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Augmenting the cytotoxic effect of the aerial parts of *Carissa macrocarpa* (Eckl.) A. DC. cultivated in Egypt by using surfactant-free nanoemulsion.

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Abstract

Carissa macrocarpa (Eckl.) A. DC. was previously reported for its anticancer and cytotoxic activities due to its high phenolic and flavonoid contents. The crude methanolic extract of the aerial parts of *Carissa macrocarpa* (Eckl.) A. DC cultivated in Egypt proved a potent cytotoxicity against HEPG2 and HCT116. The biologically active extract was encapsulated in a nanocellulose-stabilized nanoemulsion (NC-NE) in a trial to enhance its aqueous solubility and ameliorate its effect while avoiding the use of surfactants. The selected NC-NE had a particle size of 194 ± 18 nm with a uniform dispersion (PDI= 0.2) and a good physical stability (Z = -32 mV). Also, the release enhancement was about 3 fold compared to the crude extract. NC-NE had an almost spherical outline (as detected by TEM). The cytotoxicity of the prepared nanoemulsion was studied to determine the difference in efficacy between it and the crude methanolic extract of the plant under investigation. Percentage of inhibition of the selected nanoformulation (NC-NE) against HePG2 and HCT116 was 100% (IC_{50} 20.62 µg/mL, IC_{90} 33.13 µg/mL) and 99.3% (IC_{50} 32.80 µg/mL, IC_{90} 58.22 µg/mL) respectively at 100 ppm, while that of the methanolic extract (E) was 36.5% (IC_{50} was more than 100 µg/mL) and 71.3% (IC_{50} 74.11 µg/mL, IC_{90} 117.54 µg/mL) respectively at 100 ppm. It is obviously clear from our results that preparation of nanoformulation (NC-NE) increased the cytotoxicity of *carissa macrocarpa* crude methanolic extract. Phytochemical investigation of the active crude methanolic extract revealed the presence of many phenolic and flavonoid compounds. The major phenolic compounds were chlorogenic acid, protocatechuic acid and caffeic acid which represented 1024.33 µg/g, 257.48 µg/g and 179.90 µg/g respectively, while the three identified flavonoids were rutin, apigenin-7-glucoside and quercetin that represented 299.22 µg/g, 254.90 µg/g and 16.39 µg/g respectively. These compounds were separated and identified by spectroscopic and chromatographic techniques. As far as we know, it is the first time to study the effect of nanoparticle preparation on cytotoxic effect of *Carissa macrocarpa* methanolic extract.

Keywords: *Carissa macrocarpa* (Eckl.) A. DC; cytotoxicity, nanoformulation (NC-NE); nanocellulose; phenolic compounds.

1. Introduction

Carissa is a genus of small trees or shrubs of Apocynaceae family that can grow from 2 to 10 m high. It belongs to subtropical and tropical regions of Australia, Africa and Asia. About 100 species are listed up until recently, but the majority of them have been downgraded to synonym status or placed in other genera, such as *Acokanthera*. They possess strong, smooth, sharp thorns that are true botanical thorns because they are morphologically speaking and modified branches. The leaves are simple, smooth, entire, elliptic to ovate to almost lanceolate, and they are rich, glossy, waxy green. They are typically thick and leathery and range in length from 2 to 8 cm, depending on the species. Some plants flower throughout the majority of the year in suitable climates **[1]**.

Some species flowers have a scent resembling gardenia, which adds to their appeal as garden plants. The fruit is a berry that resembles a plum and has a prolate spheroid form similar to a rugby ball. The color varies depending on the species. When ripe, some species turn red, while others turn a glossy purple-black. The plant extensively branching roots make it useful for stabilizing eroding slopes. The plant *Carissa macrocarpa* (Eckl.) A. DC. is a

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member of the Apocynaceae family. It is also an edible plant. To be consumed raw, the fruit must be fully ripe, dark red, and slightly tender to the touch.

Bioactive compounds with therapeutic potential for this species have been previously identified, including flavonoids, flavanones, polyphenolics, lignans and sesquiterpenes **[2].** In several *in-vivo* and *in-vitro* models, flavonoid compounds have been examined for their protective action against oxidative mechanisms, which appears to be related to their antioxidant properties **[3].** By using several *in-vitro* assays, the antioxidant capacity and radical scavenging activity of *Carissa macrocarpa* extract have been assessed and compared **[4].** It is crucial to emphasize that flavonoids are potent antioxidants **[5, 6, 7]**. Additionally, they have a wide range of pharmacological properties, such as hepatoprotective **[5]**, cytotoxic **[5,7],** anti-inflammatory **[7,8]** and antimicrobial actions **[8]** .

