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Antioxidant Activity of Curcumin and Neem (*Azadirachta indica*) Powders: Combination Studies with ALA Using MCF-7 Breast Cancer Cells

T. K. N. Cheung[1], P. Singh Nigam[1] and R. Owusu-Apenten[1*]

[1]School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine BT52 1SA, UK.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ROA and PSN designed the study and wrote the protocol. Author TKNC wrote the first draft of the manuscript. Author TKNC managed the literature searches and the experimental process. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Breast cancer is the most common cancer affecting women globally. The essential fatty acid α-Linolenic acid (ALA) and its oxidation products inhibit cancer cell proliferation. The effect of natural antioxidants on ALA anticancer effects has not been well characterized.
Aims: To assess the effect of curcumin and neem leaf powder extract, on ALA cytotoxicity activity towards MCF-7 breast cancers.
Study Design: *In-vitro* testing.
Methodology: Antioxidant activity of neem extract and curcumin were evaluated using, four assays: Total phenolic content, Ferric reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay or 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay. Cytotoxicity activity was assessed using MCF7 cells grown in DMEM (+10% FBS) and evaluated using Sulforhodamine B colorimetric assay for cell cytotoxicity.

**Corresponding author: Email: r.owusu-apenten@ulster.ac.uk;*

Results: Curcumin and neem leaf extract had significant antioxidant power, and values varied between the four assays. Treatments of MCF7 cells with ALA, (0-500 µM) curcumin (0-50 µM), and neem leaf extract *(0-88 µM)* individually produced a concentration-dependent decrease in MCF-7 cell viability. Combination treatments using ALA with curcumin and ALA with neem were significantly less effective compared to individual treatments.
Conclusion: Combinations studies indicate that the natural antioxidants curcumin and neem reduce the inhibitory effect of α-Linolenic acid towards MCF-7 breast cancer cells.

Keywords: Antioxidant; total phenols; curcumin, neem (Azadirachta indica), linolenic acid; MCF-7 cell; anticancer.

1. INTRODUCTION

Cancer is a debilitation disease that afflicts a noticeable proportion of the world population in all generations. Breast cancer is the most common cancer among women globally with 1.67 million new cases diagnosed in 2012 and accounting for 25% of all cancers [1]. Diet is a moderating factor for cancer risk and high intakes of marine and fish derived n-3 fatty acids were associated with reduced risk of cancer though the relations are controversial [2]. Past investigations showed that Essential Fatty Acids (EFAs) inhibit the proliferation breast cancer cells [3,4] and that lipid peroxidation products may be implicated; reviewed in [5-8]. Alpha-Linolenic acid (ALA) is an unsaturated fatty acid that is essential for humans since it is not produced within the human body. Dietary ALA is converted to Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA) but the conversion rate may be variable depending on a range of factors including age [9]. Breast cancer cells have an increased requirement for n-3 fatty acids owing to a low D6 desaturase activity for converting ALA to EPA and DHA [3,8].

Recent attention has focused on antioxidants, herbals and traditional medicines from natural sources [10]. Curcumin from the spice turmeric expresses anticancer activity via multiple pathways linked with inflammation signaling, cell proliferation, invasion, cell death and gene expression (reviewed in [11-14]) and is currently undergoing Phase I-II clinical trials [13]. Neem (*Azadirachta indica*) leaf has been a traditional herbal agent for therapeutic and agricultural uses [15-17]. Recent investigations noted significant *in-vitro* antioxidant and free radical quenching activity for curcumin [18-22] and neem [23-27]. However, no single study comparing the antioxidant capacity of curcumin and neem using a range of antioxidant assays have been published.

The anticancer effects of n-3 polyunsaturated fatty acids (PUFA) are partly attributed to lipid hydroperoxides formed by enzymatic or non-enzymatic oxidation, which processes are inhibited by the antioxidant vitamin E [28-30]. Currently, the majority of investigations of n-3 fatty acids and MCF-7 breast cancer cells focused on DHA and EPA rather than ALA. *In-vitro* tests using MCF-7 cells treated with curcumin [31-35] or neem leaf extract [36] showed anti-proliferative activity but the modes of action are not understood. No reported studies have considered the effect of curcumin or neem leaf extract on ALA anticancer activity. Herbal agents may exhibit a pro-oxidant or antioxidant effect depending on their concentrations and so the consequences of combining such compounds with ALA are uncertain. The hypothesis tested in this study was that, combination treatments with ALA and natural antioxidants will affect cytotoxicity activity towards MCF-7 breast cancer cells. To address current research gaps, the aims of this study were; (a) to examine antioxidant activity of curcumin and neem leaf powder using a variety of in-vitro assays, and (b) to examine the effect of ALA, curcumin and neem leaf extracts on breast cancer cell proliferation individually and in combination.

