JOURNAL OF
EMERGING INVESTIGATORS

Article

Combating insulin resistance using medicinal plants as a supplementary therapy to metformin in 3T3-L1 adipocytes

Diya Jayram[1] and Leya Joykutty[1]
[1] American Heritage School, Plantation, Florida

SUMMARY

One in eleven people will have a form of diabetes during their lifetime (1). In 2012, diabetes and its life-threatening side effects cost 245 billion dollars in the United States (2). Averting type 2 diabetes (T2D) will significantly reduce health care costs and engender a healthier population. A primary cause of diabetes is insulin resistance, which is caused by disruption of insulin signal transduction. The objective was to maximize insulin sensitivity by creating a more effective, early intervention-based treatment to avert severe T2D. This treatment combined metformin, "the insulin sensitizer", and medicinal plants, curcumin, fenugreek, and nettle. To conduct this study, insulin resistance was induced using free fatty acids (FFAs). Insulin sensitivity in adipocytes (propagated and differentiated from 3T3-L1 fibroblasts) was measured using assays specific for leptin concentration, glucose uptake, and Akt phosphorylation. These three proteins/pathways served as quantitative measurements for insulin sensitivity, since they are essential to insulin signaling. It was hypothesized that a dual therapy would maximize insulin sensitivity. This hypothesis was supported as each plant-enhanced combination treatment attenuated the effects of FFAs, increasing and reversing each biological marker of functional insulin signaling to normal function. This indicates that each treatment has the potential to combat obesity, augment glucose uptake, and heighten Akt/PI3K pathway function. This study, therefore, offers an improved multifunctional early intervention-based diabetes treatment that maximizes insulin sensitivity.

INTRODUCTION

Diabetes, a devastating chronic disease, is one of the world's rising problems. The number of people with diabetes has risen from 108 million in 1980, to 422 million in 2014 (1). In 2015, diabe-tes was the 7th leading cause of death in the United States. New cases are constantly being diagnosed: 1.5 million Americans are diagnosed with diabetes every year. Not only is it prevalent, but it is also a costly disease. In 2012, diabetes cost 245 billion dollars total in the United States (2). This study's objectives were to discover a treatment that has the potential to prevent severe type 2 diabetes (T2D), reducing health care costs and saving lives.

T2D is a disease in which the body is incapable of responding to insulin, caused by damage or disruption in the insulin signaling transduction system. Insulin signaling occurs when insulin binds to the tyrosine kinase insulin receptor (3). Leptin and insulin signaling converge at the level of IRS-PI 3-kinase (PI3K); therefore, they are closely related in glucose homeostasis (4). This convergence phosphorylates enzymes that activate the Akt/PI3K signaling pathway (5). The PI3K pathway plays essential functions in the cell, which includes lipid, protein, glycogen synthesis, and cell proliferation (6). The enzymes also activate GLUT4, which allows glucose to enter the cell by passive diffusion (7). Downstream metabolic enzymes break down glucose to generate ATP (**Figure 1**). Therefore, leptin, GLUT4, and Akt/PI3K were used as markers to measure insulin sensitivity. Furthermore, dysregulation of free fatty acid metabolism is a key event responsible for insulin resistance and T2D. (8). Therefore, free fatty acids (FFAs) were used to induce insulin resistance.

Prediabetes and mild T2D are commonly treated by metformin, an oral diabetes medicine that aids in preventing diabetes in people who are high risk and reduces the majority of diabetic complications (9). Metformin can restore leptin sensitivity, regulate GLUT4 translocation, and modulate Akt/PI3K pathway signaling. Curcumin, (10), fenugreek (11), and nettle (12) all have been proven to increase glucose uptake by the GLUT4 pathway, augment Akt phosphorylation by the Akt/PI3K pathway, and increase leptin concentration (10). Therefore, it was hypothesized that the synergistic effects of metformin and a medicinal plant may be able to reverse insulin resistance to a normal insulin sensitivity. The objective of this study was to examine the potential of the possible synergistic effects of medicinal plants (curcumin, fenugreek, and nettle) and metformin as an early intervention-based diabetes treatment to prevent diabetes and its complications, save lives, and reduce health care costs.

