Preparation of Phytochemicals from *Moringa oleifera* for Preventative Health

by

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Abstract

Moringa oleifera (MO) is a plant species native to Africa and India that is rich in nutrients and bioactive materials. The phytochemicals within MO have been found to possess significant anti-inflammatory, anti-cancer, and anti-microbial properties, which make it a useful natural product to characterize. Our aims are to **1)** extract plant derived chemicals from MO leaves, **2)** provide standardization for the extraction process, **3)** identify bioactives in fresh and oxidized MO samples that initiate positive responses to health conditions, and **4)** test bioactivity of MO samples using *C. elegans* models and reactive oxygen species (ROS) generation. Ethanol (80%) was found to be the best solvent for phytochemical extraction, with greater Total Phenolic Content (TPC), and absorbance measurements resulting from polar solvents. GC-MS and LC-MS identification of MO samples returned varying anti-inflammatory, anti-cancer, anti-microbial, and anti-oxidant compounds, such as morin, quercitrin, rutin, and syringic acid. Oxidized and fresh MO samples reported differing phytochemicals, with fresh samples containing more phenolics and anti-inflammatory compounds. Variation in oxidized and fresh samples could be due to autoxidation, or differences in growth environment. *C. elegans* treated with MO dilutions showed slight declines in ROS production.

Keywords: *Moringa oleifera, anti-inflammatory, oxidation, phenolic content, reactive oxygen species*

Introduction

Moringa oleifera Lam. (MO) is a soft tree species native to Africa, the Middle East, and Asia[1]. MO has previously been used as a nutritional supplement, ingredient in regional dishes, cosmetic product, and medicine, leading to its increased popularity among the general public, obtaining the label "the miracle tree"[2]. MO can be used as a treatment for many ailments, including hypertension, ulcers, bacterial infection, and inflammation[3,4]. All parts of the plant (bark, seed, leaf, and root) may be utilized for natural products, but the primary focus has been on plant leaves[3]. One hundred sixty-eight compounds have been previously identified in MO, 66 of which are bioactive[2]. Many of these compounds are phenols, which have been found to possess anti-inflammatory, anti-microbial, anti-oxidant, anti-cancer, anti-ulcer, anti-diabetic, and anti-tumor properties[3]. With such a wide range of health benefits MO should be further studied as widely useful and versatile nutraceutical candidate.

Phenols as Phytochemicals of Interest

Structural features of the chemicals within plants, referred to as phytochemicals, are differentiated into five major groups as shown in Figure 1A. Many of the previously identified bioactive components of MO are classified as flavonoids which belong to the phenol structural group and are recognized by the presence of three aromatic rings labeled A, B and C in Figure 1B. Phenolic compounds are important defense systems for plants, but little is known about the oxidation of the compounds related to MO that provide some of the plant's health benefits. Oxidation is a form of protection against stressors that the plant might encounter: nutrient reductions, temperature fluctuations, drought, pathogens, or physical wound[5]. Phenolic oxidation processes can be enzymatic, during typical metabolism reactions, and via molecular oxygen.

Phenolic oxidation is associated with browning observed in tea[6], wine[7], fruits[8,9], and vegetables[10,11], factors that often contribute to a decrease in food acceptability by consumers[12,13]. Various phenolic compounds have been identified previously in MO, and flavonoids like quercetin, kaempferol, and catechin are well known for their anti-oxidant health benefits. Radical scavenging is one such strength of flavonoids, and structural features present on A, B, and/or C rings contribute to this activity as shown in Figure 1B. These may include: 1) the presence of the C2-C3 double bond with the 4-oxo group to delocalize electrons from the B ring, 2) hydroxyl groups on the 3- and 5- carbons for greatest radical scavenging potential, 3) the ortho dihydroxy structure, located on the B ring for increased stability and electron donating abilities, and 4) the presence of hydroxyl groups on the A ring when hydroxyl groups on the C ring are not present[14,15].

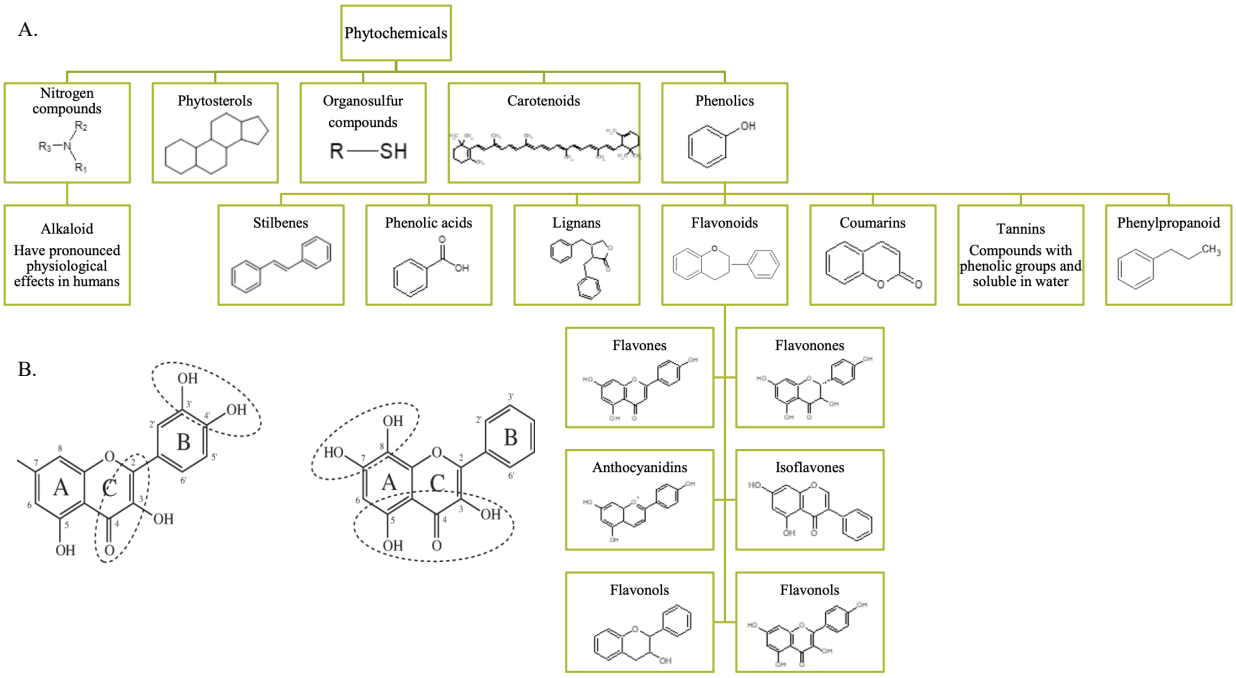


Figure 1. *A.* Classification of phytochemicals. *B*. Structure of flavonoids and their corresponding anti-oxidant indicators. Rings are labeled A, B, and C.

Antioxidants like quercetin retain their ROS scavenging abilities despite structural changes that take place after oxidation occurs[16]. In MO rather than other *Moringa* species, flavonoids are more likely to be glycosylated[17]. Glycosylation allows MO to adapt more successfully to environmental stressors, due to a potential increase in evolutionary metabolite quantity[17]. Glycosylation of flavonoids also aids in their digestion among humans, allowing for greater absorption and use in pharmaceuticals[17].

Differences in Growth Environment

MO species thrive in tropical and subtropical climates and are relatively drought resistant[18]. While MO has previously been cultivated in parts of Africa, the Middle East, and Asia[19], more recently the plant has been distributed to parts of Central, North, and South America, as well as Southeast Asia[20]. Various growing conditions have been tested to elucidate the differences in antioxidant and phenolic content, including: geographical location, plant age, time of harvest, soil pH, light intensity, and pruning methods[21]. MO leaves harvested in Pakistan were found to have higher antioxidant concentrations than previously reported values, and also reported differences in phenolic content and superoxide anion radical scavenging activity amongst regions and the month of harvest in Pakistan, with a cooler harvest month yielding greater phenolic content[22]. Meanwhile, leaves harvested in Niger, India, and Nicaragua all boasted varying anti-oxidant concentrations- with leaves grown in India having the highest antioxidant activities using the β-carotene-linoleic acid system, while leaves grown in Nicaragua contained the greatest concentration of phenolic compounds[23]. UV radiation was found to increase the concentration of bioactives, which has been corrugated in other plant species[24], while increased levels of potassium and phosphorus in the soil also correlated with higher concentrations of phenolics and

carotenoids, respectively[21]. Growing conditions and tree characteristics in tropical and subtropical climates vary leading to a wide range of anti-oxidant and total phenolic content values. Thus, standardization of growing practices is necessary to ensure comparable results in future studies. Furthermore, methods to test bioactive phytochemical concentrations are necessary.

Motivation for extraction and purification using biological safe methods

With a goal of developing MO as a nutraceutical, the choice of solvent and methods of extraction must be carefully developed. MO leaves have been extracted in methanol, ethanol, and water historically. Our lab has shown greater yield of total phenolic compounds with an 80% ethanol/20% water mixture most likely due to greater solvent polarity range[4]. With downstream purification and analysis, solvent removal is difficult especially if animal model work is planned. Traditional solvents of ethanol and methanol must be removed before sample dose administration due to toxicity concerns[25,26]. The addition of methanol or ethanol into the animal model subjects them to stress that could outweigh the health benefits of MO[25,26]. Thus methods such as desiccation or evaporation of solvents are needed to continue with ethanolic extracts.