2. MATERIALS AND METHODS

2.1 Materials

Curcumin powder (>98% pure) was purchased from Sigma-Aldrich. All additional reagents were analytical grade, purchased from Sigma-Aldrich and used as received. Neem (*Azadirachta indica*) leaf powder was originally produced in India, and supplied by TOP-OP (Foods) Ltd, MIDDX, UK (www.top-op.com) and phosphate buffered saline (PBS) was obtained from Oxoid Ltd.

2.2 Antioxidant Assays

2.2.1 Instrumentation

Colorimetric measurements were recorded using a UV/ Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Uppsala Sweden) in conjunction with 1-cm polystyrene cuvettes (Sarsted Ltd., Leicester, UK). All microplate assays involved a 96-microplate reader (VERSAmax; Molecular devices, Sunnydale, California, USA) used with flat-bottomed 96-well microplates (NUNC, Sigma Aldrich, UK).

2.2.2 Sample extractions and reference antioxidants preparation

Curcumin powder (51 mg) was dissolved in 50 ml of methanol and the mixture was centrifuged. Neem leaf extract was prepared by stirring 1 g of power with 9 g of distilled water then transferring 1 ml of the mixture to another 9 g of distilled water. The mixture was centrifuged and the solids-content for the supernatant was determined by oven drying. Curcumin or neem leaf extracts were diluted using distilled water or PBS and analyzed for total antioxidant capacity (TAC) and total phenolic content (TPC) as described below.

2.2.3 Ferric Reducing Antioxidant Potential (FRAP) assay

The ferrozine ferric reducing antioxidant power (FRAP) assay is based on the reducing power of a sample. It measures the reduction of Fe3+ (ferric iron) to Fe2+ (ferrous iron) and detection using ferrozine as dye. A ferrozine FRAP assay was used in this study as described by Butts and Mulvihill [37] with slight modifications. The assay was performed at pH 7.0 using Tris buffer and using ferric ammonium citrate in place of ferric chloride. Curcumin extracts (diluted 1-16 fold, D_F = 1-16) and neem leaf extracts (D_F= 10-320) were prepared as previously described. Diluted samples (20 µl) were added to 96 micro-well plates, 280 µl of ferrozine working solution was added, and samples were incubated for 30 min at 37℃. Absorbance measurements were recorded at 562 nm using a microplate reader.

2.2.4 The 2.2-diphenyl-1-picryhydrazyl free radical scavenging assay

The 2.2-diphenyl-1-picryhydrazyl (DPPH) assay was modified from [38,39]. Briefly, a DPPH working solution was prepared by diluting 10 ml of DPPH stock (24 mg in 100 ml methanol) with 45 ml methanol to reach the initial absorbance of 0.7±0.03 at 515 nm using a 1 cm spectrophotometer. Curcumin extracts (D_F =1-16) and neem leaf extracts (D_F = 40-1000) were prepared as previously mentioned. Diluted samples (20 µl) were added to 96 micro-well plate and 280 µl of DPPH solution was added into the plate. The mixtures were incubated in darkness for 30 min at 37℃ and then absorbance measurements were recorded at 515 nm using a microplate reader.

2.2.5 The 2,2-azinobis (3-ethyl-benzothrazoline-6-sulfonic acid radical cation de-colorization assay

The 2,2-azinobis (3-ethyl-benzothrazoline-6-sulfonic acid (ABTS) assay was modified from [40]. Briefly, 27.4 mg of ABTS and 2 mg of sodium persulfate were dissolved with 90 ml and 10 ml of phosphate-buffer saline (PBS), respectively. The ABTS working solution was prepared in 100 ml volumetric flask by mixing ABTS and sodium persulfate stock solutions and storing in the dark overnight at room temperature. Before use, ABTS solutions were diluted with PBS until to an initial absorbance value of 0.85 at 734 nm using a 1 cm spectrophotometer. For sample analysis (20 µl) of curcumin (D_F=1-75) or neem leaf extracts (D_F=10-1000) were added to 96 micro-well plate and 280 µl of ABTS solution was added. The mixtures were incubated for 30 min at 37℃ and then absorbance measurements were recorded at 734 nm using a microplate reader.

2.2.6 Total phenolic content

Total phenolic contents (TPC) were determined using the Folin-Denis method modified from [41]. The diluted samples (50 µl) of curcumin (D_F = 1-16) or neem leaf extracts (D_F= 10-160) were added to micro-centrifuge tube, followed by 100 µl of Folin-Denis regent, 800 µl of sodium carbonate reagent and 50 µl of methanol. The samples were vortexed gently, incubated at 37℃ for 20 min and centrifuged at 11,000 rpm for 5 min. The clear supernatant (200 ul) were transferred to 96 micro-well plates and absorbance was measured at 760 nm using a microplate reader.

