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Original Research

Quercetin encapsulated polymeric nanomicelles substantially chemosensitising antiproliferation by branched chain amino acids with enhanced antiinflammation capacities: A new strategy against adherent cancer monolayers

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Abstract

Background: Quercetin as a bioflavonoid with high anticancer potential, it has been proved to have a prospective applicability in chemotherapy for a series of cancers. ***PANC1, MCF7, CACO2, A375, A549 and PC3 cancer cell lines*** represent malignant neoplasms originating from respective pancreatic, breast, colorectal, skin, lung and prostate. The poor hydrophilicity of quercetin hinders its clinical usage in cancer therapy. Therefore, a strategy to improve the solubility of quercetin in water and/or enhance the bioavailability is desired. **Aims**: In attempting to present positive evidences that this drug delivery system of polymeric micelles is effective; Quercetin loaded polymeric nanomicelles vs. free Quercetin were coincubated with selected 3 branched chain aminoacids (BCAAs; Val, Leu and Ile)) to examine synergy in chemosensitizing a panel of 6 cancer cell lines, thus reducing the dose used to submicro-nanomolar affinities of greater antiproliferation potencies than cisplatin's. **Results:** Quercetin loaded nanomicelles were proved of significantly less DPPH radical scavenging activity vs. both the antioxidative ascorbic acid and free quercetin. Unlike indomethacin; in lipopolysaccharide induced RAW264.7 macrophages inflammation; quercetin loaded nanomicelles had picomolar affinity for nitric oxide (NO) radical scavenging activity. Free quercetin posed inferior micromolar antiinflammation capacity vs. quercetin loaded nanomicelles, though more potent substantially vs. indomethacin. Remarkably nanocarrier formulation of quercetin was proved of significantly more potent antineoplastic bioactivity with micromolar affinities in the adherent monolayers 6 cancer cell lines vs. both free quercetin's and cisplatin's. Exquisitely in PC3 monolayers; free quercetin was more potent than cisplatin (IC_{50} values (μM) 46 vs. 102; P<0.01) and chemosensitized cotreated less potent aminoacids (IC_{50} values (μM) 119-557); quercetin loaded nanomicelles posed substantially greater synergy in growth suppression potencies of cotreating aminoacids vs. both free quercetin and cisplatin's (IC_{50} values (μM) <50-100 vs. cisplatin's 102 and free quercetin; P<0.05-0.001) thus reducing their doses used against PC3 tumour cells. Val, Leu and Ile exhibited inferior reduction of viability capacities when compared to cisplatin in adherent monolayers of all 6 cancer cells. Equally free quercetin had suboptimal micromolar growth inhibition (200-300μM range except for skin A375 cancer cells) as compared to antineoplastic proapoptogenic cisplatin in all 4 cancer cell lines (>100μM). Quercetin cotreated PANC1 wells of Val exhibited significantly chemosensitizing antiproliferation affinities (P<0.05 vs. cisplatin's 25.6). Remarkably most appreciable synergy trends were obtainable in resistant CACO2 and MCF7 for all quercetin cotreated 3 BCAAs separately. Surprisingly; although quercetin loaded nanomicelles could chemosensitize quercetin cytotoxicity in all 6 cancer adherent monolayers; quercetin loaded nanomicelles failed to perform similarly in aminoacids incubations (except for PC3 cancer cells). **Conclusion:** Our study indicated that Quercetin loaded polymeric nanomicelles were a novel submicro-nanoagent of Quercetin with an enhanced antitumor activity, which could serve as a promising potential candidate for chemotherapy of a diversity of cancers.

