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Dr. Vijay Gokarn
Abhinav Horizon, Plot No. 100, Jankidevi School Rd, Abhinav Healthcare Products Pvt. Ltd., MHADA, 4 Bunglows, Andheri (W), Mumbai, Maharashtra, India

Dr. Sanjay Tamoli
Abhinav Horizon, Plot No. 100, Jankidevi School Rd, Abhinav Healthcare Products Pvt. Ltd., MHADA, 4 Bunglows, Andheri (W), Mumbai, Maharashtra, India

Corresponding Author:
Dr. Vijay Gokarn
Abhinav Horizon, Plot No. 100, Jankidevi School Rd, Abhinav Healthcare Products Pvt. Ltd., MHADA, 4 Bunglows, Andheri (W), Mumbai, Maharashtra, India

Comparative *in vitro* evaluation of lipophilic oxygen radical absorbance capacity of Standardized *Curcuma longa* extract vs standardized FMB Curcu^{Zn} extract

Dr. Vijay Gokarn and Dr. Sanjay Tamoli

Abstract
Curcumin from commonly known Turmeric (*Curcuma longa*) rhizome extract is well recognized as an anti-oxidant and reported to have free radicals scavenging activity. The present research work is focused on comparative study of evaluating antioxidant activity of FMB Curcu^{Zn} extract powder (Standardized extract powder of *Curcuma longa* for Curcuminoid 95% & elemental Zinc NLT 1000ppm) vs *Curcuma longa* extract (95% Curcuminoid). Curcumin being a lipid or fat soluble molecule Lipophilic Oxygen Radical Absorbance Capacity assay was selected as a tool for establishing the antioxidant potentials between the two samples *viz.*, FMB Curcu^{Zn} extract powder vs Standardized *Curcuma longa* extract (95% Curcuminoid). Based on the results of the Invitro assay it was concluded that FMB Curcu^{Zn} extract powder was having better antioxidant activity than the conventional *Curcuma longa* extract.

Keywords: *Curcuma longa*, FMB Curcu^{Zn}, ORAC L, essential trace element (ETE)

1. Introduction
Plants and their parts encompass a blend of specific phytoconstituents along with various cofactors including trace elements that determine the therapeutic potential of the plant [1]. Phytoconstituents along with a fixed proportion of essential trace elements (ETE) together helps to achieve the desired therapeutic action from the plant material.
Standardized Herbal Extracts (SHE) is based on the principal of concentrating phytoconstituents using various solvents (Water, alcohol etc.) SHE ensures quality and consistency and helps avoiding batch to batch variations [2].
However, standardization may not address the efficacy of that respective extract. Conventional standardized herbal extracts, though concentrated, are either devoid of or have left out with negligible content of ETE's in comparison to the parent herb due to the fact that ETE's like Zinc (Zn), Iron(Fe) etc. have negligible solubility in the solvents (Water, alcohol etc.) used for extraction. Trace elements are very important for cellular functions at biological, chemical and molecular levels. These elements mediate vital biochemical reactions by acting as cofactors for many enzymes, as well as act as centers for stabilizing structures of enzymes and proteins. Some of the trace elements control important biological processes by binding to molecules on the receptor site of cell membrane or by alternating the structure of membrane to prevent entry of specific molecules into the cell. The functions of trace elements have a dual role. In normal levels, they are important for stabilization of the cellular structures, but in deficiency states may stimulate alternate pathways and cause diseases. These trace elements have clinical significance [1] which plays a critical role in the therapeutic activity of the parent herb [3]
As a proof of Concept to demonstrate that trace element Zinc (Zn) is significantly lost during solvent extraction, a small experiment of estimating the Essential trace element, Zinc (Zn) was performed using ICP OES technique. The elemental Zinc in raw turmeric rhizome powder and its extract, *viz*., *Curcuma longa* extract 95% was evaluated. The results obtained are tabulated in Table 1, Figure 1 showing elemental Zinc (Zn) content in the Extract was almost negligible proving the hypothesis of losing the essential trace minerals due to solvent extraction.

Table 1: Elemental Zinc Content in Turmeric (*Curcuma longa*) Rhizome Powder & in Standardized *Curcuma longa* Extract Powder.

