



A publication of College of Natural and Applied Sciences, Fountain University, Osogbo, Nigeria.

Journal homepage: www.fountainjournals.com

ISSN: 2354-337X(Online), 2350-1863(Print)

Assay-Guided Extraction and Kinetics of Radical Scavenging Activity of Curcumin from the Rhizome of *Curcuma longa* Linn

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Abstract

Extraction of phytochemicals from medicinal plants need to be optimized to produce standardized, dose-dependent and reliable extracts to meet food or drug administration regulations for translation into approved nutraceutical or pharmaceutical products. Effect of selected limiting extraction variables on yield and antiradical activity of curcumin-rich extract from *Curcuma longa* rhizomes was investigated. Assay-guided response (free radical scavenging activity) was used to determine the optimized set of extraction parameters. Curcumin-rich extract was produced using solvent boiling process at 65 °C with absolute methanol of weight ratio 1:50 (w/v) within 1 h. Relative radical scavenging activity of the extract derived using the optimized set of parameters recorded an IC₅₀ of 497 µg/ml and its total antiradical capacity determined within 6 h (76 - 85 % at a concentration of 400 – 600 µg/ml) was not significantly different from that of commercially available natural curcumin used as reference. Time-dependent kinetic analysis revealed an initial fast burst in rate of activity (2.27 – 7.77 min⁻¹) followed by a slow reaction rate in a steady second phase (0.12 – 0.54 min⁻¹). Conclusively, appreciable amount of curcumin-rich extract of enhanced antiradical activity was extracted from the rhizome of *C. longa* using assay-guided procedure.

Keywords: assay-guided extraction; antioxidant; curcumin; *Curcuma longa*; free radical; kinetics

Introduction

Curcuma longa L. (Turmeric; Zingiberaceae family) is widely distributed throughout the tropical and subtropical regions (Stankovic, 2004). It is majorly used as a spice or coloring substance in foods and traditionally in phyto-medicine for treatment of wide range of diseases. Curcumin (diferuloyl methane) is an orange-yellow crystalline hydrophobic polyphenolic constituent of the

rhizomes of *C. longa*. Curcumin and its derivatives such as demethoxycurcumin and bisdemethoxycurcumin are referred to as curcuminoid. Due to its colorimetric nature, curcumin has broad absorption around 420 nm in organic solvents and UV-Visible spectrophotometric

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techniques have been employed to quantify the phytochemical either in its natural, pure or pharmaceutical form (Jasim and Ali, 1989; Tang *et al.*, 2002). Curcumin exhibit a keto-enol tautomerism with about 95 % enol conformation in certain solvents. The phenolic functional group with hydrogen donating ability had been implicated in the mechanism of curcumin antioxidant action (Priyadarsini *et al.*, 2003). Due to these outstanding properties, *C. longa* rhizomes have been widely accepted as one of the spices with the highest inhibitory activities against free radical induced oxidation and lipid peroxidation (Khopde *et al.*, 2000; Wojdyło *et al.*, 2007). *C. longa* is also rich in lipid soluble turmerones (Jain *et al.*, 2007), however the quality and value extract from the plant lies in its pharmacological properties which is based on the percentage of curcuminoid in the extract. Curcumin has been shown to exhibit a wide range of biological activities such as antioxidant, antimicrobial, antidiabetic, antimalarial and anticarcinogenic as well as protective effect against hepatotoxicity and neurotoxicity with detailed documented reviews by various authors (Anand *et al.*, 2008; Goel *et al.*, 2008; Perrone *et al.*, 2015).

Development of herbal extracts to meet drug administration regulations and approval rate requires optimization of extraction methods and standardization of botanicals and herbal formulations (Ong, 2004). Several extraction methods have been used to extract *Curcumin* from *C. longa* rhizomes including maceration, supercritical fluid extraction, soxhlet extraction process, microwave assisted process among others (Kulkarni *et al.*, 2012; Mandal *et al.*, 2008; Paulucci *et al.*, 2013; Sogi *et al.*, 2010).

Yield, composition and quality of botanical extracts from *C. longa* are greatly influenced by processing technologies as well as operating conditions. Extraction variables such as solvent used for extraction, concentration of solvent, solid sample to solvent ratio, extraction time and temperature are assumed to significantly influence the yield and activity of curcumin extraction (Wakte *et al.*, 2011). Optimization of these extraction

variables using assay-guided procedure will provide standardized extracts of high quality.