2.3 Cytoxcity Assay

2.3.1 Cell culture

MCF-7 cells (American Type Cell Culture) were cultured in Dulbecco's modified eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (FBS), 1% w/v penicillin streptomycin (Pen Strep) and 1% minimum essential medium non-essential amino acids (MEM NEAA). Culture flasks and 96-microwell plates were incubated in a humidified incubator at 37℃ in O2 95% 5% CO2. (LEEC research incubator, LEEC, UK). Cells were trypsinised, counted using a NucleoCounter (NC-3000, ChemoMetec, Denmark) and seeded (10,000 cells/well) in 96-microwell plates with 50 µl of culture medium overnight to allow cell attachment. Cell growth was monitored using the Sulforhodamine B assay for cell cytotoxicity (see below).

2.3.2 *In-vitro* cytotoxicity tests

Curcumin and ALA (≥99% pure) were diluted in methanol (HPLC grade, ≥99.9%) to make 10mM stock solutions. Neem leaf extracts were prepared as above (section 2.2.2) and their concentrations were determined by the TPC method in terms of gallic acid equivalence. Stock solutions were then diluted with culture medium 10-fold for curcumin and neem leaf extracts and 5-fold for ALA, and filter sterilized with 0.20-µm cellulose acetate filters. The sterile solution of curcumin, neem leaf extract or ALA was further diluted with culture medium. Cells were treated with various concentrations of curcumin (0-100 µM), neem leaf extracts (0-176 µM) or ALA (0-1000 µM; 50 µl) and incubated at 37℃ for 72 hr. The final concentration of methanol for treated cells was less than 0.1% which is non-toxic to MCF-7 cells. For the control study, cells were treated with culture medium only. For combination studies, cells were treated with 50:50 mixtures prepared using 4x the desired "within-well" concentrations of ALA with neem extract, or ALA with curcumin. All other techniques were as described previously.

2.3.3 Sulforhodamine B (SRB) Assay for cell cytotoxicity

The sulforhodamine B (SRB) assay for cytotoxicity is a colorimetric assay to determine cell numbers based on the detection of cell proteins [42]. Cells were treated as previously mentioned. The cells were fixed with 100 µl of cold 10% (w/v) Trichloroacetic Acid (TCA) and incubated at 5℃ for an hour. After four washings with tap water and air-drying, the cells were stained for 30 min at room temperature with 0.06% SRB dye solution dissolved in 1% acetic acid (100 µl/well) and subsequently rinsed four times with 1% (v/v) acetic acid to remove unbound stain. After drying, Trizma–base (200 µl/well, 10 mM) was added to the plate to solubilize SRB dye, and the plates were shaken using an Orbital Shaker for 5 min (Speed: 180 revs/min). Absorbances were measured at 564 nm using a microplate reader and the data was transferred to MS excel and SPSS for further analyses. Cell viability was calculated as a percentage of absorbance readings for cells treated with vehicle.

2.4 Data Analysis

2.4.1 ABTS and DPPH data reduction

The following equations illustrate IC_{50} determination for the ABTS and DPPH methods;

$$\Delta\text{Absorbance} = [(A_{CONTROl} - A_{SAMPLE}) \quad (1)$$

$$\% \text{ inhibition} = [(A_{CONTROl} - A_{SAMPLE})/ A_{CONTROL}]x\ 100\% \quad (2)$$

where $A_{CONTROL}$ is the initial absorbance of the ABTS or DPPH working reagent and A_{SAMPLE} is the absorbance after incubation with curcumin or neem leaf extracts for 30 minutes. The concentration of antioxidant that neutralizes 50% of ABTS or DPPH radicals (IC_{50}) was determined from the relation, IC_{50}= 50/ GRAD, where GRAD is the gradient for a graph of % ABTS inhibition (Y-axis) plotted versus concentration (x- axis) [43].