To ascertain whether the fruits could serve as a supply of essential fatty acids, lipid profiling has been also carried out. The fruit linoleic acid to α - linolenic acid ratio comply with the prescribed range for cardiac health, and it is rich in monounsaturated and essential fatty acids. The fruit elements concentrations have been discovered to be in the following order: Ca > Mg > Fe > Mn ≈ Cu ≈ Pb > Se > Cr > Ni > Zn. **[9].**

Previous studies reported that *Carissa* species are used due to its cytotoxic properties, antioxidant, anti-inflammatory, anti-cancer, anti-convulsant, analgesic, anthelmintic, anti-ulcer, anti-nociceptive, cardiovascular, anti-diabetic, hepatoprotective, antipyretic, neuropharmacological, diuretic, antimicrobial activities, *in-vitro* anti-oxidant, DNA damage inhibition, constipation and diarrheal activities in different parts of the world for the treatment of several diseases **[10].** They exhibit hypolipidemic, wound healing, antiepileptic, nephrotoxicity amelioration, and hepatoprotective activities in both *in-vitro* and *in-vivo* studies. This amazing plant extract has also proven successful in treating veterinary conditions **[2]**. It has been used in numerous traditional treatments for a variety of ailments, including skin infections, fevers, rheumatism and biliary dysfunction **[11]**. The fruit is frequently used in the treatment of anemia since it is a rich source of iron. It is an antiscorbutic since it has a significant quantity of vitamin C in it. It can be used as a topping for cakes, puddings, and ice cream as well as fruit salads, gelatins, and other desserts. They can be used to make jam, other preserves, syrup, or sweet pickles whether they are peeled or not. To enhance the color of the jelly, slightly under ripe fruits are used, or ripe and unripe fruits are combined **[12].**

Recently, the advancement in the field of nanotechnology led to the development of nanoparticles, which are very attractive and have different applications in the nanomedicine field. Nanoparticles are particles with a nanometric range of size, hence are capable to act as carriers for different active agents in order to improve their solubility, permeability, bioavailability and effectiveness. Nanoemulsions have attracted the attention of researchers for the design of different dosage forms. Pickering **[13]** has reported, in 1907, that solid colloidal particles can be used as stabilizers between two immiscible phases. Particle-stabilized emulsions avoid the use of surfactants which are necessary for the preparation of different types of nanoparticles. Therefore, this type of surfactant-free emulsions provides many advantages including safety, environmental- friendly and non-carcinogenicity. Nanocellulose is a multi-beneficial biopolymer which has been successfully implemented in some advanced pharmaceutical formulations including nanoemulsions **[14, 15, 16, 17].**

The study aims to improve the cytotoxic activity of the crude methanolic extract of *Carissa macrocarpa* (Eckl.) A. DC. aerial parts by encapsulation in a nanocellulose-stabilized nanoemulsion (NC-NE) as well as phytochemical investigation of the biologically active extract using chromatographic and spectroscopic techniques.

2. Materials and methods:

Plant materials:

The aerial parts (leaves and stems) of *Carissa macrocarpa* (Eckl.) A. DC. were collected from Prince Mohammed Ali Palace in EL-Manial, Cairo, Egypt. They were dried in shade and reduced to No.36 powder then stored in containers with tight lids. Dr. Trease Labeeb (plant taxonomy consultant at the Orman Garden, Giza, Egypt) verified the authenticity of the collected plant. Voucher specimen (No. 6-3-23) was kept in the museum of Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Methanolic extraction:

One kilogram of *Carissa macrocarpa* aerial parts powder was repeatedly extracted with 100% methanol (analytical grade) until complete exhaustion at room temperature. The resulting extract was filtered and the solvent was removed using a rotatory evaporator (Heidolph, Germany) under reduced pressure until dryness at 55 °C.

Determination of total phenolic and flavonoid contents:

The Folin-Ciocalteu procedure was used to determine the total phenolic content. The absorbance was measured at 725 nm against the solvent blank. A calibration curve made with gallic acid was used to calculate the total phenolic content, which was represented as milligrams of gallic acid equivalent (mg GAE) per gram of sample. If the measured absorbance value was greater than the linear range of the standard curve, additional dilution was carried out [**18].**

Using a colorimetric assay with aluminium chloride ($AlCl_3$), the total flavonoid content was determined. At 510 nm, absorbance was measured in comparison to the solvent blank. By using a calibration curve created using catechin, the total flavonoid content was calculated and expressed as milligrams of catechin equivalent (mg CE) per gram of sample. If the observed absorbance value was higher than the linear range of the standard curve, more dilution was performed [**18].**

Qualitative and quantitative estimation of phenolics and flavonoids by HPLC analysis:

Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector was used to perform HPLC analysis. Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) analytical column with a C18 guard column (Phenomenex, Torrance, CA) was used. Acetonitrile (solvent A) and 2% acetic acid in water (*v/v*) (solvent B) made up the mobile phase. The gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min, and 0% B to 100% B in 5 min. The flow rate was maintained at 0.8 mL/min for a total run period of 70 min. Peaks for the benzoic acid and cinnamic acid derivatives were simultaneously measured at 280 and 320 nm respectively, using an injection volume of 50 µL. Flavonoids were measured at 360 nm. Before injection, all samples were filtered with a 0.45 µm acrodisc syringe filter (Gelman Laboratory, MI). Peaks were identified by matching UV spectra and retention times, and they were then compared with the standards **[19]**.

Nanoformulation materials:

Spray-dried nanocellulose crystals (NC) with a hydrodynamic diameter of 70 nm were bought from Celluforce in Canada. Other reagents were of analytical grade. Only olive oil was of edible grade.

