total flavonoid content against alpha-glucosidase enzyme inhibition and on macrophage respectively from *Andrographis paniculata* (Burm. F) wall. Ex Nees. Methodology: The isolation of the constituents was done using column while the *in vitro* anti-inflammatory and antidiabetic was done using nitric oxide and α-glucosidase enzyme inhibition assay while anticancer assessment was done performed using cell viability on various human hepatocellular carcinoma cell) and Chang liver (normal cell line) were determined by 3-(4,5-dimethylthiazolzyl-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Results: Although, the total flavonoid content was low, ethyl acetate extract indicated the highest total flavonoids content and it's statistically different from methanol, ethanol: water (1:1 v/v) and aqueous extracts. Ethyl acetate extracts exhibited the highest percentage inhibition (29.8 %) against nitric oxide scavenging activity ($NaNO_2$) compared to other extracts. Also, the isolated crystals showed a significant inhibition against $NaNO_2$. Moreover, the ethyl acetate extract showed the highest percentage inhibition of α-glucosidase enzyme with optimal concentration of 950µg/ml for 50 % inhibition (IC_{50}) while the other three extracts (methanol, 50 % ethanol: water (1:1 v/v) and aqueous) indicated activity below 50 % inhibition which might be due to total flavonoid content. The potential anti-cancer effect indicates that both the methanol extract and crystals (AP02 & AP03) may have the same compound. Conclusion: The compound isolated might be Andrographolide and the activity might be due to flavonoid content for the extract.

Keyword: *Andrographis paniculata*, anti-inflammatory, antidiabetic, and multiple therapeutic effects.

INTRODUCTION

A long history of natural products in ancient times and in folk medicine around the world, is the basis for the use of many therapeutic agents in modern day medicine[1]. The quest to find new therapeutic candidate compounds from natural biodiversity, particularly plants, has been the prime interest among researchers[2]. Furthermore, a growing worldwide interest in the use of phytopharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, has been noted in recent years[3]. It is believed that about 80% of world's population use plants as their primary source of medicinal agents[3-5].

Natural products have been used as a potential source of anti-inflammatory drugs. Endotoxins such as lipopolysaccharide-induced pro-inflammatory mediators and cytokines production contribute to the sequence of events in activated macrophage cells, which lead to the kill of microorganisms and causing tissue damage during the inflammatory process[6]. Overproduction of nitric oxide (NO) has been implicated in the pathogenesis of inflammation via inducible nitric oxide synthase (iNOS), which reflects the degree of inflammation process[7]. The liver is the largest internal organ in vertebrates, including humans. It is vital to survival, as it is the principal organ of metabolism and excretion, and support every other organ[8]. Due to continuous exposure to xenobiotics,

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chemicals, it is prone to many diseases including cancer[8]. Cancer is one of the most common devastating diseases affecting millions of people per year. Cancer has been estimated as the second leading cause of death in humans. There has been an intense search of anti-cancer drugs from various biological sources[9].

Insulin is a hormone produced in the pancreas that allows glucose from the food to enter the body's cells and convert into energy needed by the muscles and tissues for proper functioning. In a diabetic person, there is in proper absorption of glucose that remains circulating in the blood a condition called hyperglycaemia, which causes damage to the body tissues and muscles over time. This damage can lead to disability and life-threatening health complications for the patients[10]. Many natural products or their extracts have claimed to have multiple therapeutic benefits against life-threatening diseases.

Andrographis paniculata (Burm.F) wall. Ex Nees belonging to the family Acanthaceae or Kalmegh, commonly known as "king of bitters" and in Malaysia called "Hempedu bumi"[1]. It is widely distributed throughout tropical Asian countries. Is extensively used in Ayurveda, Unani and Siddha medicines as a home remedy for various diseases in India[1]. 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[11]. The plant extracts are known to contain diterpene, and flavonoids that have been reported to exhibit many pharmacological benefits. The present study involved investigating the multiple therapeutic benefit of *A. paniculata* extracts and isolated compound via *in vitro* antidiabetic effect, nitric oxide scavenging activity (antioxidant) and anti-cancer potential against alpha-glucosidase enzyme, macrophage and hepatoma (HepG2) cells respectively.