2.4.2 Sensitivity, precision, and minimum detectable concentrations for antioxidants

The assay sensitivity was determined from the gradient (GRAD) of calibration graphs where absorbance is plotted versus gallic acid or trolox concentration. Values for the GRAD (M^{-1}) were subjected to pathlength correction to convert to the molar absorptivity (ε, M^{-1} cm^{-1}) as described recently; ε = GRAD/ L (cm) where L = pathlength for microplate reader [44]. Under the conditions of this study *L = 0.5 cm (TPC method) or L= 0.8 cm (FRAP, DPPH, ABTS methods).* The error of analysis was determined by the average coefficient of variation (CV%), where CV%= (SD/Mean) x 100%) using values for mean and

standard deviation (SD) for measurements. All analytical procedures had a measure for within and between assay precision. The minimum detectable concentration (MDC) is the least concentration of antioxidant that is detectable above the background noise. MDC was determined from [45] the relation,

$$MDC = 3.0\ SD_0 / GRAD \quad (3)$$

where SD_0 is the standard deviation for analysis using a reagent "blank" (0 µM) and GRAD is the gradient of calibration graph. The upper limit of detection (ULD) for antioxidants was determined as the highest concentration of reference antioxidant, for which r-squared (R^2) is close to 1.0. The concentration range between MDC and ULD represents the linear dynamic range for assays.

2.4.3 Total antioxidant capacity for neem and curcumin samples

The antioxidant capacity of curcumin and neem leaf extracts were determined in terms of trolox equivalents (TE: mmol/g) and gallic acid equivalents (GAE: mmol/g) using the relation,

$$TEAC\ /GAEAC = \frac{\Delta A}{GRAD} * \frac{A_v}{Sp_v} * D_F * \frac{1}{C_{ext}} * 10^5 \quad (4)$$

where ∆A = absorbance change corrected for the reagent blank, A_v = total assay volume (300 µl), Sp_v = sip volume (20 µl) of sample analyzed, C_{ext}= concentration of curcumin / neem leaf extracts (g/l), D_F = dilution for extracts prior to analysis (D_F = 1 for undiluted extract). To express antioxidant activity in terms of trolox/ gallic acid equivalent antioxidant activity (TE/ GAE; mmol/g) then GRAD from the trolox/gallic acid calibration graph was inserted into equation 4.

2.4.4 Statistical analysis

All experiments were repeated on 3 different occasions with 12-48 replicates per drug concentration. Routine data analysis were conducted using MS excel. One-way analysis of variance ANOVA tests were performed by Microsoft SPSS version 22 (IBM Corporation) with Tukey post hoc analysis for the separation of means. $P < 0.05$ was considered to be statistically significant. Paired t-tests were performed on a calculator at www.graphpad.com.

3. RESULTS

3.1 Antioxidant Activities

Extracts of curcumin prepared using methanol and neem prepared using water had a solids content of 0.9 (mg/ ml) and 30.2 (mg/ ml). The corresponding percentage yield of extraction was 90% and 30%. Fig. 1 shows four calibration graphs for antioxidant assays used in this study, with gallic acid reference. All assays had linear responses with coefficients of regression (R^2) > 0.96. The data were fitting a straight-line equation (Y = x. GRAD) where, Y = absorbance and x = concentration of antioxidant, and GRAD = slope of the line. Based on the molar absorptivity (ε, (M^{-1} cm^{-1}) value for gallic acid (Table 1) the order of assay sensitivity was; DPPH > TPC > ABTS > FRAP assay. By comparison, the order of sensitivity using trolox as reference antioxidant was; ABTS > DPPH > TPC > FRAP assay (Table 1). The MDC and other assay parameters are reported in Table 1. For gallic acid as reference antioxidant the within-assay precision (CV%) for the TPC, FRAP, DPPH or ABTS assay was 6.0%, 1.3%, 4.5% or 2.3%, respectively. Using trolox as reference, the within-assay CV% for TPC, FRAP, DPPH or ABTS assay was, 7.5%, 1.9%, 4.4% or 1.4% respectively. The between-days CV% for the assays is shown in Table 1.

The antioxidant capacity for curcumin and neem leaf extract expressed as TE (Fig. 2) or GAE (Table 2) varied according to the type of assay adopted.

From Table 2, the highest antioxidant capacity estimates were observed with the FRAP assay for neem. The other assays produced an antioxidant capacity of 22-30.4 (mmol GAE/100 g) for neem powder and 16-54 (mmol GAE/100 g) for curcumin. For neem leaf extract, antioxidant capacity determined with the TPC and ABTS assay were not significantly different (Table 2). Likewise, for curcumin, the TPC / FRAP and also ABTS/ DPPH were statistically similar. In Table 2, the numbers of samples (N‡) varied between the four different assays. All measurements involved at least 3 replicate experiments and multiple dilutions of test samples to reduce the effect of sample colour. Data showing no significant difference for the results from different dilution were averaged. Alternatively, where there was a significant difference due to sample dilution then

the highest antioxidant capacity (GAE/TE) estimates, normally obtained at higher dilutions, were used as representative data (Table 2 and Fig. 2).