Elemental Zinc Content in PPM	
Curcuma longa Rhizome Powder	Standardized *Curcuma longa* Extract
22.03 PPM	1.61 PPM

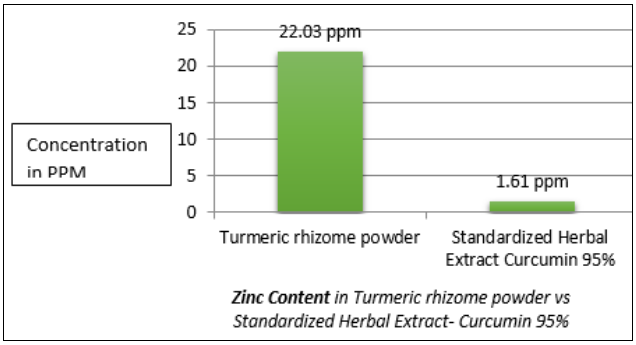


Fig 1: Elemental Zinc Content in Turmeric (*Curcuma longa*) Rhizome Powder & in Standardized *Curcuma longa* Extract Powder.

Hence in the present research FMB Curcu^{Zn} extract powder was prepared by FMB process, where the essential trace element (ETE) lost is replenished back into the Extract. The name FMB Curcu^{Zn} is coined by FMB process being used for meticulously fusing the known amount Essential element Zinc (Zn) in the extract powder of standardized *Curcuma longa* having 95% Curcuminoids. FMB process is based on the knowledge of chemistry, character and energetics of respective plant gained from traditional wisdom. This knowledge is combined and balanced with leading modern research and decades of clinical experience. FMB process thus is THE Optimized sequential process of fusing the trace element zinc (Zn) in the standardized *Curcuma longa* extract. Both the extracts *viz*. *Curcuma longa* extract & FMB Curcu^{Zn} extract were subjected to ORAC Lipophilic assay for comparative evaluation of Antioxidant potentials between them.

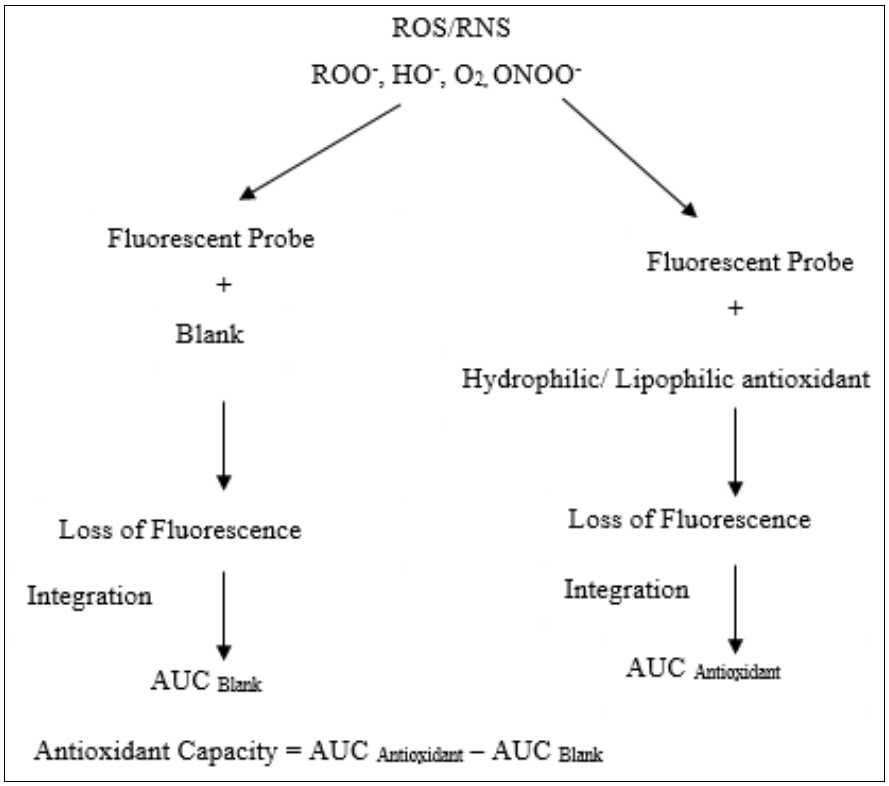
Oxygen Radical Absorbance Capacity (ORAC) assays has been widely accepted as a standard tool to measure the antioxidant activity [4]. Free radicals are generally very reactive molecules possessing an unpaired electron which are produced continuously in cells either as by-products of metabolism or by leakage from mitochondrial respiration [5].