This objective was accomplished by propagating and differentiating 3T3-L1 adipocytes, indu-ing insulin resistance through free fatty acids (FFAs) (13), treating with the appropriate metformin and medicinal plant combination therapy, and measuring effectiveness/insulin sensitivity through significant protein and pathway markers in the insulin signal transduction system. The results accomplished by these methods demonstrated that this study offers a significantly more effective, multi-functional early intervention-based treatment for a costly, widespread disease: diabetes. Not only does this treatment combat diabetes, it also has the potential to combat obesity, another increasingly prevalent and destructive disease, by increasing leptin and attenuating

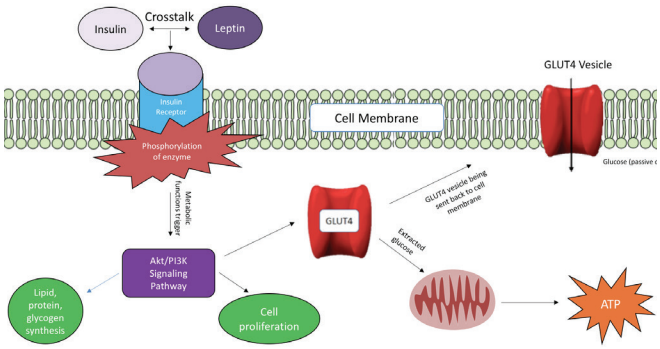

Figure 1: Insulin Signal Transduction. Simplified diagram conveying complex processes of insulin signal transduction that are significant to this study and explains the motive behind choosing leptin, GLUT4, and the Akt/PI3K pathways as biomarkers for measuring insulin sensitivity by describing the importance of the pathways to insulin signal transduction.

the effects of FFAs. Leptin, the appetite hormone, controls hunger, a symptom of diabetes; FFAs This paper also examines the effects of curcumin, fenugreek, and nettle on the insulin signal transduction system.

RESULTS

To accurately measure insulin sensitivity, it was necessary to differentiate the preadipocytes before experimentation. Preadipocytes were cultured in T75 flasks and grew rapidly with proper adherence (**Figure 2A**). Preadipocytes were transferred to and differentiated in 12-well and 24-well plates successfully. Successful differentiation was signified by the presence of lipid droplets, observed as bright yellow spheres. The differentiated adipocytes accumulated yellow lipid droplets and had low adherence (**Figure 2B**). The Oil Red O assay was conducted to confirm differentiation and show clear presence of lipid droplets. The Oil Red O assay performed resulted in blue nuclei and bright red lipid droplets, demonstrating that differentiation was successful (Figure 2C). These differentiated cells were then treated with FFAs to induce insulin resistance and with combination therapies in an attempt to attenuate insulin resistance

Treatment	Leptin (pg/mL)	Glut4 (pg/mL)	phospho-AKT (pg/mL)
Normal	45.98	240.02	14.58
Insulin-Resistant (IR)	40.89	213.44	10.00
Metformin (IR)	49.24	235.31	16.11
Curcumin	52.54	222.24	11.99
Curcumin (IR)	54.90	246.60	16.41
Curcumin w/ Metformin	57.63	338.86	21.36
Fenugreek	54.38	232.96	11.58
Fenugreek (IR)	52.10	247.08	17.81
Fenugreek w/ Metformin	68.57	313.65	25.12
Nettle	48.61	225.12	22.96
Nettle (IR)	54.20	256.05	24.45
Nettle w/ Metformin	65.77	286.58	38.20

Table 1: Raw quantitative results obtained from measuring leptin concentration, GLUT4 pathway function, and Akt phosphorylation.