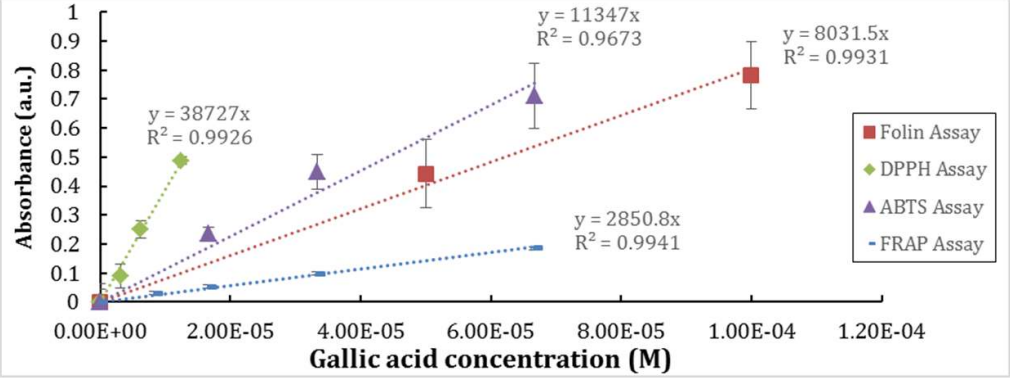

Fig. 1. Antioxidant capacity assays using gallic acid

Notes: Gallic acid as standard compound. TPC =Folin-Denis assay, FRAP =Ferric reducing antioxidant potential assay, DPPH = 2, 2-diphenyl-1-picrylhydrazyl assay, ABTS = (2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assay. For all assays absorbance were read different wave-lengths of 760 nm (TPC), 562 nm (FRAP assay), 515 nm (DPPH assay) and 734 nm (ABTS assay)

Table 1. Characteristics of four different antioxidant assays used in this study

Assays ♣	Gallic acid					Trolox standard				
	ε, (M^{-1} cm^{-1})		R^2‡	CV (%)	MDC† (µM)	ε, (M^{-1} cm^{-1})		R^2‡	CV (%)	MDC† (µM)
	Mean	SEMΘ				Mean	SEMΘ			
FRAP	3564	90	0.994	7.8	6.5	450	13	0.995	3.4	50.4
ABTS	14184	896	0.967	10.8	2.5	12169	205	0.997	7.5	2.8
TPC	16062	582	0.993	15.9	23.2	4464	206	0.981	8.8	20.8
DPPH	48409	1609	0.997	6.3	4.8	10325	564	0.974	7.7	16.6

♣ Abbreviations: TPC assay= Folin-Denis assay, FRAP assay= Ferric reducing antioxidant potential assay, DPPH assay= 2, 2-diphenyl-1-picrylhydrazyl assay, ABTS assay = 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assay; (Θ) Molar absorptivity (ε) is mean (+/-) standard error (SEM) , (‡) R^2 equal to r-squared values that range from 0 to 1, while 0 representing no statistical correlation between the data and a line, and 1 representing completely fit between the data and the line drawn through them, (†) MDC = Minimum Detectable Concentration

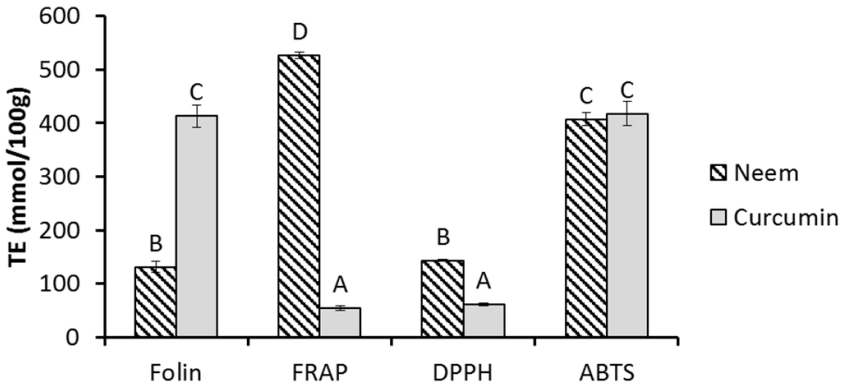

Fig. 2. Effect of assay method on antioxidant capacity of neem (*Azadirachta indica*) extract and curcumin expressed as trolox equivalent (TE)

Assays were, total phenols (TPC-), FRAP (Ferric reducing antioxidant potential), DPPH (2, 2-diphenyl-1-picrylhydrazyl), and ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) methods. Bars show means+/- SEM, different letters show significant differences (P <0.05)