MATERIALS AND MATHODS

Sodium nitric oxide ($NaNO_2$) and Griess reagent were obtained from Merck Ltd. (Germany), Macrophage (ATCC; Manassas, VA USA), HepG2, Chang liver (ATCC; Manassas, VA USA), T75 flask (Costar), 10% fetal bovine serum (Gibco USA), Penicillin and Streptomycin (Nacalai-Tesque, Japan).

Collection of Plant Material

paniculata plant was obtained in September from Kemaman in Terengganu, Malaysia. The plant was identified, authenticated by Norhaslinda Haron from Faculty of Agriculture and Animal Sciences, Universiti Sultan Zainal Abidin, and deposited at the herbarium unit with specimen voucher number 00266.

Preparation of Plant Extracts

The plant was washed with distilled water, dried at 40ºC and then grinded into powdered form. The powdered plant was weighed and soaked in different solvents (methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous) in a ratio of 1:10 respectively. The extracts were then decanted and filtered through filter paper. The filtrate obtained was concentrated using rotary evaporator at temperature 40ºC. Extracts were then dried at 50ºC and kept in a freezer at 4ºC untill use.

Total Flavonoid Content (TFC)

A method was employed with little modification to determine the TFC[12]. A 0.25ml of the diluted extracts of 250µg/ml concentration were mixed with solution containing 50 µl of 1 M aqueous potassium acetate, 50 µl of 10 (%w/v) aluminium chloride and 2.15 ml of 95% ethanol. The mixtures were mixed and incubated at room temperature in a dark for 40min, and the absorbance (*A*) was recorded spectrophotometrically at 415nm. The samples were assayed in triplicate. TFC was calculated using quercetin as standard and expressed as mg per gram from the calibration curve.

Nitric Oxide Scavenging Assay

The nitric oxide (NO) scavenging activity of the crystals were measured with little modification[10]. This method involved addition of 50µl of the sample with 50µl of 10 mM sodium nitroprusside solution into a 96-well flat-bottomed flask and the flask was incubated under light at room temperature for 90 min. Finally, an equal volume of Griess reagent (1% of sulphanilamide and 0.1% of napthylethylenedlamine in 2.5 % HPO_3) was added into each well to measure the nitrite content immediately at 546 nm. L-ascorbic acid was evaluated as a reference standard. All experiments were carried out in triplicate. The percentage scavenging activity was calculated

% scavenging activity = [1- (sample absorbance/control absorbance)] x 100

Cell line and Culture

HepG2 (human hepatocellular carcinoma cell) and Chang liver (normal cell line) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were in a sterile T75 flask (Costar) containing, D-MEME and RPMI-1640 media (Gibco, USA) respectively, supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 units/ml penicillin and streptomycin) (Nacalai-Tesque, Japan) in 5% CO_2 humidified air at 37ºC. Exponentially growing cells were used for all the experiments according to the method[13] with little modifications.

Assessment of Cell Viability

The assessment of cell viability on various human hepatocellular carcinoma cell) and Chang liver (normal cell line) were determined by 3-(4,5-dimethylthiazolzyl-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, human cell lines were seeded in 96-well microplates at a concentration of $1.0x10^5$ cells/well. After incubation for 24 hrs, the treatment of methanol extract, AP02, APO3 crystals and hydrogen peroxide (Sigma, USA) were added to 100 µg/ml followed by 2-folds dilutions. The treatment plate was prolong incubated for 72hrs. 20.µl of MTT solution (5mg/ml, Nacai-Tesque, Japan) was added to each well and was incubated at 37ºC for 4 hrs. All medium was removed from each well and replaced with 100 µl dimethylsulfoxide (DMSO) (Sigma, USA). The solubilized formazan produced by metabolically active cells were measured by scanning the 96-well plates at 570 nm with reference at 630 nm wavelength (Infinite M200, Tecan Switzerland). The IC_{50} (50% inhibition concentration) values were determined as the concentration of the crystals to result in 50% growth

