CHARACTERIZATION OF ALTERNATIVE ANTI-DIABETIC COMPOUNDS FROM *Moringa oleifera*

By

BRYAN GACHOMO

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Dr. Joseph Martin

And approved by

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Dr. Joseph Martin

_____

Dr. Angélica L. González

_____

Dr. David Salas-de la Cruz

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THESIS ABSTRACT

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Thesis Director:
Dr Joseph Martin

Diabetes mellitus is a growing health challenge across the world affecting millions of people particularly in developing nations. There are two main forms of diabetes: Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D). T2D is the most common of all types of diabetes and was the main focus of my research. To counter this disease, there is an urgent need to find alternative antidiabetic drugs to add to the current arsenal of drugs. The hypothesis of this research was that a potential source of new antidiabetic drugs could be found traditional remedies, used for many years in some societies, which are currently unknown and/or untested by modern science. To test my hypothesis, I used Hexane, Methanol + 1% Acetic Acid, 95/5 Water/Acetonitrile, and Ethyl Acetate to extract compounds from crushed *Moringa oleifera* seeds. I then tested these extracts for their ability to reduce glucose levels in C57BL/6J mice that had been fed a high fat diet to induce obesity. The results showed that the Methanol + 1% Acetic Acid had the greatest effect in reducing blood glucose levels in the mice. HPLC/MS analysis of the Methanol + 1% Acetic Acid extract yielded many unknown compounds but also indicated the presence of some compounds that are known to have an effect on glucose homeostasis based on current knowledge. Therefore, this research

showed that *Moringa oleifera*, a tropical plant that has been used for its nutritional and medicinal properties, has potential as a source for novel antidiabetic drugs.

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3. Introduction

3.1 Types of diabetes:

Diabetes is a metabolic disease characterized by hyperglycemia due to the inability to regulate blood glucose levels [1, 25]. There are two major forms of diabetes mellitus;

1. Insulin dependent diabetes mellitus (IDDM) also known as type I or juvenile diabetes
2. Non-insulin dependent diabetes mellitus (NIDDM) also known as type II diabetes.

Type 1 diabetes mellitus:

Type 1 diabetes mellitus is caused by failure to release insulin from the β-cells of the islets of Langerhans in the pancreas. Type I diabetes is also called *Juvenile type* because it often first manifests itself in the children and young adults [3]. It is characterized by β-cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency.

The destruction of the β-cells occurs via apoptosis. The process of apoptosis involves direct contact by activated immune cells, and/or exposure to mediators secreted by the cells such as oxygen free radicals, cytokines and nitric oxide (NO) [13, 14]. The cytokines (IL-1β and/or IFN-γ) induce stress response genes which are necessary for the cytokine-induced apoptosis. The activation of the stress response genes is mediated mainly by the transcription factors NF-κB (activated by IL-1β) and STAT-1 (activated by IFN-γ) as seen in Figure 1 [12, 13]. Some proposed mechanisms of cytokine-induced apoptosis include: activation of the

stress-activated protein kinases c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK); triggering of ER stress; and the release of death signals from the mitochondria [12].

The onset of Type 1 diabetes is usually acute, developing over a period of a few days to weeks and often before the age of 25. Type 1 diabetes is a chronic disease in which there are high levels of glucose in the blood [2]. A Type 1 diabetic does not produce insulin hence requires insulin injections.

Type 2 diabetes mellitus:

Type 2 diabetes mellitus is also known as *non-insulin dependent diabetes mellitus* (NIDDM) or *maturity-onset type* because it often first manifests itself in the adult, after 40 years of age especially if obese. Type 2 diabetes is the most common form of diabetes. In type 2 diabetes, either the body does not produce enough insulin or the cells lose sensitivity to insulin. The causes of type 2 diabetes include; genetic factors and diet (increased intake of saturated fats and decreased intake of dietary fiber).

The mechanism of type 2 diabetes is more complex and involves factors such as glucotoxicity and lipotoxicity [12] (See Figure 2).

The complications of type 2 diabetes include; chronic and severe hypoglycemia. This is characterized by fatigue. The long-term complications are hypertension, loss of eyesight, kidney failure and diabetic foot disorders. The management of

type 2 diabetes is through oral hypoglycemic agents and in severe cases, insulin injection is used.