Keywords: quercetin; branched chain aminoacids; sulforhodamine B; cisplatin; nanomicelles; Indomethacin; Ascorbic Acid; Lipolysaccharides; Macrophages; Adherent Monolayers; Antiinflammation; Chemosensitising Antiproliferation

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INTRODUCTION

The fight against cancer is an unbreakable continuous challenge. Cancer statistics are overwhelming and new cases are arising each year. The cost, resistance and serious side effects of existing therapy made such battle more complicated. The need for new cheaper agents with new mechanism or lesser side effects is substantiated urgently especially to overcome

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resistance issues. Quercetin as new anticancer agents might be the answer for such problems.[1] Several reports have mentioned that quercetin have anti-proliferative activity by inhibiting of PI3K, NF-B, and other kinases,[2a-b] inhibiting mTOR activity,[3] and inducing apoptosis in cell cancer.[4] Quercetin also works as antiinflammatory by inhibiting the in vitro production of enzymes that is regularly induced by inflammation (i.e. cyclooxygenase [COX] and lipoxygenase [LOX]).[5]

Amino acids are the building blocks of proteins and have important metabolic and physiological roles in all living organisms. BCAAs (branched chain amino acids) are composed of valine (Val), isoleucine (Ile), and leucine (Leu).[6] BCAAs work as anti-inflammatory by significantly reducing the levels of pro-inflammatory cytokines and macrophages' mediators.[7] They also work as anti-oxidative by suppressing oxidative stress via downregulation of the ER (endoplasmic reticulum) –related stress pathway.[8a-b] Nano-system classified polymeric nanaoparticle and polyrmeric micelles, polymeric micelles (PMs) are nanocarriers that are formed by spontaneous arrangement of amphiphilic block copolymers in aqueous solutions. These nanoparticles have a hydrophobic core–hydrophilic shell architecture that facilitates the loading of hydrophobic drugs into the core.[9] Additional rationalization for this research comes from the fact that polymeric micelles technology is superior to traditional pharmaceuticals methods because it improves the safety and efficacy of the drugs and increases patient compliance.[10] Given the emerging evidence for the anti-cancer activity of many flavonoids, and the advantages of nanotechnology in targeted drug delivery, we propose to design nano-scale polymeric micelles for efficient and targeted delivery of flavonoids to cancer cells.[10,11a-b] The project will start from the preparation and characterization of the flavonoid-loaded micelles (quercetin loaded nanomicelles), followed by determination of their combined anti-tumor activity with bioactive BCAAs in adherent monolayers of pancreatic PANC1, mammry MCF7, colorectal CACO2, skin A375, lung A549 and prostate PC3 cancer cell lines compared to the free compounds and reference drugs. So we also hypothesize that possible molecular antineoplastic action mechanism can be via antioxidation/antiinflammation.[11a-b,12a-i]

EXPERIMENTAL

All cancer cell lines were cultured in DMEM containing 10% FBS (Bio Whittaker, Verviers, Belgium), HEPES Buffer (10 mM), gentamicin (50 µg/mL), L- glutamine (100 µg/mL), streptomycin (100 mg/mL), penicillin (100 µg/mL), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Buffer, (Sigma, St. Luis, MO, USA) whereas Sulforhodamine B was from Santa Cruz Biotechnology, Inc. Texas, USA. Quercetin (Sigma), Valine (Val), Leucine (Leu), Isoleucine (Ile) and Arginine (Arg; as a straight (unbranched) chain amino acid) were supplied from local manufacturers.

Preparation and characterization of quercetin-loaded polymeric micelles

Micelles were prepared using the amphiphilic copolymer Pluronic P123 (P123, Sigma, St. Louis, MO, USA) by the direct dissolution method. P123 is a triblock copolymer that consists of poly(ethylene oxide) (PEO) and poly (propylene oxide) (PPO) repeating units. The amphiphilicity of this copolymer leads to the formation of micelles in aqueous solutions, where the hydrophobic core contains PPO blocks and the hydrophilic surface layer is comprised of PEO blocks.[10-11] To prepare the micelles, P123 was dissolved in ultrapure water at 5% w/v. Five milligrams of quercetin hydrate (Sigma) was weighed in a 20 mL glass vial, to which 10 mL of the P123 solution was added. The mixture was stirred vigorously for 48 h to solubilize quercetin. After 48 h, the contents of the vial were centrifuged at 4,000 rpm for 10 min (Hermle Z326K centrifuge, Wehingen, Germany) to precipitate undissolved drug. The supernatant containing quercetin -loaded P123 micelles was removed and stored at 4 °C. For the characterization, 100 µL of the micelle solution was diluted with 900 µL DMSO to breakdown the micelles and release quercetin. The UV absorbance of the sample was measured at 378 nm (Shimadzu UV-1800 spectrophotometer, Kyoto, Japan). The concentration of quercetin in the micelles was calculated based on a calibration curve of quercetin absorbance at 378 nm versus concentration in DMSO (y = 0.0801x – 0.0065; R2 = 1). Quercetin loading efficiency was calculated based on the following equation:

