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Nanocapsulation of curcumin and its protective effects against oxidative stress and carcinoma HepG2, MCF7 cells

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Abstract

Curcumin, the highly active compound has restricted in the medical and nutritional applications due to its poor bioavailability and solubility. Using Ball milling technique for grinding curcuma powder at 30, 90 min and capsulated the natural extracts in micro and nano forms. Chemical components were compared between control (0 min) powder with 90 min grinded time. Comparing the non-capsulated (CM), Microencapsulated (CMm) and nanoencapsulated (CMn) as biological and chemical agents. Using HPLC-MS, it was found three main components; curcumin, curcumin glucuronid and curcumin sulfate at different ratios. Moreover, SEM microscope showed average particle size more than 450-550 µm for micro CMm 90 capsules, while TEM microscope showed spherical micelles ranged between 7 – 42 nm for nano CMn 90 capsules. Microencapsulated (CMm) and nanoencapsulated (CMn) were investigated as antioxidant and anticancer agents. Samples (66.6 µg/ml) were showed obvious DPPH scavenging activity for micro- then nanoencapsuled, and that was the same in slightly trend for Reducing power compared with non-encapsulated and ascorbic acid. Against human hepato cancer HepG2 cells, nanoencapsules were more active than micro capsules followed by non-capsuled samples in a bright selective anticancer activity. Against human breast cancer MCF7, the anticancer potential has higher IC_{50} (µg/ml) but in the same activity trend.

Keywords; Curcumin – micro capsulation – nano capsulation – alginate - HPLC MS – SEM - TEM – HepG2 – MCF7

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1. Introduction

Curcumin the natural pigment derived from *Curcuma longa* is an efficient drug for the treatment of colon cancer, breast, prostate and bladder cancers [1]. As been mentioned in many studies, curcuma potency facing disadvantages such as instability, poor aqueous solubility and rapid metabolic elimination by reduction and conjugation in the presence of mild temperature and light. For these reasons, the poor bioavailability of curcumin causes a primary barrier to achieve adequate plasma levels with favorable pharmacological effects [2, 3]. Solving the pharmacological significance and therapeutic feasibility of curcumin is restricted due to intrinsic physicochemical characteristics including low bioavailability and short half-life.

Micro and nano-delivery modified systems showed promising efficiency in achieving antioxidant and anticancer specific targets, maximizing

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internalization of drugs into cancer cells, as well as improving anticancer efficacy. The potential cytotoxic and alginate curcumin Alg-CM encapsulations have been discovered at different shape and particles in our study. The natural copolymer Alg, is highly useful for biomedical applications due to its striking characteristics like biodegradability, non-toxicity, and chelating ability [4]. Alg-CM conjugate was synthesized to obtain a potential curcumin conjugate for therapeutic applications with improved aqueous solubility and stability of curcumin.

Aim of the research is investigating the nanocapsules and microcapsules in improving the nutritive and pharmacokinetics as correlated with another research [5]**.** Curcumin CM non-capsulations were compared with CM capsulations in analyzing with LC-MS, SEM, TEM, free radical scavenger, human liver and breast carcinoma cell lines through its phenolic, diketone and methoxy groups present in curcumin structure components.

3. Material and Methods

The normal grinded turmeric sample was purchased from Egyptian local market in 2018. Ball milling mechanical method (Model: PQ-N2 Planetary Ball Mill, Gear Drive 4- station – planetary Ball mill, 220 v) was used for equal amounts of stainless steel balls in ratio (10:1) to CM powder for different periods of 0, 30 and 90 minutes in spin speed about 40,000 rpm [6]. Powder of each grinded curcuma powder (100 g) has been extracted with ethanol alcohol 95%, then concentrated and the yield of extract was 6.66mg/100g.

3.1. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS):

A three-fold XEVO TQD quadruple instrument (Water Corporation, Milford, MA01757 USA mass spectrometer) was used to acquire ESI-MS positive and negative ion mode. ACQUITY UPLC-BEH reverse phase C-18 column, 1.7 µm particle size -2.1 × 50 mm, and mobile phase elution flow rate 0.2 mL/min. Elution was performed by (eluent A) Water acidified with 0.1 % formic acid & (eluent B) Methanol acidified with 0.1 % formic acid in gradient mobile phase. UPLC for extracts 0 and 90 min grinded times was then subjected to LC-ESI-MS analysis. Mass spectra were detected as well in the ESI between *m/z* 100–1000. The peaks identified using the Maslynx 4.1 software.