To measure the efficacy at which the combination therapies attenuate insulin resistance, GLUT4, leptin, and Akt phosphorylation were measured using ELISAs specific to these markers. GLUT4, leptin, and Akt phosphorylation are significant in insulin signaling and are indicative of potential glucose uptake, hunger control, and Akt/PI3K function, respectively. Based on these markers, all combination treatments were effective at increasing insulin sensitivity (**Table 1**).

Curcumin (p=0.0110), fenugreek (p=0.0041), and nettle (p=0.0126) combination treatments were each able to recover Akt levels (**Figure 3**). It was hypothesized that a dual therapy of a medicinal plant and metformin would restore the Akt concentrations to normal levels. The data show that each medicinal plant used with metformin produced an average Akt concentration higher than the normal value. The data also show that a nettle adjunct therapy is the most effective at enhancing Akt/PI3K pathway function because it produced the highest Akt concentration. Thus, curcumin, fenugreek,

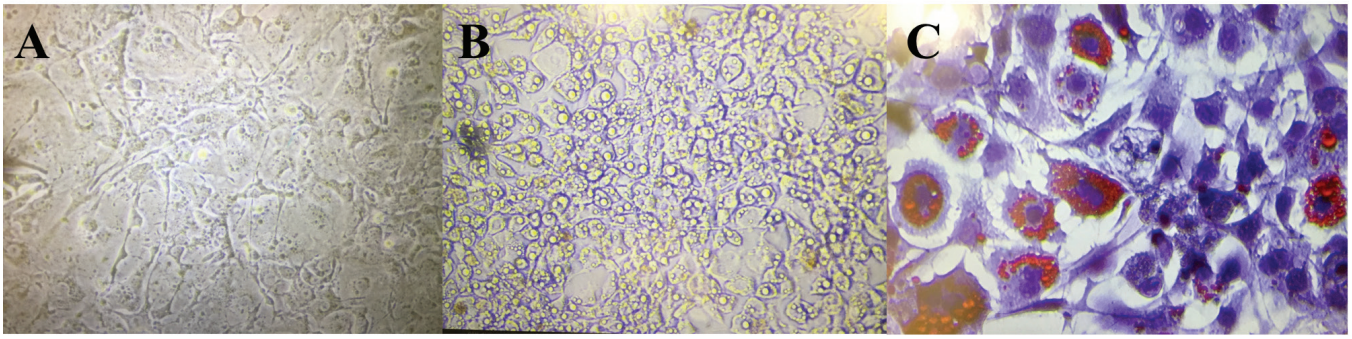

Figure 2: Differentiation of 3T3-L1 Adipocytes (A) 3T3-L1 preadipocytes prior to differentiation. (B) 3T3-L1 preadipocytes after differentiation prior to Oil Red O stain. Cells successfully differentiated, as depicted by the lipid droplets. (C) 3T3-L1 fully-differentiated adipocytes after Oil Red O Lipid Staining. Lipid droplets are stained red and nuclei are stained blue.

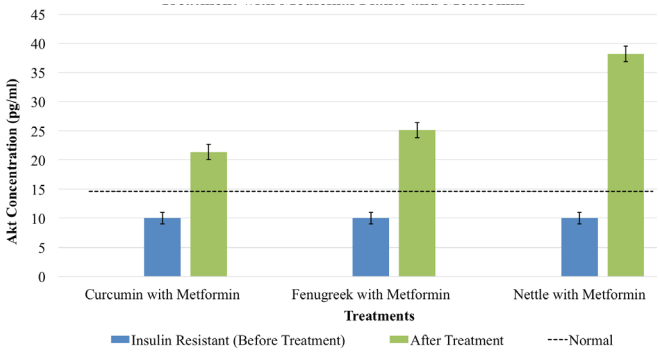

Figure 3: Measurement of Akt phosphorylation in Adipocytes Following Treatment with Medicinal Plants and Metformin. Bars represent the mean of phospho-Akt concentration of 4 samples. Error bars represent the standard deviation. The dashed line represents untreat-ed/normal group. The dashed line acts as the baseline to measure treatment efficacy. * indi-cates p<0.05 as determined by a Student's t-test. 1mM of metformin, 20 μM of curcumin, 5 μg/ml of fenugreek, and 5 μg/ml of nettle was used.