Reactive Oxygen Species using a *C. elegans* Model

The *C. elegans* serves as a whole organism model for in vivo assays and despite the nonmammalian classification, the model shares many mammalian characteristics, including human disease pathways and genes[27]. To assess the bioactivity of MO leaf extracts, we examined the ability of extracts to control the production of reactive oxygen species (ROS) in the *C. elegans* model. Thermal shock is known to promote formation of ROS in the nonmammalian

model and historical ROS generation is known to lead to states of inflammation similar to those prevalent in metabolic diseases, cancers, and arthritis[28]. Using dyes that only fluoresce when oxidized, we expected to find a decrease in fluorescence reporting from the mitochondrial ROS (MitoTracker Red CM-H2Xros) which detects $O_2^{\bullet -}$ or H_2O_2. The red dyes were chosen as we expected blue/green fluorescence from flavonoids present in the MO extracts to cause interference with traditional green fluorescing ROS dyes[29].

Our aims for this paper are to **1)** extract plant derived chemicals from MO leaves, **2)** provide standardization for the extraction process, **3)** identify bioactives in fresh and oxidized MO samples that initiate positive responses to health conditions using TPC, optical spectroscopy, GC-MS, and LC-MS and **4)** test bioactivity of MO samples using *C. elegans* models and ROS generation.

Materials and Methods:

Moringa oleifera Preparation and Extraction

Extraction of MO leaf samples was standardized to the following method. Oxidized MO leaf samples were harvested in Nicaragua (gift from Linda Gable with New Song Ministries) in 2016 and allowed to air dry. Oxidized samples were stored in an airtight container at -4°C until use. Fresh leaf powder samples were collected from Nicaragua (gift from Linda Gable with New Song Ministries) in 2022 and allowed to air dry and stored at 4oC in the dark prior to use. Fresh and oxidized samples (1.0 g) were ground to a uniform particle size and extracted with 80% ethanol for 4 hours, shaken at room temperature. Samples were then filtered twice using 0.22 μm filter paper before storage at -25°C.

Analysis of Whole Leaf Extract

Total Phenolic Content TPC of whole leave MO extracts were determined using Folin Ciocalteu reagent[30, 31]. Standard solutions of 0, 1, 3, 5, 8, 11, and 15 μL of gallic acid stock solution (CAS 149-91-7) were diluted to 15 μL with 10 mM borate buffer. Samples solutions were prepared in triplicate with 15 μL of either fresh or oxidized MO sample. To each solution, 40 μL of DI water and 15 μL Folin Ciocalteu reagent was added, and after 5 minutes at room temperature, 65 μL of 8% sodium carbonate was added to quench the reaction. Samples were diluted to 200 μL and absorbance was measured at 595 nm using a BioTek Synergy H1 microplate reader.

Absorbance Spectroscopy Optical spectroscopy measurements were conducted on fresh and oxidized MO extracts using a Thermo Scientific Nanodrop 2000 Spectrophotometer. UV-Vis detection with a Thermo Scientific Nanodrop 2000 Spectrophotometer was carried out with standard 1 cm glass cuvettes washed twice with 95% ethanol after each trial and with a 100 M Borate buffer (pH 9) blank. MO samples were diluted 1:200 with a final volume of 1 mL.

Fluorescence spectra. Fluorescence spectra of 1:100 dilutions of MO extractions were determined on Horiba Jobin Yvon FluoroMax-4. Excitation wavelengths of 325, 355, and 390 nm were utilized with excitation and emission slit widths of 5 nm. An emission wavelength of 460 nm was utilized for the excitation scan with excitation and emission slit widths of 5 nm. The signal was corrected by the reference lamp spectra.

GC-MS Analysis of phytochemicals present in MO 100% ethanol whole leaf extract samples was carried out using the Agilent 6890N GC, coupled with the Agilent 5973 MS and C18 column. Split ratio was 10:1, with N_2 set to a flow rate of 1 mL/min. A sample volume of 1 μL was injected, with an initial temperature of 100°C and ion source temperature of 260°C, with a rate of 10°C/min.

LC-MS

For LC-MS detection, an Agilent LC-MS QTOF 6540 was used and equipped with HPLC Agilent 1260 HPLC, HiP Sampler, binary pump, column comp, and Dual Agilent Jet-Stream Electrospray Ionization (ASJ-ESI). HPLC separation was performed with a C18 column with the mobile phase containing solvent A (0.1% formic acid (v/v) in water) and solvent B (0.1% formic acid (v/v) in acetonitrile) at 0.8 mL/min and a pressure limit of 600.0 barr. The injection volume was 1.00 μL. The elution conditions were as follows: 0.0–2.0 min 2% B. 2.0 -12.0 min 2-100% B, 12.0-15.0 min 100% B, 15.1–18.0 min 100-2% B. The temperature of the column was 30°C. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) scanned from *m*/*z* 110 to 1700 Da. ESI-MS was performed in positive ion mode with gas temperature at 250oC, glow of 8 L/min, nebulizer pressure at 35 psi, sheath gas temperature at 300oC and sheath gas flow at 11 mL/min, nozzle voltage of 500 V, and capillary voltage of 3500 V. Reference masses of 121.050 and 922.009 Da were employed and the Mass Hunter Workstation, and Mass Profiler Professional software were used to analyze spectra.

Reactive Oxygen Species in *C. elegans* Wildtype N2 *C. elegans* were obtained from the University of Minnesota Caenorhabditis Genetics Center. Nematodes were age synchronized to

stage L4 on bacterial lawns with OP50 bacteria heat inactivated at 65 ºC for 30 minutes. The heat inactivated OP50 was concentrated prior to use (Mechanisms of Ageing and Development 133 (2012) 1–10) and resuspended in 100 micrograms/mL MO leaf extraction samples or control of water/ethanol for use in experiments. Nematodes were harvested from plates, resuspended in M9 buffer, and thermally stressed at 35ºC for 2 hours in a 96 well plate. The dye Mitotracker Red CM-H2XRos (Invitrogen, Germany) was dissolved in dimethyl sulfoxide and 0.5 micromolar was added to each well of the 96 well plate[32]. Fluorescence of each well was obtained every 10 minutes for one hour with 579 nm excitation and 599 emission on a BioTek plate reader. Ten nematodes per treatment was obtained.

Results and Discussion:

Analysis of Whole Leaf Extract

Total Phenolic Content The total phenolic content of MO samples indicates the bioactivity of the sample, and can be used to predict many of the health benefits of the leaves. TPC was calculated using Figure 3. TPC values for fresh and oxidized MO samples are reported in Table 1.

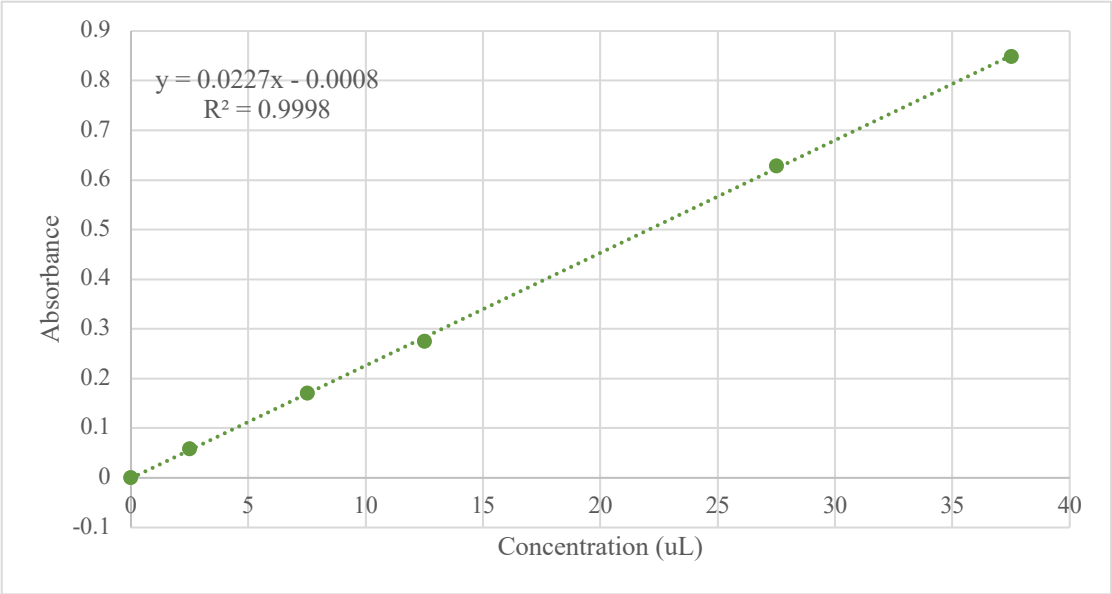


Figure 3: MO Total Phenolic Content Standard Addition Curve. Gallic acid equivalents were used to study triplicate samples.