3.2 Treatment of diabetes in developing countries:

Globally, about 400 million people are affected by diabetes with over 70% of them in developing countries [4]. The vast majority of people in developing countries suffering from diabetes have limited resources and access to drugs. Therefore they have to spend a great deal from their own pockets to obtain health treatment [5]. As a result of the high cost and poor access to health care, many people often turn to cheaper sources, such as traditional remedies [6 – 7].

In Benin and other developing countries, one such remedy involves the use of the seeds from the *Moringa oleifera* plant. *Moringa oleifera* grows best in tropical or subtropical regions within an altitude of 0 – 2000m and an average rainfall of 250 – 3000m. It grows best in loamy, sandy or sandy-loam soil with a pH of 5 – 9 [8]. It is also well known for its medicinal and nutritional benefits and is a good source of Vitamin C, calcium and β-carotene (see Figure 3) [9].

In the treatment of diabetes, seeds are eaten whole or crushed and used to make “tea” which is then drank The goal of this research is to identify the bioactive compounds in *Moringa oleifera* by extracting and testing the anti-diabetic effect different compounds on diabetic mice. I hypothesize that if the bioactive compounds found in Moringa oleifera have an anti-diabetic effect, then these would lower the blood glucose in the diabetic mice.

4. Materials and Methods

4.1 Plant Sample Collection:

The *Moringa* seed samples were collected several sites in Benin, West Africa. Multiple sites across different parts of the country were chosen in order to account for differences in growth conditions. This eliminated any potential bias that would have inadvertently affected the results of the experiments such as presence of toxins and nutrient poor soils. The seeds were dried and packaged in plastic bags for storage and transportation. The plastic bags provided a moisture free environment that is better for long term storage.

4.2 Compound Extraction:

Extraction of compounds from *Moringa* was performed using the following solvents: hexane, methanol (with 1% Acetic Acid), ethyl acetate, and 95/5 Water/Acetonitrile. These solvents were chosen for their differences in polarity which would allow for extraction of the most compounds (see Figure 4). The dried *Moringa* seeds were crushed and weighed before adding the solvent. Once the solvent was added, the mixture was shaken for 5 days at room temperature on a VWR Standard Analogue Shaker set at Speed 3. After shaking, the mixture was filtered using cotton wool and a funnel. The filtrate was air-dried at room temperature and the end product weighed. The residue was also air dried and reused in the next solvent extraction.

4.3 HPLC/MS, Compound Identification and Analysis:

To determine the compound composition of the bioactive extract, I used the Thermo Fisher Scientific UltiMate 3000 High Performance Liquid Chromatography (HPLC) system along with the Eclipse Plus C18 Column. The dry bioactive extract was diluted in a 200uL solution of 95/5 Water/Acetonitrile. The sample was run on a 25 minute long, 2-solvent, multi-step gradient of Water + 0.1% Formic Acid (Solvent A) and Acetonitrile + 0.1% Formic Acid (Solvent B) at a flow rate of 400uL/min. Solvent B was run at 2% for the first 2 minutes increasing gradually to 98% by the 15 minute mark. The solvent was maintained at 98% until the 19 minute mark and gradually lowered to 2% by the 21 minute mark where it was maintained to the 25 minute mark. Solvent A made up remaining percent difference at each stage. To account for background noise a blank with 95/5 of Water/Acetonitrile was used. Ten uL of the eluted sample was injected into the Bruker UHR-QqTOF (Ultra-High Resolution Qq-Time-Of-Flight) mass spectrometer at a flow rate of 400uL/min to generate a chromatogram profile.

Data Analysis was conducted using software provided along with the MS device. The chromatogram profiles of the blanks were used as a baseline for establishing background interference in the results. The sample data was then used to create a dissected compounds spectra. Compound identification was done using SmartFormula**3D**™ software which compared the m/z (mass over charge) data from the chromatogram profile to known data from databases such as ChemSpider (chemspider.com), PubChem (https://pubchem.ncbi.nlm.nih.gov/), and KEGG (Kyoto Encyclopedia of Genes and Genomes) to determine the

structure and function of the compounds identified in the extract by generating a similarity score. The variation of the matched peaks was set at a threshold of 10ppm to ensure the most accurate matching. The compounds with the highest similarity score were chosen.