Preparation of the extract-loaded nanocellulose-stabilized nanoemulsion (NC-NE):

The nanoemulsion was prepared following the procedure previously reported by Kamel *et al*. **[15]**. Briefly, the oil phase (olive oil) was gradually added (10% *w/w*) after the nanocellulose crystals (1, 2 or 4% *w/w*) were dispersed in distilled water to create the o/w emulsion. The nanoemulsion was then homogenized with a Heidolph Homogenizer (Germany) for one minute at 20,000 rpm after the crude extract (1 mg/g) had been added. Until further investigations, the prepared NC-NE were stored dry and at room temperature.

Quantitative assessment of Carissa macrocarpa extract:

Using a Shimadzu UV spectrophotometer, model number 2401/PC, Japan, the calibration curve was assessed using the absorbance values of a series of alcoholic aqueous solutions (70%) containing various concentrations of the extract.

Stability study:

Three steps were used to evaluate thermodynamic stability **[20]**. The NC-NE were briefly centrifuged (Centurion Scientific Ltd., UK) for 30 minutes at 3,500 rpm after six heating/cooling cycles at 45/4 ˚C. They were subjected to three freeze/thaw cycles in all.

The NC-NE was put through the following testing after passing the stability study successfully.

a. Particle size analysis, polydispersity index and zeta potential:

ZetaSizer Nano ZS (Malvern Instruments Ltd., UK) was used for analysis. The particle diameter and size distribution were given as the mean particle size (PS) and polydispersity index (PDI), respectively. Deionized water was used to dilute the NC-NE before measurement.

b. ***In-vitro*** **release study:**

Using the dialysis bag diffusion technique, the release of the active agent from the selected NC-NE was evaluated. The nanoemulsion (1g) was put into a cellulose dialysis bag (Dialysis tubing cellulose membrane, Sigma Co., USA; Molecular weight cutoff 12,000–14,000) that was already pre-soaked. Additionally, the release profile of *Carissa macrocarpa* aqueous suspension that contained an equivalent quantity of the active agent was examined for comparison. Aqueous alcohol (70%) was used as the release medium **[21, 22]** kept in a thermo-stated shaking water bath (Memmert, SV 1422, Germany) at 37 °C ± 0.5 °C and 50 rpm. Four milliliter samples (4 mL) were taken out of the release medium at various time intervals and replaced with an equivalent volume of fresh medium. Using a Shimadzu UV spectrophotometer, 2401/PC, Japan, the obtained samples were spectrophotometrically examined at 280 nm. Curves were drawn showing the cumulative amount of active agent released as a percentage versus time. Data were provided as the mean ± S.D. after the experiment was carried out in triplicate. The area under the release curve (AUC) at

a specific time was compared to the area corresponding to complete release in order to determine the release efficiency using the trapezoidal rule **[23]**.

c. Transmission electron microscope:

Transmission electron microscopy (TEM; JEM-1230, Jeol, Tokyo, Japan) was used to examine the morphological characteristics of the NC-NE. Phosphotungstic acid aqueous solution (2% *w/v*) was used to negatively stain the examined formulation. After the stained samples were air dried, TEM at 70 kV was used to examine them.

Determination of in-vitro cytotoxic activity:

The cell line technique was used to test the cytotoxic activity of *Carissa macrocarpa* crude methanolic extract and the prepared nanoemulsion against human hepatocellular carcinoma cell line (HePG 2), human Caucasian breast adenocarcinoma (MCF7), colon cell line (HCT116), and lung carcinoma cell line (A549) according to Cordero *et al*. **[24]**. The National Research Centre in Cairo, Egypt has these cancer cell lines on hand. From the ATCC, USA, cancer cell lines were obtained. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), (Sigma Aldrich, MO). Dimethyl sulfoxide (DMSO) solvent (Lab Scan, Ireland). All procedures were performed according to the guidelines of the Medical Research Ethics Committee of the National Research Centre, Cairo, Egypt (Ethical Approval Certificate No. 16-473).

- Procedure:

The mitochondrial-dependent reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan was used to measure the vitality of the cells **[25]**. The following operations were all carried out in a biosafety class II level laminar flow cabinet (Baker, SG403INT, and Sanford, ME, USA) in a sterile environment. At 37 ºC and 5% carbon dioxide, cells were suspended in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium) [(for HePG2, MCF7, and HCT116)], 1% antibiotic-antimycotic mixture (10,000 U/mL potassium penicillin, 10,000 µg/mL streptomycin sulfate and 25 µg/mL amphotericin B) and 1% L-glutamine.

Using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA), cells were batch cultured for 10 days before being seeded at a concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2. Cells were incubated either alone (negative control) or with various sample concentrations to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 µg/mL). While media was aspirated, fresh media (without serum) was added, and cells were then incubated. Medium was aspirated after 48 hours of incubation, 40µL of MTT salt (2.5 µg/mL) was added to each well, and the incubation process was continued for a further four hours at 37ºC with 5% CO_2. 200 µL of 10% sodium dodecyl sulphate (SDS) in deionized water were added to each well and incubated overnight at 37 ºC to stop the reaction and dissolve the crystals that had formed. A known cytotoxic natural substance at a concentration of 100 µg/mL was utilized as a positive control since it causes 100% fatality under the identical conditions **[26, 27].** A microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) was then used to measure the absorbance at 595 nm and a reference wavelength of 620 nm. Using independent T-test and the SPSS 11 program, a statistical significance was examined between the samples and the negative control (cell with vehicle). Plant extracts are dissolved using DMSO, and its final concentration on the cells was less than 0.2%.