In this study, effect of time, temperature and concentration of solvent as well as solid sample to solvent volume ratio on the yield and antiradical activity of curcumin from *C. longa* rhizomes were investigated using DPPH radical scavenging assay as bioactivity response. The objective is to produce curcumin-rich extracts using optimized set of conditions and used time-dependent kinetic data to analyze the antioxidant properties of the optimized extracts.

Materials and Methods

Plant sample

The rhizomes of *Curcuma longa* was obtained from the premises of Sheda Science and Technology Complex, Abuja, Nigeria. Identification was carried out at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, where voucher specimen was deposited (FHI 106920).

Chemicals

Commercially available natural curcumin used as reference was obtained from Santa Cruz Biotechnology (Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was purchased from Sigma-Aldrich Chemical Company (USA), while the solvent of extraction methanol, ethanol, chloroform and acetone were from BDH Laboratories (UK).

Preparation of sample

Fresh rhizomes were washed with distilled water, air-dried for a week and further oven-dried at 40 °C for six hours. The dried rhizomes were sliced, pulverized and sieved.

Extraction of curcumin and effect of extraction parameters

Curcumin was extracted from rhizomes of *Curcuma longa* (turmeric) and the effect of following limiting extraction parameters (Solvent, temperature and time of extraction as well as concentration of solvent and solid sample to solvent volume ratio) on yield and quality of the curcumin extracted were evaluated.

Solvent of extraction

Powdered *C. longa* rhizomes (5 g) were weighed into 500 ml conical flasks and the solvent of extraction (250 ml of acetone, chloroform, ethanol or methanol) was added and extracted separately at room temperature (27 °C) in a shaker (250 rpm) for 6 h.

Extraction temperature

C. longa rhizome samples (5 g) were weighed into 1 L conical flasks; 250 ml of methanol was added and kept at room temperature (27 °C) for 1 h. For cold extraction (4 °C), samples were flushed with liquid nitrogen and mixed with cold 250 ml of methanol (refrigerated prior to use) and kept in a cold room. For elevated temperature regime (65 °C), extraction was carried out for 6 h using a Soxhlet set-up (Kulkarni *et al.*, 2012).

Concentration of solvent

Samples (5 g) were weighed into a thimble and 250 ml of various concentration of methanol (60, 70, 80, 90 and 100 %) was added. The extraction was carried out at 65 °C for 6 h.

Solid sample to solvent ratio

The volume of the solvent of extraction was varied to give 1:50, 1:100 and 1:200 w/v ratios of *C. longa* rhizome sample to amount of methanol used. The extractions were carried out at 65 °C for 1 h using 250 ml of methanol.

Extraction time

The effect of extraction time within a 12 h period (0.25, 0.5, 1, 2, 3, 4, 5, 6 and 12) was also evaluated. The extractions were carried out at 65 °C for 1 h. All experiments were carried out in triplicates. The various extracts were concentrated to about 5 ml using a rotary evaporator and dried using evaporating dish at 40 °C.

Quantification of curcumin

Extracts from turmeric rhizomes was reconstituted in 1:10 with Methanol to a total of 4 ml. Peak plasmon resonance absorbance of curcumin was determined using a UV-VIS scanning spectrophotometer. Maximum absorbance for curcumin was 405 nm. Standard curves of curcumin (1-100 µg) in methanol were prepared.

Concentrations of total polyphenols were derived by regressing absorbance readings of test samples onto the standard curve of curcumin.

Antiradical assay

Bioassay guided extraction of curcumin from the rhizomes of *C. longa* was carried out to determine the optimized set of extraction parameters. The extracted curcumin were evaluated using DPPH radical scavenging activity according to the method reported by Terpinic *et al.* (Terpinic *et al.*, 2009) with slight modification. DPPH radical solution (0.1 mM) was prepared in 90 % v/v ethanol. The DPPH solution (2.9 ml) was added to 0.1 ml solution of the extracted and reference curcumin at concentrations ranging from 0.05 to 10 mg/ml. The resulting solution was mixed and incubated in the dark for 30 min. The absorbance (Abs) was determined at 517 nm. The control solution consisted of 0.1 ml of 90 % ethanol in lieu of the phytochemicals. Ethanol (90 % v/v) was used as a blank. Analyses were carried out in triplicates. Percentage inhibition of DPPH radical was calculated as $\% \text{ DPPH inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / (\text{Abs}_{\text{control}}) \times 100$.