4.4 Animal model, Diet Induced Diabetes and the Oral Glucose Tolerance Test (OGTT)

25 C57BL/6J mice from The Jackson Laboratory (600 Main Street Bar Harbor, ME USA 04609) were used as the animal model and handled according to current best practices of ethical experimental animal treatment [15 -21, 26] . The mice were all male and approximately 3 - 4 weeks old. The mice were each given a unique identification number and housed in cages with a maximum of 3 mice each at room temperature conditions (25ºC and 50% humidity) and normal 12-hour day and night cycle (light cycle was from 8am to 8pm). They were fed and watered *ad libitum*.

The mice were then divided into 2 groups: the diet-induced obesity (DIO) group consisting of 20 randomly chosen mice; and the non-DIO/control group consisting of 5 randomly chosen mice. The DIO group of mice was fed a high fat diet (Catalog ID D12492) composed of 60% kcal fat, 20% kcal protein and 20% kcal carbohydrate while the control group was fed a low fat diet (Catalog ID D12450B) composed of 10% kcal fat, 70% kcal carbohydrate and 20% kcal protein [24]. The mice were fed these diets throughout the duration of the research (12weeks) The diet was from Research Diets Inc. (20 Jules Lane New Brunswick, NJ 08901). Diet induction was chosen as opposed to chemical

induction because it resembles the process of acquisition of T2D in humans [10, 22, 23].

The Oral Glucose Tolerance Test (OGTT) was used to determine if the diet induced diabetes method worked. OGTT involved administering a glucose solution in Phosphate Buffered Saline (PBS) at a dose of 2g/kg after a 6-hour morning fast. The blood glucose concentration was then monitored for 2hrs after administration of the glucose solution. The blood glucose concentration data for the DIO and non-DIO groups was compared.

4.5 Blood Glucose Measurement and Data Analysis:

Blood glucose was measured using a standard glucometer using test strips (TRUEtest disposable test strips and TRUEresult glucometer. The blood glucose concentration was measured in mg/dl. Blood sampling was done via tail vein sampling technique. In this technique, a small incision was made at the tip of the tail from which blood was drawn. A test strip was then inserted into the glucometer and a drop of blood was then placed on the test strip. The glucometer then gave a reading of the blood glucose. More blood could be sampled from the same incision by gently rubbing with a clean cotton ball until the wound bled. The blood was sampled before the extract was administered (time 0), one hour after the extract was administered (time 60) and 2hrs after the extract was administered (time 120).

Bioactivity was determined by the ability of the extract to lower blood glucose concentration in comparison to the DIO control group. The greater the bioactivity

the lower the blood glucose levels in the mice.

Data analysis was done using GraphPad Prism software and Microsoft Excel. The experimental groups were compared to the DIO control group using a Two-way Repeated Measures ANOVA test -- with an accounting for mice ID's and randomization of the experimental group mice in before each trial -- and Bonferroni posttest. In addition to the above, effect sizes were calculated determine overall effectiveness of each treatment dose.

4.6 Fasting and Treatment Administration:

Prior to administering the treatment, the mice were fasted for a 6-hour morning fast to stabilize their blood glucose levels before addition of the treatment [27]. The plant extracts were then solubilized in Phosphate Buffered Saline (PBS) prior to administration via the oral gavage technique [11] (see Figure 5). The mice were weighed individually to determine the dosage necessary for each mouse. The maximum oral gavage volume for mice is 10ml/kg, however, only 5ml/kg was used.

The DIO/diabetic group was further subdivided into two groups: the DIO control group consisting of 5 mice and a diabetic experimental group consisting of 15 mice (see Figure 6). To test multiple doses, the experimental group mice were randomized and divided into 3 groups of 5 mice each. This allowed for a maximum of 3 different doses to be tested in each trial. The experimental mice were always randomized prior to each new trial. Only the DIO experimental groups were administered with the plant extracts. The control groups (both DIO

and non-DIO) were administered with plain PBS. In addition, metformin, a well-known antidiabetic drug was used as a positive control.

