Mistletoe infested *Moringa oleifera* and *Terminalia catappa* leaves supplemented diet enhances antioxidant and insulin-like peptide mRNA levels in *Drosophila melanogaster*

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**ABSTRACT**

Moringa and Almond are common plants of medicinal and economic value which are often infested with mistletoe. Host plants' infestation could result in major differences in their phytoconstituents and biological activities. Thus, effects of mistletoe infestation on Moringa and Almond host plants supplemented diets on mRNA expression levels of *Drosophila* insulin-like peptide-2 (*Dilp2*), heat shock protein-70 (*Hsp70*) and superoxide dismutase (*Sod*) in diabetic-like flies were evaluated using quantitative real-time PCR system. Mistletoe infestation on host leaves caused significant upregulation of *Sod* and significant downregulation of *Hsp70* and *Dilp2* genes. Hence, we opined that infestation of Moringa and Almond trees with mistletoe resulted in improved expression level of antioxidant and insulin-like peptide genes. This may be the mechanism by which host plants caused enhanced regulation of circulating glucose and oxidative stress. Therefore, consumption of mistletoe infested Moringa and Almond host leaves could possibly offer better antioxidant and hypoglycemic effects.

1. Introduction

Mistletoes are parasitic leaves that grow on several host plants, however, they possess chlorophyll, and this makes it possible to carry out photosynthesis but still depend on host plants for water and minerals. These parasites are capable of supplying their host plants with several constituents due to the capability of infested host plants to activate several defense mechanisms when parasitic plants infest them, and this could alter their biological activities (Adodo, 2004; Malami, Mainasara, Alieri, Alieri, & Maishanu, 2017). Mistletoes grow on plants of nutritional, medicinal and economic value like moringa and almond. *Moringa* (*Moringa oleifera* Lam.) is a fast-growing common pan-tropical plant whose various parts are used in the treatment of a long list of several ailments. The leaves are the most utilized part of this plant, it can be eaten fresh or cooked as vegetables and/or process dried and powdered; the powdered moringa leaves can be added as supplements to juices, ice creams, stews, vegetable soups, cereals (e.g. pap) and flour products (Ademiluyi, Aladeselu, Oboh, & Boligon, 2018; Zaku, Emmanuel, Tukur, & Kabir, 2015). *Almond* (*Terminalia catappa* Linn.) is a common prominent tropical important plant that is highly cultivated for its shade, ornamental purpose, and the nutritional and medicinal properties of its leaf, bark and fruit (Mallik, Faruq, & Banik, 2013). Most importantly, moringa and almond leaves are used in traditional medicine in the management of Diabetes mellitus (DM) as prolonged use of synthetic drugs may cause many side effects such as liver disorders, flatulence, abdominal pain, abdominal fullness and diarrhea (Kim et al., 2014; Kumari, Lakshmi, Jyothi, & Prasanthi, 2016). DM are metabolic disturbances that cause hyperglycemia which results from either insulin deficiency or insulin resistance (Maher & Schubert, 2009). The incidence of DM has increased exponentially over the years and is projected to rise above 592 million by 2035, likewise, the mortality rate was found to increase from 1.6 to 2.2 million in 2012 and 2016, respectively (Ayadurai, Hattingh, Tee, & Md Said, 2016; WHO, 2020). Cellular damage caused by the oxidative ability of reactive species (i.e., reactive oxygen species: ROS and reactive oxygen species: RNS) have been implicated in the pathogenesis of DM. Thus, oxidative event is heightened in diabetic condition in response to a lot of factors including increased polyol and hexosamine pathways flux, activation of protein.

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kinase C (PKC) isoforms, increased formation of advanced glycation end products (AGEs), oxidation/reduction imbalances and reduction in antioxidant defense systems (Brownlee, 2001; Penkofer, Schwertz, & Florczak, 2002; Rahimi, Nikfar, Larijani, & Abdollahi, 2005). These plant foods (i.e., moringa and almond leaves) are rich in polyphenols (Oyeniran, Ademiluyi, & Oboh, 2021, 2022), and polyphenols have been reported to elicit antioxidant and insulin-like effects (Cao et al., 2018; Su, Hung, & Chen, 2006).