Table 1: Total Phenolic Content of Fresh and Oxidized MO samples in varying extraction techniques. Ethanol (100%) and ethanol (80%) were the two parameters tested to determine the best extraction method for phenolics in MO.

	100%		80% / 20%	
	Fresh	Oxidized	Fresh	Oxidized
TPC (uM)	30.37 ± 0.04	38.88 ± 0.04	216.27 ± 0.04	140.21 ± 0.04

TPC values were highest for 80/20 extraction methods in fresh samples, suggesting how phenolics degrade via autoxidation over time. In the 80/20 extraction, fresh MO samples exhibited significantly greater TPC levels compared to oxidized samples, but this trend is reversed in 100% ethanol extraction. This could be attributed to the presence of molecular oxygen in oxidized samples, or typical metabolic pathways. Phenols are polar molecules, due to

the presence of alcohol groups on the ring structures, proving that aqueous extracts (with higher polarity than alcohol extracts) are the preferred method for nutraceuticals. Harvest date, location, and plant age were dissimilar between the two samples and could factor into differences in TPC content between fresh and oxidized samples.

Optical Spectroscopy UV-Vis and fluorescence analysis of MO leaf extracts in both 100% ethanol and 80/20 ethanol indicated the presence of bioactives in MO leaves Figure 4.

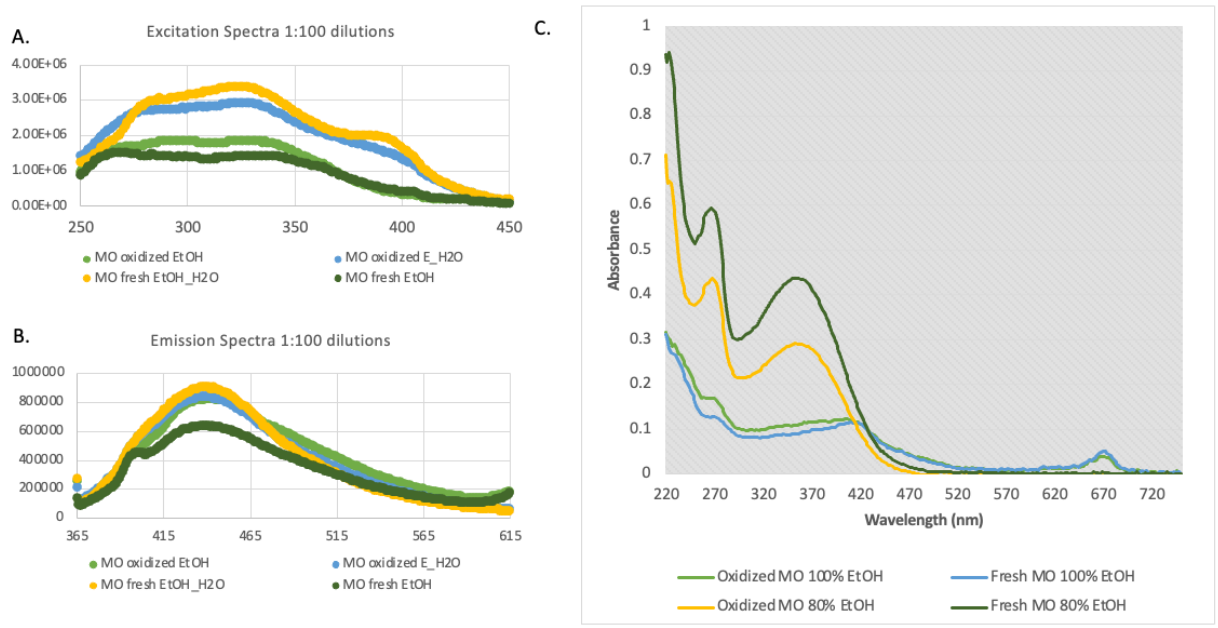


Figure 4. *A.* Excitation spectra of 1:100 dilutions of fresh and oxidized MO under 100% ethanol and 80% ethanol 20% water extraction methods. A wavelength of 460 nm was specified. *B.* Emission spectra of 1:100 dilutions of fresh and oxidized MO under 100% ethanol and 80% ethanol 20% water extraction methods. A wavelength of 355 nm was utilized. *C.* UV-Vis absorbance spectra of 1:100 dilutions of fresh and oxidized MO under 100% ethanol and 80% ethanol 20% water extraction methods.

UV-Vis peaks at 270 nm and 360 nm indicate the highest phenolic and flavonoid concentrations. The 80% ethanol solvent remains the optimal extraction procedure, with fresh, then oxidized 80% ethanol samples showing the highest absorbance peaks. Oxidized MO samples extracted in 100% ethanol continued to show higher absorbances than fresh 100% ethanol samples. Recent research has shown that some flavonoids still retain anti-oxidant properties even after ROS oxidation has occurred[16, 33]. In quercetin, conformational changes due to oxidation have shown limited decline of anti-oxidant capacities, with retention largely based on the presence of a catechol in ring B and an enol in ring C[33]. These findings may provide insight to why the oxidation of MO samples does not have as pronounced an effect on fluorescence as extraction medium. Similar to differences in TPC, comparisons between the absorbance and fluorescence measurements of oxidized and fresh samples are limited because of variation in environmental growth factors.

GC-MS The GC-MS identified compounds present in oxidized and fresh MO samples in ethanolic extraction are listed in Table 2 and Table 3. Structures and uses of key compounds are located in Table 4. Oxidized compounds in the highest abundance were 9,12,15-octadecatrienoic acid (zzz), ethyl 9,12,15-octadecatrienoate, [17.09] and, benzophenone, respectively. The most abundant compounds found in the fresh MO sample were benzophenone, 9,12,15-octadecatrienoic acid (zzz), and n-hexadecanoic acid.

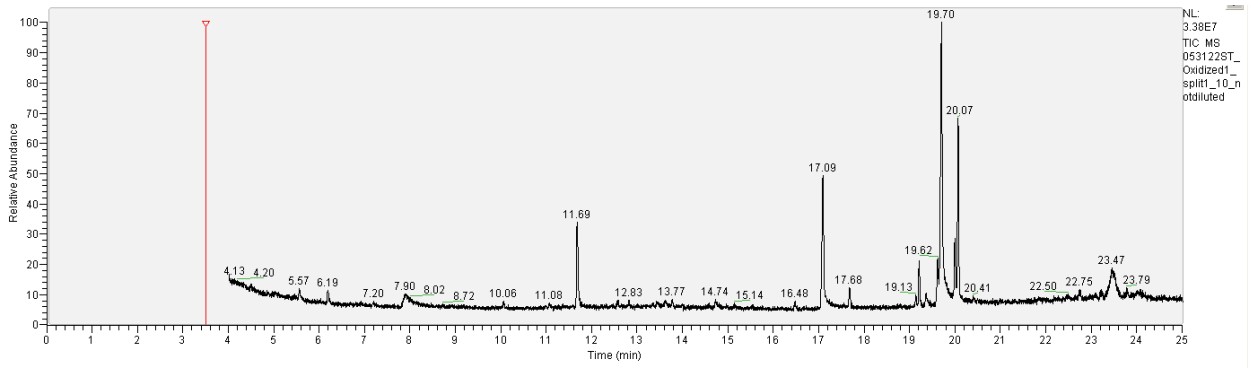


Figure 5: GC-MS of Ethanolic Extract Oxidized MO Leaves.

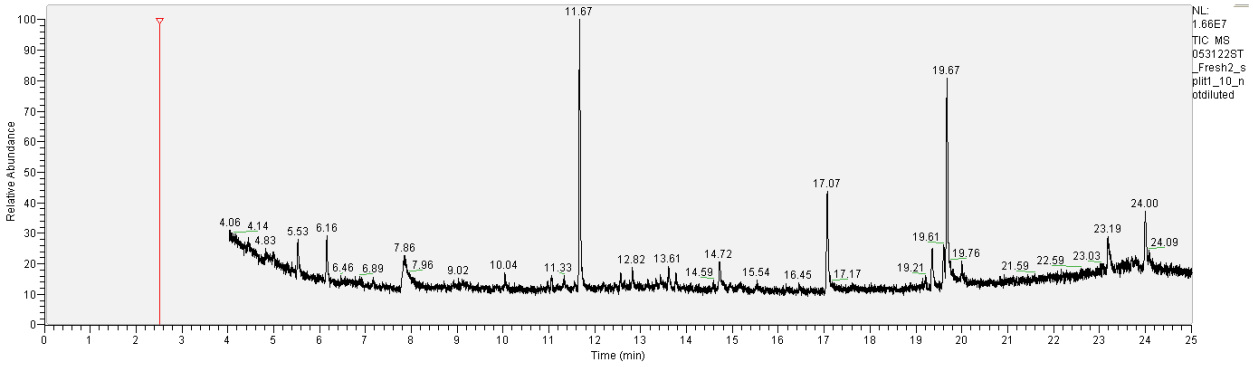


Figure 6: GC-MS of Ethanolic Extract Fresh MO Leaves.

Table 2: Tentatively identified compounds present in ethanolic MO extract of oxidized leaf samples analyzed by GC-MS.