- The formula used to determine the visibility change percentage:

((reading of extract / reading of negative control)-1) x100)

Using the SPSS 11 program, a probit analysis was performed to determine the IC_{50} and IC_{90} values. The present study uses the formula $SI=IC_{50}$ of the pure compound in a normal cell line/IC_{50} of the same pure compound in a cancer cell line, to calculate the degree of selectivity of the synthetic compounds.

Where: - Sample concentration ranges between (100 to 0.78 µg/mL) using MTT assay.

- IC_{50}: Lethal sample concentration that kills 50% of cells within 48 hours
- IC_{90}: Lethal sample concentration that kills 90% of cells within 48 hours

3. Results and discussion:

Methanolic extraction:

The crude methanolic extract of the aerial parts of *Carissa macrocarpa* yield was 231.7 gm. The yield was 23.17% *w/w*. The crude extract was used in the phytochemical, biological and pharmaceutical studies.

Total phenolic and flavonoid contents:

Total phenolic content calculated as gallic acid equivalent (GAE), was determined by applying the modified Folin-Ciocalteau method. The result is 31.57 mg GAE/g plant extract while the total flavonoid content was expressed as milligrams of catechin equivalent (mg CE) per g of plant extract. The result was 28.66 mg CE/g plant extract. Total phenolic content was previously measured and found

to be ranging from 575.40 to 1134 mg GAE/100 g dry weight of the plant. That study showed clear seasonal variation in the total phenolic content that also reduced with fruit ripening **[28].**

Qualitative and quantitative estimation of phenolics and flavonoids by HPLC analysis:

Ten phenolic compounds were identified from HPLC analysis of phenolic compounds as illustrated in table 1 which revealed that the major compounds were chlorogenic acid, protocatechuic acid and caffeic acid that representd 1024.33 µg/g, 257.48 µg/g and 179.90 µg/g respectively. In general, there are lots of chlorogenic acids (CGAs), a class of hydroxycinnamates, in common foods and drinks, most notably in some varieties of coffee. One of the most prevalent and extremely useful polyphenolic substances in the human diet is chlorogenic acid (CGA), also known as 5- *O* -caffeoylquinic acid (5-CQA). There is an inverse relationship between 5-CQA consumption and a lower risk of metabolic syndromes and chronic diseases, according to the health benefits evidence from clinical trials and basic research **[29].** Heterocyclic compounds known as flavonoids and polyphenols have been linked to positive impacts on human health, including lowering the risk of conditions including cancer, diabetes, cardiovascular, and brain illnesses. A common form of phenolic acid that occurs naturally is protocatechuic acid (PCA). The well-known antioxidant chemicals caffeic acid, gallic acid, syringic acid and vanillic acid share structural similarities with PCA. Many plants have PCA as an active component, and this substance has a wide range of pharmacological effects. These effects are a result of PCA antioxidant characteristics as well as other potential mechanisms such its anti-inflammatory effects and interactions with other enzymes. Numerous studies on polyphenols and flavonoids have been published in the last two decades, highlighting how crucial it is to comprehend the chemistry underlying the antioxidant activities of both natural and synthetic compounds when taking both their dietary and pharmacological benefits into account **[30]**. A bioactive substance called caffeic acid can be found in a wide range of plants, including fruits, vegetables, herbs, and beverages. It is a significant representative of the polyphenol subgroup of hydroxycinnamic acids and a member of the enormous group of compounds known as polyphenols. Caffeic acid primarily manifests itself in food as the quinic acid ester called chlorogenic acid. Similar to other polyphenols, caffeic acid is thought to provide several health advantages brought on by their antioxidant properties, including the reduction of inflammation, diabetes, neurological illnesses, and cancer. The usage of naturally occurring bioactive compounds, such as caffeic acid, is becoming increasingly popular in modern society. Therefore, knowledge of their characteristics and roles is crucial **[31].**

The total number of identified flavonoids was 3 compounds (Table 1), which were rutin, apigenin-7-glucoside and quercetin that represent 299.22 µg/g, 254.90 µg/g and 16.39 µg/g respectively. Rutin has a wide spectrum of pharmacological qualities that have been used in human nutrition and medicine, such as its antioxidative activity. As a matter of course, it functions as an antibacterial, antifungal, and anti-allergic agent. Recent studies have nonetheless demonstrated its broad-spectrum pharmacological advantages for the management of a number of chronic illnesses, including cancer, hypertension, diabetes and hypercholesterolemia **[32].** Apigenin is a flavonoid known to have anti-apoptotic, anti-inflammatory and antioxidant activities **[33].** The most significant flavonoid in the flavonol class is quercetin. Many foodstuffs contain quercetin which is a crucial biologically active substance. It is extensively utilized in pharmaceutics and medicine. It is used to treat cancer in particular because it slows the proliferation of cancer cells. Although they were made at a low theory level, earlier computational studies of this molecule were mentioned in the literature. Quercetin has a variety of health-promoting qualities, including anti-inflammatory, asthma, cancer prevention especially colon cancer, and cardiovascular capabilities [34].