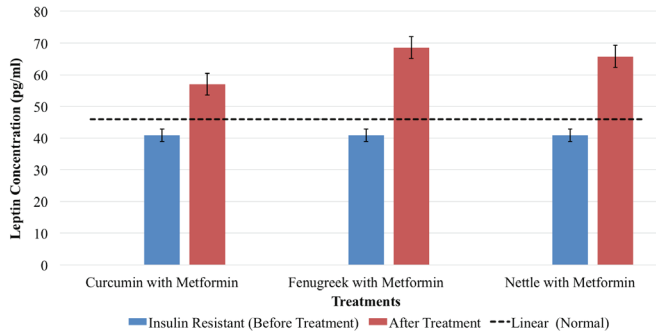

Figure 4: Measurement of Leptin Concentration in Adipocytes Following Treatment with Medicinal plants and Metformin. Bars represent the mean of leptin concentration of 4 samples. Error bars represent the standard deviation. The dashed line represents untreat-ed/normal group. The dashed line acts as the baseline to measure treatment efficacy. * indi-cates p<0.05 as determined by a Student's t-test. 1mM of metformin, 20 μM of curcumin, 5 μg/ml of fenugreek, and 5 μg/ml of nettle was used.

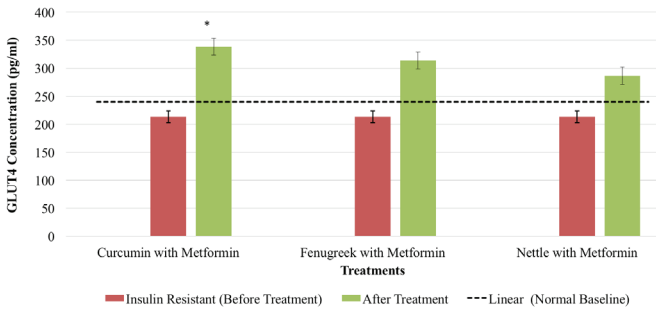

Figure 5: Measurement of GLUT4 Concentration in Adipocytes Following Treatment with Medicinal plants and Metformin. Bars represent the average of GLUT4 concentration of 4 samples. Error bars represent the standard deviation. The dashed line represents un-treated/normal group. The dashed line acts as the baseline to measure treatment efficacy. * indicates p<0.05 as determined by a Student's t-test. 1mM of metformin (21), 20 μM of curcumin, 5 μg/ml of fenugreek, and 5 μg/ml of nettle was used.

and nettle combination therapies were effective in maximizing Akt phosphorylation and attenuating the effects of free fatty acids (**Figure 3**), especially nettle, since Akt levels increased to match the normal baseline

Curcumin (p=0.0329), fenugreek (p=0.0262), and nettle (p=0.0072) used as an adjunct therapy to metformin were each also able to significantly recover leptin levels (**Figure 4**). It was hypothesized that a dual therapy of a medicinal plant and metformin would restore leptin levels to normal concentrations. The data shows that each medicinal plant produced an average leptin concentration higher than the untreated group. The data also shows that a fenugreek supplementary treatment has the most potential to effectively satiate hunger as it produced the highest leptin concentration. Thus, curcumin, fenugreek, and nettle combination therapies were effective in maximizing leptin and attenuating the effects of free fatty acids (Figure 4), especially fenugreek, since leptin levels increased to match the normal baseline.