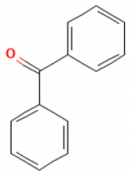
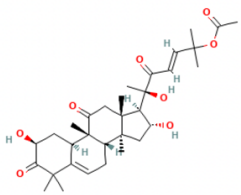
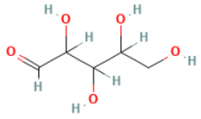
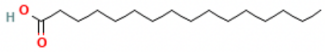
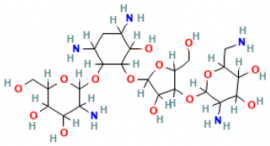
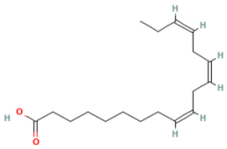
Peak	**RT (min)**	**Tentative Identification**
3	5.17	Acetic acid, hydroxy, ethyl ester
4	6.19	1,2,3 propanetriol, diacetate
6	7.90	DL-arabinose
11	11.69	benzophenone
17	17.68	n-hexadecanoic acid, ethyl ester

19	19.62	5,8,11,14 eicosatetraenoic acid, methyl ester
21	19.70	9,12,15-octadecatrienoic acid (zzz)
23	20.07	ethyl 9,12,15-octadecatrienoate
28	23.47	octasiloxane

Table 3: Tentatively identified compounds present in ethanolic MO extract of fresh leaf samples analyzed by GC-MS.

Peak	**RT (min)**	**Tentative Identification**
4	5.53	hydrazine carboxylic acid, ethyl ester
5	6.16	diethyl azodicarboxylate
8	7.86	L-glucose
16	11.67	benzophenone
20	14.72	Z- 4-octadecen-1-ol acetate
23	17.07	n-hexadecanoic acid
26	19.61	paromomycin 1
27	19.67	9-12-15 octadecatrienoic acid (zzz)
28	19.76	10,13 octadecadienoic acid methyl ester
32	23.19	9,12,15-octadecatrienoic acid
33	24.00	cucurbitacin b

Table 4: List of phytochemicals and their proposed bioactivity in ethanolic extract of oxidized and fresh MO leaves.

Compound	Structure	Sample Present	Known Applications
benzophenone		Fresh & Oxidized - More abundant in fresh MO samples.	Anti-tumor[34] UV protection[35] Treatment for Parkinson's Disease[36]
cucurbitacin b		Fresh	Anti-cancer[37] Anti-inflammatory[38] Anti-proliferative[39]
DL-arabinose		Oxidized	Hypercholesterolemia[40]
n-hexadecanoic acid		Fresh	Anti-microbial[41] Anti-oxidant[42] Anti-inflammatory[43]
paromomycin 1		Fresh	Anti-microbial[44]
9,12,15-octadecatrienoic acid (zzz)		Fresh & Oxidized	Cardioprotective[45] Anti-inflammatory[45] Antithrombotic[45]

(α-linolenic acid)		- More abundant in oxidized MO samples.	Neuroprotective [46] Micronutrient[45]

The effects of oxidation on the phytochemical content in MO has not been closely examined in previous literature. Fresh MO extracts contain a greater variety of phytochemicals compared to oxidized samples, indicating that the bioactive properties of fresh and oxidized MO samples are dissimilar. Two compounds, benzophenone and α-linolenic acid, are present in both oxidized and fresh samples, with benzophenone having a higher concentration in fresh samples and α-linolenic acid in oxidized samples. The presence of 9,12,15-octadecatrienoic acid (zzz) and ethyl 9,12,15-octadecatrienoate in oxidized samples signify that autoxidation is occurring, with the addition of the ethyl group and loss of a hydrogen forming the conjugate base of 9,12,15-octadecatrienoic acid (zzz). Acidic compounds were more present in oxidized samples, which could indicate that antioxidant activity is retained even during compound oxidation[16, 33]. Fresh samples also contain photo-oxidized compounds in hydrazine carboxylic acid, ethyl ester, commonly found in fermented food and beverage items. Previous studies in fresh samples have also identified propionic acid[47], benzeneacetonitrile[47], ethanamine[48], and heptadecanoic acid[47]. This indicates that MO samples begin to deteriorate soon after harvest, and should be stored and sealed properly after harvest to prevent rapid autoxidation of bioactives.

LC-MS The LC-MS identified compounds present in oxidized and fresh MO samples in polar extraction are listed in Tables 5, 6, and 7. Structures and uses of key health-related compounds are located in Table 8.

Table 5: Tentatively identified compounds present in ethanolic MO extract of five year oxidized leaf samples analyzed by LC-MS.

Cpd	**RT**	**m/z**	**Identity**
1	1.076	145.1577	spermidine
9	1.169	131.1289	agmatine
11	1.283	147.0529	o-acetylserine
17	1.391	133.0737	1,4-dideoxy-1,4- imino-D-arabinitol
19	1.406	195.1105	meglumine
22	1.459	117.0788	isoamyl nitrite
23	1.471	145.1101	2R-aminoheptanoic acid
24	1.472	159.0893	2- methylbutyrylglycine
28	1.506	161.105	bis (2- hydroxypropyl) amine
30	1.507	209.1263	minoxidil
33	1.526	155.0581	ethosuximide M5
36	1.564	241.1175	sapropterin
37	1.572	179.0804	glucosamine
39	1.575	153.0647	4,6-diamino-5- formamidopyrimidine
48	1.782	205.1313	dexpanthenol
52	1.837	140.0583	1,4- methylimidazoleacetic acid

54	1.891	173.0685	2,6- piperidinedicarboxylic acid
55	1.895	149.105	trolamine
68	2.153	153.0788	octopamine (p- hydroxyphenylethanolamine)
69	2.154	182.0577	veratric acid
71	2.178	225.1	L-4-hydroxy-3- methoxy-a- methylphenylalanine
74	2.190	216.0786	6-methoxy-2- naphthylacetic acid
75	2.195	166.0627	atrolactic acid
76	2.197	178.0627	8S-hydroxy-2- decene-4,6-diynoic acid
78	2.197	150.0678	4-ethylbenzoic acid
79	2.199	270.1103	idebenone metabolite
89	2.791	267.0968	vidarabine
98	3.292	183.0893	racepinephrine
108	3.671	181.1101	2-hepten-4-yn-1- amine, N,6,6-trimethyl-, (E)- metabolite
109	3.673	227.1157	hydroxyphenoxyethylaminohydroxypropanol
116	4.475	188.0682	ethyl oxalacetate
117	4.476	128.0471	6-hydroxy-2-hexynoic acid
118	4.476	146.0576	alpha- ketopantoic acid
123	4.730	399.1894	diethylpropion
124	4.792	253.1313	4-(2-hydroxy-3- isopropyl- aminopropyl)benzoic acid
126	4.901	222.1003	4- aminohippurate
127	4.901	205.0735	deschlorobenzoyl indomethacin
128	4.901	163.0629	p-acetaminobenzaldehyde
129	4.930	169.0753	Pyridoxine (Vitamin B6)

131	4.958	354.095	Chlorogenic acid
132	4.598	180.0419	m-hydroxyphenylpyruvic acid
139	5.175	148.0521	p-hydroxycinnamaldehyde
140	5.176	176.0471	10-hydroxy-8E- decene-2,4,6-triynoicacid
143	5.176	188.0834	5,8,11- dodecatriynoic acid
145	5.258	170.1304	2- decylenic acid
149	5.305	164.047	o-coumaric acid
151	5.499	424.1346	ginkgolide B
154	5.907	434.1764	lonchocarpic acid
156	6.195	432.106	1-Methyl-4-nitro- 5-(S-Gluctathionyl) Imidazole
158	6.328	236.1048	carboxyibuprofen
159	6.368	302.0427	morin
160	6.368	464.0958	demeclocycline
164	6.368	464.0958	Benzenemethanol, 2-(2- aminopropoxy)-3-methyl-
165	6.610	448.1007	quercitrin
166	6.611	304.0583	dihydroquercetin
173	6.893	140.1198	2-nonenal
182	7.386	375.1353	tiagabine
183	7.681	584.2818	ouabain
186	8.273	453.3091	10- deoxymethymycin
188	8.351	417.1495	mefenamic acid metabolite
190	8.468	528.3777	isorenieratene (leprotene)
198	8.676	290.1881	8-hydroxy-17- octadecene-10,12-dinoic acid

200	8.680	348.1911	sodium chlorovulone III
203	8.997	324.1936	12-oxo-14,18- dihydroxy-9Z,13E,15Z- octadecatrienoic acid
204	8.999	306.183	tetranor iloprost
209	9.434	390.2379	gitoxigenin
210	9.859	294.2195	12-oxo-9- octadecynoic acid
212	9.990	336.23	11-deoxy-PGE2
213	9.993	376.2226	resolvin D2
215	10.052	318.2195	5S-HEPE
217	10.413	352.2249	thromboxane A2
220	10.485	576.3629	1- hydroxyvitamin D3 3-D- glucopyranoside
222	10.642	360.2275	pregn-4-ene- 3,20-dione, 6a,17- dihydroxy-6-methyl-
225	11.334	322.2509	12R-HETrE
226	16.604	141.0899	histidinol

Table 6: Tentatively identified compounds present in ethanolic MO extract of fresh leaf samples analyzed by LC-MS.