Loading efficiency (%) = (Total amount of Quercetin in micelles / Added amount of Quercetin)100%

Quercetin loading efficiency was determined from three different batches of micelles and reported as mean ± SD. In addition, particle size of the micelles was measured by diluting 200 µL of micelles solution with an equal volume of ultrapure water and analyzing the sample using a Nicomp Nano Z3000 instrument (Particle Sizing Systems, Santa Barbara, CA, USA). Measurements were reported as mean intensity diameter ± SD from three different batches of micelles.

Antiinflammatory (Nitrite) Determination in Vitro

RAW 264.7 mouse macrophage cell line (ATCC® TIB-71) were cultured in high glucose DMEM supplemented with 10% (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamate (100 µg/mL) in a 37 °C humidified atmosphere with 95% air and 5% CO_2. Confluent macrophages (2 x 10^5 /well) were incubated with macrophage prompting lipopolysaccharide (LPS; 20 µg/mL; Sigma, St. Luis, MO, USA) added simultaneously with indomethacin (25-200 µg/mL) as the positive control[11a-b,12a-i] BCAAs, quercetin, and quercetin loaded nanomicelles at different concentrations (5-200 µg/mL) for 24 hour incubations. Griess reagent were mixed with aliquots of 100 µL of cell culture media and incubated at R.T. for 10 minutes. Absorbance at 550 nm was determined using microplate reader (Biotekmultiwell plate reader MQX200, USA). The concentration of nitrite was determined by comparison with sodium nitrite standard curve. SRB cytotoxity protocol was performed for evaluation of the effect of studied test compounds on RAW 264.7 viability.[11a-b,12a-i]

DPPH free radical scavenger assay

This method depends on the reduction of the radicals resulting in a color change from oxidized purple to reduced yellow.

Principally Diphenyl-2-picryl-hydrazyl (DPPH) undergoes reduction in methanol (MeOH) solution, in the presence of a hydrogen-donating compound due to the formation of the non-radical form DPPH-H. This change in color can be quantitatively measured using a spectrophotometer at 515–520 nm. In contrast to other radical scavenging assays, a DPPH radical is stable and can provide reproducible spectroscopic values.[11a-b, 12a-i,13a-f] A DPPH solution (0.2 mM) was diluted with MeOH and then mixed with BCAAs, quercetin, quercetin loaded nanomicelles as well as ascorbic acid in a concentration ratio of 1:1 using a 96-well plate (so that a final concentration range 6.25-200 µg/mL was obtained for test agents); the treated solution was incubated one hour isolated from light. Finally, a change in absorbance at 517 nm wavelength was measured using microplate reader (Bio-Tek Instrument, USA). Ascorbic acid was the robust and classical standard radical scavenging reference agent for comparison purposes. The calculation of the DPPH radical scavenging activity inhibition was determined by the following equation where A represents photometric absorbance: in % = (A control – A sample) / A control x 100%.[11a-b, 12a-i, 13a-f]

Viability assays for antiproliferative capacities of test compounds: Sulforhodamine B (SRB) assay