The common fruit fly (Drosophila melanogaster Meigen) has globally emerged as a beneficial research model due to their strong evolutionary conservation with human (Morris et al., 2012; Scott, Schuldiner, & Neufeld, 2004). Of significant importance is that the endocrine architecture and mechanisms involved in sugar homeostasis in mammals are also found in the fruit flies. They possess insulin-producing cells, insulin-like peptides and insulin receptor, thereby, the insulin and insulin-like growth factor signaling pathways are conserved (Musselman et al., 2011). Studies had shown that Drosophila genome possesses eight insulin-like–peptides (Dilps 1–8), however, Dilp2 peptide has the closest homology to the vertebrate insulin gene (Alvarez-Rendon, Salceda, & Risgo-Escovar, 2018). Thus, fruit flies are excellent model organisms for studying DM, most especially, type-2 diabetes (Broughton et al., 2005; Ecker et al., 2017). Type 2 diabetes is characterized by insulin resistance or reduced insulin sensitivity which alters the utilization of endogenously produced insulin at the target cells (WHO, 1999). Musselman et al. (2013) reported that rearing fruit flies on high sucrose diet resulted in higher expression of Dilps mRNA levels coupled with higher circulating sugar levels; this feature resembles the mammalian insulin resistance.

Studies underlying the possible influence of mistletoe infestation on the phytoconstituents and anti-diabetic activities (particularly enzymatic and non-enzymatic antioxidants, enzymes linked with diabetes, glucose, trehalose, triglycerides and Drosophila insulin-like peptide contents) of the host plants, particularly, moringa and almond leaves had been done (Oyeniran et al., 2021, 2022; Oyeniran, Ademiluyi, & Oboh, 2020). However, there is still paucity of information on influence of mistletoe infestation on antioxidants and insulin-like peptides mRNA levels. Thus, the present study explored the effect of mistletoe infested moringa and almond supplemented diets on gene expression levels of Drosophila insulin-like peptide-2 (Dilp2), heat shock protein-70 (Hsp70) and superoxide dismutase (Sod) in fruit flies.

2. Materials and methods

2.1. Materials

2.1.1. Collection of leaves

The fresh leaves of mistletoe-infested and non-infested moringa and almond were gathered in a farm garden in South gate, Akure and authenticated in the Center for Research and Development, FUTA, with voucher numbers 0151 and 0152.

2.1.2. Fruit fly source

Oregon-R strain of wild fruit fly was originally gotten from the Drosophila Research Laboratory of the Department of Biochemistry, University of Ibadan. Cooled culture medium (prepared from cornmeal, 1 % agar, 0.08 % nipagin and 1 % brewer’s yeast) in glass vials was used for raising the flies at constant room temperature (25 °C) in the Drosophila Research Laboratory of Functional Foods, Nutraceuticals and Phytochemistry Unit, FUTA.

2.1.3. Chemicals

Quick-RNA™ MiniPrep plus kit, cDNA Synthesis kit, primers, nuclelease-free water and Luna Universal qPCR mix were sourced from Invaba Biotec West Africa Ltd., (Ibadan, Nigeria). Every other reagent was of analytical grade.

2.2. Methods

2.2.1. Processing of moringa and almond leaves

Fresh mistletoe-infested and non-infested moringa and almond leaves were washed in a plastic sieve and dried in a tray freeze dryer sourced from Carrier Vibrating Equipment, Inc. (Louisville, KY, USA. Ademiluyi et al. (2018) reported that freeze drying could preserve the phytoconstituents and nutraceutical properties of leaves (particularly, moringa) better than other methods. The freeze dried leaves were powdered in an electric VTCL kitchen blender at an average speed for about 5 min at 1 min interval, and kept in a well-covered jar at 25 °C. The leaves extracts were prepared at 20 % concentration (i.e. 20 g/100 mL distilled water) for 12 h. The solutions were filtered, centrifuged, clear liquids were concentrated and subsequently freeze-dried. The obtained powder was used to supplement the flies’ diet.

