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The effect of amaranth oil on autoantibodies in lupus prone mice

Danik Martirosyan[1,2], Jack Hutcheson[1], Deena Sajitharan[1], Samantha Williams[2], Chandra Mohan[1]

[1]Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, TX, USA; [2]Functional Food Center/Functional Food Institute, Dallas, TX, USA

***Corresponding Author:** Danik Martirosyan, PhD, Functional Food Center/Functional Food Institute, Dallas, TX, 75254, USA

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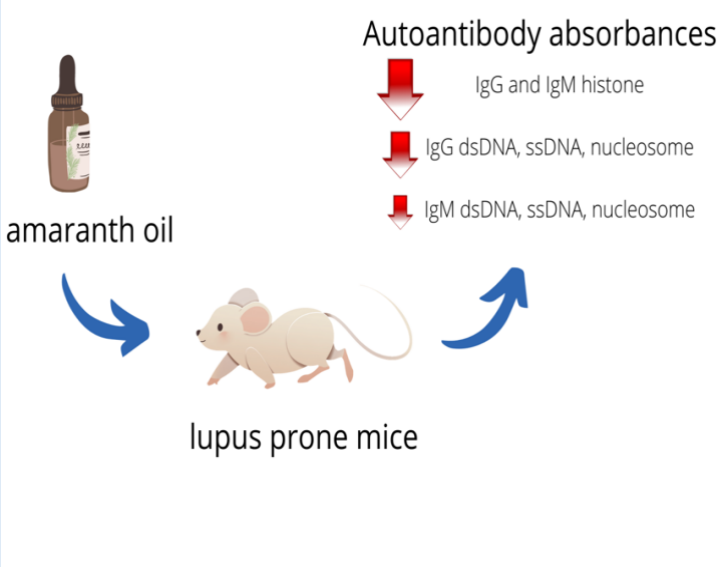
ABSTRACT

Background: Autoimmune diseases, such as systemic lupus erythematosus, can have severe impacts on quality of life. They are one of the leading causes of death for women in the United States. Distinguished by the body damaging its own tissues and organs, they are often classified and diagnosed based on autoantibody levels. Treatments often include immunosuppressant drugs, which can have adverse effects.



Aim of Study: Amaranth is a good functional food candidate, possessing antioxidants, bioactive compounds, and a variety of health benefits, such as lowering cholesterol, and aiding diabetes and hypertension. Previous studies have largely focused on the grain or seed, but amaranth oil is less explored. This study examines whether orally

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administered amaranth oil had any effects on autoantibodies and splenic immune cell populations in murine subjects.

Methods: Mice in the experimental group (n = 3) were given 4µl of amaranth oil per gram of mouse weight for 5 days a week over 84 days. Control mice (n = 2) were sham treated on the same schedule with no oil. To determine autoantibody levels, enzyme-linked immunosorbent assays (ELISAs) were first conducted on wells pre-coated with double stranded DNA, single stranded DNA, histones, or double stranded DNA and then histones (nucleosomes). Autoantibody presence was quantified by measuring absorbance at 405nm. Splenic cell populations were examined with flow cytometry and compared using a student's t-test.

Results: Compared to the control group, the mice receiving amaranth oil showed decreased IgG and IgM histone autoantibody absorbance levels throughout the whole study. IgG dsDNA, ssDNA, and nucleosome autoantibody absorbances were lower than that of the control group for the first 42 days. IgM dsDNA, ssDNA, and nucleosome autoantibody absorbances were lower only for the first 14 days. There were no significant differences found amongst splenic immune cell populations between the control and experimental groups.

Conclusion: These preliminary data show that amaranth oil may help decrease autoantibody levels in lupus prone murine subjects. However, given the small number of subjects in this study, further research is needed to confirm observed effects and determine the most effective dose and administration schedule.

Keywords: autoantibody, IgG, IgM, histone, dsDNA, ssDNA, nucleosome, amaranth oil, immunoglobulin, lupus

INTRODUCTION

Autoimmune diseases, such as systemic lupus erythematosus (SLE), can have chronic debilitating effects and thus significant impacts on quality of life. They are one of the leading causes of death for young and middle-aged women in the United States [1-2]. Autoimmune diseases encompass a wide variety of diseases that afflict people from all backgrounds and are estimated to affect 2.5-5% of the general population [3-6]. Treatments generally include immunosuppressants. Immunosuppressant drugs have a range of side effects; after receiving high doses, side effects can become more severe and treatments can lose their efficacy [7]. Finding functional food natural alternatives with bioactive compounds and potentially less severe side effects would therefore be beneficial.