Cpd	**RT**	**m/z**	**Tentative Identity**
1	1.074	145.1573	spermidine
9	1.146	228.1704	10-keto tridecanoic acid
12	1.245	314.1825	pergolide
14	1.285	146.0686	beta-ureidoisobutyric acid

15	1.288	147.0527	o-acetylserine
18	1.300	225.1232	4'- hydroxyminoxidil
21	1.314	143.07	cyclocreatine
23	1.391	133.0735	1,4-dideoxy-1,4- imino-D-arabinitol
24	1.449	140.0582	1,4- methylimidazoleacetic acid
26	1.466	117.0786	isoamyl nitrite
27	1.472	145.1096	2R-aminoheptanoic acid
33	1.527	155.0578	ethosuximide M5
36	1.557	145.0847	4- (diaminomethylideneamino) butanoic acid
39	1.570	153.0646	4,6-diamino-5- formamidopyrimidine
40	1.579	215.0815	kinetin
42	1.583	309.1057	N-acetyl-a- neuraminic acid
49	1.710	210.037	mucic acid
52	1.728	141.0421	2- aminomuconate 6- semialdehyde
54	1.736	146.021	2-ketoglutaric acid
61	1.872	149.1047	trolamine
72	2.140	182.0573	veratric acid
73	2.140	153.0785	octopamine (p- hydroxyphenylethanolamine)
75	2.169	178.0625	10-hydroxy-8E- decene-4,6-diynoic acid
76	2.173	216.0782	6-methoxy-2- naphthylacetic acid
79	2.177	270.1102	idebenone metabolite
80	2.183	166.0626	atrolactic acid
81	2.191	208.073	benzylsuccinic acid

84	2.214	150.0676	4-ethylbenzoic acid
91	2.259	216.0781	euparin
96	2.760	161.0683	L-2-aminoadipic acid
100	2.841	267.096	neuraminic acid
101	3.196	136.0379	hypoxanthine
104	3.256	144.0999	4-guanidinobutanamide
106	3.274	183.089	racepinephrine
118	3.560	309.1208	tranylcypromine glucuronide
119	4.476	129.0787	vigabatrin
120	4.508	378.2031	18-hydroxycortilsol
122	4.819	362.1715	thyrotropin releasing hormone
124	4.903	205.0736	deschlorobenzoyl indomethacin
125	4.904	222.1003	4-aminohippurate
126	4.904	163.063	p-acetaminobenzaldehyde
127	4.936	169.0735	pyridoxine
128	4.962	354.0951	chlorogenic acid
129	4.962	180.042	caffeic acid
131	5.167	544.2662	7- deacetylkhivorin
134	5.309	164.047	o-coumaric acid
136	5.427	234.1005	p-hydroxyprimidone
137	5.428	161.0838	indole-3-ethanol
138	5.671	198.054	syringic acid
139	6.200	432.106	cosmosiin

140	6.226	610.1543	rutin
141	6.313	432.1061	1-methyl-4-nitro- 5-(S-gluctathionyl) imidazole
142	6.370	221.0574	histidinol phosphate
143	6.371	320/0533	dihydromyricetin
144	6.487	264.0576	enoximone sulfoxide
145	6.590	196.1097	4-(2- hydroxypropoxy)-3,5- dimethyl-phenol
146	6.597	275.0479	phenylacetic mustard
147	6.615	304.0581	dihydroquercetin
148	6.615	448.1001	quercitrin
149	6.778	267.05	1-alkenyl-2-acyl- glycerophosphoethanolamine
156	7.388	375.1349	tiagabine
157	7.686	329.2172	8-hydroxyamoxapine
160	8.480	273.2662	C16 sphinganine
161	8.490	506.3954	(20S)-24- hydroxy-19- norgeminivitamin D3
162	8.511	596.3904	26-o-[beta-d- glucopyranosyl]-25R- furostan 3beta,22alpha,26- triol
164	8.611	290.1877	8-hydroxy-17- octadecene-9,11-diynoic acid
165	8.666	256.1036	dinorpromazine
166	8.671	300.0674	sulfaquinoxaline
167	8.694	371.1031	nedocromil
168	9.046	297.2666	(Z)-N-(2- hydroxyethyl)hexadec-7- enamide
171	9.803	422.2662	lovastatin acid (mevinolinic acid)
172	9.858	294.2192	alpha-9(10)- EpODE
175	10.115	268.1494	equilin

176	10.487	594.3731	7,8-didehydroastaxanthin
178	10.814	381.2875	5,6-DiHETrE-EA
179	10.860	321.2663	anandamide (18:3, n-3)
180	11.335	322.2504	12R-HETrE
181	11.415	341.2926	N-acetylesphingosine
183	10.098	512.2812	3-deoxo-3beta- acetoxydeoxydihydrogedunin
184	14.045	558.3229	cucurbitacin B
185	16.604	141.0898	histidinol

Table 7: Tentatively identified compounds present in ethanolic MO extract of one year oxidized leaf samples analyzed by LC-MS.

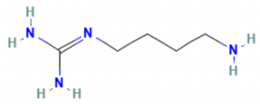
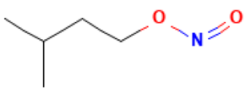
Cpd	**RT**	**m/z**	**Tentative Identity**
1	1.059	145.1574	spermidine
10	1.166	130.1214	agmatine
11	1.176	129.0786	vigabatrin
16	1.284	146.0686	B-ureidoisobutyric acid
17	1.285	147.0527	o-acetylserine
19	1.307	225.1231	4'- hydroxyminoxidil
22	1.391	133.0735	1,4-dideoxy-1,4- imino-D-arabinitol
23	1.426	253.1308	8- azaspiro[4.5]decane-8- butanoic acid, 7,9- dioxo (buspirone metabolite)
25	1.467	117.0786	isoamyl nitrite
26	1.527	155.0578	ethosuximide M5
29	1.559	145.0846	4- (diaminomethylideneamino) butanoic acid

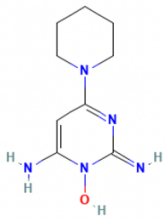
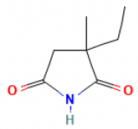
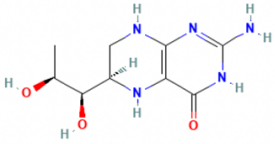
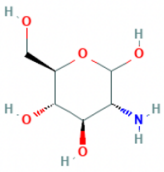
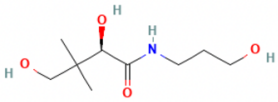
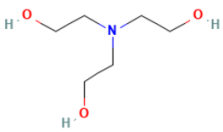
31	1.573	226.1048	carnosine
32	1.573	180.0629	D-(+)-mannose
38	1.738	210.037	D-saccharic acid
43	1.835	140.0581	1,4- methylimidazoleacetic acid
44	1.880	149.105	trolamine
48	2.139	140.0469	2-methoxyresorcinol
49	2.141	153.0786	octopamine (p- hydroxyphenylethanolamine)
51	2.142	182.0574	veratric acid
52	2.176	270.1098	idebenone metabolite
54	2.183	178.0624	8S-hydroxy-2- decene-4,6-diynoic acid
55	2.183	216.0782	euparin
58	2.226	216.0782	6-methoxy-2- naphthylacetic acid
60	2.229	150.0675	4-ethylbenzoic acid
63	2.237	166.0625	atrolactic acid
65	2.767	153.0645	4,6-diamino-5- formamidopyrimidine
66	2.769	161.0683	L-2-aminoadipic acid
73	3.211	183.089	racepinephrine
75	3.258	155.0999	4-guanidinobutanamide
79	3.289	148.0519	p-hydroxycinnamaldehyde
90	4.483	188.0682	ethyl oxalacetate
91	4.483	128.0469	6-hydroxy-2-hexynoic acid
92	4.719	260.0502	suprofen
93	4.902	163.0627	p-acetaminobenzaldehyde

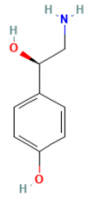
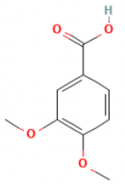
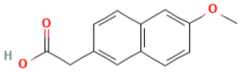
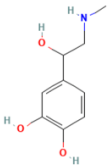
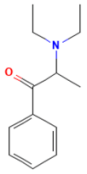
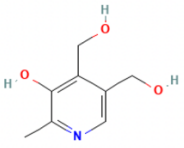
95	4.903	205.0734	deschlorobenzoyl indomethacin
96	4.904	222.1	4-aminohippurate
97	4.937	169.0734	pyroxidine (B6)
98	4.946	180.0417	m-hydroxyphenylpyruvic acid
99	4.962	354.0948	chlorogenic acid
102	5.180	176.0467	10-hydroxy-8E- 10-hydroxy-8E-decene-2,4,6-triynoicacid
105	5.308	164.0468	o-coumaric acid
107	5.426	234.1001	p-hydroxyprimidone
108	5.428	161.0836	indole-3-ethanol
111	6.196	432.105	cosmosiin
112	6.225	320.0528	rutin
113	6.225	320.0528	dihydromyricetin
115	6.369	442.1132	S-(4- nitrobenzyl) glutathione
122	6.587	196.1095	benzenemethanol, 2-(2- aminopropoxy)-3-methyl-
124	6.613	286.0473	6-chloropurine riboside
133	7.385	375.1347	tiagabine
135	7.688	329.093	8-hydroxyamoxapine
137	8.350	417.1454	mefenamic acid metabolite
139	8.486	506.395	(20R)-24- hydroxy-19- norgeminivitamin D3
140	8.536	528.3766	isorenieratene (leprotene)
142	8.607	290.1877	8-hydroxy-17- octadecene-9,11-diynoic acid
143	8.660	530.3088	L-oleandrosyl-oleandolide
145	8.690	371.1035	nedocromil