2.2.2. Larva collection and groupings

Female flies of five (5) days old were quickly transferred to 6.5 by 16.5 cm sterilized glass vials which contain fresh basal diet. The adult flies were removed after laying eggs for a period of 12 hrs, then, the first instar larva (L1) were collected the next day and used for this experiment because of their higher feeding rate, fast development and sexual immaturity when compared to adult flies. The L1 flies were further grouped, each vial consisted of 50 L1 and nurtured for 14 days in 2.5 by 6.5 cm sterilized plastic bottles. Diabetic-like phenotypes were induced in the flies using a high sucrose diet (15 % and 30 % w/v) and the treated groups received the mistletoe-infested and non-infested moringa and almond leaves (10 mg/g diet). Flowchart of the experimental design is presented in Fig. 1.

Note: The medium for diabetic-like flies consisted of cornmeal, 1 % agar, 0.08 % nipagin, 1 % brewer’s yeast containing the different sucrose concentrations.

2.2.3. RNA isolation and mRNA expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

Three separate samples containing 30 newly eclosed adult flies (both sex) from each vial in a group were pooled together, RNA was isolated using Quick-RNA™ MiniPrep plus kit and processed using manufacturer’s instruction. Total RNA was estimated using nano-400A micro-spectrophotometer and cDNA was synthesized from the total RNA with FIREScript reverse transcriptase (RT) cDNA Synthesis kit and processed using manufacturer’s protocol. The gene-specific primer sequences in Table 1 were used as based on published sequences in GenBank Overview (https://www.ncbi.nlm.nih.gov/genbank/). The primers were designed using Primer3 program version 0.4.0 (https://frodo.wi.mit.edu/prime3/). The qRT-PCR was done in 20 μL reaction mixtures which contained 1 μL RT product (cDNAs) as template, 10 μL of 1 × Luna Universal qPCR mix, 0.5 μL of 0.25 μM forward and reverse primers of interest respectively and 8 μL nuclelease-free water in 48-well plates. Thermal cycle was done in a StepOne Plus qRT-PCR system (Applied Biosystems StepOne™ Instrument). Taq DNA polymerase activation was done at 95 °C for 1 min, then, 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 15 sec at 95 °C. After the polymerization step at 60 °C, the amplification produced a fluorescent signal. StepOne Software V.2.2.3 was used to determine the threshold and baselines and cycle threshold (CT) value was calculated using 2 –ΔΔCT (Livak & Schmittgen, 2001). Each well was analyzed in replicate and the ΔCT value was obtained by subtracting the GAPDH CT from CT of gene of interest. The mRNA levels in each group were shown as a proportion of the control.

All the equipment used were sourced from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

2.3. Data analysis

We expressed the results as average ± standard deviation of triplicate readings. The mean was analyzed using one-way Analysis of
Variance, Turkey's post hoc test was done and significant difference was taken at $p < 0.05$ using Graph pad Prism Statistical software V.5.0.

3. Results

The effect of diets supplemented with 10 mg/g of mistletoe-infested (a) moringa and (b) almond leaves on mRNA expression level of *Drosophila* insulin-like peptide-2 (*Dilp2*) gene of sucrose-induced diabetic flies is presented in Fig. 2. This revealed that neither high sucrose diet nor treatment with mistletoe-infested and non-infested moringa and almond leaves caused any substantial modification in mRNA expression of *Sod* gene in relation to both normal and sucrose controls. Nevertheless, diet supplementation of 15% sucrose-induced diabetic flies with mistletoe-infested moringa leaf gave rise to significantly ($p < 0.05$) marked upregulation of *Sod* gene when likened to both normal and sucrose controls, and the non-infested moringa leaf. Likewise, treatment of the non-sucrose fed flies with parasitized almond leaf resulted in significantly ($p < 0.05$) elevated mRNA expression of *Sod* gene when equated to non-sucrose control.

Fig. 3 depicts the effect of diets supplemented with 10 mg/g of mistletoe-infested (a) moringa and (b) almond leaves on superoxide dismutase (*Sod*) gene of sucrose-induced diabetic flies. The result revealed that neither high sucrose diet nor treatment with mistletoe-infested and non-infested moringa and almond leaves caused any substantial modification in mRNA expression of *Sod* gene in relation to both normal and sucrose controls. Nevertheless, diet supplementation of 15% sucrose-induced diabetic flies with mistletoe-infested moringa leaf gave rise to significantly ($p < 0.05$) marked upregulation of *Sod* gene when likened to both normal and sucrose controls, and the non-infested moringa leaf. Likewise, treatment of the non-sucrose fed flies with parasitized almond leaf resulted in significantly ($p < 0.05$) elevated mRNA expression of *Sod* gene when equated to non-sucrose control.