Table 2. Antioxidant capacity of neem (*Azadirachta indica*) and curcumin as Gallic Acid Equivalents (GAE)

Assay method ♣	N‡	Neem leaf powder (GAE, mmol/100 g)		Curcumin (GAE, mmol/100 g)	
		Mean	SEMΘ	Mean	SEMΘ
TPC	96	27.1 [a]	2.0	51.4 [b]	2.0
FRAP	46	540.8 [c]	7.0	54.2 [b]	4.0
DPPH	40	30.4 [b]	0.0	16.0 [a]	0.0
ABTS	87	22.0 [a]	1.0	23.4 [a]	1.0

♣ Abbreviations: TPC assay = Folin-Denis assay, FRAP assay = Ferric reducing antioxidant potential assay, DPPH assay= 2, 2-diphenyl-1-picrylhydrazyl assay, ABTS assay = 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assay, GAE = Gallic acid equivalent antioxidant capacity, TE = Trolox equivalent antioxidant, capacity, (Θ)SEM = standard error of mean, (‡)N = number of samples. (a-e) Columns with different letters are significantly different (p <0.05)

In other studies, we found the concentration of curcumin and neem leaf extract necessary for 50% inhibition (IC_{50}) of ABTS was 7.5 (µg/ ml) and 1.7 (µg/ ml) respectively. By comparison, the IC_{50} values for inhibition of DPPH radicals was 7.8 (µg/ ml) and 3.8 (µg/ ml) for curcumin and neem leaf extracts, respectively. Based on the values for IC_{50}, neem leaf extract showed ~4-times greater ABTS inhibition or 2-times greater DPPH inhibition compared to the equivalent concentration (µg/ml) of curcumin. The IC_{50} values are given as the actual concentrations in the reaction system.

3.2 Cell Viability

In Fig. 3, treatment of MCF-7 cells with ALA (0-500 µM) or neem leaf extract (0-88 µM, GAE) produced a concentration-dependent decrease of cell viability. The final panel in Fig. 3 shows that the losses of cell viability were less severe if cells were treated with a combination of neem plus ALA as compared to each of these agents individually.

Fig. 4 shows MCF-7 cell treatments with curcumin (0-50 µM), ALA (0-500 µM), or their combinations. The concentrations of the individual agents required to inhibit MCF-7 cells by 50% (IC_{50}) were estimated (from Figs. 3 and 4) as 50 µM for ALA whilst IC_{50} = 17.8 µM GAE for neem extract and IC_{50} = 7.2 µM for curcumin. There were significantly *lower* decreases of cell viability for combination treatments, whilst the individual treatments using ALA, or curcumin produced higher inhibitions of MCF-7 cells.

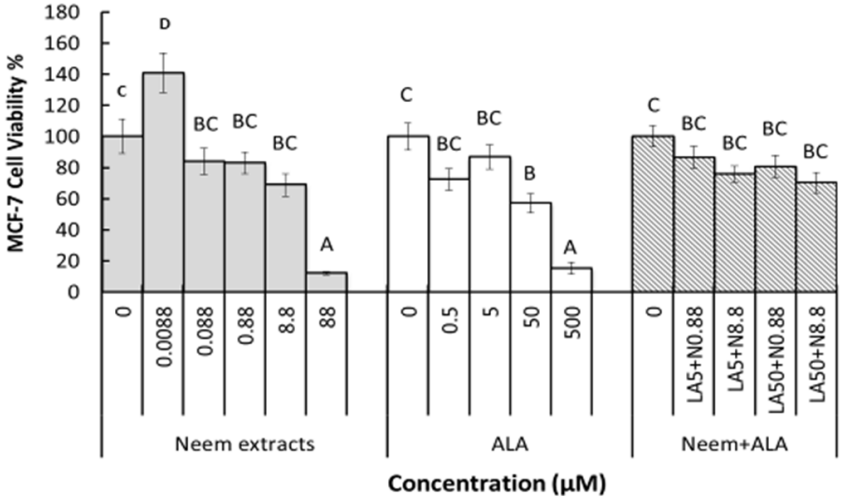

Fig. 3. Effect of neem, α-Linolenic acid or their combination on MCF-7 cell viability

MCF-7 cells were treated with neem leaf extract (0-88 µM), ALA (0-500 µM), or combinations for 72 hr. Bars show means +/- SEM. Different letters indicate significant differences (P < 0.05)