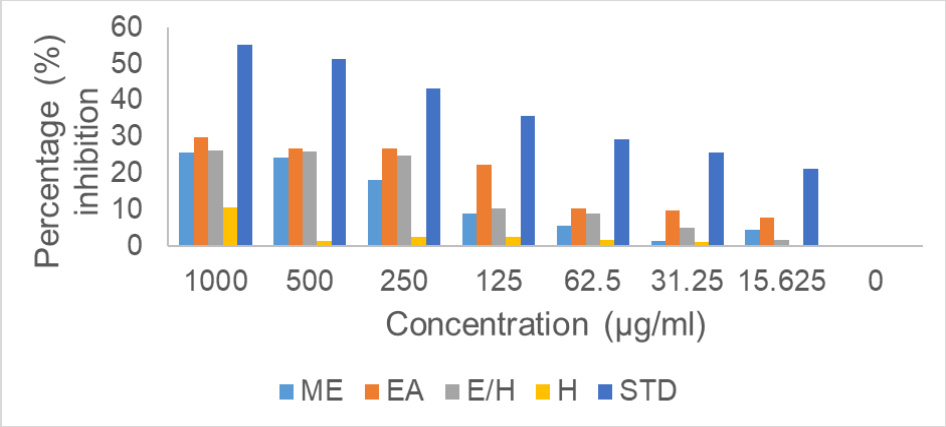

Figure 1: Percentage Inhibition of $Nano_2$ By *A. Paniculata* Extracts Upon Nitric Oxide Inhibition.
Key terms: ME = methanol, EA = ethyl acetate, E/H = ethanol: water (1:1 v/v), H = aqueous and STD = standard sample.

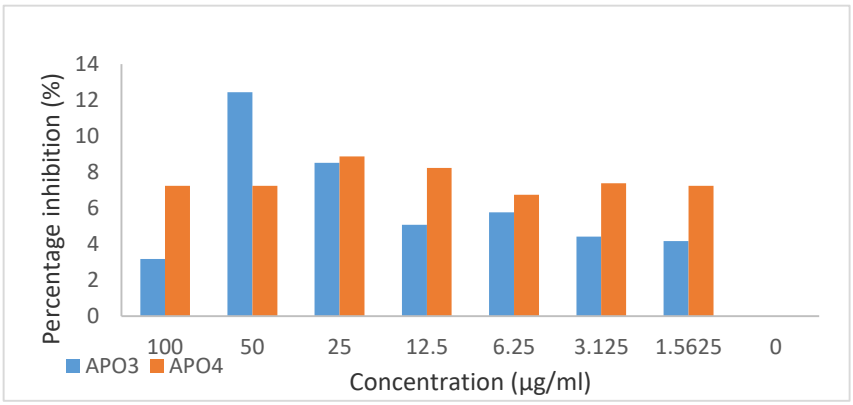

Figure 2: Percentage Inhibition Rate of $NaNO_2$ By Crystals Obtained From *A. Paniculata* (AP03 And AP04) Respectively**.**

Table 1: Total flavonoid content of methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous extracts of *A. paniculata*.

Sample	Total flavonoid content (mg of QE/g of extract)
Methanol	0.43 ± 0.00^a
Ethyl acetate	0.44 ± 0.01^b
Ethanol: water (1:1 v/v)	0.43 ± 0.00^a
Aqueous	0.43 ± 0.00^a

inhibition of the cancerous cells. Data expressed as mean±SE of three experiments.

In Vitro Alpha-Glucosidase (A-Glucosidase) Inhibitory Assay

A method was adopted to test the antidiabetic effect of *A. paniculata* by assessing the α-glucosidase enzyme percentage inhibition[14]. Using Acarbose as standard (positive control), appropriate dilution of the extracts and 25 µl of α-glucosidase in 0.1 M phosphate buffer (pH 7), was incubated at 37ºC for 10 minutes. Then, 25 µl of 5mM p-nitrophenyl-α-glucopyranoside solution (*p*NPG) in 0.1 M phosphate buffer (pH 7) was added. The mixtures were incubated at 37ºC for 30 min, after which 100 µl of 0.2 M $NaCO_3$ was added to terminate the reactions. The experiment was done in a dark due to the sensitivity of the enzymes and sample (standard) to light, and the plates were covered with tissue paper. Absorbance (*A*) was read at 410 nm in the Elisa Microplate reader and the α-glucosidase inhibitory activity was expressed as percentage inhibition

RESULTS AND DISCUSSION

The highest inhibition (%) was shown by ethyl acetate extract followed by ethanol: water (1:1 v/v), methanol and aqueous extracts (29.8, 26, 18, and 10.7%) respectively as compared to standard 35.8 %. TFC screening indicate that ethyl acetate extract has the highest TFC, this might be attributed to its highest percentage inhibition against $NaNO_2$.