For cytotoxicity screening, Breast cancer cell lines MCF7 (ATCC® HTB-22) and A375 human skin cancer cell line ((ATCC® CRL-1619), PANC1 pancreatic cell line (ATCC® CRL-1469), A549 lung cancer cell line ((ATCC® CCL-185) and CACO-2 colorectal cancer cell line (ATCC® HTB-37) and PC3 prostate cell cancer (ATCC® CRL-1435) were procured. Surviving cancer cell lines provide numerous advantages; they provide a pure and continuous supply of cells, thus overcoming any ethical barriers concerning human's tissue usage, and cost effective.[14] The cytotoxicity measurements were determined using Sulforhodamine B (SRB; Santa Cruz Biotechnology, Inc. Texas, USA) colorimetric assay for cytotoxicity screening (using Spectro Scan 80D UV-VIS spectrophotometer (Sedico Ltd., Nicosia, Cyprus)).[11a-b,15a-e] Cells were coincubated with selected BCAAs, free quercetin, and quercetin loaded nanomicelles at different concentrations (5-200 µg/mL). As a robust and classical antineoplastic apoptogenic reference agent;16 cisplatin (1-200 µM) was recruited for comparison purposes.[11a-b,12a-i,17a-c] The mechanism of reduction of cell viability was adopted so that Dose–response curves were plotted and values were expressed as percentage of control optical density and IC_{50} values 50% inhibitory concentration were estimated by regression analysis.[18] Triplicate assay approach was performed and the calculated anti-proliferative activities were reported as as mean IC50 values of tested agents ± SD (n=3) on any specific pathological cell line.[11a-b,12a-i,17a-c,19]

Statistical analysis

The values were presented as mean ± SD of 4 independent experiments. Statistical differences between reference agent and different treatment drugs were determined using GraphPad Prism software unpaired t-test [version 5.01 for Windows; GraphPad software, San Diego, CA, USA]. Values were considered significantly different if P value< 0.05 and highly significantly different if P value<0.001.

RESULTS

Preparation and characterization of quercetin-loaded polymeric micelles

Successful incorporation of quercetin in P123 micelles was confirmed by measuring drug loading and particle size. Quercetin loading in micelles was achieved with a loading efficiency of 89.3 ± 15.1%, producing an aqueous micelle solution equivalent to 1478 ± 250 µM of quercetin and it is a very good loading dose. Moreover, the particle size of the micelles was found to be 18 ± 2.

DPPH radical scavenging effects and antiinflammatory properties in LPS-prompted RAW264.7 macrophages of tested BCAA, Querctin, Quercetin loaded nanomicelles vs. respective reference agents (Table 1)

In comparison to the reference ascorbic acid, Val (unlike remaining BCAAs Leu and Ile and unbranched Arg) had reasonably more appreciable greater radical scavenging capacities in micromolarity range. BCAAs Leu and Ile and unbranched Arg had inferior capacities to those of ascorbic

Table 1. IC_{50} values (µM; µg/mL) of in vitro DPPH-radical scavenging properties and antiinflammatory activities of the tested BCAA, Quercetin, Quercetin loaded nanomicelles vs. respective reference agent

Treatment	DPPH radical scavenging IC_{50} value µM (µg/mL)+	P- values	NOS- IC_{50} value µM (µg/mL)++	P- values
Val	**5.32± 2.04** (0.623±0.24)	NS	395.80±10.75 (46.36± 1.26)	**<0.0007**
Leu	16.03±0.83 (2.10±0.10)	**<0.0007**	189.60±21.59 (24.86±2.83)	**<0.0149**
Ile	**10.42±0.29** (1.3±0.03)	**<0.0161**	**10.05±1.77** (1.32 ±0.23)	**<0.008**
Arg	20.06±1.5 (3.49±0.26)	**<0.0004**	372.50±6.93 (64.88±1.21)	**<0.004**
Quercetin	**0.43±0.08** (0.13±0.03)	**<0.0001**	22.87±4.32 (6.91±1.31)	**<0.0037**
Quercetin micelles	17.41±1.46 (5.26±0.44)	**<0.0001**	$2.6*(10^{-6})\pm0.2*(10^{-6})$ $(7.85*(10^{-6})\pm6.04*(10^{-7})$_	**<0.0003**
Reference agent	**Ascorbic acid** 7.23±1.35 (1.27±0.24)	-	**Indomethacin** 60.88±6.21 (21.78±2.22)	-