Curcumin (p=0.0093), fenugreek (p=0.0387), and nettle (p=0.0235) as an addition to metformin were each able to recover GLUT4 levels, as well (**Figure 5**). It was hypothesized that a dual therapy of a medicinal plant and metformin would restore GLUT4 levels to normal concentrations. The data show that each medicinal plant used with metformin produced an average GLUT4 concentration higher than the normal values. A fenugreek adjunct therapy proved to be the most potentially effective at maximizing glucose uptake because it produced the highest GLUT4 concentration. Thus curcumin, fenugreek, and nettle combination therapies were effective in attenuating the effects of free fatty increasing GLUT4 levels to match the GLUT4 concentration before treatment with free fatty acids. All data was statistically significant based on a Student's t-test (α=0.05) and an ANOVA (α=0.01).

Using a medicinal plant with metformin maximized insulin sensitivity and produced synergistic effects (Figure 6). Each dual treatment produced an improvement in each biomarker's (leptin, GLUT4, and phospho-Akt) concentration when compared to the concentration of these biomarkers after treating with solely metformin. This controlled experimental setup directly attributes the improvement in insulin signal transduction biomarkers to the addition of medicinal plants. This showed that each adjunct treatment is more effective than only metformin at reversing insulin resistance. Fenugreek was the most effective at maximizing leptin (133.40%) and GLUT4 (129.70%) concentration, and nettle was the most effective at maximizing phosphorylated Akt concentration (237.11%). In fact, nettle doubled the efficiency of metformin as a diabetes treatment, in terms of the Akt/PI3K pathway.

DISCUSSION

Diabetes, a chronic and costly metabolic disease, is one of the world's rising problems (14). Metformin, the current early-intervention based treatment, is usually used to treat severe prediabetes and mild T2D and is the go-to medication before insulin, but after other modifications, such as diet and

exercise, have been made.

The combination of metformin and the medicinal plants curcumin, fenugreek, and nettle have the potential to reduce constant hunger and combat obesity, augment glucose uptake, and heighten the Akt/PI3K pathway significantly more than solely metformin, therefore this experiment offers a more effective early-intervention diabetes treatment than solely metformin. This combination treatment is most applicable to the early stages of diabetes because these dual treatments improve an early-intervention-based drug, metformin. If diabetes is diagnosed in its early stages, this treatment could be utilized to return to a normal insulin sensitivity and avert severe T2D. In 2012, this disease cost the United States 245 billion dollars, and this number will only continue to increase because new cases are being diagnosed every day (2). Averting T2D will significantly reduce health care costs and engender a healthier population.

Furthermore, these dual treatments have the potential to combat obesity. Obesity leads to increased levels of circulating FFAs (fatty acids) that are converted into metabolites that induce insulin resistance (15). Restoring leptin levels maintain weight loss (16). This study offers a possible multi-functional treatment for obesity and diabetes by combatting the effects of FFAs and restoring leptin levels. Moreover, not much is currently known about fenugreek and nettle's efficacy in improving health condition, although these diet supplements are available across the world. This study offers new information on the effects of fenugreek, curcumin, and nettle in adipocytes.

This study could be further expanded to provide a more comprehensive perspective of the combination treatments' effects on insulin signaling: observing other important pathways in the insulin signal transduction system, such as IL-6, using different cell lines, utilizing other medicinal plants, and expanding to an animal model. Furthermore, metformin is currently being tested in cancer patients as a cancer prevention drug people at increased risk, including cancer survivors who have a higher risk of a second primary cancer, because of its association in the decrease of secondary cancers. (17). Curcumin (18), fenugreek (19), and nettle (20) have each been shown to be anticarcinogenic. This study could be expanded to observe how these dual treatments function as a cancer prevention therapy for cancer survivors who have a higher risk of a second primary cancer. In summary, this study offers a significantly more effective, multifunctional, insulin-sensitizing, early intervention-based treatment for diabetes, a costly, widespread disease.