The effect of diets supplemented with 10 mg/g of mistletoe-infested (a) moringa and (b) almond leaves on heat shock protein (*Hsp70*) gene of sucrose-induced diabetic flies is presented in Fig. 4. The result revealed that the sucrose-induced diabetic groups showed substantial ($p < 0.05$) marked increase in mRNA expression of *Hsp70* gene in comparison to control. Nonetheless, diet supplementation of 30% sucrose-induced diabetic flies with mistletoe-infested and non-infested moringa leaves brought about substantial ($p < 0.05$) down-regulation in the transcript levels of *Dilp2* gene in comparison to 30% sucrose control group. Likewise, treatment of diabetic flies with infested almond leaves led to significantly ($p < 0.05$) notable downregulation of *Dilp2* transcript levels in relation to sucrose controls.
4. Discussion

Our previous studies have revealed that mistletoe infestation may cause enhanced phytochemical constituents coupled with improved antioxidant and antidiabetic activities of moringa and almond host plants at proteomics level (Oyeniran et al., 2020, 2021, 2022). Here, we further inquired into the influence of mistletoe infestation on the mRNA expression of antioxidants and insulin-like peptides genes. Considering the hyperglycemic state associated with the consumption of high sucrose diets, this condition disrupts insulin signaling pathways and triggers oxidative events; hence, the mRNA transcript levels of insulin responsive and oxidative stress genes were investigated.

The *Drosophila* insulin-like peptides (most importantly, *Dilp2*) share the same sequence, functional and structural resemblances with the vertebrate insulin and insulin receptors. They are involved in the regulation of both growth and glucose homeostasis (Kang et al., 2020; Musselman et al., 2011). The increased expression of *Dilp2* mRNA transcripts in adult flies which eclosed from larva fed with sucrose-rich diets corresponds with earlier studies where enhanced *Dilps* mRNA levels were reported in flies raised on rich sucrose diet (Musselman et al., 2011; Pasco & Leopold, 2012). This further supports the findings that linked high sugar diets with insulin signaling dysregulation and the induction of type-2 diabetes mellitus. This is in tandem with our recent reports (Oyeniran et al., 2020, 2021) whereby high sucrose diet fed flies had elevated levels of glucose, trehalose, insulin-like peptides, triglycerides and insulin-like peptides when compared with their controls. These catastrophic effects of high sucrose diets on regulation of carbohydrate metabolism were ameliorated by treatment with the mistletoe-infested and non-infested moringa and almond leaves. The downregulation of *Dilp2* mRNA transcripts by *Moringa* and *Almond* leaves could be related to the antioxidant properties of their phytochemical compounds (Ademiluyi et al., 2018; Ahmed, 2005). Studies have shown that phytoconstituents are crucial in insulin signaling pathways as they are able to enhance glucose uptake by improving translocation of glucose transporter-4 (GLUT4), secretion of adiponectin and peroxisome
proliferator-activated receptor gamma (PPAR-γ) activity in human adipocytes (Scacciachio et al., 2011). Furthermore, the observed higher downregulation of Dilp2 by mistletoe-infested moringa and almond leaves could be due to the higher phenolic constituents in these leaves (Tables 2a and 2b), as foods rich in polyphenols have been identified as potent anti-diabetic agents (Ademiluyi et al., 2018; Djeridane et al., 2015).