The adaptive immune system utilizes a group of leukocytes, the lymphocytes. Particularly, T and B lymphocytes (T cells and B cells) recognize antigens; B cells produce immunoglobulins (Ig), or circulating antibodies, while T cells are more involved with

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intracellular pathogens and regulating immune responses [8]. The five isotypes include IgG, IgM, IgA, IgD, and IgE. IgM and IgG in particular are known to fight against infections [9-10]. Autoantibodies are defined as immunoglobulins that attack self-antigens and are a distinguishing factor in autoimmune diseases [9, 11]. While autoantibodies are also observed in healthy individuals, their presence also plays an important role in disease likelihood, diagnosis, prognosis, and even tissue damage [3, 6, 12]. Diseases such as type 1 diabetes and adrenalitis are identified as autoimmune based on autoantibody levels. Antinuclear antibodies (ANAs) and IgG antibodies to dsDNA are often present in cases of systemic lupus erythematosus (SLE) and rheumatoid arthritis [12]. They have even previously been shown to precede clinical onset of SLE and rheumatic diseases [13]. Because of their high specificity, antibodies against double-stranded (ds) DNA are considered the most characteristic marker for diagnosing SLE [14-17]. However, they are also associated with renal disease, rheumatoid arthritis, HIV, type 1 autoimmune hepatitis, myeloma, parvovirus B19 [15, 18-19]. Similarly, anti-nucleosome antibodies are associated with SLE, rheumatoid arthritis, Sjögren's syndrome, and systemic sclerosis [15, 17, 20-21]. Anti-histone antibodies have been associated with SLE, rheumatoid arthritis, systemic sclerosis, Alzheimer's disease, dementia, autoimmune hepatitis, drug-induced lupus, primary biliary cirrhosis, infection, as well as linear sclerosis severity [15, 17, 22-25]. Anti single-stranded (ss) DNA antibodies are an indicator of flares in SLE, linear sclerosis severity, and have been correlated to preeclampsia and malaria [24, 26-28]. Enzyme-linked immunosorbent assay (ELISA) is one of the most common methods used in detecting autoantibodies [29].

Amaranth is an increasingly popular tropical plant dating back to the Aztecs, Inca, and Maya, who consumed it in their daily diet [30-31]. Amaranth has previously been shown to have antioxidants and possess a variety of health benefits, such as lowering cholesterol, and aiding diabetes and hypertension [32-34]. These properties stem from amaranth being rich in monosaturated fatty acids, polyunsaturated fatty acids, squalene, linoleic acids, tocopherols, and essential amino acids [32-33, 35]., Squalene and tocopherol are antioxidative, resulting in high oxidative stability in amaranth oil [35]. Past researchers have shown that amaranth can decrease total cholesterol and low density lipoprotein; the hypocholesterimic effect has been attributed to squalene, fatty acids, proteins, amino acids, as well as a combination of constituents [33]. Amaranth was shown to decrease blood glucose and increase serum insulin in streptozocin-induced rats [33- 34]. Notably, conjugated linoleic acid has previously been shown to decrease signs of SLE, including autoantibodies and splenomegaly [36]. Moreover, compared to other cereals, amaranth has a high proportion of lysine and tryptophan, rivaling that of animal sources [33, 35, 37].

It was also recently found that amaranth proteins contain bioactive peptides, making amaranth a strong functional food candidate [38-39].

A few studies examined foods containing amaranth and found that consuming these products still resulted in antihypertensive effects [39-43]. Additionally, amaranth somewhat maintains its favorable composition even after being cooked, raising the possibility that it can be incorporated in functional food products. Puffing or popping *Amaranthus cruentus* seeds reduced unsaturation levels (75.5 to 62.3%), and linoleic acid (46.8 to 27%), but increased squalene by 15.5% [44-45]. Popping and cooking decreased lipid contents in *A. cruentus* seeds by 1.7 and 3.7%, respectively [45-46]. *A. cruentus* flour maintained its unsaturated fatty acid levels at 75.44%, and amaranth oil exhibited oxidation stability better than sunflower oil [45, 47]. In terms of immune effects, Hibi et al. found that amaranth can inhibit IgE production and therefore possibly allergies [48-49]. This study examines the effect of amaranth oil on autoantibody and splenic cell population levels.