Time-dependent (kinetic) study

Curcumin was extracted from *C. longa* using set of the extraction parameters that gave the highest bioactivity and re-evaluated for DPPH radical scavenging activity. The mean inhibitory concentration (IC₅₀) was determined. Based on the IC₅₀, a range of concentration was prepared and at various time intervals (Terpinic *et al.*, 2009), the disappearance of the purple coloration of DPPH radical was observed for 6 h. The activity coefficients (relative and total antiradical activity), mean inhibitory time (MIT), rate constants (regression coefficients), rate of activity at 5, 30 and 360 min as well as time-dependent activity indices were calculated from accrued data.

Statistical analysis

Statistical evaluation of data was performed by Graph pad prism version 5.02. All data are expressed as the mean of at least three replicates \pm standard error of mean. Student's t-test was used to compare between control and test experiments, while one way analysis of variance followed by

Turkey's post hoc test was used for multiple comparison.

Results and Discussion

Development of herbal extracts to meet drug administration regulations and approval rate requires optimization of extraction methods and standardization of botanicals as well as herbal formulations (Ong, 2004; Thakur *et al.*, 2011). Several methods have been used to extract Curcumin from *C. longa* rhizomes including maceration, supercritical fluid extraction, soxhlet extraction and microwave assisted process (Kulkarni *et al.*, 2012; Mandal *et al.*, 2008; Paulucci *et al.*, 2013; Sogi *et al.*, 2010). Yield, composition and quality of botanical extracts from *C. longa* are greatly influenced by processing technologies as well as operating conditions. Extraction variables such as the type of solvent used for extraction, concentration of the solvent, time and temperature of extraction as well as solid sample to solvent ratio influence the yield and bioactivity of extracted curcumin. Assay-guided optimization of extraction parameters are employed not only to increase yield and process efficiency but also to enhance or at least sustain antiradical activity of the extracts (Weller, 2012).

Effects of solvent of extraction

Table 1 shows the effects of various solvents used for extraction on the yield and antiradical activity of curcumin extracted from *C. longa* rhizomes. Extraction with methanol gave significantly ($p < 0.05$) lower yield when compared to any of the other solvents used for extraction. In contrast, DPPH radical scavenging (antiradical) activity of the methanolic extract was significantly ($p < 0.05$) higher than the other extracts. Yield of curcumin were not different when extracted with either acetone, chloroform or ethanol. Extraction with acetone yielded twice as much curcumin as the methanolic extracts but of less antiradical activity. Curcumin, the major curcuminoid of *C. longa* extracts is easily degraded to give feruloylmethane and other low molecular weight products; hydrolysis of feruloylmethane produces vanillin and acetone (Wang *et al.*, 1997; Tsuda, 2018). Extraction

with acetone may promote such hydrolysis and degradation of the extracted curcumin resulting in loss of activity.

Table 1. Yield and free (DPPH) radical scavenging activity of curcumin extracted from turmeric rhizomes using different solvents

	Yield (mg/g)	DPPH Inhibition (%)
Acetone	16.79±1.47 ^b	40.25±1.30 ^a
Chloroform	15.08±0.16 ^b	63.84±1.51 ^c
Ethanol	15.32±0.03 ^b	55.11±1.11 ^b
Methanol	8.25±0.63 ^a	67.61±1.09 ^d

*Values are expressed as mean of three replicates ± SEM; Values along a column with different superscript letters are significantly ($P < 0.05$) different from each other.

Chloroform extracts also exhibited lower activity; chloroform is generally used to precipitate or extract lipid soluble fractions. Tumerones, which are more lipid-soluble phyto-constituents of turmeric are likely to form the bulk of the chloroform fractions (Lee *et al.*, 2006). The antioxidant activity of turmeric extracts is dictated by the amount of curcuminoids present in the samples (Priyadarsini *et al.*, 2003). Lower percentage of curcuminoids or higher concentration of tumerones in the extract will result in concomitant lower activity. Also, extraction with ethanol yielded high amount of curcumin of moderate antiradical activity. Curcumin and its derivatives are totally soluble in ethanol (Stankovic, 2004), the absolute solubility may enhance modification to less active forms or derivatives and this may be the cause for the moderate to low DDPH activity recorded by the ethanolic extract. The solvent (methanol) that gave the highest free radical activity was used as for further evaluation of the other extraction parameters.