HPLC chromatogram of phenolic and flavonoid compounds is represented in figure (1).

The concentration of different phenolic and flavonoid compounds of C. macrocarpa was previously reported. It was found that the major hydroxylcinnamic acids in Carissa macrocarpa were caffeic acid, ferulic acid, coumaric acid, dicaffeoyl tartaric acid and chlorogenic acid. Quercetin 3-O-rhamnosyl glucoside was the most abundant flavonol. They stated that concentration of phenolic and flavonoid compounds vary according to the season of harvesting [28]. Table (2) is showing the difference in concentrations of some phenolic and flavonoid compounds between that previous study and the current study.

Quantitative assessment of Carissa macrocarpa extract:

Calibration curve (figure 2) was assessed from the values of absorbance of a series of alcoholic aqueous solutions (70%) containing various concentrations of the extract, and the slope as well as the regression equation were computed. The regression equation was $y = 0.0038x + 0.0219$ and $R^2 = 0.9995$.

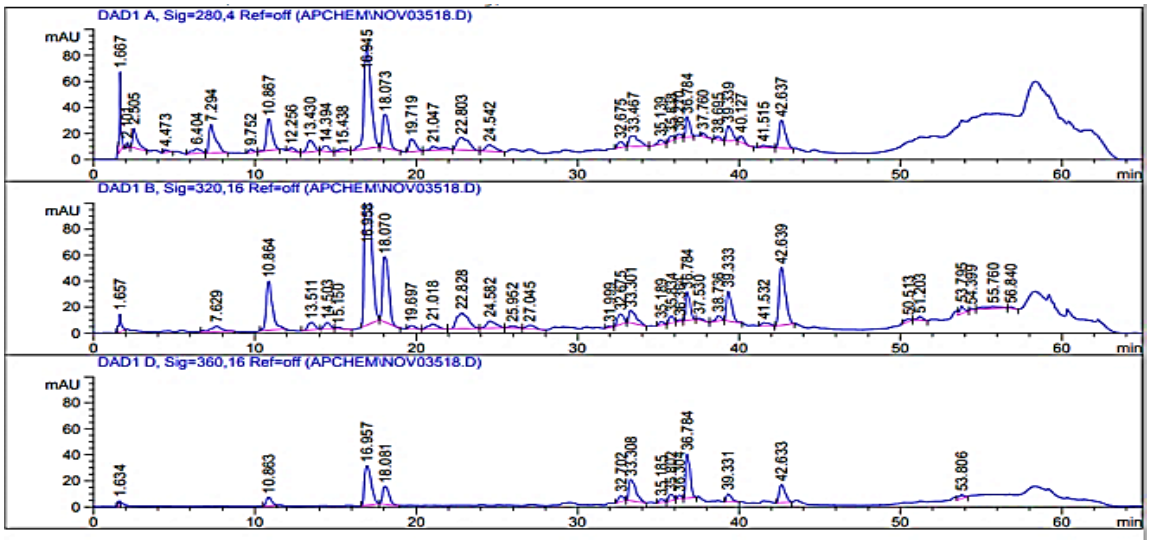

Figure (1): HPLC chromatogram of phenolic and flavonoid compounds of *C. macrocarpa* methanolic extract

Table (1): Phenolic and flavonoid compounds identified in the methanolic extract of *C. macrocarpa* aerial parts.

Compound	Concentration represented as (µg/g)
Gallic acid	ND
Protocatechuic acid	257.48
p-hydroxybenzoic acid	23.27
Gentisic acid	ND
Catechin	72.45
Chlorogenic acid	1024.33
Caffeic acid	179.90
Syringic acid	64.95
Vanillic acid	23.26
Ferulic acid	ND
Sinapic acid	45.50
p-coumaric acid	24.80
Rosmarinic acid	48.80
Cinnamic acid	ND
Chrysin	ND
Rutin	299.22
Qurecetin	16.39

Apigenin	ND
Apigenin-7-glucoside	254.90
Kaempferol	ND

µg/g= microgram of the compound per gram C. macrocarpa crude methanolic extract, ND= not detected.

Table (2): Comparison between the concentrations of some previously reported phenolic and flavonoid compounds of *C. macrocarpa* and our study.

Compound	Concentration (µg/g)	Concentration (µg/g) [28]
Catechin	72.45	79-136
Chlorogenic acid	1024.33	1.04-2.64
Caffeic acid	179.90	0.87-3.67
Ferulic acid	ND	1.04-5.91
p-coumaric acid	24.80	0.87-1.74
Apigenin	ND	3.33-11.08

µg/g= microgram of the compound per gram *C. macrocarpa* extract, ND= not detected.

