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Synergistic Antidiabetic Activity of Extracts of *Asystasia gangetica* and *Morus alba*

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ABSTRACT

Diabetes mellitus, with cases globally rising at an alarming rate, has been the focus of several types of medical research and oxidative damage has been linked to its development. From the foregoing, *Asystasia gangetica* (L.) T. Anderson of Acanthaceae family, whose ethanol extract has been proven to possess antioxidant and antidiabetic properties was subjected to reflux extraction using water, methanol, ethanol, acetone, ethyl acetate, dichloromethane, and hexane and 20% v/v yeast cell treatment to determine its effect on the glucose uptake of yeast cells at different concentrations (1, 2, and 5 mg/mL) and varying amount of glucose (2, 4, and 10 mg/mL). The most promising extract, *A. gangetica* DCM extract, was also combined with Methanol extract of *Morus alba* (L.) leaves of Moraceae family, which has also been found to possess antidiabetic property, to assess possible synergistic effect. The results showed that 5 mg/mL of *A. gangetica* dichloromethane extract have a significant and highest increase in glucose uptake, 44.46 ± 1.64% ($p < 0.05$), at 0.5 mg glucose as compared to that of metformin: 30.24 ± 2.52%. Upon analyzing the antidiabetic effect of the combined extracts, the most favorable increase in glucose uptake was observed at the combination ratio of 8:2 at 5 mg/mL and 0.5 mg glucose, setting a record of 72.78 ± 0.62% ($p < 0.05$) increase as opposed to 30.24 ± 2.52% increase in with metformin. These findings confirm the ability of *A. gangetica* to act as an antidiabetic agent either alone or in combination with *M. alba*.

Keywords: Antidiabetic activity, *Asystasia gangetica*, Glucose uptake, *Morus alba*, Yeast cells.

Introduction

Natural products research significantly involves the exploration of active components present in biological sources. Another important aspect in the study of natural products that plays a major role is often the next stage after the chemical constituent analysis, which is the determination of pharmacological applications of these constituents where bioassay techniques are applied. Bioassay techniques must be simple, rapid, reproducible, and comparable.[1]

In choosing which pharmacological property to be assessed, it is important to understand which diseases are in dire need of attention and treatment, how such diseases occur, and how they may be treated. One of the most widely studied causes of a disease is the excessive oxidation that occurs in the human body. Oxidation is a natural reaction in the body, but an imbalance between the supply of reactive oxygen species (ROS) and free radicals and the availability of antioxidants leads to the development of oxidative stress, leading to damaged tissues and cells.[2,3] Studies have linked oxidative damage to the development of several diseases including diabetes mellitus and cancer. It has been observed that enzymatic systems, lipid peroxidation, and glutathione metabolism are altered in different experimentations for diabetes.[4-5] Diabetes also causes many changes in the body, including inflammation that makes the body susceptible to the evolution of cancer.[6] With the level of cases of diabetes rising at an alarming rate,[7] the attempt to find cure and treatment for diabetes continue to be the focus of medical research.

Asystasia gangetica (L.) T. Anderson, also referred to as Chinese Violet [8], is a weed that is part of the Acanthaceae family and is known to exhibit an invasive nature because of its ability to produce a large quantity of seeds and carry out rapid germination. Despite its invasive nature, *A. gangetica* has been used as a cover crop and has been found to help in pest control because of the ability of its flowers and trichome to attract parasitoid caterpillar bag, consequently protecting the valuable crops. Aside from these uses, *A. gangetica* possesses biological properties which it owes to its active components.[9,10] Pharmacological studies on *A. gangetica* revealed its antioxidant, anti-asthmatic, and antidiabetic properties.[11-15] This means that *A. gangetica* may be a potential source of promising natural products and may have a wide variety of applications in relation to the medical field. However, studies on the plant are relatively limited, hence our study aims to obtain crude extracts of *A. gangetica* from different solvents via reflux extraction to explore the antidiabetic activity each extract exhibits and to determine which among them has the most potent ability to become an antidiabetic agent. In addition to the above, another goal of this study will be to ascertain the synergism between the most promising *A. gangetica* extract and the methanol extract of *Morus alba* (L.) leaves of Moraceae family, which has been reported to exhibit antioxidant and antidiabetic activities and whose methanol extract has been proven to contain chemical compounds that contribute to its antioxidant property.[16,17] Ultimately, all of these are geared towards providing a new direction for natural products research.