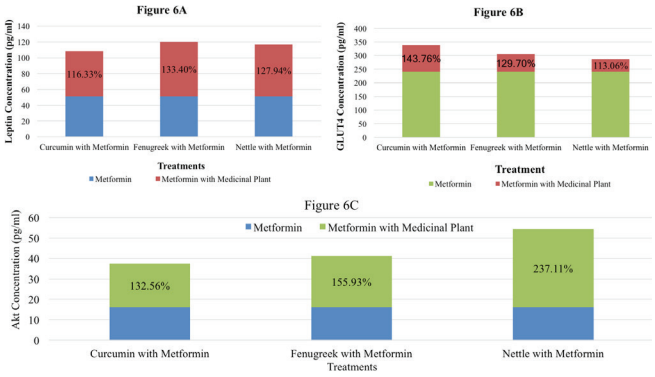

Figure 6: Percent improvement After Medicinal Plant Addition to Metformin. Compares the combination treatment groups with the metformin treatment group to show synergistic effects. The value on each bar shows the percent improvement of each adjunct treatment. Bottom bar represents treatment with solely metformin. Top bar represents the improvement that occurred with the addition of a medicinal plant.

Leptin						
Source of Variation	**SS**	**dF**	**MS**	**F**	**P-value**	**F crit**
Between Groups	2012.23	11	182.93	6.53	7.74 E-06	2.067
Within Groups	1008.87	36	28.02			
Total	3021.10	47				
GLUT4						
Source of Variation	**SS**	**dF**	**MS**	**F**	**P-value**	**F crit**
Between Groups	66580.7	11	5961.9	12.48	3.11 E-09	2.067
Within Groups	17199.8	36	477.77			
Total	82780.42	47				
Phospho-AKT						
Source of Variation	**SS**	**dF**	**MS**	**F**	**P-value**	**F crit**
Between Groups	2906.03	11	264.18	15.06	2.35 E-10	2.067
Within Groups	631.44	36	17.54			
Total	3537.47	47				

Table 2: Results of a one-way ANOVA conducted to analyze variance between and within groups. Differences between means of the treatment groups statistically significant (α=0.01).

METHODS

3T3-L1 Mus musculus fibroblasts were cultured in complete preadipocyte medium (DMEM, supplemented with 10% BCS, 1% penicillin-streptomycin) in T75 flasks in a humidified incubator at 37˚C with 5% CO2. All cell culture practices were conducted with good laboratory practices, with proper personal protective equipment, proper engineering controls, and within the parameters of the purchaser's chemical hygiene plan. All cell culture practices were performed under a cell culture laminar-flow hood. Preadipocytes were subcultured in a medium with 10% bovine calf serum and medium was changed every 2-3 days.

To prepare for differentiation, 3T3-L1 preadipocytes were transferred to 12-well and 24-well plates. Medium was replaced with fresh preadipocyte medium 48 hours prior to induction of differentiation. Differentiation was initiated at 80% confluency by replacing the preadipocyte medium with the differentiation medium (1.5 µg/ml insulin, 1 µM dexamethasone, 500 µM IBMX, and 1 µM rosiglitazone in DMEM:F12 (1:1) with 10% FBS). Differentiation medium was filter sterilized with a 0.22 µM syringe filter. Preadipocytes with differentiation medium were incubated for 3 days in a humidified incubator at 37˚C with 5% CO2. Differentiated cells were maintained in maintenance medium (100 µl of a stock solution of insulin (1.5 mg/ml) was added to 100ml of DMEM/F12 (1:1) with 10% FBS). Maintenance medium was filter sterilized with a 0.22 µM syringe filter. Differentiation medium was removed and replaced with maintenance medium. Medium was changed every 2-3 days. Lipid droplet accumulation was visible by light microscopy 7-10 days after the addition of differentiation medium. Differentiation was confirmed using the Oil Red O Lipid Staining Kit (K580, BioVision). Cells were stained at a density of 0.1x10^6 in a 24 well plate. The presence of red stained lipid droplets indicated successful differentiation.