Effects of concentration of solvent and sample to volume ratio

Effects of concentration of methanol and the volume of methanol used for extraction per gram of

sample on yield and antiradical potential of curcumin from turmeric extracts are shown in Figures 1 and 2 respectively. Reducing the concentration of methanol for extraction caused significant ($p < 0.05$) reduction in yield and antiradical activity of the extracted curcumin, although the antioxidant capacity of 90 % methanolic extract was not significantly different from those of the curcumin extracted with absolute methanol. Since curcumin is insoluble in water, extraction with lower concentration of methanol may result in lower yield. Although, Paulucci *et al.* (Paulucci *et al.*, 2013) reported that extraction via maceration process using 70 % ethanol gave maximum curcumin yield when compared to extraction with 96 % ethanol. In this study, reduction in the concentration of absolute methanol by 10 % gave about 58 % reduction in the amount of curcumin extracted. Choice of solvent (ethanol vs. methanol) and processing method (maceration vs. solvent boiling) used for extraction may be responsible for the discrepancy in findings.

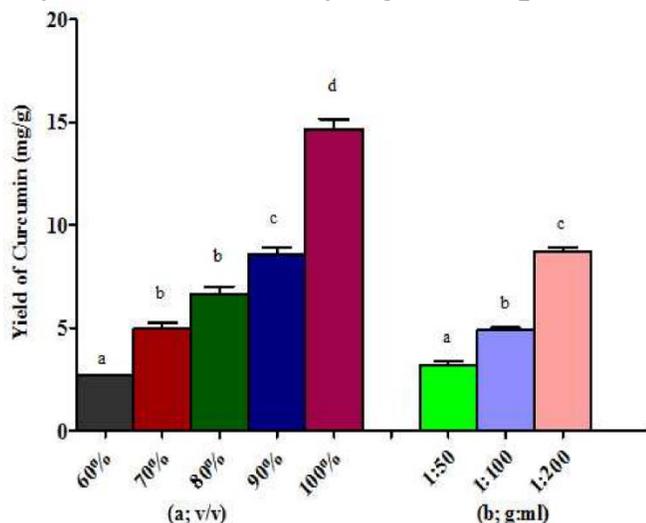


Figure 1: Yield of curcumin from turmeric rhizomes as affected by varying (a) concentration of methanol and (b) weight of sample to volume of methanol used for extraction

*Bars are expressed as mean of three replicates \pm SEM; Bars with different superscript letters are significantly ($P < 0.05$) different from each other.

Previous study by Sogi *et al.* (Sogi *et al.*, 2010) reported that the solid sample to solvent volume

ratio exhibited marginal effect on yield of curcumin from *C. longa* rhizomes. However, in this study, there was significant ($p < 0.05$) increase in the yield of curcumin as the solid to solvent ratio increases,

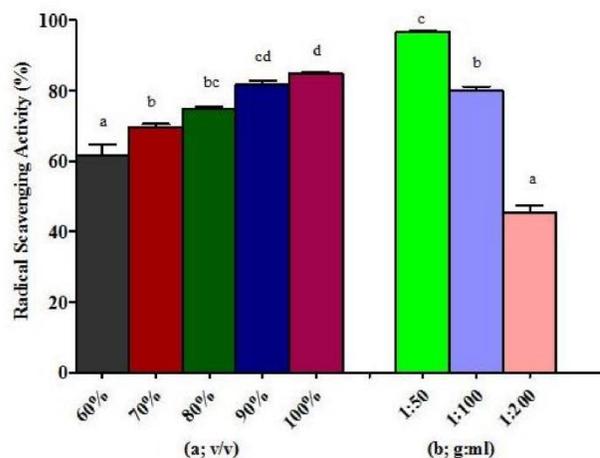


Figure 2: Free (DPPH) radical scavenging activity of curcumin extracted from turmeric rhizomes using different (a) concentration of methanol and (b) weight of sample to volume of methanol ratio *Bars are expressed as mean of three replicates \pm SEM; Bars with different superscript letters are significantly ($P < 0.05$) different from each other.