METHODS and MATERIALS

Mice: B6.Sle1.Sle2.Sle3 mice (n = 5) were bred and housed at University of Texas Southwestern Medical Center. Mice were aged to 7-9 months of age prior to the start of the study and all experiments were performed on male mice. All studies were conducted with the prior approval of the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Amaranth Oil Treatment: Amaranth oil extracted from *Amaranthus hybridus* L. (Amaranthaceae) was obtained from Russian Oliva. Mice in the experimental group (n = 3) received 4µl of amaranth oil per gram of mouse weight by oral gavage 5 days per week over the course of 84 days. Control mice (n = 2) were sham treated on the same schedule with an oral gavage needle containing no oil.

Autoantibody ELISAs: Serum was collected from the mice biweekly and assayed for the presence of nuclear autoantibodies. Briefly, samples were added into 96-well plates pre-coated with double stranded DNA, single stranded DNA, histones, or double stranded DNA and then histones (nucleosomes). An alkaline phosphatase-conjugated anti-IgG or anti-IgM secondary antibody was then added to the plate and autoantibody titers were determined by the absorbance read at 405nm on an ELx808 plate reader (BioTek, Winooski, VT) horseradish peroxidase-conjugated rabbit anti-mouse IgG or IgM antibodies were added.

Flow cytometry: At the conclusion of the 84 days of study, spleens were isolated from mice following sacrifice and perfusion. A single cell suspension was obtained by crushing the spleen through a 100-micron mesh filter and washing the cells with DMEM. Red blood cells were lysed using PharmLyse (BD Biosciences, San Diego, CA). Cells were stained with antibodies against AA4.1, B220, CD4, CD5, CD8, CD11b, CD11c, CD21/35, CD23, CD45, CD62L, CD69, CD80, CD138, GL-7, Gr-1, I-A/IE (BD Biosciences, San Diego, CA) and F4/80 (Invitrogen, San Diego, CA). All samples were run on an LSRII flow cytometer (BD

Biosciences, San Diego, CA) in the University of Texas Southwestern Medical Center Flow Cytometry Core.

Statistics: All statistical analyses were done using a two-tailed Student's T-test with Welch's correction.

RESULTS

Autoantibodies: The group receiving 4µl of amaranth oil for 5 days a week showed decreased absorbances for all autoantibodies studied for the first 14 days. However, only the IgG (Figure 1A, Table 1A) and IgM (Figure 1B, Table 1B) histone autoantibody absorbances were lower throughout the entire 84 days of study. For IgG dsDNA, ssDNA, and nucleosome, absorbances were lower for only the first 42 days. After the 42nd day, as depicted in Figure 2A and Table 2A, IgG dsDNA autoantibody absorbance increased to higher than that of the control before decreasing after the 70th day; the same was observed for IgG ssDNA (Figure 2B and Table 2B). Illustrated in Figure 2C and Table 2C, IgG nucleosome autoantibody absorbance was lower in the experimental group for the first 56 days before increasing to above that of the control, then decreasing after the 70th day. As shown in Figure 3A and Table 3A, IgM dsDNA absorbance was lower in the experimental group for the first 28 days before increasing to approximately the same or higher than the control. Displayed in Figure 3B and Table 3B, IgM ssDNA absorbances were lower in the experimental group for only the first 14 days; the same was seen with IgM nucleosome (Figure 3C and Table 3C). After the 14th day, these absorbances generally were higher or the same as that of the control group.