To induce insulin resistance, cells were treated with free fatty acids (FFAs). FFAs were prepared by dissolving 13.75 mg of palmitic sodium salt (Sigma Aldrich) in 600 µl MQ water (Sigma Aldrich) on a hot plate, heated and stirred till dissolved. 25 µl of dissolved FFA was added to 50 ml of maintenance medium. The solution was filter sterilized using a 0.22 µM filter. Combination treatments were made up of metformin and a medicinal. Metformin and each medicinal plant were dissolved in maintenance medium at different concentrations: 1mM of metformin (21), 20 µM of curcumin, 5 µg/ml of fenugreek, and 5 µg/ml of nettle. The treatments were administered to the cells according to the well plate diagram in **Figure XX**. Cell extracts were prepared for protein analysis. Cells were washed with pre-cooled PBS and a cell scraper was used to dissociate the cells. The cell suspension was transferred into a centrifuge tube. Suspension was centrifuged for 5 minutes at 1000xg. The medium was discarded and cells were washed 3 times with ice-cold PBS. The cell suspension was centrifuged for 10 minutes at 1500xg. Supernatants were aspirated and centrifuged at 1500xg for 10 minutes. The supernatants were used for the following assays. Akt quantification was carried out on treated cells using an ELISA kit (ab126433, Abcam) according to the manufacturer's instructions. Quantification of GLUT4 was carried out on treated cells using an ELISA Kit (E-EL-M0564, Elabscience) according to the manufacturer's instructions. Quantification of leptin was carried out on treated cells using an ELISA Kit (ADI-900-019A, Enzo) according to the manufacturer's instructions.

The data collected was analyzed using a single factor ANOVA using Microsoft Office Excel (version 2016) with at a significance level of α=0.01. Once the one-way ANOVA showed a significant difference among the means of the treatments, a Student's t-test was used for each treatment to evaluate statistical difference between the treatments.

ACKNOWLEDGEMENTS

Enzo Life Sciences provided the Leptin ELISA. Abcam provided a discount for the pSer472 phospho-Akt ELISA. Elabscience provided a discount for the GLUT4 ELISA. BioVision provided a discount for the 3T3-L1 Differentiation Kit and the Oil Red O Assay Kit.

Received: May 22, 2018
Accepted: November 5, 2018
Published: March 13, 2019

REFERENCES

1. "Diabetes." World Health Organization, World Health Organization, 30 Oct. 2018, www.who.int/news-room/fact-sheets/detail/diabetes.
2. "The Cost of Diabetes." American Diabetes Association, www.diabetes.org/advocacy/news-events/cost-of-diabetes.html.
3. Wang, Haitao, et al. "Proline-Rich Akt Substrate of 40kDa (PRAS40): A Novel Downstream Target of PI3k/Akt Signaling Pathway." Cellular Signalling, vol. 24, no. 1, 2012, pp. 17–24, doi:10.1016/j.cellsig.2011.08.010.
4. Kraus, Daniel, et al. "Leptin Secretion and Negative Autocrine Crosstalk with Insulin in Brown Adipocytes." Journal of Endocrinology, vol. 24, no. 1, 2002, pp. 185-91.
5. Kadota, Yoshito, et al. "Metallothioneins regulate the adipogenic differentiation of 3T3-L1 cells via the insulin signaling pathway." PLOS One, vol. 12, no. 4, 2017, doi: 10.1371/journal.pone.0176070.
6. Li, et al. "Silibinin Improves Palmitate-Induced Insulin Resistance in C2C12 Myotubes by At-tenuating IRS-1/PI3K/Akt Pathway Inhibition." Brazilian Journal of Medical and Biological Research, vol. 48, no. 5, 2015, doi: 10.1590/1414-431X20144238.
7. Saini, Vandana. "Molecular Mechanisms of Insulin Resistance in Type 2 Diabetes Mellitus." World Journal of Diabetes, vol. 15, no. 3, pp. 68-75, doi: 10.4239/wjd.v1.i3.68.
8. Delarue, Jacques, and Christophe Magnan. "Free Fatty Acids and Insulin Resistance." Current Opinion in Clinical Nutrition and Metabolic Care, vol. 10, no. 2, pp. 142-48,