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Materials and Methods

Materials, Sample Preparation, and Extraction

A. gangetica whole plants were collected in September, 2019 in Chang Jung Christian University, Tainan, Taiwan, while *M. alba* leaves were purchased from a local herbal store. The identities of both plants were authenticated by Dr. Chia-Jung Lee, Ph.D. Program in Clinical Drug Development of Herbal Medicine, College of Pharmacy, Taipei Medical University. Voucher specimens were deposited as #CJCU-AG-001 and #CJCU-MA-001 for *A. gangetica* and *M. alba*, respectively, at the Department of Medical Sciences Industry in Chang Jung Christian University, Taiwan. All chemical reagents used in the experiments were bought from Sigma-Aldrich.

Sample Preparation and Extraction

A. gangetica whole plants were oven-dried at 40 °C and were powdered using a blender after three days. Approximately 50 g of the ground *A. gangetica* was weighed and extracted through reflux using seven different solvents, which are deionized and distilled water ($DD\ H_2O$), methanol (MeOH), ethanol (EtOH), acetone, ethyl acetate (EA), dichloromethane (DCM), and hexane (2 hrs, 65°C, 1 L), while *M. alba* leaves were extracted using 50% MeOH in water [16]. Decoction was also performed on *A. gangetica* using 4 L of $DD\ H_2O$ to obtain an aqueous extract. The *A. gangetica* aqueous, MeOH, EtOH, acetone, EA, DCM, and hexane crude extracts and the 50% MeOH *M. alba* crude extract were collected via vacuum filtration and the solvents were removed from the filtrate using rotary evaporation in preparation for freeze-drying. The *A. gangetica* samples were then prepared in a microcentrifuge tube by dissolving 10 mg of each of the dried extracts in 1 mL of ethanol, except for the aqueous extract, which was prepared using distilled and deionized water. The combined *A.gangetica* and *M. alba* extracts were prepared by mixing 50% MeOH *M. alba* extract (MA) dissolved in EtOH (10 mg/ml) to the previously prepared *A. gangetica* DCM extract (AG) with 0 to 100 percent volume to give a total of 11 solutions: 100% AG, 90% AG-10% MA, 80% AG-20% MA, 70% AG-30% MA, 60% AG-40% MA, 50% AG-50% MA, 40% AG-60% MA, 30% AG-70% MA, 20% AG-80% MA, 10% AG-90% MA, and 100% MA. The sample solutions prepared were kept in the freezer at -20 °C.