The free radicals produced *in-vivo* include the active oxygen species such as super-oxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [5].

Oxygen free radicals have been shown to be responsible for many pathological conditions. Free radicals and Reactive Oxygen Species (ROS) cause DNA damage, lipid per-oxidation and protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders as cancer, cardiovascular diseases, inflammatory diseases, asthma and aging [6]. Free radicals like the hydroxyl radical, hydrogen peroxide, superoxide anion mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Superoxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorphonuclear leucocytes to the endothelium and stimulation of platelet aggregation [7].

2. Principle of ORAC assay

The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. In the present assay, AAPH [2, 2'-azobis (2-methyl propionamidine) dihydrochloride] is used as free radical generator to reduce the fluorescence characteristics of sodium fluorescein, which is used as the fluorescence probe. The change of fluorescence intensity (reduction in fluorescence) is an index of the degree of free radical damage. In the presence of an antioxidant, there is decrease in the change of fluorescence induced by AAPH. In the ORAC assay, the antioxidant activity of a sample is expressed relative to Trolox [(±) 6-hydroxy-2, 5, 7, 8-tetra methylchromane-2-carboxylic acid], a water soluble analog of Vitamin - E. The area under the curve (AUC) for TROLOX and sample is calculated from the data obtained. The net AUC corresponding to a sample is calculated by subtracting the AUC corresponding to the control (blank).



Materials and Methods

Reagents / chemicals used:

1. Sodium phosphate, monobasic, anhydrous, A.R. (NaH_2PO_4, RM3964, Himedia, India)
2. Di-sodium hydrogen phosphate dihydrate, A.R. ($Na_2HPO_4.2H_2O$, RM257, Himedia, India)
3. Fluoresceinsodium salt (F6377, Sigma, USA)
4. AAPH [2,2'-azobis(2-methylpropionamidine) dihydrochloride, 44091-4, Aldrich, USA]
5. Trolox[(±)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid, 56510, Fluka, USA]
6. Acetone, A.R. (AS025, Himedia, India)
7. Randomly Methylated β – cyclodextrin DS-12 (RMCD) (Cat # CY-2004.1, Cyclolab, Budapest)

Miscellaneous

Microwell plate: Costar96 well plate, polystyrene, non-treated, black round bottom wells, non-sterile (Cat. no. 3792, Corning, USA)

Sample preparation

The samples FMB Curcu^{Zn} & *Curcuma longa* extract 95% a stock solution of 5000 µg/mL was prepared.

Briefly, 10mg of samples were dissolved in 1.0ml of 100% acetone. For calculation purposes the samples were considered to be 100% soluble. The 100% acetone stock was further diluted with 1:1 acetone water mixture containing RMCD to get 5000µg/mL stock solution.

Trolox (standard) preparation

For trolox, a stock solution of 5000µg/mL was prepared.
Briefly, 5mg of trolox was dissolved in 1000µL of 100% acetone.This stock was further diluted with 1:1 acetone water mixture containing RMCD.

Procedure

This assay was performed as per Huang *et al.* (2002) [8] and Ganske, F. (BMG LABTECH, Application Note 148 Rev. 12/2006) [9]. A pre-incubation mixture contained acetone water mixture containing RMCD / test solution / trolox of various concentrations and sodium fluorescein. The plate was mixed and pre-incubated. Following pre-incubation, AAPH was added and mixed. The reaction was carried out and the fluorescence measurements were taken at 485nm excitation and 520nm emission filters with the following settings:

1. Mode: Fluorescence intensity
2. Filters: Excitation 485nm, emission 520nm
3. Plate Used: Costar 96, Code 3792 from Corning

Data reduction and ORAC value calculation was done as per Davalos *et al.* 2004 [10].

Results

Results for ORAC Lipophilic assay are tabulated in Table 2 & standard curve of Trolox is shown in Figure 2.