Chronic extracellular hyperglycemia causes elevated production of reactive oxygen species by the mitochondrial electron-transport chain and thus leads to disturbed cell redox state (Lushchak, Zayachkivska, & Vaiserman, 2015; Zephy & Ahmad, 2015). Therefore, increased oxidative stress levels and chronic inflammation are metabolic effects associated with type-2 diabetes (Rovira-Llopis et al., 2017; Tangvarasittichai, 2015). Thus, the mRNA expression of the antioxidant enzyme; superoxide dismutase (Sod) gene, was also disrupted in the newly eclosed adult flies raised on high sugar diets. Nevertheless, treatment with the leaves enhanced the mRNA transcript level of Sod, however, the elevated expression level of Sod gene by mistletoe-infested moringa leaf might be due to the greater ability of the leaf to activate coordinated transcriptional upregulation of Sod antioxidant enzymes as the mistletoe infested moringa leaf had higher phenolic compounds (Table 2a). A corresponding increase in the level of mRNA transcripts of antioxidant enzymes following treatment with phenolic compounds have also been observed in previous studies (Spanier et al., 2009). This corresponds with the activities of some antioxidant enzymes (particularly Sod) reported in our earlier studies (Oyeniran et al., 2020, 2021).

Hsp70 is the principal heat shock protein in Drosophila with 70 Kd protein as its product. It is a crucial intermediate of the heat shock factor-1 (HSF-1)-stress response pathway. It functions majorly in protein folding, trafficking and degradation where they inhibit the formation of protein aggregates and act as molecular chaperones in non-stressed cells (Parsell & Lindquist, 1993). The activation of Hsp70 gene in sucrose-induced diabetic flies could probably relate with cellular response to

Table 2a

| Compounds           | Infested | Non-infested (Min) | Retention Time |
|---------------------|----------|-------------------|---------------|
| Catechin            | 1.94 ± 0.00b | 0.01 ± 0.00b | 9.74         |
| Protocatechaeic acid| 17.59 ± 0.22b | 0.02 ± 0.01b | 10.65     |
| P-coumaric acid     | 96.43 ± 0.64a | 133.58 ± 0.59a | 11.06     |
| Vanillic acid       | 16.22 ± 0.15a | 18.49 ± 0.60a | 11.68     |
| P-hydroxybenzoic acid| 16.73 ± 0.26a | 0.01 ± 0.00a | 13.29    |
| Gallic acid         | 1.88 ± 0.02a | 0.01 ± 0.00a | 13.75     |
| Caffeic acid        | 6.89 ± 0.05a | 7.05 ± 0.11a | 14.16     |
| Ferulic acid        | 128.41 ± 0.43a | 124.89 ± 0.39a | 14.84     |
| Syringic acid       | 58.45 ± 0.26a | 105.92 ± 0.15b | 15.51     |
| Apigienin           | 1.74 ± 0.00a | 0.00 ± 0.00a | 17.55     |
| Naringenin          | 94.67 ± 0.41a | 123.36 ± 0.41a | 18.06     |
| Kaempferol          | 49.00 ± 0.82a | 45.98 ± 0.15a | 19.52     |
| Quercetin           | 147.92 ± 0.48a | 338.49 ± 0.20b | 22.60     |
| Myricetin           | 79.51 ± 0.59b | 62.42 ± 0.11a | 24.20     |
| Isohamnetin         | 131.23 ± 0.15b | 117.21 ± 0.49b | 24.62     |
| Chlorogenic acid    | 47.44 ± 0.58b | 13.50 ± 0.18b | 25.70     |
| Rutin               | 41.68 ± 0.23a | 35.44 ± 0.51a | 27.80     |

Values represent mean ± standard deviation of triplicate readings. Superscripts with different alphabets along the same row are significantly (p < 0.05) different.