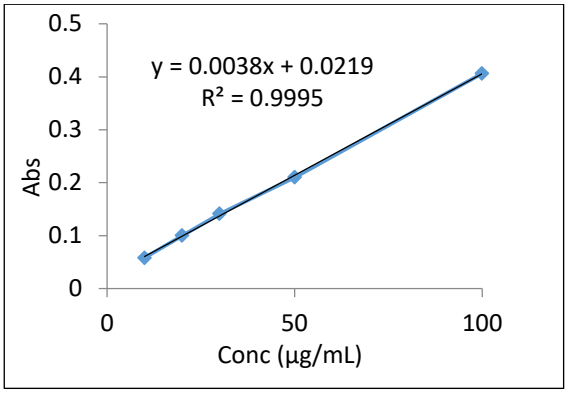

Figure (2): Calibration curve of *C. macrocarpa* in alcoholic aqueous solution (70 %) at 280 nm.

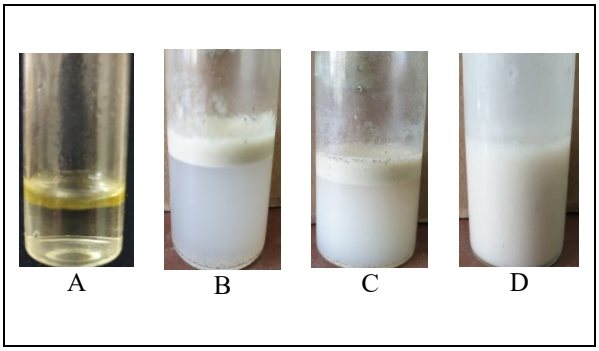

Figure (3): Photographs of the prepared formulations. A: no NC, B: NC 1% *w/w*, C: NC 2% *w/w* and D: NC 4% *w/w*

NC= Nanocellulose

Preparation and examination of the NC-NE:

The formulation prepared without the addition of nanocellulose (NC) didn't form a nanoemulsion and showed an instant and persistent phase separation with the oil phase clearly appearing above the aqueous phase (Figure 3A). While the formulations prepared with different concentrations of NC formed a single-phase with milky-white appearance. During formulation studies, the selection of the suitable concentrations of the used ingredients (notably the stabilizers) is of great importance. Stable nanoemulsions must be free of any signs of phase separation, creaming or cracking. Hence, before further investigations, the thermodynamic stability of the preparations was tested. As seen in figure 3, preparations containing less than 4% NC didn't have enough stability, the oil and water phases appeared separated, this may be explained by that the amount of NC used was insufficient to stabilize the oil/water interface consequently. Therefore, the preparation containing 4 % *w/w* NC was selected to continue the investigations as it passed successfully the three-steps stability testing which indicates its physical and thermodynamic stability **[15]**.

a. Particle size analysis, polydispersity index and zeta potential:

As shown in figure 4A, the tested NC-NE mean particle size was found to be in the nanometric range (194 ±18 nm). The polydispersity index (PDI) was equal to 0.2 which indicates the uniform size distribution of the nanoemulsion as it was documented that low PDI values (<0.3) reflects a homogenous distribution and higher stability **[35].**

Zeta potential is considered as an important parameter which can predict the nanoparticles stability. It is well known that high surface charges

(about |30| mV) cause inter-particulate electric repulsion forces which ensure a higher stability **[36].**

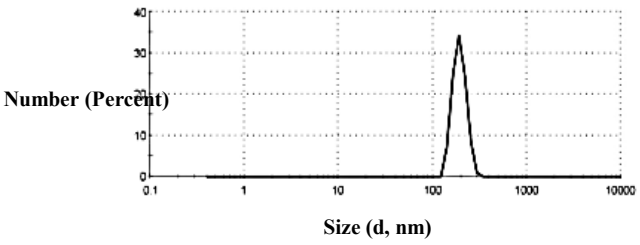

Figure (4A): Particle size of the selected formulation.

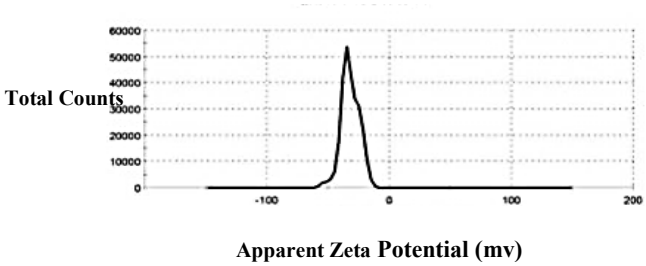

Figure (4B): Zeta Potential of the selected preparation (-32 mV)

The zeta potential value of the tested NC-NE was equal to -32 mV (figure 4B), this high negative charge is due to the sulfate as well as the hydroxyl groups of the used emulsion stabilizer (nanocellulose crystals) **[37]**. These anionic moieties afforded NC a negatively charged surface creating an inter-particulate steric hindrance forming a stable colloidal emulsion.

b. *In-vitro* release study:

Figure 5 is showing the release profile of the active agent from the prepared nanoemulsion (NC-NE) compared to the crude extract, the release enhancing and solubilizing effect of the formulated NC-NE is clear, as it attained an almost complete release at about 6h and that of the crude extract was only about 31% ($p < 0.05$). The release efficiency of the crude extract and the NC-NE was 20.16 % and 62.66 %, respectively; hence, release enhancement was about 3 fold.

c. Transmission electron microscope:

Figure 6 is displaying a photograph showing the micro-morphological structure of the prepared NC-NE. The nanoparticles appeared as well-separated dark spots with an almost spherical outline and a size approaching that detected by the ZetaSizer. preparation of different types of nanoparticles. Therefore, this type of surfactant-free emulsions provides many advantages including safety, environmental- friendly and non-carcinogenicity.