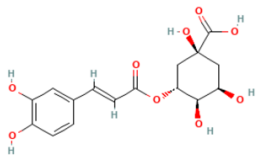
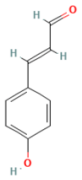
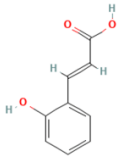
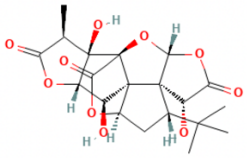
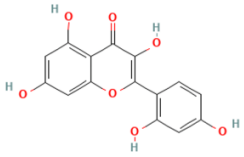
147	9.433	386.2669	6-deoxyerythronolide B
148	9.435	350.2457	U46619
149	9.905	294.2191	9S,10-epoxy- 10,12Z-octadecadienoic acid
151	9.981	330.2789	1-hexadecanoyl-sn-glycerol
152	9.995	354.2404	13,14-dihydro- 15-keto-PGF2alpha
157	10.486	594.3726	7,8-didehydroastaxanthin
158	11.338	322.2504	12R-HETrE
159	12.200	666.4469	GPGro(14:0/14:0)[U]
165	16.606	141.0898	Histidinol

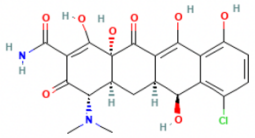
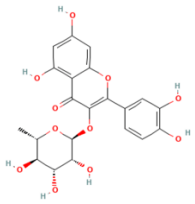
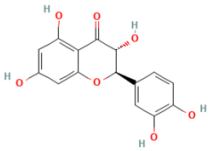
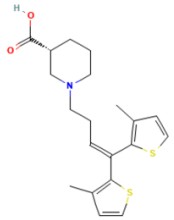
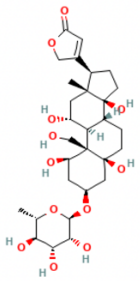
Table 8: List of phytochemicals and their proposed bioactivity in ethanolic extract of oxidized and fresh MO leaves. (5) indicates a five year oxidation period. (1) indicates a one year oxidation period.

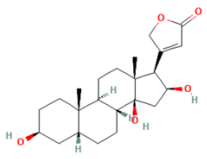
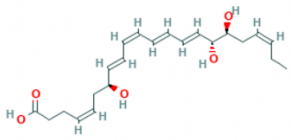
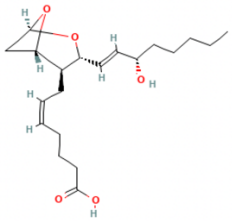
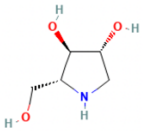
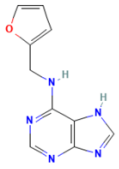
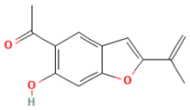
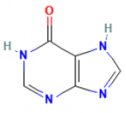
Compound	**Structure**	**Sample Present**	**Known Applications**
agmatine		Oxidized (5) Oxidized (1)	Neuroprotective[49] Anti-depressant[49]
isoamyl nitrite		Oxidized (5) Fresh Oxidized (1)	Anti-hypertensive[50] Vasodilator[51]
minoxidil		Oxidized (5)	Anti-hypertensive[52] (severe cases)

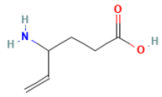
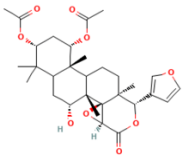
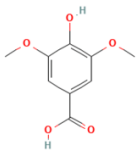
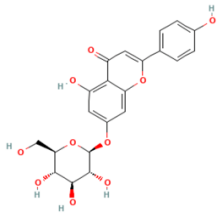
			
ethosuximide M5		Oxidized (5) Fresh Oxidized (1)	Anti-convulsant[53]
sapropterin		Oxidized (5)	Medication for PKU[54]
glucosamine		Oxidized (5)	Joint and cartilage supplement[55]
dexpanthenol		Oxidized (5)	Anti-inflammatory[56] Anti-oxidant[56]
trolamine		Oxidized (5) Fresh Oxidized (1)	Osteoarthritis[57] Anti-inflammatory[58] Reduces radiation-induced skin damage[58]

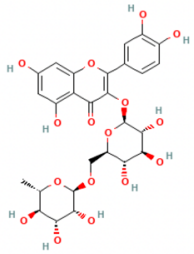
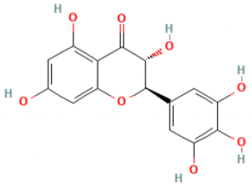
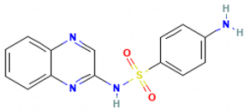
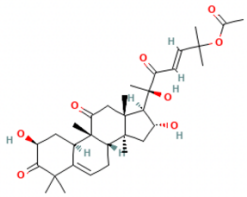
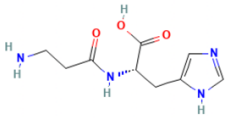
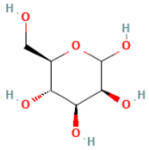
octopamine (p-hydroxyphenylethanolamine)		Oxidized (5) Fresh Oxidized (1)	Hypotension[59]
veratric acid		Oxidized (5) Fresh Oxidized (1)	Anti-inflammatory[60] Anti-oxidant[60]
6-methoxy-2- naphthylacetic acid		Oxidized (5) Fresh Oxidized (1)	Non-steroidal anti-inflammatory[61]
vidarabine		Oxidized (5)	Anti-viral[62]
racepinephrine		Oxidized (5) Fresh Oxidized (1)	Bronchodilator[63]
diethylpropion		Oxidized (5)	Anti-obesity[64]
pyridoxine (Vitamin B6)		Oxidized (5) Fresh Oxidized (1)	Medication for nausea and

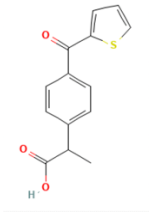
			vomiting during pregnancy[65]
chlorogenic acid		Oxidized (5) Fresh Oxidized (1)	Anti-oxidant[66, 67] Cardioprotective[67]
p-hydroxycinnamaldehyde		Oxidized (5) Oxidized (1)	Anti-inflammatory[68] Osteoarthritis[68]
o-coumaric acid		Oxidized (5) Fresh Oxidized (1)	Anti-oxidant[69] Anti-obesity[70] Anti-carcinogenic[71]
ginkgolide B		Oxidized (5)	Anti-oxidant[72] Anti-inflammatory[72] Neuroprotective[73]
morin		Oxidized (5)	Anti-oxidant[74] Anti-inflammatory[75] Anti-cancer[76] Anti-hypertensive[77] Anti-bacterial[78] Neuroprotective[79]

			Hepatoprotective[80]
demeclocycline		Oxidized (5)	Anti-biotic[81]
quercitrin		Oxidized (5) Fresh	Anti-oxidant[82] Anti-inflammatory[82]
dihydroquercetin		Oxidized (5) Fresh	Anti-oxidant[83] Anti-cancer[83] Anti-tumor[83] Anti-cardiovascular[83]
tiagabine		Oxidized (5) Fresh Oxidized (1)	Anti-convulsant[84]
ouabain		Oxidized (5)	Cardiotonic[85]

gitoxigenin		Oxidized (5)	Anti-cancer[86]
resolvin D2		Oxidized (5)	Anti-inflammatory[87, 88] Anti-cancer[89] Anti-obesity[88]
thromboxane A2		Oxidized (5)	Prothrombotic[90]
1,4-dideoxy-1,4- imino-D-arabinitol		Fresh Oxidized (1)	Anti-hyperglycemic[91]
kinetin		Fresh	Anti-oxidant[92] Anti-aging[93]
euparin		Fresh Oxidized (1)	Anti-oxidant[94] Anti-viral[95] Anti-inflammatory[96]
hypoxanthine		Fresh	Anti-inflammatory[97]

			Anti-cancer[97]
vigabatrin		Fresh Oxidized (1)	Anti-convulsant[98]
7- deacetylkhivorin		Fresh	Anti-protozoal[99]
syringic acid		Fresh	Anti- inflammatory[100] Anti-cancer[100] Anti-oxidant[100] Anti-microbial[100] Neuroprotective[100] Hepatoprotective[100] Prevention of: Diabetes[100], CVD[100], Cerebral ischemia[100]
cosmosiin		Fresh Oxidized (1)	Non-steroidal anti- inflammatory [101] Anti-diabetic[101] Anti-cancer[102] Neuroprotective[102]

rutin		Fresh Oxidized (1)	Anti-oxidant[103] Cardioprotective[103] Neuroprotective[103]
dihydromyricetin		Fresh Oxidized (1)	Anti-oxidant[104] Anti-bacterial[105]
sulfaquinoxaline		Fresh	Anti-biotic[106]
cucurbitacin B		Fresh	Anti-inflammatory[107] Anti-cancer[107, 108] Anti-microbial[107] Hepatoprotective[107] Anti-diabetic[107]
carnosine		Oxidized (1)	Anti-oxidant[109]
D-(+)-mannose		Oxidized (1)	Treatment for urinary tract infections[110]

suprofen		Oxidized (1)	Non-steroidal anti-inflammatory [111]