Nitric oxide (NO) is one of an essential bioregulatory molecule required for several physiological conditions like natural signal transmission, cardiovascular dilatation, immune response and blood pressure. Despite these possible beneficial effects of NO on the human body system, its contribution to oxidative damage is rapidly becoming evident. This might be due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potential strong oxidant that can decompose to produce OH and NO_2. Thus, the NO released from

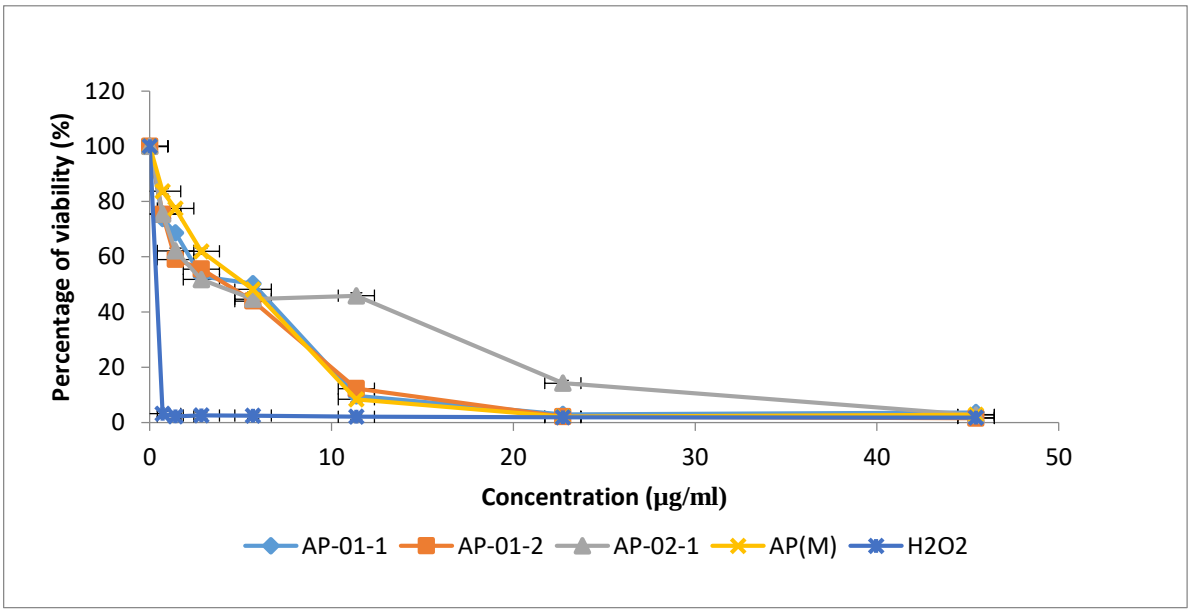

Figure 3: MTT assay for *A. paniculata* crystals on HepG2 cell line.