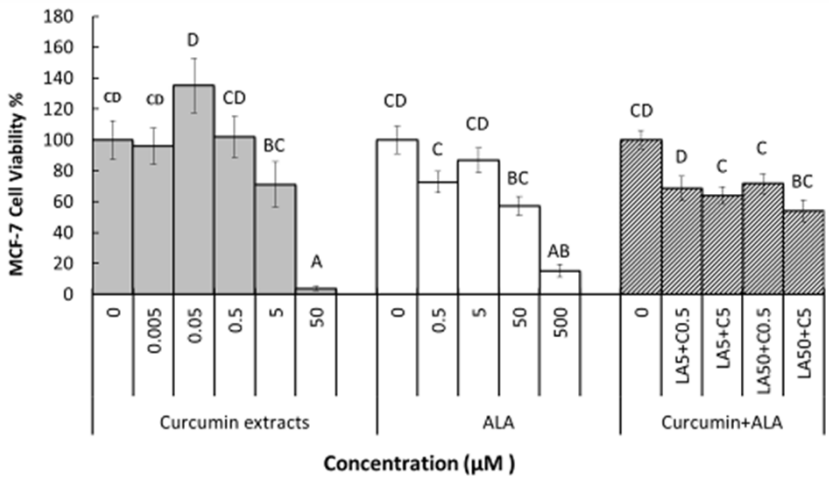

Fig. 4. Effect of curcumin, α-Linolenic acid or their combination on MCF-7 cell viability

MCF-7 cells were treated with curcumin ALA or combinations for 72 hr. Bars show means +/- SEM. Different letters indicate significant differences (P < 0.05)

4. DISCUSSION

4.1 Extraction Yield and Antioxidant Activity

Under the conditions of this study, curcumin was insoluble in water and 90% soluble in methanol. The weight-yield for neem extraction with water (30%) compares with previous reports of 58%, 19%, 17% and 4% with water, butanol, ethyl acetate, or hexane solvent [25,26]. The high yield obtained in polar solvent was probably due to high solubility of major components of neem in high polarity solvent. Methanol is expected to extract more nonpolar components compared to water.

In the assays described here, multiple dilutions were adopted a precautionary measure for colorimetric analysis, to ensure sample absorbances occur within the linear range of the assay. Secondly, dilutions were adopted to address a more stealthy issue which is the inadvertent precipitate formation when a concentrated extract is added to an assay system. Where a system behaves ideally, then dilutions should have no effect on final TAC results.

Estimates for TAC (Fig. 1, Table 2) differ according to different assays which are expected since different assays employ different chemistries, and solvent conditions [39,46]. For such reasons, multiple methods are recommended for TAC assessment [46]. The performances of different assays may be difficult to compare quantitatively also because each laboratory has developed its own standard operating procedures for analysis whereas a wide variety of conditions affect assay results, e.g. assay time, sample volume, wave-length, and the type of reference antioxidants used. We found the antioxidant activity for trolox was unaffected at pH 4.0-pH 10.0 whereas gallic acid showed rising antioxidant activity with increasing pH so the former maybe preferable as a standard [47]. The Folin assay, formerly used to determine TPC in food products, is a suitable general assay for antioxidants [41]. Both the ABTS and DPPH assays are widely used also due to their high sensitivity, short analysis times and high reproducibility. Studies using ABTS and DPPH assay typically adopt trolox as reference antioxidant [39,40]. Here, all 4-assays were calibrated with gallic acid and trolox as reference compounds to allow a comparison of results.

The antioxidant methods could be ranked according to molar absorptivity values (Table 1), but the ranking differed when trolox or gallic was used as references. A further ranking was possible based on values for the MDC which takes account of the precision for different methods (eq. 3). Data from Table 1 is should not pit one assay method against another because different assays emphasise different antioxidant characteristics [46].

The antioxidant capacity of neem leaf extract was reported previously [23-27] but no previous

study employed all the four assays used in the current paper. The free radical quenching capacity for neem leaf water extract for DPPH and ABTS radicals produced IC_{50} estimates of 26.5 µg/ml and 96 mmol/100 g [23] which are 3-4 fold higher than values reported in the current study. We found also that IC_{50} values were 2-fold lower for ABTS as compared to the DPPH assay (Table 2), which suggests that former may be more sensitive towards water extractable antioxidants from neem leaf. Ghimire et al. [26] reported the total phenols content for neem leaf water extract as 66.37 (mg GAE/g) equivalent to 39 (mmol GAE/100g) or 6.6% w/w. By contrast, the total phenols content for neem leaf extracts was, 38% (w/w), 20% (w/w) or 15% (w/w) using ethanol, ethyl acetate or methanol as solvent, respectively [24]. Others reported a total phenols content ranging from 9.6% - 11.5% w/w for water, methanol and ethyl acetate neem leaf extracts [25]. The total phenols value from the current study was 4.5% (w/w) or 27 (mmol GAE/100 g) which is in fair agreement with reported literature values. Moreover, extraction using nonpolar solvents is expected to increase the recovery of Total Phenols and polyphenols [25,26].