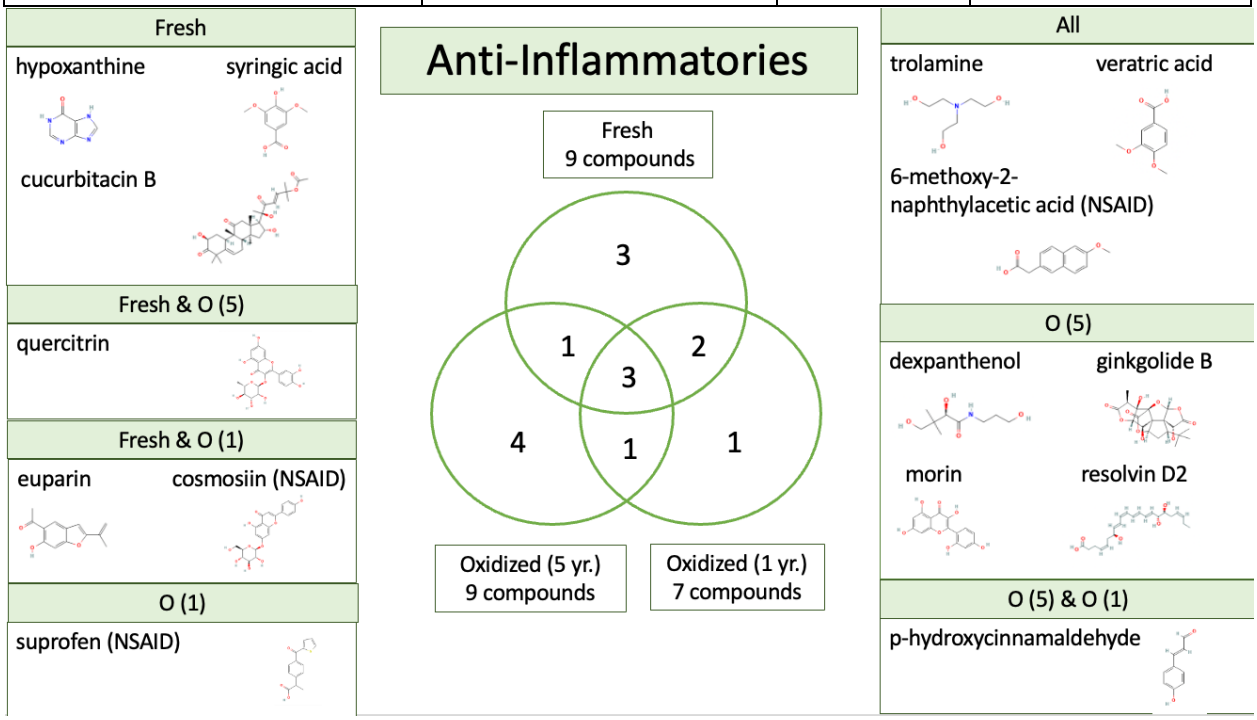


Figure 7: Classification of Identified Anti-Inflammatory Compounds by the Samples they are Present In.

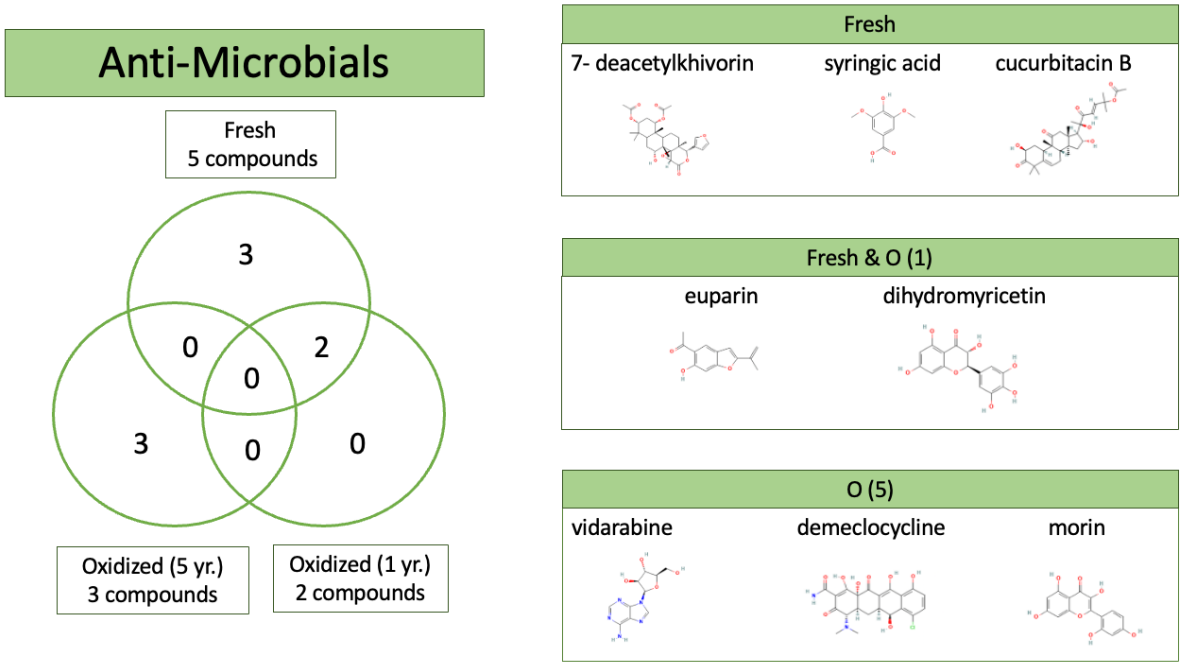


Figure 8: Classification of Identified Anti-Microbial Compounds by the Samples they are Present In.

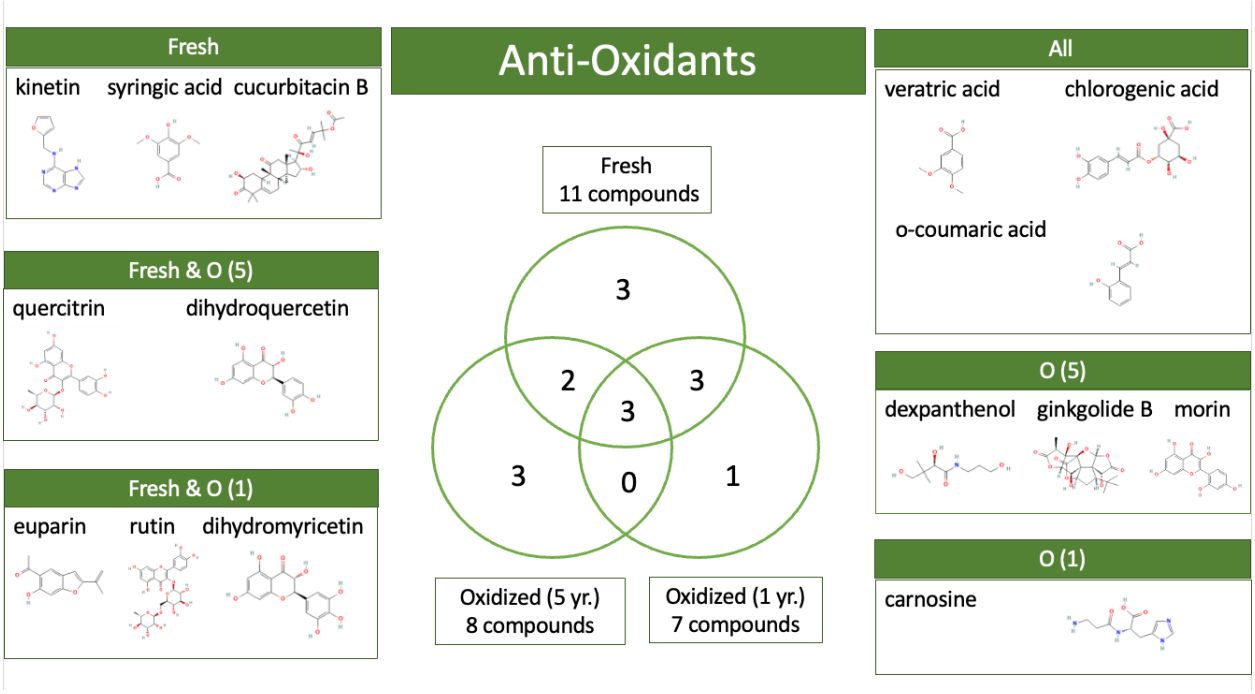


Figure 9: Classification of Identified Anti-Oxidant Compounds by the Samples they are Present In.