but the antiradical activity of the extract significantly reduced as the volume of methanol used for extraction increases. Dispersion in solvent is improved and leaching is enhanced, when large volume of extracting medium is used. However, this may cause reduction in activity due to modification, isomerization and alterations in the composition of solubilized curcumin and its derivatives (Stankovic, 2004). Most curcuminoids (except tetra-hydro-curcuminoid) can easily be degraded during extraction process and curcumin had been shown to have higher inhibitory effects on DPPH radical and lipid peroxidation of liver microsomes than its closest derivative demethoxycurcumin (Priyadarsini *et al.*, 2003); the phenolic $-OH$ was demonstrated by the authors using density-functional theory to be responsible for both its antioxidant activity and free radical kinetics.

Effects of temperature and time of extraction

The effects of extraction conditions (temperature and time) on yield and antiradical activity of curcumin from *C. longa* are shown on Figures 3 and 4 respectively. Solvent boiling process gave significantly ($p < 0.05$) higher yield of curcumin-rich extract when compared to extraction at lower temperature regimes, Also, the Soxhlet-derived extracts gave the highest DPPH radical scavenging activity but the activity was not significantly different from those extracted at room temperature. Extracts derived via cold process recorded significantly ($p < 0.05$) lower *in vitro* antioxidant capacity when compared to the other methods. In correlation with this study, temperature ≥ 60 °C had been reported to enhance leaching of curcumin resulting in higher yield (Sogi *et al.*, 2010). In addition, extraction at elevated temperature did not affect the bioactivity of the curcumin extracts as observed in this study.

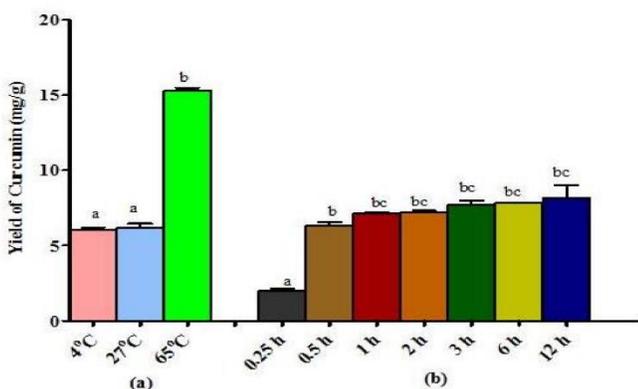


Figure 3: Yield of curcumin from turmeric rhizomes as affected by (a) temperature and (b) time of extraction

*Bars are expressed as mean of three replicates \pm SEM; Bars with different superscript letters are significantly ($P < 0.05$) different from each other.

Yield of curcumin increased by two folds when the time of extraction was extended from 15 to 30 min and the antiradical activity of the extract was not significantly affected. However, extending the time of extraction to 1 h significantly ($p < 0.05$) increased both yield and antiradical activity.

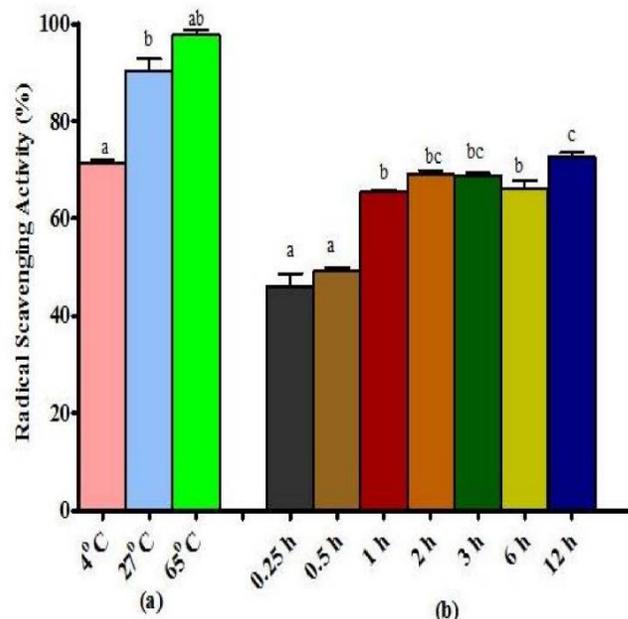


Figure 4: Free (DPPH) radical scavenging activity of curcumin derived from turmeric rhizomes at different (a) temperature and (b) time of extraction. *Bars are expressed as mean of three replicates \pm SEM; Bars with different superscript letters are significantly ($P < 0.05$) different from each other.