doi:10.1097/MCO.0b013e328042ba90.
9. Nasri, Hamid and Mahmoud Rafieian-Kopaei. “Metformin: Current Knowledge.” Journal of Research in Medicial Sciences, vol. 19, no.1, 2014, pp. 658-664
10. Zhang, Dong-wei, et al. “Curcumin and Diabetes: A Systematic Review.” Hindawi, 2013, doi: 10.1155/2013/636053.
11. Sundaram, Gopalakrishnan, et al. “Fenugreek, Diabetes, and Periodontal Disease: A Cross-Link of Sorts!” Journal of Indian Society of Periodontology, vol. 22, no. 2, 2018, pp. 122-126, doi: 10.4103/jisp.jisp_322_17.
12. Mahmoodnia, Leila, et al. “Traditional Uses of Medicinal Plants to Prevent and Treat Diabe-tes; An Updated Review of Ethnobotanical Studies in Iran.” Journal of Nephropathology, vol. 6, no. 3, 2017, pp. 118-125, doi: 10.15171/jnp.2017.20.
13. Su, Li-qing, et. al. “Effect Of Curcumin on Glucose and Lipid Metabolism, FFAs And TNF-a in Serum of Type 2 Diabetes Mellitus Rat Models.” Saudi Journal of Biological Sciences, vol. 24, no. 8, 2017, pp. 17776-1780, doi: 10.1016/j.sjbs.2017.11.011.
14. Tanaka, Shiro, et al. “Cohort Profile: The Japan Diabetes Complications Study: A Long-Term Follow-Up Of A Randomised Lifestyle Intervention Study Of Type 2 Diabetes.” Inter-national Journal of Epidemiology, vol. 43, no. 4, 2014, pp. 1054-1062, doi: 10.1093/ije/dyt057.
15. Aristizabal, Juan, et al. “Concentrations of Plasma Free Palmitoleic and Dihomo-Gamma Lin-oleic Fatty Acids Are Higher in Children with Abdominal Obesity.” Nutrients, vol. 10, no. 1, 2018, doi: 10.3390/nu10010031.
16. Pala, Laura, et al. “The Therapy of Insulin Resistance in Other Diseases Besides Type 2 Dia-betes.” Eating and Weight Disorders, vol. 19, no. 3, 2014, pp. 275-283, doi: 10.1007/s40519-014-0139
17. Kasznicki, Jacek, et al. “Metformin in Cancer Prevention and Therapy.” Annals of Transla-tional Medicine, 2014, vol. 2, no. 6, 2014, doi: 10.3978/j.issn.2305-5839.2014.06.01.
18. Bachmeier, Beatrice E., et al. “The Role of Curcumin in Prevention and Management of Metastatic Disease.” International Journal of Molecular Sciences, vol. 19, no. 6, 2018, doi: 10.3390/ijms19061716.
19. Shabbeer, Shabana et al. “Fenugreek: A Naturally Occurring Edible Spice as an Anticancer Agent.” Cancer biology & therapy, vol. 8, no. 3, 2009, pp. 272-278
20. Fattahi, Sadegh, et al. “Nettle Inhibits Cell Growth and Induces Apoptosis by Targeting Orni-thine Decarboxylase And Adenosine Deaminase As Key Regulatory Enzymes In Adenosine and Polyamines Homeostasis in Human Breast Cancer Cell Lines.” Cellular and Molecular Biology, vol. 64, no. 3, 28 Feb. 2018, pp. 97-102, doi:10.14715/cmb/2018.64.3.16.
21. Chen, Dian et al. “Dual Effects of Metformin on Adipogenic Differentiation of 3T3-L1 Preadipocyte in AMPK-Dependent and Independent Manners” International Journal of Mo-lecular Sciences vol. 19, no. 6, 2018, doi: 10.3390/ijms19061547.