Table 1A. Average IgM histone autoantibody absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.5075	0.65	0.6925	0.4415	0.672	0.527	0.58
4µl/g	0.3575	0.401333	0.363	0.403	0.4615	0.3735	0.4505
Differences	0.15	0.248667	0.3295	0.0385	0.2105	0.1535	0.1295

Table 1B. Average IgG histone autoantibody absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.2035	0.353	0.324	0.249	0.3295	0.1855	0.2885
4µl/g	0.125	0.112333	0.127	0.1875	0.1995	0.142	0.1465
Differences	0.0785	0.240667	0.197	0.0615	0.13	0.0435	0.142

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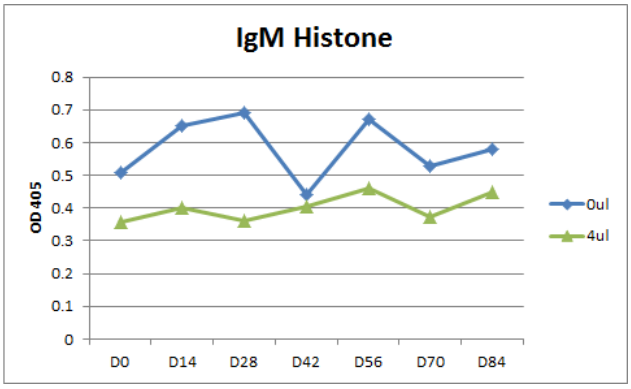

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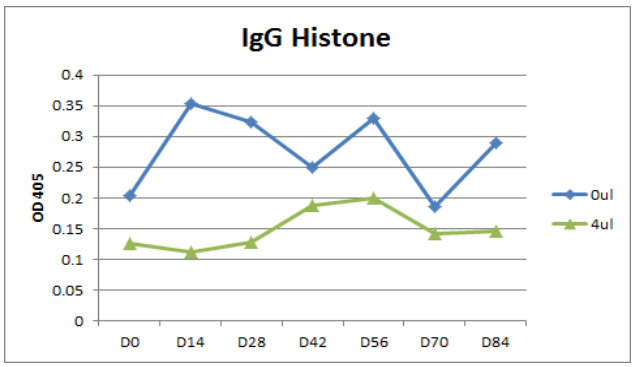

Figure 1A, 1B. Average IgM and IgG histone autoantibody absorbance levels for control and experimental groups over the 84 days of experimentation.

Table 2A. Average IgG dsDNA absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.708	0.69	0.612	0.622	0.6005	0.754	0.587
4µl/g	0.3945	0.388667	0.436	0.5015	0.682	0.701	0.6675
Differences	0.3135	0.301333333	0.176	0.1205	-0.0815	0.053	-0.0805

Table 2B. Average IgG ssDNA absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.8985	0.925	0.8525	0.8485	0.8255	0.877	0.823
4µl/g	0.614	0.612	0.725667	0.7255	0.8525	0.9495	0.793
Differences	0.2845	0.313	0.126833	0.123	-0.027	-0.0725	0.03

Table 2C. Average IgG nucleosome absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.753	0.752	0.645	0.712	0.574	0.6125	0.6665
4µl/g	0.5235	0.471333	0.483	0.535	0.52	0.6645	0.671
Differences	0.2295	0.280667	0.162	0.177	0.054	-0.052	-0.0045