Further increase in time did not result to significant increase in antiradical activity or yield. Extraction time of 1 h was enough to achieve optimum yield and bioactivity; this was faster than the optimum extraction time reported by Kulkarni *et al.* (Kulkarni *et al.*, 2012) and Paulucci *et al.* (Paulucci *et al.*, 2013).

Comparative study with commercially available natural curcumin

Based on the parameters that gave maximum antiradical activity, curcumin was extracted from turmeric rhizome at 65 °C for 1 h with 50 ml of absolute methanol per gram of sample. Approximately 17 mg of curcumin was quantified. The free radical scavenging activity of varying concentration of the curcumin extract derived using optimized set of extraction parameters is shown in Figure 5.

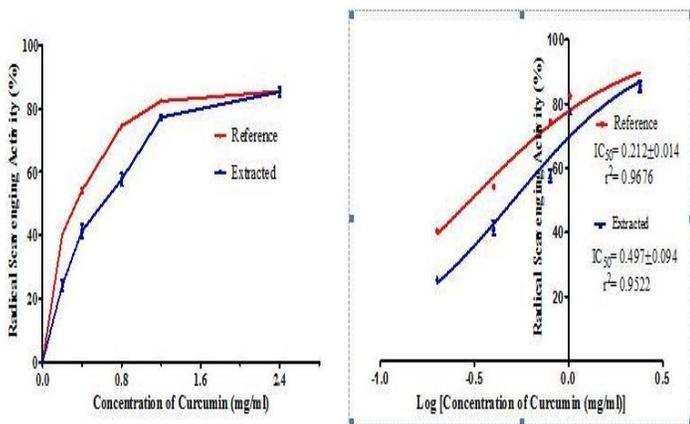


Figure 5: Free (DPPH) radical scavenging activities of varying concentration of the extracted and reference curcumin

*Values are expressed as mean of three replicates \pm SEM; IC_{50} = Mean Inhibitory Concentration; r^2 = Regression coefficient.

The antioxidant activity of the extracted curcumin was concentration dependent and competes favourably with commercially available curcumin used as reference for comparison. The DPPH radical scavenging activity at a high concentration of 2.4 mg/ml of the extracted curcumin was not significantly different from that of the reference at the same concentration. The DPPH radical is widely used to assess the free radical scavenging capacity of antioxidants (Terpinc *et al.*, 2009) and it is the most frequently employed *in vitro* methods to evaluate antioxidant activity (Alam *et al.*, 2013). Assay-guided extraction and optimization of curcumin from *C. longa* rhizomes yielded high quality DPPH radical scavenging extracts with mean inhibitory concentration (IC_{50}) value of 497 μ g/ml; the lower the IC_{50} value, the higher the inhibitory or radical scavenging capacity.

Time-dependent (kinetic) study

When studied statically, the computed IC_{50} value for the extracted curcumin (497 μ g/ml) was higher than the reference curcumin (212 μ g/ml) indicating lower activity. Similarly, relative antiradical capacities of the extracted curcumin at lower concentrations (0.2 - 0.6 mg/ml) were lower than

those of the reference curcumin (Table 2). Kinetic parameters have been proposed to provide better information on antioxidant status than static studies (Akolade *et al.*, 2017; Terpinc *et al.*, 2009). Antioxidant capacities are derived at a fixed endpoint, but time dependent (kinetic) studies provide better insight to reaction rate, rate constants and activity index after the initial burst activity. Time-course study of the DPPH radical scavenging activity within a 6 h incubation period showed that relative antioxidant capacity (RAC; 30 min incubation) of 0.4 mg/ml of extracted curcumin was significantly lower than reference curcumin, while total antioxidant capacities (TAC; 6 h incubation period) of both were approximately 80 % (TAC; Figure 6).