3.2. Preparation of micro- and nano-encapsulation of curcumin extract:

Micro-encapsulation: Extract (0 or 30 or 90) was conducted using emulsion extrusion technique [7]. Sodium alginate Alg was dissolved in distilled water to produce polymer solutions with a concentration of 2 % w/v [8], Alginate solution (100 ml) and curcumin extract (1 ml) were homogenized for 10 hrs using magnetic stirrer. Product alginate - curcumin extract emulsion were sprayed into a collecting water bath containing calcium chloride solution (2 w/v %).

Nano-encapsulation: According to the modified method of using homogenization (Homogenizer PRO 400 PC, Germany) model in a matrix comprised the Alg and Tween 20 (T_{20}) [9]. The proportion of curcumin extract: Alg: T_{20} was 3:10:1, respectively gave optimum yield of powder with desirable attributes. In a high-pressure homogenizer at 18.000 rpm for 30 min, the emulsion was created.

3.3. SEM and TEM for curcuma encapsulations

Scanning electron microscopy SEM: The morphology and particle size of the micro capsules (90 min) was pipetted onto a gloter aldehyde and left to dry. Afterwards, the sample was sputter coated with a gold layer of 15 nm at a current of 20 mA for 50s (SPI-Module Control, Sputter Coater), before images were taken using (ISM-5200. JEOL, Japan).

Transmission Electron Microscopy TEM: The morphology charachterization of Curcumin nanoencapsules (milled 90 min) was resuspended by pipetting it with making grids (carbon-coated 400 mesh copper grids) on specimen [10] in the microscope (JEOL JEM-1400 Electron Microscope).

3.4. Antioxidant activity for curcuma encapsulations

Radical scavenging of 2,2-diphenyl-1-picryl hydrazyl (DPPH). According to published research [11], 6.66 µg of ethanol extract was mixed with one ml DPPH (0.2 mM) and kept in the dark before reading the absorbance at 517 nm, compared with ascorbic acid with the equation;

% Inhibition = $(A_b - A_s) / A_b$ X 100 [33]

Where A_b mean absorbance of blank at 517 nm, and A_s mean absorbance of sample at 517 nm.

Reducing Power (RP) Assay. According to previous research [12] with some modifications, ethanolic extract (100 µL, 2.7 µg) mixed with destilled water until 1 ml solution comparing to ascorbic acid (100 µL, 0.03% w/v) as standard reducing agent before reading the absorbance at 700 nm.

Increase in reducing power (%)=$A_{test} - A_{blank} / A_{blank}$ X 100

Where A_{test} is absorbance of test solution; A_{blank} is absorbance of blank at 700 nm

3.5. Anticancer cell line activity

Using sulpho rhodamine B (SRB) 0.4% dye in acetic acid as a protein dye was done for RPM1-1640 medium. Two human cell lines were used; HepG2 liver carcinoma, and MCF7 breast carcinoma. The SRB's binding is stoichiometric the amount of dye extracted from stained cells and proportional to

the cell mass directly.Cells were plated in 96 multiwell plate (10^4 cells/well) for different concentrations (2.5, 5, 10, 20 µg/ml for tested capsules. Color intensity was measured using an ELISA reader with wavelength 570 nm. Experiment was repeated three times and cell survival fraction has been counted [13].

Survival fraction ***S.F.*** = wavelength (treated cells) / O.D.
(control cells)

IC_{50} values or the concentration of tested sample required to produce 50% inhibition of cell growth.

3.6.Statistical analysis: Mean values have been calculated with excel for the DPPH and reducing power, and the standard deviations were calculated as well. Values are presented as mean ± SD, One-way analysis of variance (ANOVA), and Tukey multiple comparison tests showed statistically significant differences compared to ascorbic acid result.

4.Results and Discussion

Physical transformation "top to down technique" is required to enhance solubility especially for drugs with limited aqueous solubility. Moreover, Alginate, the hydrophilic polymer was finely dispersed to enable C-6 alginate carboxylate functionality with the phenolic –OH group of curcumin via esterification forming yellow colored Alg-CM.