Glucose Uptake by Yeast Cells

The antidiabetic property of *A. gangetica* aqueous, MeOH, EtOH, acetone, EA, DCM, and hexane extracts was determined by using a modified method described by Vani *et al.* (2018) and originally designed by Cirillo *et al.* (1963) [18-19], which analyzes the increase in the glucose uptake by yeast cells. Approximately 4 g of yeast cells were soaked in 200 mL of ddH_2O in a beaker and was set aside for 24 hours. After the soaking period, the mixture was centrifugated at 1000 rpm for 5 mins and washed five times. A final 20 v/v% yeast suspension in water was obtained. For the treatment of the cells, *A. gangetica* extracts with concentrations 1, 2, and 5 mg/ml were first prepared along with glucose solutions with concentrations 2, 4, and 10 mg/ml from a 1 mg/ml glucose stock solution. A mixture of 625 µL of the yeast suspension and 1 mL of each extract solution was done in triplicates. Extract solutions were replaced with distilled water for mixtures that served as the control group. The mixtures were incubated at 37 ºC for 10 mins then 250 µL of the glucose solutions were added. Incubation was repeated for an hour before proceeding with the 3,5-dinitrosalicylic acid (DNS) analysis. A DNS reagent was prepared by combining a mixture of 20 mL of 2 N NaOH and 1 g of DNS in 50 mL of $DD\ H_2O$ with 30 g of KNa tartrate dissolved in 20 mL of $DD\ H_2O$, diluting it to 100 mL and heating it at 95 ºC water bath. This reagent was added to the solutions of treated yeast cells to obtain a mixture containing 60% analyte and 40% DNS solution. Both reagent and method blanks were also made. The reagent blank contained 600 µL of ddH_2O and 400 µL of DNS, while the method blank contained 750 µL of ddH_2O, 100 µL of sample extracts, and an appropriate amount of DNS solution to achieve the 60:40 analyte to DNS volume ratio. All solutions were subjected to a boiling water bath for five minutes. After cooling down, a volume of 250 µL of the solution was transferred to the wells in triplicates and absorption reading was carried out at 540 nm. The same procedures were also applied to the 11 combined solutions of AG and MA mentioned in the previous section with concentrations 1, 2, and 5 mg/ml. To understand the significance of the results, a commercially available antidiabetic drug, metformin, was used as a reference drug and underwent the same glucose uptake assay as done with the samples, with concentrations 1, 2, and 5 mg/mL. The percentage increase in glucose uptake was used to express the findings and was calculated using the following equation:

$$\%\ increase = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100\%$$

Statistical Analysis

The calculated percentage increase in the glucose uptake of yeast that underwent *A. gangetica* aqueous, MeOH, EtOH, acetone, EA, DCM, and hexane extract treatment, as well as the yeast suspension that was treated with combined AG and MA extracts, were presented as mean ± SEM. These values were then compared against that of the yeast treated with metformin using a two-tailed test. Data values with $p < 0.05$ were considered significant.

Results and Discussion

Diabetes mellitus is characterized by chronic hyperglycemia either caused by a failure in the production of insulin or the action of the hormone. As a result, it becomes difficult for glucose molecules to enter the cells from the blood vessels, which leads to several other complications. In 2015, a study claimed that far too many people have been diagnosed with metabolic disease to a point that it is almost an epidemic [7]. Because of its alarming progression, studies that focus on finding cure and treatment for diabetes are continuously being performed and in fact, several methods have been made available. Obesity, along with many other factors, is also being examined as such factors play a major role in the development of diabetes [20]. In this study, the transport of glucose molecules into yeast cells is observed for different extracts of *A. gangetica* and in combination with *M. alba* 50% MeOH extract at different concentrations (1, 2, and 5 mg/mL). This glucose transport into yeast cells by the sample extracts was compared with that of a commercially available drug known as metformin, which served as the standard in assessing the potential of *A. gangetica* extracts and of combined AG-MA extracts in exhibiting antidiabetic activity. The cell treatment was carried out at varying glucose concentrations: 2, 4, and 10 mg/mL.

Antidiabetic Activity of A. gangetica

Seven different extracts of *A. gangetica* were used to study the plant's antidiabetic activity. Table 1 displays the effect of metformin in the glucose uptake of yeast cells in terms of percentage increase as well as the percentage increase in glucose uptake of yeast cells after treatment using the extracts of *A. gangetica* at different concentrations. For metformin, the effect in glucose uptake is observed to be increasing in a dose-dependent manner. It is also seen that the drug is relatively more effective at lower loads of glucose, 2 and 4 mg/mL, than at 10 mg/mL. At 1 mg/mL of metformin, the percent increase was recorded to be as low as 6.54% (at 10 mg/mL glucose load), while at 5 mg/mL, regardless of the glucose concentration, percent increase values were all above 20% and were as high as 30.24% (at 2 mg/mL glucose load). For *A. gangetica* extracts, only acetone, EA, DCM, and hexane extracts manifested an effect on the glucose uptake of yeast cells. No coherent data were obtained from the other solvent extracts. Similar to the observed trend in the study using metformin, the transport of glucose into yeast cells generally occurs more easily at higher concentrations of *A. gangetica* extracts and a lower amount of glucose. In fact, at 1 mg/mL extract concentration and 10 mg/mL glucose load, an increase in the uptake was no longer detected in any of the extracts. This observation, however, is not exactly applicable and consistent for all extracts. For example, in the case of acetone extract, no increase is recorded at its highest concentration (5 mg/mL) at the lowest glucose load concentration (2 mg/mL). The same inconsistency is noted for