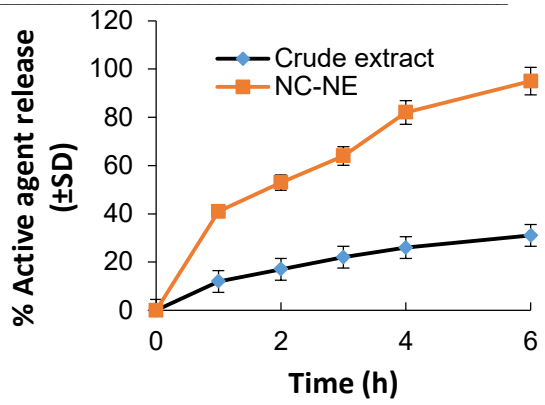

Figure (5): *In-vitro* release profile.
NC-NE=the nanoformulation

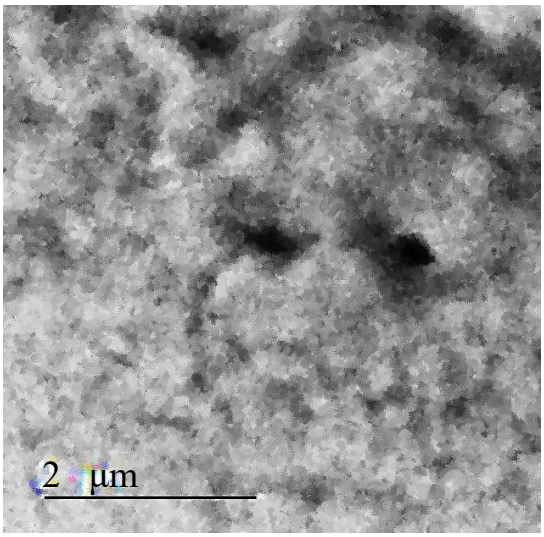

Figure (6): TEM photograph of the prepared NC-NE

Nanocellulose is a multi-beneficial polymer obtained from biomass which showed great success in the preparation of many advanced pharmaceutical formulations and can provide a great economic, ecological and industrial value **[16].**

In this study, oil-in-water nanoemulsions which are free of surfactants, were stabilized using nanocellulose, and were used as vectors for *C. macrocarpa* extract to enhance its solubility and biological effect.

Determination of in-vitro cytotoxic activity:

The prepared nanoformulation (NC-NE) gave high activity against HePG 2 and HCT116 compared to that of the methanolic extract (E) of the plant under investigation. Percentage of inhibition of NC-NE against HePG2 was 100% at 100 ppm while that of E was 36.5% at 100 ppm. Percentage of inhibition of DMSO was 1% at 100 ppm and that of negative control (Cells incubated alone with vehicle) was 0%.

IC_{50} of NC-NE was 20.62 µg/mL and IC_{90} was 33.13 µg/mL, while IC_{50} of E was more than 100 µg/mL.

Percentage of inhibition of NC-NE against HCT116 was 99.3% at 100 ppm compared to 71.3% at 100 ppm of E. Percentage of inhibition of DMSO was 1% at 100 ppm and that of negative control was 0%. IC_{50} of NC-NE was 32.80 µg/mL and IC_{90} was 58.22 µg/mL. IC_{50} of E was 74.11µg/mL and IC_{90} was 117.54 µg/mL

Activity of NC-NE and E against MCF7 and A549 was not promising. Percentage of inhibition of NC-NE against MCF7 was 8.6% at 100 ppm compared to 7.2% at 100 ppm of E. Percentage of inhibition of DMSO was 3% at 100 ppm and that of negative control was 0%. Percentage of inhibition of NC-NE against A549 was 11.2% at 100 ppm compared to 14.3% at 100 ppm of E. Percentage of inhibition of DMSO was 5% at 100 ppm and that of negative control was 0%. Results are represented in figure (7).

Dhatwalia *et al.* [38] reported that, various studies using a variety of cell lines, including breast cancer (MCF-7), human cervical cell line (Hela), bone sarcoma (MG-63), hepatocellular carcinoma (HepG2), prostate cell (PC-3), ovarian cell (OVCAR-5), human leukemia (HL-60), non-small cell lung carcinoma (NCI-H460), human lung cancer (A549), normal human (WI38) and colon cancer (SW480) cell lines, have shown the anticancer potential of the crude extracts and the purified constituents of *Carissa* species. The aerial parts of the hydroethanolic extract of different organs of *C. macrocarpa* was used to test against (HeLa), (MCF7), (HepG2) and (NCI-H460) cell lines; the IC_{50} ranged from 52 to 400 µg/mL, while IC_{50} for non-tumor porcine liver primary cells (PLP2) was more than 400 µg/mL **[39,40]**. However, more research is required to evaluate the exact mechanism by which extracts and compounds work to inhibit cancer cell lines **[38]**. Comparison between our results and the previous ones are represented in table (3).