4.1. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS):

Liquid chromatography comparison between control and 90 min grinded ethanolic extracts was showed the difference in main compounds and their ratios in positive (Figures 1 & 2) and negative mode. Previous results showed that methanolic extract of *Curcuma longa* L. rhizomes was subjected to GC-MS analysis[14]to identify curcumin with 50 compounds such as ar-turmerone (20.50%), ß-sesquiphellandrene (5.20%) and curcumenol (5.11%). On the other side, LC-MS/MS method has been used for simultaneous determination of curcumin (CM), and its metabolites [15].

Curcumin and derivatives such as curcumin glucuronid (COG) and curcumin sulfate (COS) have been decrlared. Compounds (COG and COS) have been detected in the negative electrospray ionization mode. Curcumin in the negative mode appeared at t_R 16.09 min with MW 367, and the fragmentation has been occurred as follows; one fragment represent the removal of one methoxy group (m/z 337) and the second is the removal of bis-methoxy groups (m/z 307) as was mentioned in the MS fragments (Figure 3). Results were correlated with other researchers [16], whovalidated simultaneous quantification of curcumin using LC-MS/MS.

The second identified compound; curcumin glucuronid (COG) with MW 543 has been found in the negative mode at t_R 19.89. This compound has been found both in the control and the highly blended sample. It contains carbohydrates moieties, and its mass fragments were m/z 293 and 250, and it seems that the bond cleavage has been occurred (Figure 3) as the arrow focused to give the glucuronic attached with the methoxy aromatic ring.

The present results are in agreement with those reported earlier [17], where the reductive anti-inflammatory and anticarcinogen activities have a significant proportion of sulfate conjugates. In the positive ionization mode, MW 448 curcumin derivative known as curcumin sulfate (COS) has been found at t_R 20.89 min both in control and grinded samples. Sulfonic group might be involved in thiol-amino acid structures as in cysteine, cystine and methionine. Toluenic ring fragments appeared in mass spectrum at m/z 125, and 219 fragments. Peak area ratios has been varied between control and 90 min chromatograms for curcumin 41 and 37%, respectively; for COG 16 and 14%, respectively and for COS 9 and 8%, respectively. These compounds were stable at room temperature for at least 24 hrs [15].

4.2. Scanning (SEM) and Transmission electron microscopy (TEM):

SEM micrographs of curcuma micro particles showed that microparticles were approximated spherical and appeared in a homogenous size distribution with larger particles (Figure 4a,b). The present results are in agreement with those previously reported [18. 19], where the average particle size was more than 450-550 µm. In the cutting across the capsules, holes have been detected between 10-50 µm.

