Measuring Antioxidant Properties through FRAP, Xanthine Oxidase, and ORAC Assays

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ABSTRACT

The original goal of this project was to identify possible antioxidants from Costa Rican plant extracts from bark of the species *oligantus*, *orotinus*, and *monteviridis* from genus *Lonchocarpus* through xanthine oxidase assay which allows for quantitative measurement of the reduction in the build-up of uric acid with an antioxidant compared to enzyme solution without the antioxidant. The project was extended to employing different assays—FRAP, xanthine oxidase, and ORAC—to test first known flavonoids and then plant extracts. Incorporated with further extractions and NMR structures, the assay data would allow for optimized assay methods and for identification of structure and composition of new antioxidants. While the FRAP assay easily yielded results, further work on xanthine and ORAC assays are required before obtaining reliable results.

BACKGROUND

Natural Products Research involves procuring possible medicinal plants, extracting crude oil and superfractions, testing for antioxidant, cytotoxic, or various properties, and identifying the structure and composition of the pure extract.

Bark extracts from Species *oligantus* (LOOLA), *orotinus* (LOORBA), and *monteviridis* (LOMOBA) all from Genus *Lonchocarpus* were extracted through distillation in acetone and large column chromatography. The crude extract for LOOLA and LOMOBA, which showed the indication of most antioxidant activity under DPPH spray, went under smaller column chromatography to yield 14 and 11 super fractions, respectively.

LOOLA gave 1 crude and 14 super fractions. For LOORBA, there was 1 crude extract. LOMOBA gave 1 crude extract and 11 super fractions.

FRAP, which stands for Ferric Reducing Ability of Plasma, measures the absorbance change when Fe^{+3} ions (colorless) in the solution are reduced to FE^{+2} (blue). Xanthine oxidase assay measures the change in absorbance from xanthine (clear) to uric acid (yellow). The ORAC, which stands for oxygen radical absorbance capacity, involves transfer of hydrogen atom where the antioxidant and substrate s compete for the free radicals generated from AAPH (2,2'-azo-bis-(2-methylpropionamidine). The spectrophotometer measures fluorescence as radicals are quenched.

ASSAY METHODS

FRAP Assays (End Point):

Blank	Test Compound
20 uL MeOH	15 uL MeOH & 5 uL Test compound
150 uL FRAP Reagent at 37 °C (1 TPTZ: 1 FeCl3: 10 Acetate Buffer)	
Endpoint was measured after 30 minutes at 37 °C	

TPTZ had concentration of 3.125 g/L, $FeCl_3$ had concentration of 5.4 g/L, and acetate buffer had concentration of 3.1g/L.. The endpoint absorbance was used to compare. Good antioxidants will have high Fe (II) concentration due to higher reductive properties.

Xanthine Oxidase Assays (Kinetics):

Enzyme Blank	Enzyme Test Compound
133, 66.5, 33.3, or 16.6 uL Xanthine (0.0228 g/L)	
0, 66.5, 99.7, or 116.4 uL dH_2O	
3 uL DMSO	3 uL Test compound in DMSO (6.55nM)
64 uL Xanthine Oxidase (.077g/L)	
Read kinetics immediately for 30 minutes at 37 °C (290nm)	

Xanthine solutions at different concentrations were used. Another set of these wells were made to measure the buffer blank. In the buffer blanks, the 64 uL of enzyme was replaced with phosphate buffer. The kinetic rates (V_{max}) will be compared. Lower Vmax is expected for the most inhibiting extract, due to production of less uric acid.

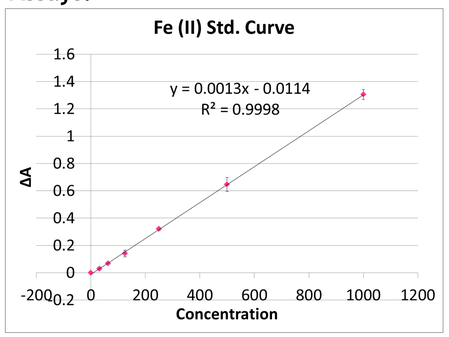
ORAC Assays (Kinetics):

Blank	Test Compound (Flavonoid/Extract)
20 uL Deionized water	20 uL Test compound (1%)
120 uL 89nM Nile blue	
Incubate for 15 minutes at 37 °C	
60 uL AAPH (0.012M)	
Read kinetics immediately for 60 min at 37 °C (excitation 620nm and emission at 680nm)	

The 89nM Nile Blue was made fresh for every test. AAPH is very sensitive to light energy, so all precautions were taken to keep the solution in dark. Cutoff of 630 nm was used instead of the automatic 665nm cutoff. The area under the curve will be compared.