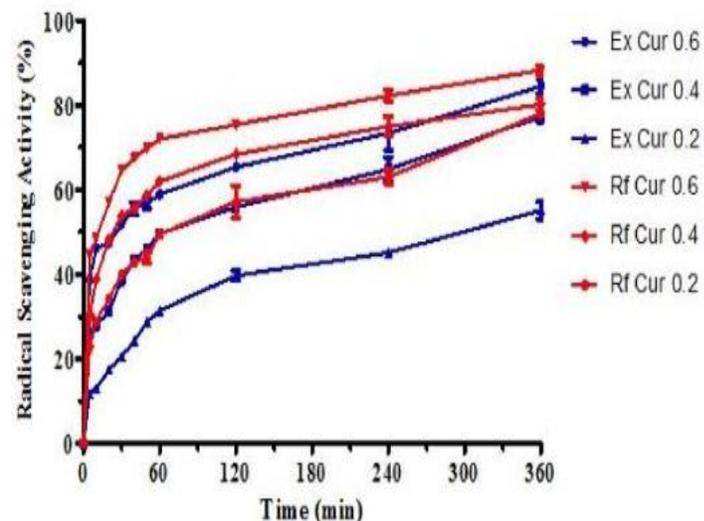


Figure 6: Time-dependent free (DPPH) radical scavenging activities of 0.2 - 0.6 mg/ml of extracted (Ex Cur) and reference curcumin (Rf Cur)

*Values are expressed as mean of three replicates \pm SE

The discrepancy can be attributed to the time required to scavenge the radical. The mean inhibitory time (MIT) for the extracted curcumin at 0.4 mg/ml to scavenge 50 % of the DPPH radical (~ 62 min) was significantly slower when compared to the reference (~ 24 min; Table 2). Data from this study showed that results determined at a static endpoint do not strongly correlate to the kinetic data. The rate of antiradical activity as well as the activity index of both the extracted and reference curcumin decreased as time increases.

Table 2. Kinetics of the DDPH radical scavenging activities of curcumin extracted from *Curcuma longa*

Curcumin	RAC	TAC	MIT	r ²	Rs (t=5)	Rs (t=30)	Rs (t=360)	α	β
Extracted									
0.2	20.50±0.34 ^a	55.08±1.21 ^a	72.26±1.26 ^d	0.9878	2.34±0.07 ^a	0.35±0.00 ^a	0.12±0.00 ^a	0.151 ^c	0.052 ^f
0.4	38.54±0.53 ^b	77.25±0.77 ^b	61.79±3.34 ^c	0.9810	5.23±0.11 ^c	0.50±0.00 ^b	0.14±0.00 ^b	0.095 ^b	0.028 ^d
0.6	51.87±0.47 ^c	84.46±1.00 ^c	61.19±1.77 ^c	0.9669	7.71±0.06 ^e	0.53±0.01 ^b	0.13±0.00 ^a	0.069 ^a	0.017 ^b
Reference									
0.2	39.89±0.28 ^b	78.00±0.31 ^b	47.46±1.40 ^b	0.9845	4.56±0.01	0.68±0.01 ^c	0.15±0.00 ^c	0.150 ^c	0.034 ^b
0.4	53.87±0.46 ^c	80.17±0.84 ^b	25.84±1.13 ^a	0.9913	6.05±0.03	0.95±0.01 ^d	0.14±0.00 ^b	0.156 ^c	0.023 ^c
0.6	64.53±0.42 ^d	88.23±0.65 ^c	23.84±1.22 ^a	0.9877	8.90±0.02 ^f	0.80±0.01 ^e	0.12±0.00 ^a	0.090 ^b	0.014 ^a

RAC = Relative Antiradical Activity; TAC = Total Antioxidant Activity; MIT = Mean Inhibition Time; r² = Regression Coefficients; R_s = Reaction Rate; t = Time in Minute; α = Relative activity index at 30 min; β = Total activity index at 360 min.

Conclusion

Assay-guided extraction using the optimized set of parameters (50 ml of absolute methanol per gram of sample at 65 °C for 1 h) afforded appreciable amount of curcumin-rich extract from the rhizomes of turmeric. The extract showed high free radical scavenging activity. Kinetic analysis of the time-dependent study showed that the rate of activity regressed with time. Overall, high quality phytochemicals (curcumin rich-extract) with enhanced antiradical activity was extracted from the rhizomes of *C. longa* and the bioactivity of the extract compared favourably with commercially available natural curcumin.

Acknowledgement

We acknowledge the memory of Late Prof. N.O. Muhammad of the Federal University, Dutsin-Ma, Nigeria, for his contribution to this work.

Conflict of Interest

The authors declare no conflict of interest.

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