Table (3): *In-vitro* cytotoxic activity of *C. macrocarpa* in the previous studies and the current study.

Part used	Solvent used	Cell lines	IC_{50} µg/mL (mean ± SD)	Reference
Leaves	Hydroethanol	MCF7	167 ± 2	
		NCI-H460	120 ± 1	
		HeLa	101 ± 1	
		HepG2	152 ± 3	
		PLP2	>400	
Stems	Hydroethanol	MCF7	70.38 ± 0.03	
		NCI-H460	58.7 ± 0.2	
		HeLa	52.1 ± 0.3	**[39]**
		HepG2	89 ± 1	
		PLP2	>400	
Flowers	Hydroethanol	MCF7	95.25 ± 0.01	
		NCI-H460	68 ± 1	
		HeLa	75 ± 1	
		HepG2	>400	
		PLP2	>400	
Fruits	Hydroethanol	MCF7	109 ± 5	
		NCI-H460	57 ± 2	
		HeLa	66 ± 4	**[40]**
		HepG2	>400	
		PLP2	>400	

Arial parts (leaves and stems)	Methanol	MCF7	>100	**Current study**
		HepG2	E >100 NC-NE 20.62	
		HCT116	E 74.11 NC-NE 32.80	
		A549	>100	

IC_{50}: Inhibitory sample concentration that kills 50% of cells within 48 hours

It was previously reported that *C. macrocarpa* has several activities related to its antioxidant activity. It has anti-inflammatory, anti-cancer, hepatoprotective and DNA damage inhibition **[10]**. It was found that nanoformulations enhance the activity of pharmaceutical preparation **[13]**. The effect of nanoformulation of *C. macrocarpa* leaves aqueous extract was previously studied on antibacterial activity by measuring zone of inhibition. The results indicated that nanoparticles showed effective antibacterial activity both in Gram negative and Gram positive bacteria in different concentrations, while the extract of the plant had no activity **[41].** We decided to study the effect of nanoformulation (NC-NE) on cytotoxic activity of *C. macrocarpa*. From the results we obtained, it was obviously clear that nanoformulation (NC-NE) increased the activity of *C. macrocarpa* extract against HEPG2 and HCT116 due to increasing the solubility, bioavailablity and effectiveness of the methanolic extract.

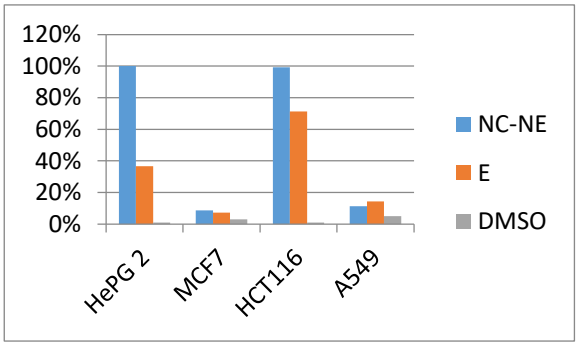

Figure (7): Percentage of inhibition against different cell lines.

DMSO=dimethyl sulfoxide solvent, E= *C. macrocarpa* methanolic extract, NC-NE= nanoformulation.
HePG 2= human hepatocellular carcinoma cell line, MCF7=human Caucasian breast adenocarcinoma, HCT116=colon cell line, A549=lung carcinoma cell line.

Since phenolic and flavonoid compounds were reported to have cytotoxic activity **[3, 4, 5, 6, 7, 8]**, so we studied the total phenolic and flavonoid contents of the plant extract. It was clearly noted that *C. macrocarpa* has high phenolic and flavonoid contents. Quantitative and qualitative estimation of phenolics and flavonoids by HPLC analysis was done to determine the concentration of the existed phenolics and flavonoids. The results of HPLC analysis of phenolic compounds revealed that the major compounds were chlorogenic acid, protocatechuic acid and caffeic acid. Protocatechuic acid and caffeic acid was known to have anticancer effect due to their antioxidant activity **[30, 31]**. The three identified flavonoids were rutin, apigenin-7-glucoside and quercetin which were previously studied and confirmed to have cytotoxic activity **[32, 33, 34].**

4. Conclusion:

The formulated surfactant-free nanocellulose-stabilized nanoemulsion (NC-NE) can be a suitable safe and low cost carrier for the extract to facilitate its solubility and hence enhance the biological effect and the anticancer activity. It is obviously clear that preparation of nanoparticles increased the cytotoxic activity of *Carissa macrocarpa* methanolic extract against HEPG2 and HCT116. It was the first time to evaluate the effect of nanoformulation of *carissa macrocarpa* methanolic extract on cytotoxic activity. It was found that nanoformulation increased the activity of the extract due to increasing the solubility, bioavailablity and effectiveness.

5. Conflicts of interest:

The authors have declared that no conflicts of interest exist.

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