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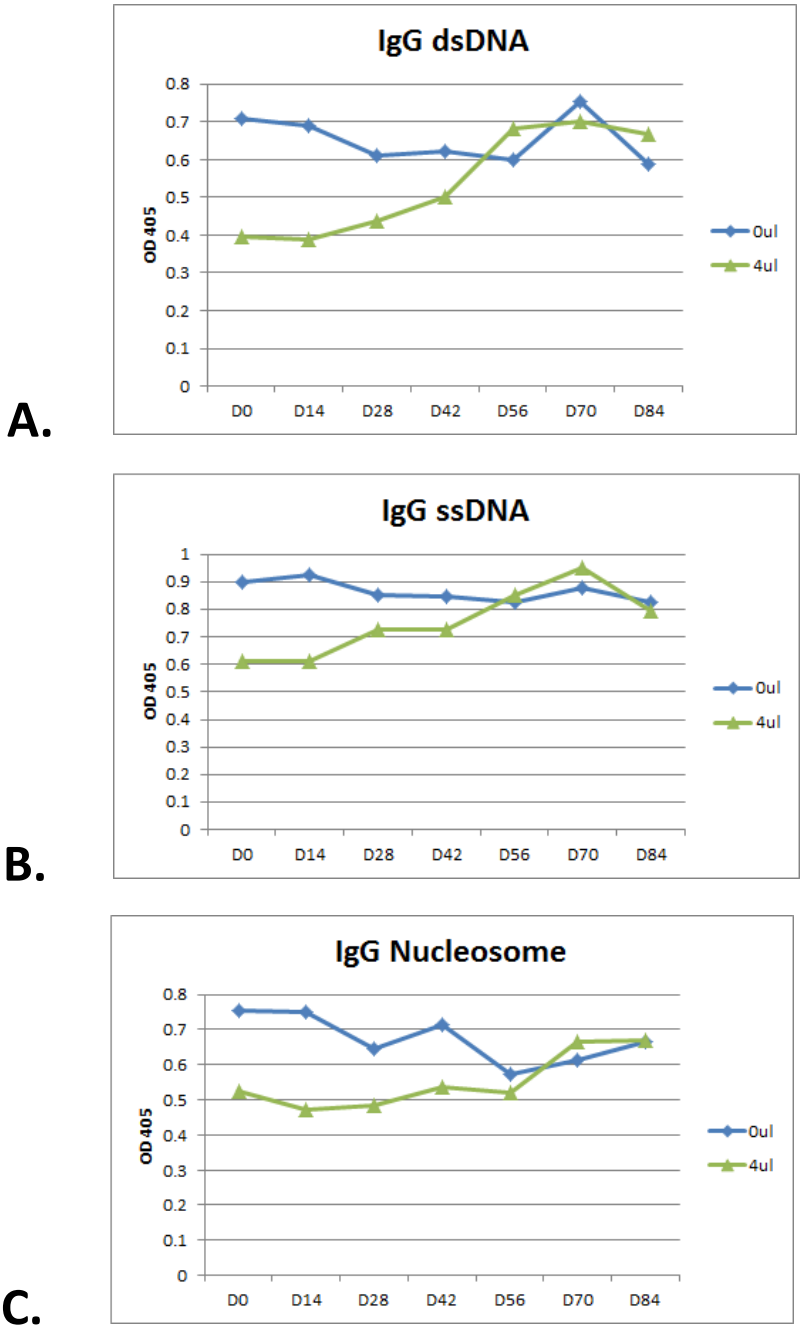

Figure 2A, 2B, 2C. Average IgG dsDNA, ssDNA, and nucleosome absorbance levels for control and experimental groups over the 84 days of experimentation.

Table 3A. Average IgM dsDNA absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.4315	0.6035	0.448	0.4265	0.4015	0.374	0.238
4µl/g	0.348	0.504667	0.424	0.4495	0.4165	0.516	0.516
Differences	0.0835	0.098833	0.024	-0.023	-0.015	-0.142	-0.278

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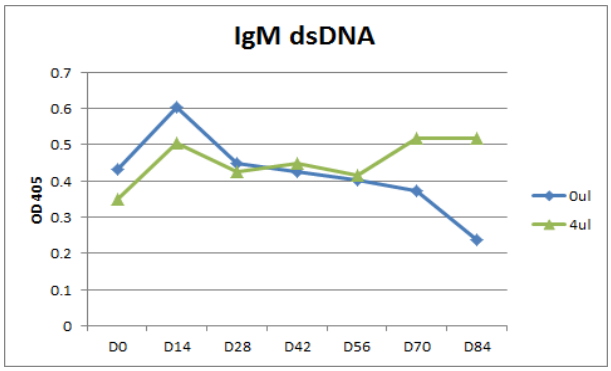
Table 3B. Average IgM ssDNA absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.5345	0.7625	0.5065	0.6325	0.5095	0.4285	0.6635
4µl/g	0.3335	0.563	0.542333	0.5905	0.527	0.6525	0.789
Differences	0.201	0.1995	-0.03583	0.042	-0.0175	-0.224	-0.1255

Table 3C. Average IgM histone absorbance levels for control, experimental group, and their differences over the 84 days of experimentation.

	D0	D14	D28	D42	D56	D70	D84
0µl/g	0.488	0.578	0.405	0.47	0.3875	0.4145	0.452
4µl/g	0.405	0.501	0.465	0.5085	0.3685	0.5355	0.455
Differences	0.083	0.077	-0.06	-0.0385	0.019	-0.121	-0.003

A.