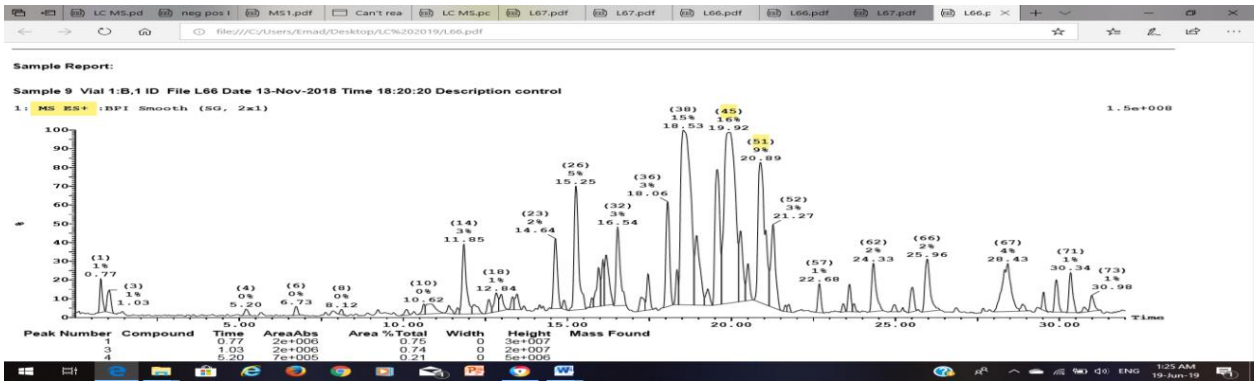


Figure 1: LC-MS spectrum for control at positive mode

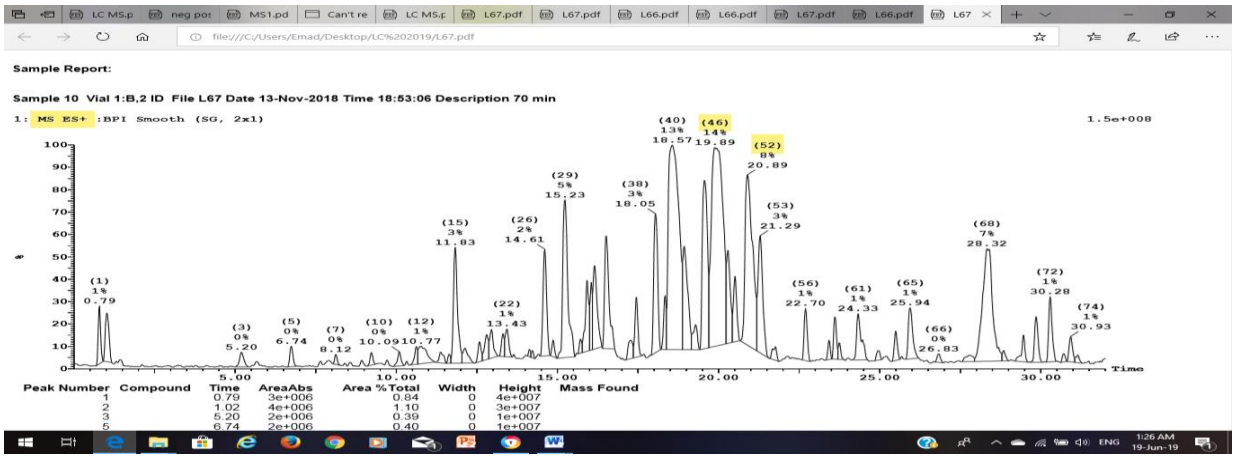


Figure 2: LC-MS spectrum for grinded 90 min at positive mode

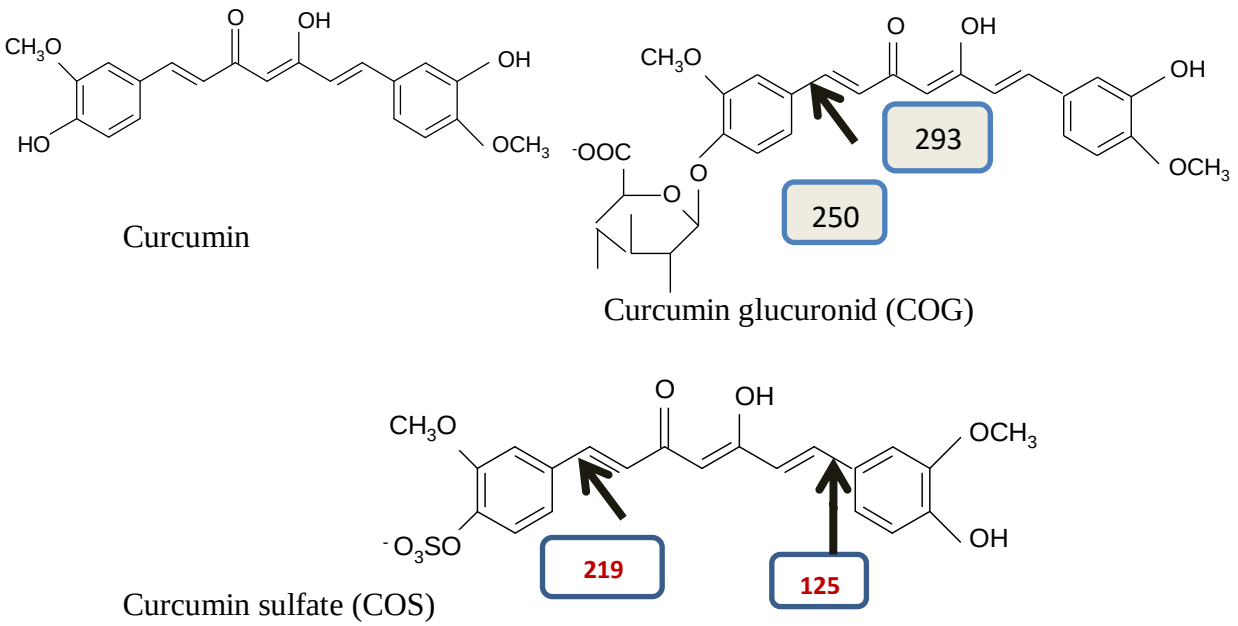
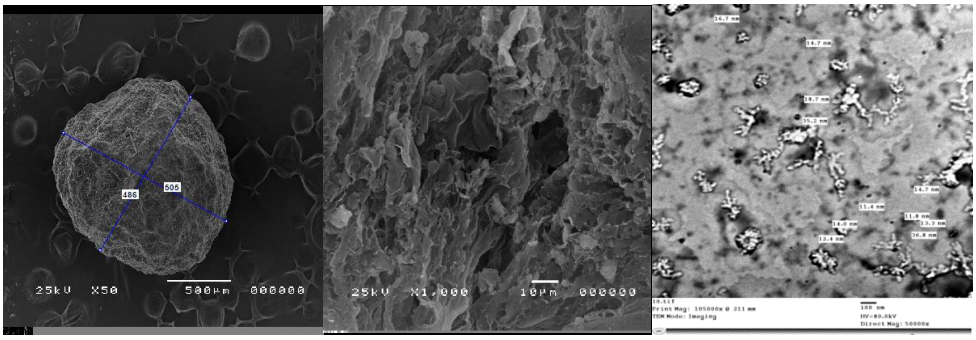