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EA extract at 5 mg/mL, at 4 mg/mL glucose load. It is possible that the extracts induced cytotoxicity against the yeast cells, which caused the variations in the results. It was also observed that only the less polar extracts had a significant effect in increasing the glucose uptake, with 44.46 ± 1.646% ($p < 0.05$) increase by the DCM extract being the highest overall at 5 mg/mL with a glucose concentration of 2 mg/mL followed by 31.96% ± 1.455% ($p < 0.05$) for the hexane extract at the same concentration with a glucose concentration of 4 mg/mL. These findings indicate that both DCM and hexane extracts are more capable of allowing glucose to enter the yeast cells than the reference drug at the specified concentrations and that they can be potentially used in addressing diabetes through this mechanism. Figures 1 and 2 display visual representations of the increase using the reference compound and using the extracts of *A. gangetica* at 2 and 4 mg/mL glucose concentration loads, respectively.

Antidiabetic Activity of combined extracts

Because *A. gangetica* DCM extract produced the highest increase of glucose uptake overall, it was used in combination with *M. alba* methanol extract to observe whether a synergistic effect would be seen with final concentrations of 2 and 5 mg/mL. The findings for this part in Table 2 revealed that the combined extracts with a lower amount of *A. gangetica* extract, from ratio 4:6 to 0:10, did not exhibit an effect in the glucose uptake by yeast cells. However, a volume ratio of 8 AG:2 MA exhibited the most favorable percent increase (72.78 ± 0.62%; $p < 0.05$) in glucose uptake. This result is found to be significantly better than metformin and even than the aforementioned *A. gangetica* extracts.

It was noted that antidiabetic property was observed only at higher concentrations of *A. gangetica* extracts and for the combination study. A possible reason for this may be ascribed to the cytotoxic effect of these extracts, another possible factor may have been the difference in solubility of the solvents used. It is important to note that *A. gangetica* DCM extract used in the combination is a non-polar extract, as opposed to the methanol extract of *M. alba*, which is a polar extract. Despite these differences, the 72.78% increase in glucose uptake is a remarkable result that portrays synergism between the extracts of two plant materials. Given that being able to increase the passage of glucose molecules through the cells is vital in the treatment of diabetes mellitus, this combined extract of 8 AG:2 MA becomes another potential source of solution in solving the rising cases of diabetes. In addition, these findings give an emphasis to the importance of synergism as a factor that should be taken into consideration.

Table 1: Percent increase in the glucose uptake of yeast cells by a reference drug and by *A. gangetica* extracts

Glucose (mg/mL)	2			4			10		
Reference/ Extract(mg/ml)	1	2	5	1	2	5	1	2	5
Metformin	12.82 ± 1.04	15.95 ± 1.51	30.24 ± 2.52	14.80 ± 1.45	19.29 ± 1.17	25.24 ± 1.72	6.54 ± 0.63	11.65 ± 0.63	21.91 ± 1.87
H_2O	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MeOH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
EtOH	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acetone	N.D.	5.31 ± 0.24	N.D.	N.D.	N.D.	9.11 ± 0.45	N.D.	N.D.	N.D.
EA	N.D.	13.94 ± 0.12	6.57 ± 0.33	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DCM	N.D.	25.97 ± 0.26	44.46 ± 1.64	N.D.	N.D.	14.30 ± 0. 37	N.D.	N.D.	N.D.
Hexane	N.D.	4.97 ± 0.12	30.25 ± 0.53	N.D.	N.D.	31.96 ± 1.46	N.D.	N.D.	N.D.