The mice were given a minimum rest period of 24hrs prior to the commencement of a new trial to help them recover. After the first 24hrs the mice were fasted again and the blood glucose level was measured to ensure that they had returned to normal fasting blood glucose levels of greater than 120 mg/dL for DIO mice and less than 100mg/dL for normal mice. The doses of plant extracts tested were: 75 mg/kg, 150 mg/kg, 300 mg/kg, 450 mg/kg, 900 mg/kg and 1200 mg/kg.

Since most diabetic humans take their medication without fasting, this means that their glucose levels are affected by the food they eat. Therefore it was important to test the effect of altered blood glucose levels due to food consumption on the extracts. To accomplish this, a glucose solution at a dose of 2g/kg was included with the extract by dissolving both the glucose and the extract in the same PBS solvent and administering both to the mice after the morning fast. These results were then compared to trials where the extract was administered without glucose.

5. Results

5.1 Compound extraction:

Table 1 shows the percent yield of each extract. The greatest yield was the hexane fraction with 18%. 95/5 water/acetonitrile yield was next with a percentage of 12.2%. The methanol + 1% acetic acid extract and the ethyl acetate extract had a close percent yield with values of 10.5% and 9.8% respectively.

5.2 HPLC/MS, Compound Identification and Analysis:

Figure 10 shows the chromatogram profile for the methanol fraction. Figure 10A shows the retention time versus intensity profile of the methanol fraction. There were three main retention time ranges that showed a high intensity of compounds. The first range was the 2 minute to 3 minute range which showed peak intensity of approximately $0.6 * 10^7$. The 6 minute to 8 minute time range also showed a high intensity which peaked at $0.5 * 10^5$. The longest range with the highest intensity was from 13.5 minutes to 23 minutes. The intensity at this range peaked at $1.0 * 10^7$. Figure 10B shows the dissected compounds from the MS profile of the methanol fraction. By using the m/z (mass over charge) data from the eluted compounds, a total of 194 individual compounds were dissected.

Table 3 shows some of the identified compounds based on the data from the dissected compounds. A wide range of compounds were identified with most being complex organic compounds with high molecular weights such as 1-[4-[(E)-pent-3-enyl]cyclohexyl]-4-[4-[(E)-prop-1-enyl]cyclohexyl]benzene which has a molecular weight of 350amu. The functions of most of the compounds were also

unknown. Figure 11 shows the percentage of known (30%) and unknown compounds (70%) with the known compounds corresponding to the compounds that had positive hits in the databases.

5.3 Animal model, Diet Induced Diabetes and the Oral Glucose Tolerance Test (OGTT)

Figure 7 shows the results of the Oral Glucose Tolerance Test (OGTT) after 2 weeks and 5weeks of diet induction of diabetes. After 2 weeks, there was a significant difference in the blood glucose levels between the diabetic group of mice and the control (non-diabetic) group of mice 60 minutes after the administration of the treatment. At this time point the control group had an average blood glucose concentration of about 100mg/dL while the diabetic group had a blood glucose level greater than 150mg/dL ($P<0.05$, $t = 3.043$). There was no significant difference between the two groups at the 0minute and 120minutes time points. The results of the OGTT five weeks after the diet administration had begun showed no significant difference at any time point. This meant that the diet had yet to take effect after 5 weeks of administration and more time was needed for it to take effect. The 2 way Repeated Measures ANOVA also revealed that the effect of the treatment and time was very significant ($F_{1,85} = 12.51$, $P = 0.0025$; $F_{5,85} = 5.729$, $P = 0.0001$, respectively).

5.4 Blood Glucose Measurement and Data Analysis:

The mean metformin effects were significantly different as shown in Figure 8A. Time and treatment all had a significant effect as sources of variation ($F_{8,22}$ = 10.37, P < 0.0001; $F_{8,22}$ = 10.37, P < 0.0001,respectively). There was a significant difference between the DIO and non-DIO control at all time points. The DIO control and the 100mg/kg dose response curve were not significant at 0, 60, or 120minutes after treatment (P>0.05; P>0.05; P>0.05, respectively). The 200mg/kg dose response curve differed from the diabetic control group at 60minutes (P<0.001, t = 4.420) and 120minutes (P<0.01, t = 3.999) after treatment. The result was similar at the 400mg/kg dose response curve with the results being significant 60minutes and 120minutes after treatment (P<0.001; P<0.001, respectively). In terms of overall effectiveness, metformin showed increasing effectiveness in blood glucose reduction as the dosage increased (see Table 2). The effect sizes were -0.1, -0.83 and -1.19 for 100 mg/kg, 200mg/kg and 400mg/kg respectively.