ASSAY DATA ANALYSIS

FRAP Assays:

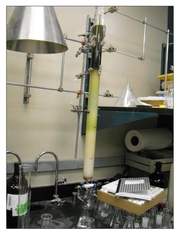


Fe^{+2} (ug/mL)	1000	500	250	125	62.5	31.25
Absorbance	1.305	0.646	0.3195	0.141	0.068	0.05
Standard Deviation	0.037	0.051	0.012	0.024	0.01	0.011

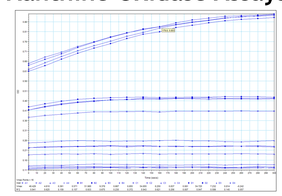
	Fe (II) (ug/uL)	% Rxn
LOORBA	7.827	80.692
LOOLA	9.665	68.256
LOMOBA	14.768	140.937
LOMOBA1	3.077	22.615
LOMOBA2	0.444	21.590
LOMOBA3	4.070	33.385
LOMOBA4	5.005	84.667
LOMOBA5	10.471	128.308
LOMOBA6	28.404	233.385
LOMOBA7	24.391	256.846
LOMOBA8	14.904	77.519
LOMOBA8s	7.833	25.981
LOMOBA9	16.631	81.846
LOMOBA9s	16.570	65.308

	Fe (II) (ug/uL)	% Rxn
LOOLA1	11.112	26.462
LOOLA2	2.209	41.269
LOOLA3	6.355	23.192
LOOLA3s	5.457	35.179
LOOLA4	2.350	26.974
LOOLA5	0.444	26.205
LOOLA6	7.048	30.747
LOOLA7	32.777	84.627
LOOLA8	10.087	75.363
LOOLA9	8.175	50.747
LOOLA10	10.868	68.330
LOOLA11	11.304	94.593
LOOLA12	29.110	137.467
LOOLA13	20.169	86.352
Trolox	275.75	1147.2

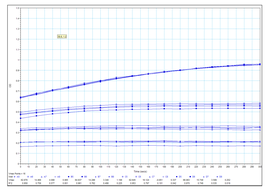
LOMOBA7 (24.391) and LOOLA11 (32.777) had the highest concentration of Fe (II), exhibiting the most antioxidant properties (reduction).



Xanthine Oxidase Assays:



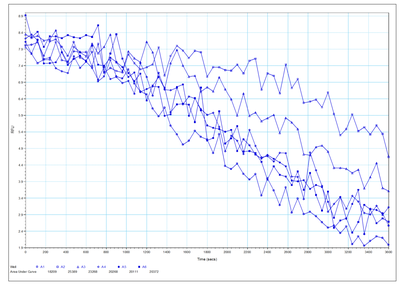
Enzyme Blank



Enzyme &Catechin

The enzyme solution without any antioxidants show high rise in the absorbance of uric acid (Vmax = 48.429 for 0.1M) and the enzyme-catechin solution shows lower rise (Vmax = 6.2).

ORAC Assays:



Trolox:	AUC:
Blank	18209
8 uM	25389
4 uM	23268
2 uM	20268
1 uM	20111
0.8 uM	20372

This is result from one assay. It shows high area under curve (AUC) for the well with the high antioxidant (trolox) concentration. Getting more data and averaging the results would allow for construction of a good example to follow for the plant extract assays.

CONCLUSIONS

Through the FRAP assays, LOMOBA7 and LOOLA11 have the highest antioxidant properties, 24.391 and 32.777 respectively. For the xanthine oxidase and ORAC assays, more assays must be done before reliable data can be found.

ACKNOWLEDGEMENT

This research was funded by the RCEU program with funds provided by the President's office, Vice President for Research, Chemistry Department through the patent account, and external funding from Alabama Space Grant Consortium.