*N.D. = not detected

Table 2: Percent increase in the glucose uptake of yeast cells by combined *A. gangetica* DCM and 50% MeOH *M. alba* extracts

Glucose (mg/mL)	**2**		**4**		**10**	
Extract ratio (AG:MA)	**2**	**5**	**2**	**5**	**2**	**5**
9:1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8:2	N.D.	72.78 ± 0.63	N.D.	ND	N.D.	N.D.
7:3	N.D.	11.50 ± 0.41	N.D.	N.D.	N.D.	N.D.
6:4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5:5	N.D.	25.63 ± 1.53	N.D.	24.66 ± 1.09	N.D.	N.D.
4:6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
3:7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2:1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1:9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10:0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

*N.D. = not detected

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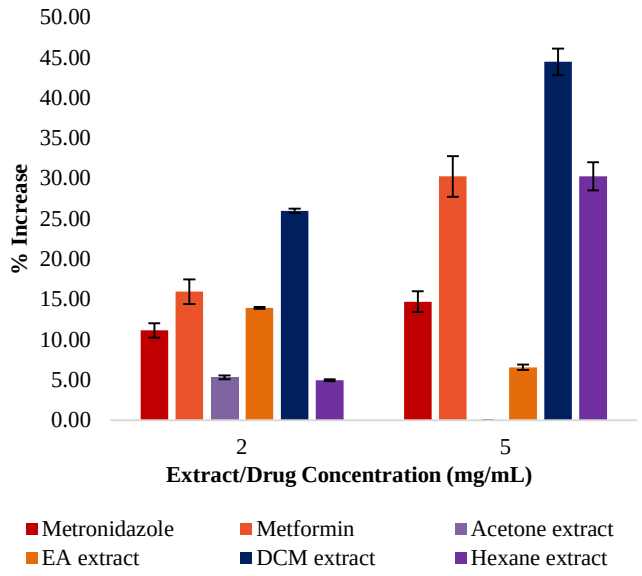

Figure 1: Percent increase uptake of *A. gangetica* DCM extract against other extracts at 2 mg/mL glucose load

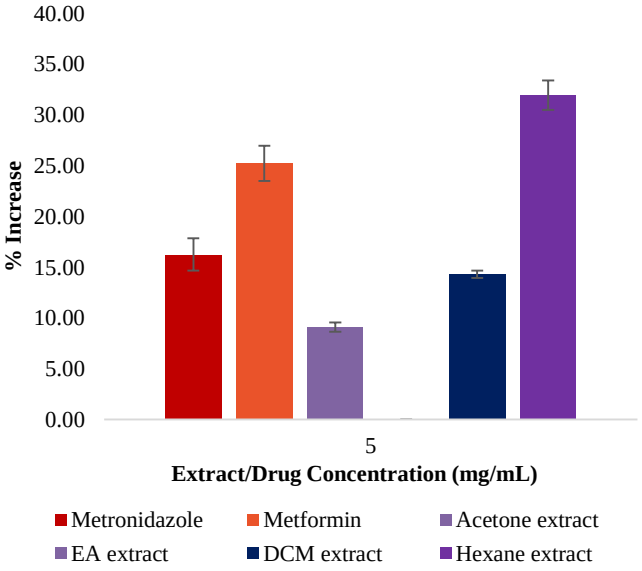

Figure 2: Percent increase uptake of *A. gangetica* hexane extract against other extracts at 4 mg/mL glucose load

Conclusion

Findings from this study showed that *A. gangetica* DCM extract had the highest percentage increase in glucose uptake overall, which was higher compared to the reference drugs metronidazole and metformin. The combined extract with a ratio of 8:2 of *A. gangetica* extract to *M. alba* extract resulted in a percent increase equal to 72.78%, which is a manifestation of the synergistic effect between the two plant materials. In conclusion, *A. gangetica* is a potential antidiabetic agent with a synergistic effect with *M. alba* extract. Further studies on both plant materials are recommended as more opportunities in natural products research exist for both plants.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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