Table 2b

| Compounds           | Infested | Non-infested (Min) | Retention Time |
|---------------------|----------|-------------------|---------------|
| Catechin            | 1.94 ± 0.00b | 0.01 ± 0.00b | 9.74         |
| Protocatechaeic acid| 17.59 ± 0.22b | 0.02 ± 0.01b | 10.65     |
| P-coumaric acid     | 96.43 ± 0.64a | 133.58 ± 0.59a | 11.06     |
| Vanillic acid       | 16.22 ± 0.15a | 18.49 ± 0.60a | 11.68     |
| P-hydroxybenzoic acid| 16.73 ± 0.26a | 0.01 ± 0.00a | 13.29    |
| Gallic acid         | 1.88 ± 0.02a | 0.01 ± 0.00a | 13.75     |
| Caffeic acid        | 6.89 ± 0.05a | 7.05 ± 0.11a | 14.16     |
| Ferulic acid        | 128.41 ± 0.43a | 124.89 ± 0.39a | 14.84     |
| Syringic acid       | 58.45 ± 0.26a | 105.92 ± 0.15b | 15.51     |
| Apigienin           | 1.74 ± 0.00a | 0.00 ± 0.00a | 17.55     |
| Naringenin          | 94.67 ± 0.41a | 123.36 ± 0.41a | 18.06     |
| Kaempferol          | 49.00 ± 0.82a | 45.98 ± 0.15a | 19.52     |
| Quercetin           | 147.92 ± 0.48a | 338.49 ± 0.20b | 22.60     |
| Myricetin           | 79.51 ± 0.59b | 62.42 ± 0.11a | 24.20     |
| Isohamnetin         | 131.23 ± 0.15b | 117.21 ± 0.49b | 24.62     |
| Chlorogenic acid    | 47.44 ± 0.58b | 13.50 ± 0.18b | 25.70     |
| Rutin               | 41.68 ± 0.23a | 35.44 ± 0.51a | 27.80     |

Values represent mean ± standard deviation of triplicate readings. Superscripts with different alphabets along the same row are significantly (p < 0.05) different.

The effect of diets supplemented with mistletoe-infested (a) moringa and (b) almond leaves on relative transcript level of heat shock protein-70 gene of sucrose-induced diabetic flies. Data are presented as plot of mean with standard deviation (n = 3). # represents significant difference at p < 0.05 from normal control; * represents significant difference at p < 0.05 from sucrose control, $ represents significant difference at p < 0.05 from non-infested leaf. Keys: CTR: Control, NIM: Non-Infested Moringa, IM: Infested Moringa, NIA: Non-Infested Almond, IA: Infested Almond.
oxidative disturbances induced by the diet. This is in tandem with previous studies where newly eclosed flies from larva grown on rich sucrose diet had increased oxidative events (Ecker et al., 2017; Pendse et al., 2013). Treatment with mistletoe-infested and non-infested moringa and almond leaves normalized the mRNA transcription of Hsp70. However, the observed higher downregulation in the expression of Hsp70 gene by mistletoe-infested moringa leaf may possibly relate to the stimulatory effects of polyphenols on transcription of genes associated with antioxidant defense system (Kang et al., 2020; Nicholson, Tucker, & Brameld, 2008). This observation is in agreement with our earlier reports whereby mistletoe-infested moringa and almond leaves had higher polyphenolic contents (Tables 2a and 2b) coupled with improved antioxidants and antiadiabetes activities at proteomics level (Oyeniran et al., 2020, 2021, 2022).

5. Conclusion

This study further established that feeding Drosophila melanogaster with high sucrose diet activates phenotypic responses that agree with insulin signaling disruption and redox status imbalance in diabetic state. However, treatment with mistletoe-infested and non-infested moringa and almond leaves brought about higher upregulation of Sod and downregulation of Hsp70 and Dlipz2 mRNA levels with the mistletoe-infested Moringa and Almond host leaves having higher positive effects. Hence, we opined that infestation of Moringa and Almond trees with mistletoe resulted in improved mRNA expression of antioxidant and insulin-like peptide genes; this may be the mechanisms involved in regulation of blood glucose and oxidative stress. Our findings suggest that consumption of mistletoe-infested Moringa and Almond leaves could possibly cause enhanced expression of antioxidant and hypoglycemic genes.

Ethical approval

Currently, there are no ethical approvals or restrictions in the use of fruit fly as a model for research. However, the use of fruit fly as a model organism is in compliance with the 3Rs protocol of the European Centre for the Validation of Alternative Methods on 3Rs-Refinement, replacement and reduction.

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CRediT authorship contribution statement

Olubukola H. Oyeniran: Resources, Investigation, Methodology, Writing – original draft. Ganiyu Oboh: Conceptualization, Project administration, Resources, Writing – review & editing. Adedayo O. Ademiluyi: Supervision, Resources, Validation, Visualization, Writing – review & editing. Haruna 1. Umar: Resources, Investigation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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