The mean hexane fraction effects showed no significant bioactivity in all the tests carried out. In figure 8B, there was no significant difference between the control group and the groups administered a hexane fraction of 75mg/kg, 150mg/kg and 300mg/kg without the addition of the 2g/kg glucose solution. In terms of variability, time was the most significant source of variability ($F_{2,28}$ = 13.31, P<0.0001). Increasing the hexane fraction dosage to 1200mg/kg and adding the 2g/kg glucose solution as shown in figure 8E still did not produce any significant bioactive effect (P>0.05). However, those results had significant variation due to time, treatment and interaction ($F_{2,26}$ = 124.16, P<0.0001; $F_{4,26}$ = 31.40, P<0.0001; $F_{8,26}$ = 2.75, P = 0.0241; respectively). Overall, the lower hexane doses showed no

effectiveness at reducing blood glucose levels (with effect sizes of 0.42, 0.45, 0.42 at doses of 75 mg/kg, 150mg/kg and 300mg/kg respectively) (see Table 2). The only exception was the 1200 mg/kg dose which had an effect size of -0.22.

The ethyl acetate did show more instances of significant bioactivity when compared to the hexane fraction. In figure 8C, the 150mg/kg concentration showed a significant difference to the control group at the 60minutes after treatment but not at any other point ($P<0.01$, $t=3.722$). However, no glucose solution was added in this case. In figure 8E, which included a glucose solution, there was a significant difference 60 minutes after treatment but not at any other point ($P<0.05$, $t=3.108$). The ethyl acetate fraction had effect sizes of -0.35, -0.4, 0.75 and -0.67 for doses of 75, 150, 300 and 1200 mg/kg respectively (see Table 2)

The aqueous fraction (95/5 Water/Acetonitrile) showed no significant bioactivity at 150mg/kg, 300mg/kg, and 450mg/kg doses as shown in figure 8D. However, once the dose was increased to 1200mg/kg there was significant bioactivity at 0 minutes, 60 minutes and 120 minutes after treatment ($F_{2,28} = 28.49$, $P<0.05$, $P<0.001$, $P<0.001$, respectively). The effect sizes for the water extract showed and increasing effect of reduced glucose levels as the doses increased. For example, at 1200 mg/kg the water extract had an effect size of -1.45 which is comparable to the methanol fraction effect size of -1.57 (see Table 2). This means that the bioactive compounds are likely to be polar compounds since both methanol and water are polar solvents.

The methanol fraction showed significant bioactivity even at doses that were lower when compared to the other extracts. This suggests that the methanol fraction is far more bioactive. The bioactivity was not clearly demonstrated at lower doses such as those below 450mg/kg (see Figure 9B-E). At higher doses (Figure 9F) there was a significant difference in bioactivity for each time point (0 – 180min) and at all concentrations (450mg/kg, 900mg/kg and 1200mg/kg) ($F_{4,39}$ = 79.82, $P<0.0001$). The only exception was 60minutes and 180minutes after treatment at 1200mg/kg dose. The effect sizes indicate that in the presence of glucose, the methanol extract had the largest effect sizes of all (see Table 2). For example, at 150 mg/kg the methanol extract treatment had effect sizes of -0.47 with glucose which was much higher than the same methanol extract without glucose (-0.18) and the water extract (-0.29), ethyl acetate extract with no glucose (-0.4), and hexane extract with no glucose (0.42).

6. Discussion

6.1 *Moringa oleifera* is a potential source of novel antidiabetic compounds:

With Type 2 Diabetes (T2D) cases projected to grow in the coming years, the need for alternative drugs has never been greater. The goal of this research was to identify novel antidiabetic compounds from *Moringa oleifera*. These novel compounds could potentially be used to create new drugs against T2D.

Moringa oleifera is a widely grown plant in the tropics that makes it advantageous as a prime candidate for novel antidiabetic compounds as it is already widely used in remedies and food consumption [9]. An additional benefit is that it is available to many people in developing countries in the tropics meaning that people in these countries, who often cannot access current medication, would benefit greatly if any novel drugs were synthesized from an easily available plant like *Moringa*. These novel drugs would provide cheaper and more accessible alternatives without compromising effectiveness.