Figure 3: Structural of main three compounds found in HPLC-MS



a **b** **c**

Fig 4. SEM micrographs showing the external 500µm (a) and the cutting across the capsules, holes have been detected between10-50µm(b) morphology of different curcumin microcapsules. TEM morphology (c) of nano-encapsulated cucrcuma ethanol in sodium alginate

Table 1: DPPH inhibition percentages & Reducing power for ascorbic acid, grinded curcuma ethanolic extracts CM & microencapsulation MCM and nanoencapsulation NCM (for 0, 30 and 90 min for blended extracts)..

Samples	DPPH Inhibition %	Reducing Power
Ascorbic acid 100 µl	97.3±5.4	62.08±2.8
grinded after 0 min. 100 µl	83.3±2.3	62.76±4.2
After 30 min. 100 µl	83.4±4.5	68.21±2.3
After 90 min. 100 µl	80.6±2.1*	73.67±1.8*
Microencaps 0min. 100µl	99.4±1.2	121.1±3.7****
after 30min. 100µl	93.3±2.2	126.6±1.7****
after 90min. 100µl	93.5±0.8	132±3.6****
Nanoencaps 0min. 100µl	94.6±5.3	102.2±6.4****
after30min. 100µl	92±2.6	107.6±3.8****
after90min. 100µl	88.7±2.1	113.1±3.4****

Mean percentages are the average of three samples, (Average ± SD) Values are presented as mean ± SD, One-way analysis of variance (ANOVA), and Tukey multiple comparison tests showed statistically significant differences in relation to ascorbic acid: (*P < 0.05 and ****P < 0.0001).

Table 2: Effect of grinded curcuma powder times and micro, nano-encapsulated formation on IC_{50} for HepG2 and MCF7 human carcinoma cells (IC_{50} µg/ml)

IC_{50} µg/ml	**Grinded**			**Micro capsules**			**Nano capsules**		
	0	**30**	**90**	**0**	**30**	**90**	**0**	**30**	**90**
HepG2	*2.2*	*2.4*	*2.1*	2	1.99	1.75	*1.76*	*1.4*	*1.3*
MCF7	*19.8*	*16.5*	*14*	12	11	9	*8*	*7.5*	*4*

Curcumin nano-capsules were imaged by TEM (Figure 4c). The pictures of spherical micelles confirmed the size of nano-capsules ranged between 7 – 42 nm. Curcumin nano-capsules exhibited a smooth surface and spherical shape [20]. In gathered particles the nano-capsules has been figured out in different shape than that fabricated and processed for curcumin nanoparticles CM NP.

4.3. Antioxidative activity of curcuma capsules:

Table 1 shows that microencapsulated curcuma (100 µl, 6.7 µg) M_0, M_{90}, have the highest DPPH activity then followed by M_{30} and for nanoencapsulated N_0, N_{30}, then N_{90}. The lowest value was for grinded uncapsulated samples in the trend B_{30}, B_0, then B_{90}in descending order. Comparing to ascorbic acid, only grinded samples at 90 min uncapsules showed significant result. These

unexpected results might be due to mechanical pressure for micro-capsules process released inreducing power or DPPH scavenging technique.

RP activity has been shown in studied capsules. Comparing to ascorbic, grinded samples for 90 min showed significant activity, while micro and nano capsules exhibited highly significant activity at P<0.0001. The high solubility and bio-released compounds from nanoparticles explains the efficiency of nanoparticles in DPPH and reducing power. Phenolic hydroxyl groups [21], besides thatTween 80 molecules increased the viscosity and decreasing the mobility with competing for the interactions with curcumin molecules, ethanol and DPPH..