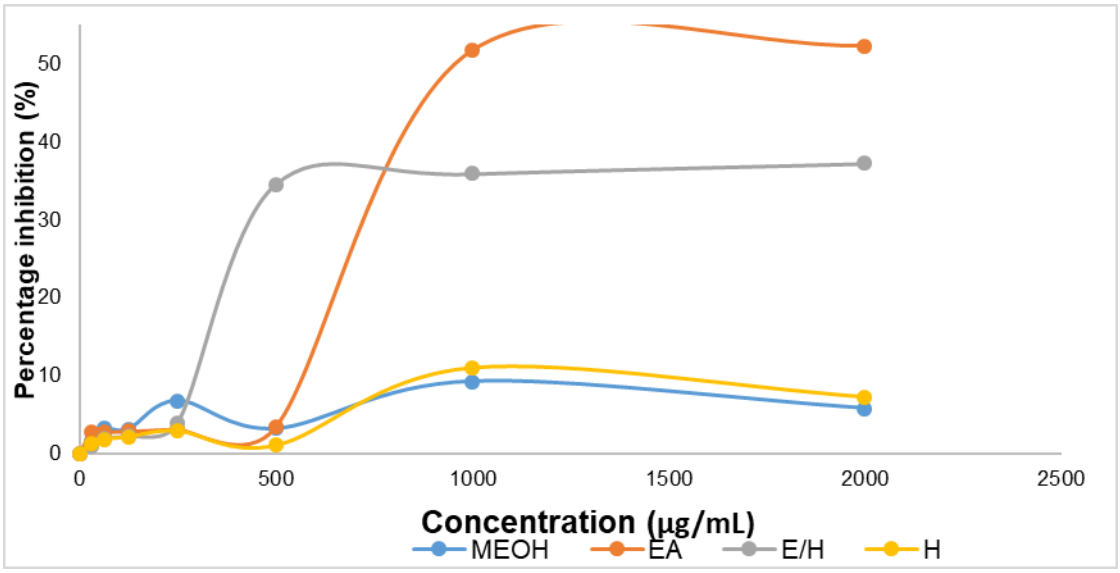

Figure 4: Alpha-glucosidase inhibitory assay of *A. paniculata* extracts.

sodium nitroprusside (SNP) has a high NO+ character which can alter the structure and function of many cellular components[10,15]. Therefore, the highest percentage inhibition was shown by ethyl acetate extract followed by ethanol: water (1:1 v/v), methanol and aqueous extracts (29.8, 26, 18, and 10.7%) compared to standard 35.8 % respectively (Figure 1). Therefore, due to its total flavonoid contents. However, the crystals APO3 also showed a significant inhibition of nitric oxide (12.4%) production (Figure 2) indicating its strong antioxidant effects and may have the substantial beneficial anti-inflammatory condition. Therefore, APO3 crystal may have an anti-inflammatory effect.

Flavonoids have been found to have therapeutic application against different diseases caused by oxidative stress[16]. This present study indicates that ethyl acetate extracts showed the highest total flavonoid content while methanol, ethanol: water (1:1 v/v) and aqueous extracts have similar TFC (Table 4). The mean flavonoid content of *A. paniculata* extracts was significantly different ($p<0.005$). The data expressed as mean ± SD (n=3 for each extract); means were compared by Bonferroni test ($p<0.05$). However, the average total flavonoid content of methanol, ethanol: water (1:1 v/v) and aqueous extracts of *A. 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. TFC of the extracts was determined using the calibration curve equation (y=216 X-23.294 with R^2=0.9934) of Gallic acid and presented as gallic acid equivalents (GAE) in mg per gram of dried weight of the extract.

The potential anticancer properties of the crystals, MTT assay, was performed on HepG2 cells (human hepatocellular carcinoma cell) and compared it with Chang liver (normal cell line). Methanol extract, AP02 and AP03 crystals were found to be significantly active (IC_{50} approximately 4–8 µg/ml). This indicates that both the methanol extract and crystals (AP02 & AP03) may have the same compound. The percentage cell viability which may indicate either same compounds or mixed compounds.

Inhibition of α-glucosidase by different classes of phenolic and flavonoid compounds have been reported in many literatures[17-19]. Therefore, the total flavonoid content (statistically different from other extracts at $p<0.005$) of ethyl acetate extract might be responsible for the optimal concentration required for the 50% inhibition (IC_{50}) against the alpha-glucosidase and showed the highest percentage inhibition (51.8%) with optimal concentration of 950 µg/ml required for the 50% inhibition (IC_{50}) of alpha-glucosidase enzyme. However, the other three extracts (methanol, ethanol: water (1:1 v/v) and aqueous) have activity below the optimal concentration required for the 50% inhibition rate, this also corresponds to their total flavonoid contents, thus signifying the effects of total flavonoid content from *A. paniculata* to exhibit different pharmacological effect.

CONCLUSION

In summary, it could be concluded that *A. paniculata* bear a potential anti-inflammatory, antidiabetic and possible anti-cancer effect. Its constituents exhibit different pharmacological effect through α-glucosidase inhibition, nitric oxide inhibition properties. Preliminary chemical examination indicated the presence of polyphenols and flavonoids, which the total flavonoid content may be responsible for anti-oxidant (anti-inflammatory) and antidiabetic (α-glucosidase inhibitory) activities. Further studies on the isolation of the active constituent(s) along with the animal studies in vivo to be investigated in detail to explore its pharmaceutical/nutraceutical potential for the development of herbal medicinal extracts and products.

CONFLICT OF INTEREST

Authors declared no conflict of interest

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