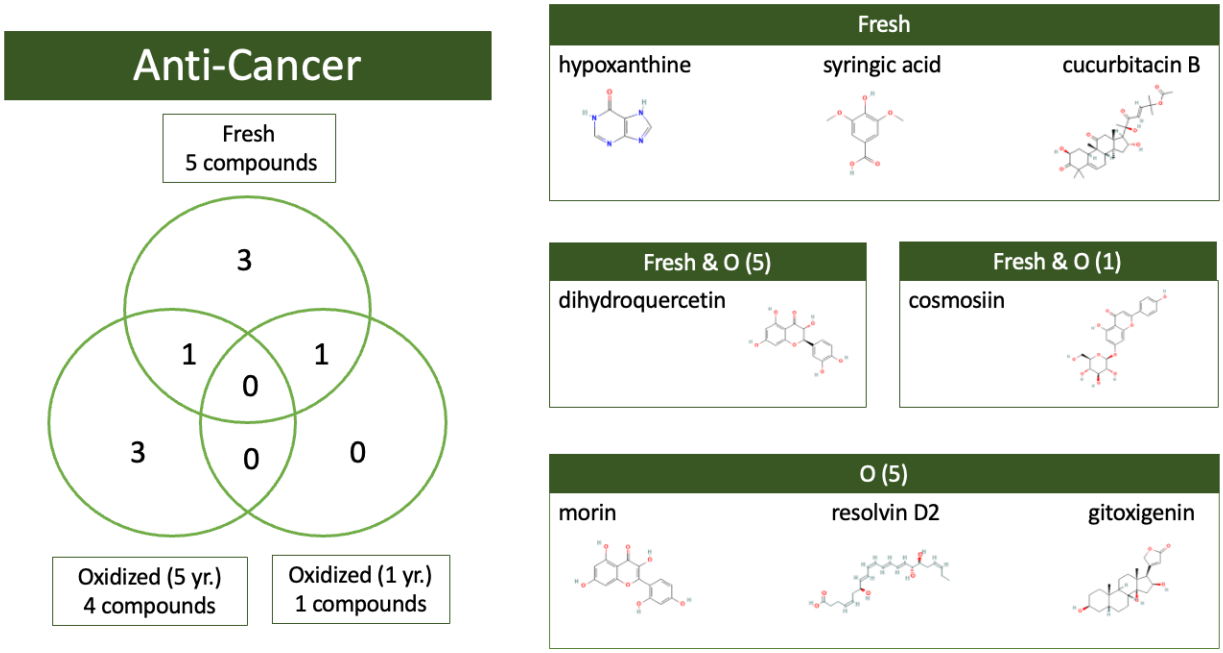


Figure 10: Classification of Identified Anti-Cancer Compounds by the Samples they are Present In.

The phytochemicals present in MO have a wide range of industrial and medicinal applications, including anti-oxidant (15), anti-cancer (8), anti-bacterial (2), neuroprotective (6), and anti-inflammatory (15). Fresh MO samples contained the most anti-inflammatory compounds of all samples tested, including trolamine, veratric acid, 6-methoxy-2-napthylacetic acid, euparin, hypoxanthine, cosmosiin, quercitrin, and cucurbitacin B. The variation in identified phytochemicals between instruments (Table 4 and Table 8) is due to differences in the polarity of extraction solvents, with a greater number of polar compounds extracted with the LC-MS methodology of methanolic and water. Cucurbitacin B was the only anti-inflammatory compound identified by both GC-MS and LC-MS, indicating that it can be extracted in both polar and nonpolar solvent for future use. Prolonged oxidation resulted in the decrease of anti-

inflammatory compounds present in MO samples. Few phytochemicals were present in both oxidized samples, indicating further degradation from one sample to the other, or a difference in growth environment and phytochemical availability. Previous studies have also identified 6 quercetin and six kaempferol derivatives[112], astragalin[113], cryptochlorogenic acid[114], and oleic acid[115] present in fresh MO samples. Research has shown that phytochemical content is dependent on harvest location and time[8], and the presence of morin, a flavonoid with great health benefits, in only the oldest sample may be due to agricultural climate variation. Another possibility is that each sample was exposed to a variation of environmental stressors that enhanced phytochemical content, found in previous articles[116]. To continue our work with MO, growth conditions and stressors must be monitored to determine standard phytochemical identities and concentrations. Overall, fresh MO extracts contain the greatest supply of phytochemicals for further study, with health benefits including: anti-inflammatory, anti-cancer, anti-oxidant, anti-microbial, anti-diabetic, cardioprotective, and neuroprotective properties.

Reactive Oxygen Species in *C. elegans* Previous C. elegans studies have shown that heat stress induces reactive oxygen species (ROS) generation. Synchronized C. elegans were grown for two weeks in liquid culture. The culture was divided into 5 treatments and cultured an additional 24 hours at 22°C in S-basal with the following conditions: a control, a 1:100 dilution of 80% ethanol/20%water, 1:200 dilution of 80% ethanol/20% water, a 1:100 dilution of fresh MO aqueous extract or 1:200 dilution of fresh MO aqueous extract. Samples were further divided and incubated either at room temperature or 33°C for 2 hours.

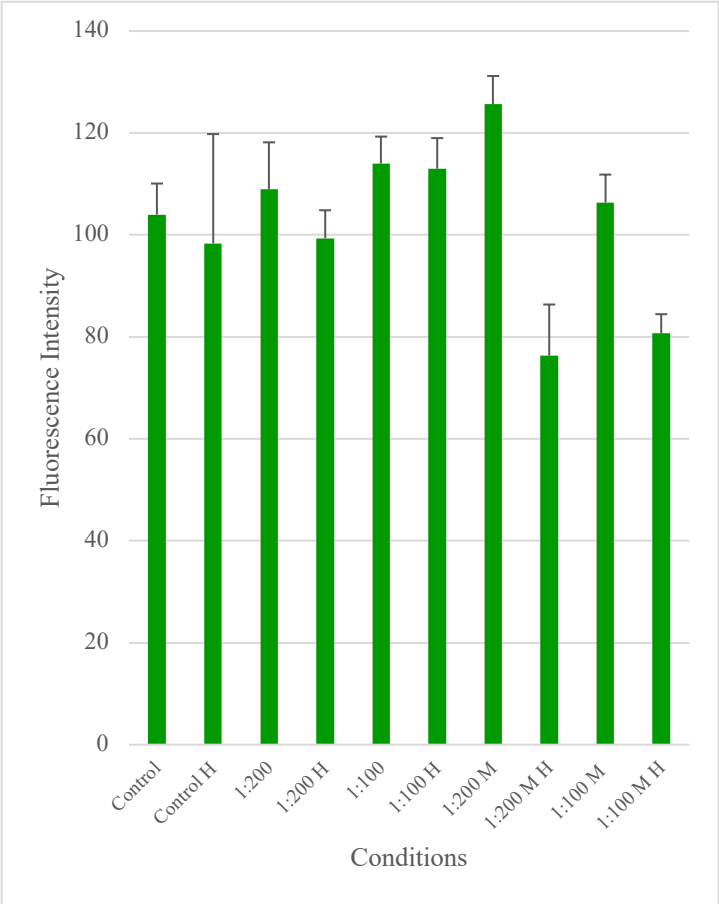


Figure 11: ROS generation in *c.elegans* species with heat stress (H) of 33°C for 2 hours applied. Solvent and fresh MO extract were diluted and applied to *c. elegans* to examine the effect on ROS generation. Fluorescence after 25 minutes is illustrated.

ROS was quantified by exposure of the C. elegans to the MitoTracker dye. Experimental data suggest that ROS generation ranges between ~100 to 125 arbitrary fluorescence units and is reduced approximately 25% when the C. elegans are stressed by heating. The data suggests solvent exposure at these dilutions does not generate ROS. Refinements on the experimental conditions are required to 1) lower the concentration of MO extract pretreatment as pretreatment

alone generated ROS, 2) reduce standard deviation, and 3) increase ROS response under heating conditions.

Conclusion:

MO samples were analyzed for phytochemical content variation amongst oxidized and fresh samples, and their subsequent effect on ROS generation in *c.* elegans. Anti-inflammatory compounds are found in both oxidized and fresh MO samples, as well as other benefits including anti-cancer, anti-oxidant, and anti-microbial benefits. TPC measurements indicated the presence of phenols in fresh and oxidized samples, with fresh MO extracts possessing higher TPC. This trend continues with our optical spectroscopy measurements, with extraction medium seen as the greater contributor to phenolic and flavonoid content than oxidation and aging of samples. GC-MS and LC-MS were employed to identify phytochemicals present in the extracts, with significant differences between methanolic and ethanolic extracts. A greater number of anti-inflammatory compounds were extracted in polar methods, but some differences between samples can be attributed to MO growing conditions and their effect on phytochemical content. Diluted MO extracts applied to *c. elegans* showed slight reductions in ROS generation when heat stress was applied. Experimental methods must be revised to reduce the effects of ethanol solvent and MO pretreatment on ROS generation. The abundance of phytochemicals present in both fresh and oxidized MO extracted samples indicates the plant's potential as a nutraceutical.

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