Comparing DPPH, FRAP and TPC for antioxidant capacity, nanosuspension or nanoparticles did not find to degrade the curcumin molecules [22], but proved more soluble than the original powder. Microcapsules in some experiments in our study proved to be impermeable and were broken apart by mechanical or chemical means, for the inner ingredients to become active [23]. For this reason microcapsules showed higher activity as antioxidants.

4.4. Anticancer effect for curcuma capsules:

Nanocapsulation has particles size directly influences the distribution of any bioactive substance [24].So that, it enhances bioavailability, improve controlled release, and enable precision targeting than microencapsulation [25]. Anticancer activity of nano-encapsulation was the most superior pioneer against HepG2 cancer cell line, especially for that previously grinded at 90 min (Table 2). In the meantime, uncapsulated curcuma was the lowest active against hepato cancer cell line. Something similar has been mentioned by Farzaei [26] who investigated that the curcumin is one of the most commonly used indigenous molecules endowed by various shielding functionalities that protects the liver through treatment oxidative stress.

Against human carcinoma cell, previous research [27] proved interesting results that curcumin showed toxicity to human breast carcinoma cells MCF7 from doses 50 to 6.25 µg/ml. In the same trend, SRB detected that more efficiency in micro- and nano-encapsulation at 90 min grinding time comparing to grinded un-capsulated curcumin (Table 2). The toxicity for capsules is increased at 20 µg/ml non-capsulated, then micro- and finally 5 µg/ml nano-encapsulation against MCF7. The toxicity against HepG2 was from 2.5 to 1.3 µg/ml in a selective model for the anticancer efficiency. On the other hand,they exhibited chemopreventive and chemotherapeutic activities towards breast cancer by the polyphenol curcumin.

The therapeutic efficiency of NPs by cancer cells was affected by their intracellular uptake activities. The IC_{50} of CM NPs of original grinded samples was lower than that for micro-encapsulates. In the present study, sodium alginate gel and beads showed thermal stability [28]. The Differential scanning calorimetry DSC of extracted non-encapsulated was 139.12^{O}C indicated the blended curcuma powder proved ability tolerance to high temperatures. On the other hand, micro-encapsulation for the same blended time was at intervals temperatures of 58.58, 84.73 and 95.06^{O}C. Nano-encapsulated samples showed thermal stability and not melted at 300^{O}C. The prospected results for nano-encapsules encourages successful food applications without any loss of its antioxidant and anticancer activities or change in its characters. These propertiesare due to the ability of nanomaterial to protect and mix well those compounds [29].

In general, the intensity or grinded time decreased IC_{50} for the original, micro- and nano-encapsulates (Table 2). CM NPs encapsulateshave a potent anticancer effect than original CM.

5. Conclusion

In conclusion, cell viability of variable cell line was used to evaluate the cytotoxicity of CM NPs and their capsules against human cell line. The progressiveantitumor activity *in vitro* against HepG2 cells has IC_{50} 1.3 µg/ml, and IC_{50} 4 µg/ml for nano (grinded 90 min) against MCF7. Carcinoma cell line increased intracellular ROS level and that explain the link between the antioxidant and anticancer activities. Moreover, cell availability decreased with increasing drug concentration.

Tween 80 under ultrasound increases the low solubility molecules and preventing particle aggregation [21]. That reason might explain the high efficiency of anticancer potential for nano-encapsulation. The decrease of particle size was according to the increase in stirring speed effect as has been mentioned [30]. Concerning solubility,the sonication procedure with ethanol generates smaller particles than other conditions tested [31]. Since the hydroxyl groups present in both molecules create a great solvation effect due to the interaction of hydrogen bonds between solvent and curcuminoid molecules.

Finally, results vary between antioxidative and anticancer activities, these propertieswere explained [32].As has been mentioned,DPPH$^{\cdot}$ mechanism depends on the structural conformation of

the antioxidant and lead to that some compounds react very quickly with DPPH· , reducing number of DPPH· molecules equal to available hydroxyl groups and that completely differ than proliferation stopping mechanism. As has been detected, microencapsulation enhances the antioxidative activity for producing protection for variable compounds without interfering the potential compounds on each other. While, nano-encapsulation technique clearly appears in ideal process in tumor cells, its potential needed to appear.

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