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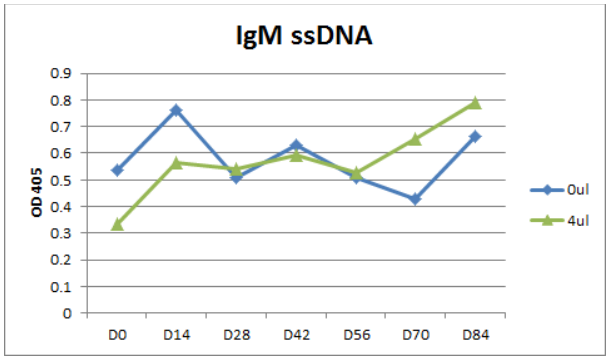

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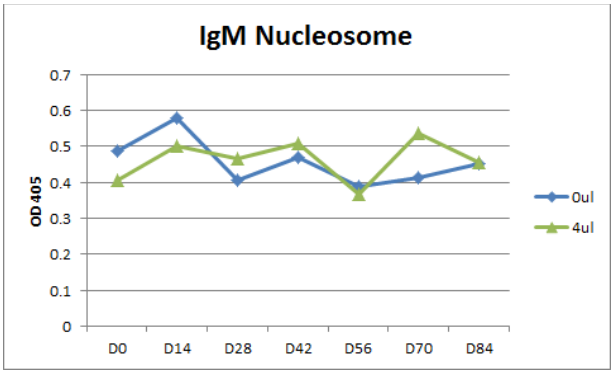

Figure 3A, 3B, 3C. IgM dsDNA, ssDNA, and nucleosome absorbance levels for control and experimental groups over the 84 days of experimentation.

Flow Cytometry: There were no significant differences found amongst the splenic immune cell populations tested between the control and experimental groups.

DISCUSSION

Since the sample sizes were so small, the data is preliminary and only limited conclusions can be drawn. The study began with two dosage groups, 4µl and 1µl, with three mice each, as well as a control group consisting of two mice. Two mice in the 1µl group passed away, leaving n = 1 for that group; the data for that one mouse are not reported, but are in line with the presented data. The presented data thus includes n = 3 for the 4µl experimental group and n = 2 for the control group. This study also utilized lupus prone mice, so it is possible that results may differ in non-lupus prone subjects. That being said, the results are intriguing: decreased absorbances for all autoantibodies were observed for the first 14 days in the group receiving 4µl amaranth oil per gram of mouse weight. It has been shown that curcumin can decrease anti-dsDNA levels [50], but similar results with amaranth oil possibly decreasing histone, nucleosome, ssDNA, and dsDNA autoantibodies have not previously been presented. Moreover, fish oil can help lipid metabolism in SLE, in turn aiding immune, atherosclerotic, and inflammatory events, due to its omega-3 polyunsaturated fatty acids [51-53].

Lupus is associated with changes in lipid metabolism, especially increased oxidative stress. Lipid alteration and oxidative stress in turn play a role in T lymphocyte dysfunctions and systemic inflammation, suggesting lipids are involved in their regulation [51]. Since amaranth oil has previously been shown to reduce oxidative stress and is rich in squalene and polyunsaturated fatty acids [32, 48, 54], the decreased autoantibody levels make sense. n-3 polyunsaturated fatty acids are known to suppress antigen presentation, as well as T cell activation and proliferation [55-58]. Including polyunsaturated fatty acids in the diet also has immunosuppressive effects [56-58].

Absorbances for IgG nucleosome, ssDNA, and dsDNA autoantibodies were lower than the control for the first 42 days. As previously mentioned, anti-nucleosome, anti-ssDNA, and anti-dsDNA autoantibodies are associated with various diseases or disease severity. Anti-dsDNA, in particular, strongly correlates to disease activity for all isotypes [59]. Normal subjects generally have IgM ssDNA antibodies, but IgG antibodies to dsDNA are less frequent [60]. IgG anti-dsDNA antibodies are a distinguishing factor in SLE and are generally believed to play a role in the pathogenesis of SLE. They also have a relationship with nephritis disease activity [61]. Though anti-dsDNA autoantibodies are specific for SLE diagnosis, anti-nucleosome autoantibodies actually have a better correlation with SLE disease activity and lupus nephritis [25]. In most studies, the sensitivity for anti-nucleosome antibodies was higher than that of anti-dsDNA. As such, it is interesting that all three autoantibodies showed a somewhat similar effect in response to the regular doses of amaranth oil.