ORAC value (Lipophilic) of sample FMB Curcu^{Zn}, was found to be 335.80 µM of Trolox Equivalent per gram.

ORAC value (Lipophilic) of sample *Curcuma longa* extract, was found to be 283.30 µM of Trolox Equivalent per gram.

- Refer Table 2 for ORAC value (Lipophilic) of tested samples.
- Refer Figure 2 for trolox standard curve.

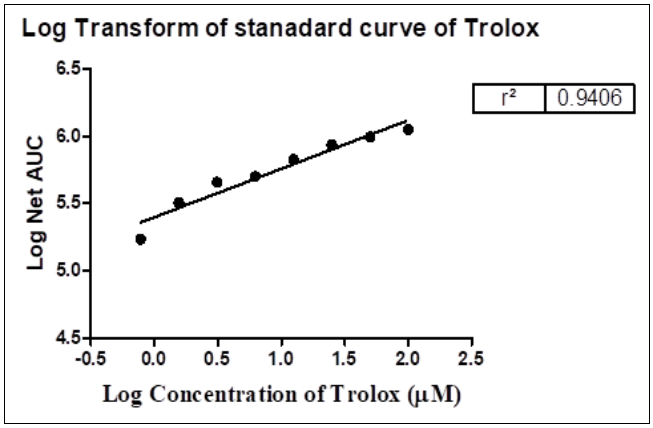


Fig 2: Standard Curve of Trolox

Table 2: ORAC values (Lipophilic) of tested samples

Sample	Conc.	Net AUC (Mean ± SD)	Trolox Equivalent (Extrapolated values) (Mean ± SD)	ORAC Value** (Mean ± SD)	Average ORAC Value**
Trolox (Standard)	0.78125µM	171075±5450	Not Applicable	Not Applicable	Not Applicable
	1.5625µM	319202 ± 51112			
	3.125µM	454093 ± 84853			
	6.25µM	502093 ± 29698			
	12.5µM	671593 ± 7778			
	25µM	960093 ± 57983			
	50µM	986093 ± 29698			
	100µM	1121000 ± 20435			
FMB Curcu^{Zn} Extract Powder	4.88µg/ml	960093 ± 57983	1.64±0.09	335.80± 17.45	335.80
	9.766µg/ml	2545593*	-	-	
	19.53µg/ml	2943093*	-	-	
	39.06µg/ml	2470593*	-	-	
	78.12µg/ml	1680093*			
Curcuma longa extract powder	4.88µg/ml	805593 ± 13647	1.38± 0.24	283.30 ± 49.15	283.30
	9.766µg/ml	2269593*	-	-	
	19.53µg/ml	1805593*	-	-	
	39.06µg/ml	1404093*	-	-	
	78.12µg/ml	2636093*			

* Values are out of range of standard curve, hence not considered.
** Expressed as micro moles of trolox equivalent per gram.

5. Discussion

The samples subjected to ORAC Lipophilic assay suggests FMB Curcu^{Zn} extract powder has 18.53% greater Antioxidant activity than the Conventional *Curcuma longa* extract. The

enhanced Antioxidant activity can be attributed to the presence of essential trace element replenished back into the *Curcuma longa* extract by the innovative technique being used *viz*. FMB Process which is not just mere admixture of the element Zinc but a meticulous three step sequential process of Fusion (F), Micronization (M) & Bioligation (B).
The present research work suggests exploring the FMB technology as a valuable tool for addressing enhanced therapeutic activity of the herbal extracts with selective or specific Essential trace element.

6. Conclusion
A significant relationship between presence of essential trace element like Zinc (Zn) and its influence in enhancing the antioxidant capacity of *Curcuma longa* extract was found indicates these trace elements definitely have a major contribution in enhancing the antioxidant property of plants.
The present research work also opens new avenues in the field of Standardized Herbal Extracts being standardized not only for the essential phyto actives but also their characteristic Essential Trace elements. The research work suggests that each herbal extract should be further investigated for its characteristic trace element that is lost during solvent extraction process of the respective herb/plant part.

7. Acknowledgement
We sincerely appreciate Natural Remedies R&D Laboratory for conducting the ORAC-L Assay of the said samples.

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