Using curcumin dissolved in ethanol the IC_{50} was 34.86 µg/ ml from the DPPH assay or 18.97 µg/ ml for the ABTS assay [19] as compared with values of 7.8 µg/ ml and 7.5 µg/ ml (this study) with methanol solvent, respectively. Interestingly the antioxidant capacities noted in Fig. 2 and Table 2 for neem and curcumin are comparable to values reported for other herbals and traditional medicines; a survey of 3500 natural agents found that herbals and traditional medicines had the highest antioxidant capacity ranging from 230-1448 mmol/100 g on a TE basis [10].

4.2 Cell Viability

Omega-3 fatty acids inhibit the proliferation of MCF-7 (ER+) cells and the order of effectiveness was EPA = DHA > ALA [3,4]. Nevertheless ALA is considered unique in terms of the ability to substantiate anticancer effects based on population studies [4]. A 24 hr. treatment with 75 µM of ALA produced 55% inhibition of MCF-7 cells [4]. However, treatment with 50 µM ALA for 5-days reduced MCF-7 proliferation by 33% [48] and a seven day treatment with 30 µM ALA reduced cell growth by 30% [3]. In the current study, the 50% inhibitory concentration for ALA (IC_{50}) was 50 µM with 72 hrs treatment (Fig. 3). The IC_{50} for curcumin inhibition of MCF-7 was reported previously as, 31.1 µM, 21.3 µM or 11.3 µM for cells treated for 24, 48 or 72 hrs [32,34]. Another literature IC_{50} value for curcumin using MCF-7 was 60 µM following 48 hr treatment [35]. These results compare with our curcumin IC_{50} of 7.2 µM for 72 hr exposure. There is only one relevant literature study of ethanoic neem leaf extract (ENLE) on MCF-7 cells [36] which found that cell viability was reduced by ~50% with 400 μg/ml extract (2.4 µM GAE). By comparison, our study found IC_{50} was 17.8 µM (GAE) using a water neem extracts.

To summarize, the present paper confirms that ALA, neem and curcumin are all inhibitory for MCF-7 cells individually. IC_{50} estimates for the individual components were similar to values from past studies for ALA [3,4], curcumin [32,34,35] or neem [36]. However, this appears to be the first published report to compare neem, curcumin and ALA anticancer properties within a single study. The results indicate that curcumin, neem leaf extract and ALA inhibit MCF-7 cell proliferation in a concentration-dependent manner and that the order of effectiveness was tentatively, curcumin > neem leaf water extract > ALA.

Synergistic interactions between DHA and curcumin were examined recently [49] but no combination studies involving ALA, neem or curcumin appear to be available. *In-vitro* studies and research using rodent models showed that n-3 fatty acids inhibit breast cancer cells via multiple pathways including, the modification of membrane composition, inhibition of cyclooxygenases or activation of PPAR [5-8]. The findings of the current combination study supports the hypothesis that lipid peroxidation may be implicated anticancer effect of ALA [5-8]. Combination treatments using ALA and curcumin or neem on MCF-7 cells found no improvements compared to individual treatments.

5. CONCLUSION

This study confirms that curcumin and neem are substantive antioxidant materials comparable to other herbal and traditional medicinal agents [10]. *In-vitro* test using MCF-7 cells indicated that ALA, curcumin and neem leaf extract produce a concentration-dependent inhibition of cancer cell growth. Combination treatments using ALA and curcumin or ALA and neem extract, resulted in a significant loss in efficacy of each agent. The findings are consistent with past literature

suggesting that lipid peroxidation and oxidative stress may be negative risk factors for breast cancer and that antioxidants may *on some occasions* impair rather than support other therapies [28-30]. It is not possible to eliminate other possible underlying causes for the observed findings. There are further limitations to this study. The design of this study did not extend to a formal analysis of interactions using isobolograms. The study was also an *in-vitro* study with all the usual well-known limitations. Though *in-vitro* findings may provide a rational for other studies, the interpretations of data should not be extrapolated to more complex systems such as animal models or human studies. The results obtained with MCF-7 cells should be examined with other cells lines including MCF-10A epithelial cells. Interestingly, a recent study showed that a combination of curcumin and ALA increased the synthesis of n-3 fatty acids in the brain [50]. More extensive studies will be needed to improve current understanding of the effect of antioxidants on ALA anticancer activity.

CONSENT

The study is an in-vitro design and no consent issues are applicable.

ETHICAL APPROVAL

The study is an *in-vitro* design and no ethical approval was needed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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