One of the most consistent and pronounced effects were observed with IgM and IgG histone autoantibodies; lower absorbances were observed for the entire 84 days of study. This is curious but encouraging since the other autoantibodies measured eventually showed an increase in absorbance. While anti-histone autoantibodies are generally not very pathogenic or useful in disease monitoring/diagnosis, they are often present in

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patients with many autoimmune diseases, particularly SLE, drug-induced lupus, and primary biliary cirrhosis [25, 62-63].

Absorbances for IgM nucleosome, ssDNA, and dsDNA were a little more erratic, but nonetheless were lower for the first 14 days. The inconsistency may be due to IgM's potential protective effects. IgM anti-dsDNA antibodies are present in SLE, rheumatoid arthritis, autoimmune liver disease, and Sjögren's syndrome [61]. While autoantibodies are a hallmark for autoimmune diseases, natural autoantibodies, mostly IgM, are found in healthy individuals [64]. It has even been suggested that mice lacking IgM antibodies could accelerate SLE [64]. IgM antibodies against dsDNA were previously shown to delay proteinuria, reduce renal pathological severity and increase lifespans of lupus prone murine subjects [61]. They also had a very strong negative correlation with glomerulonephritis, and appear to possess a protective effect for nephropathy [15, 61, 64]. Similarly, anti-ssDNA antibody may be related to the severity of hypertension and proteinuria [27].

Previous researchers thought IgM anti-dsDNA antibodies might hinder IgG anti-dsDNA antibody production, leading to milder disease activity and some protection from lupus nephritis [59, 64]. As such, while amaranth oil may not provide an immunosuppressive effect for IgM as strong as it seems to for IgG, it is not necessarily a downfall. It may end up being more optimal for prospective treatments.

Though autoantibodies exist in healthy subjects, they are the distinguishing characteristic of autoimmune diseases. Since no cure exists for these diseases, treatment involves immunosuppressants, which can have adverse effects on the patient; so perhaps incorporating amaranth oil into the treatment regimen may be beneficial. Previous researchers have largely focused on the grain or seed, but this study shows amaranth oil may also be valuable.

Amaranth oil is a promising functional food candidate. As noted earlier, it provides several health benefits, exhibits antioxidant protective effects and possesses bioactive compounds, such as squalene. Past researchers demonstrated that it can significantly decrease total cholesterol, low density cholesterol, and triglycerides [32]. It has also been shown to have hepatoprotective effects [65]. The Functional Food Center states a functional food should contain defined bioactive compounds and non-toxic dosages that provide a clinically proven and documented health benefit, utilizing specific biomarkers [66-69]. Resultantly, since our data are preliminary and based on a small sample size, we cannot definitively say whether amaranth or amaranth oil is a functional food in terms of lupus. Future studies should determine if the possible autoantibody lowering effects of amaranth oil are replicable in a larger sample size of lupus prone murine subjects. If confirmed, then clinical studies would be needed to verify amaranth oil effectiveness in decreasing autoantibodies. The most effective dosage and administration schedule should also be determined.

CONCLUSION

This study demonstrated the potential autoantibody lowering effects of amaranth oil in murine subjects. Given the small sample size, the data are preliminary and future studies are needed to confirm the observed effects. Considering the results and amaranth oil's health properties and bioactive compounds, it is a good functional food candidate.

Abbreviations: Ig: immunoglobulin, ANA: antinuclear antibody, SLE: systemic lupus erythematosus, dsDNA: double-stranded deoxyribonucleic acid, ssDNA: single-stranded deoxyribonucleic acid

Conflicts of Interest: There are no conflicts of interest associated with this study.

Authors' contribution: The original idea was conceived by DM and was discussed with CM. The main focus and ideas of the experiments finally agreed with JH and DS. The experiments were conducted and analyzed by DS, JH and DM. Experimental data was analyzed by DS and JH, and discussed with DM and CM. The main text of the paper including methods were written by SW, JH, and DM. The manuscript was revised, edited, and formatted by DM and SW.

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