6.2 The methanol fraction displays greatest bioactivity:

The data presented here shows that there is a clear bioactive effect from the methanol *Moringa* extract. From this we can glean that the bioactive compounds in *Moringa* are polar since there was no bioactivity in the less polar extract such as hexane and ethyl acetate. The water fraction while being polar did not display as much bioactivity as the methanol fraction but did display more bioactivity than hexane and ethyl acetate extracts respectively.

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6.3 The large number of unknown compounds provides great potential for the discovery of novel antidiabetic compounds:

Thanks to the combined power of High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS) over 194 individual compounds have been dissected from the bioactive methanol fraction (see Figure 10B). HPLC allows for greater separation of liquid mixtures which results in high resolution of compounds. MS allows for identification of compounds by their unique m/z (mass-over-charge) signature. When these two powerful techniques were combined, they prove to be instrumental in resolving and identifying the compounds in the methanol fraction. In addition to these two techniques, the utilization of current chemical compound databases such as PubChem and KEGG greatly aided

Figure 11 shows the percentage of known and unknown compounds wherein 70 percent of the compounds were unknown (meaning that they did not generate positive hits in current databases).

Current research has yielded several bioactive phytochemicals in *Moringa oleifera*. Phenolic acids, flavonoids and glucosinolates are of particular interest for their medicinal properties [27,28,29]. Phenolic acids isolated from *Moringa oleifera* include kaempferide 3-O-(2'',3''-diacetylglucoside), kaempferide 3-O-(2''-O-galloylrhamnoside), kaempferide 3-O-(2''-O-galloylrutinoside)-7-O-alpha-rhamnoside, kaempferol 3-O-[beta-glucosyl-(1 --> 2)]-[alpha-rhamnosyl-(1 --> 6)]-beta-glucoside-7-O-alpha-rhamnoside and kaempferol 3-O-[alpha-rhamnosyl-(1 -

-> 2)]-[alpha-rhamnosyl-(1 --> 4)]-beta-glucoside-7-O-alpha-rhamnoside, benzoic acid 4-O-beta-glucoside, benzoic acid 4-O-alpha-rhamnosyl-(1 --> 2)-beta-glucoside and benzaldehyde 4-O-beta-glucoside [27]. Figure 12 shows some of the flavonoids and glucosinolates that have been isolated from *Moringa oleifera* [27].

Some well characterized antihyperglycemic compounds found in *Moringa oleifera* include: quercetin [30,31] , moringinine [35,36,37,38] and chlorogenic acid [32,33,34]. Quercetin has been shown to have a protective effect against oxidative stress in streptozotocin-induced diabetes in rats [31]. Moringinine, also identical to benzylamine, was shown to improve glucose tolerance in 7-week-old, male, Wistar rats given an oral dose of 2.9 g/L in drinking water for 7 weeks [37]. Chlorogenic acid has been identified as an inhibitor of the glucose-6-phosphate translocase component (Gl-6-P translocase) of the glucose-6-phosphatase enzyme system [32]. Glucose-6-phosphatase is responsible for the formation of glucose originating from gluconeogenesis, therefore inhibiting this pathway decreases amounts of hepatic glucose [32].

Trinexapac-ethyl is a compound that was identified from this research. It is of particular interest due to its function as a plant growth regulator [40]. It acts as an enzyme inhibitor in the production of gibberellins in the plant cell. Gibberellins promote cell elongation. Therefore, in the absence of gibberellins plants do not grow taller and instead redirect their energy to other parts of the plant such as

reproduction [40]. Trinexapac-ethyl can also be used to increase the content of flavonoids and phenolic substances in plants [41]. This is of great interest because flavonoids and phenolic substances have shown great potential as antihyperglycemic agents.

Another compound of interest was adenosine. Current research shows that adenosine receptors can act as therapeutic agents [42]. Adenosine is also an important extracellular signaling molecule that regulates many aspects of tissue function. In regards to diabetes mellitus, adenosine is known to affect insulin secretion; regulate homeostasis of pancreatic beta-cells in inflammatory environments and control insulin signaling in muscle, liver and adipose tissue [42].

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