Results are mean ± SD (n = 4 independent replicates). IC_{50} values (concentration at which 50% inhibition of DPPH reduction or 50% inhibition of Nitric oxide synthase took place in comparison to non-induced basal 24h incubations) were calculated within testing dose range.
P-value is calculated by unpaired t-test between test compound IC_{50} values µM and reference agents IC_{50} values µM using GraphPad Prism software version 8·0·1 * When P value<0·05 and ** when P value<0·01 or 0·001, *** when P value< 0·001or 0·0001 , **** when P value<0·0001, **NS**: not significantly different from reference agent. **Bolded** numerals stand out as the least IC_{50} values (most active) among others· **NI**: Non-Inhibitory.

acid. Unlike its nanocarrier formulation, the flavonoid quercetin with IC_{50} value (0.4±0.08 µM) exhibited significantly superior affinities for nanomolar radical scavenging effects vs. ascorbic acid effectiveness (Table 1). The inhibitory bioactivities of the compounds against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW264.7 cell line adherent monolayers were analyzed using the Griess assay. Quercetin micelles had superiorly incomparable picomolar antiinflammation effectiveness vs. the classical robust reference agent indomethacin (P value=0.0003). Exceptionally the tested BCAA Ile and flavonoid quercetin had appreciably greater micromolar anti-inflammatory antiinflammatory effects vs. the reference agent (P value<0.01 vs. indomethacin's). Evidently the rest of acids; namely Val, Leu and unbranched Arg; exhibited inferior inhibition to that of indomethacin in LPS-induced inflammation in RAW264.7 macrophages adherent monolayers (Table 1).

Comparisons of antineoplastic bioactivity of selected aminoacids, cisplatin's before and after cotreating with quercetin loaded nanomicelles in PANC1, MCF7, CACO2, A375, A549 and PC3 cancer cell lines (Tables 2 and 3*)*

Using the SRB assay; HTS for antiproliferative activity of 4 tested aminoacids against cancer adherent monolayers of PANC1, MCF7, CACO2, A375, A549 and PC3 cell lines was demonstrated with respective IC_{50} values. Each cell line showed a different response profile to each of the set of tested aminoacids. Cisplatin antiproliferative efficacies in all cancer cell lines were further illustrated. All selected 4 aminoacids lacked antineoplastic bioeffectiveness in apparently highly resistant colorectal CACO2 and mammary MCF7 cancer cell lines vs. cisplatins' (IC_{50} values (µM) 32.9 and 88.7 respectively). While unbranched Arg lacked antiproliferation efficacy in pancreatic PANC1; Val, Leu and Ile exhibited inferior reduction of viability capacities when compared to cisplatin in PANC1 monolayers (IC_{50} value (µM) 25). Equally free quercetin had suboptimal micromolar growth inhibition (200-300 µM range) as compared to antineoplastic proapoptogenic cisplatin in all 3 cancer cell lines (>100µM). The modulation of cytotoxicity of promising aminoacids by free quercetin was further examined. Quercetin cotreated PANC1 wells of Val exhibited significantly chemosensitizing antiproliferation affinities of IC_{50} value (µM) from 72.5 to 14.6 (P value<0.05 vs. cisplatin's 25.6). Remarkably most appreciable synergy trends were obtainable in resistant CACO2 and MCF7 for all 3 BCAAs.

Table 2.Cytotoxicity (as of %Control) IC_{50} value in µM (µg/mL) of quercetin, quercetin loaded nanomicelles in cotreatment to IC_{50} of the selected BCAAs, vs. reference drug in 6 cancer cell lines

Treatment	PANC1			CACO2			MCF7		
	IC_{50} value in µM (µg/mL)	free quercetin IC_{50} 309.05±14.35*** (93.40±4.34) +cotreatment IC_{50}	Quercetin micelles IC_{50} 11.23±1.23** ΔΔΔ (3.39±0.37) +cotreatment IC_{50}	IC_{50} value in µM (µg/mL)	free quercetin IC_{50} 291.10±42.37** (87.98±12.80) +cotreatment IC_{50}	Quercetin micelles IC_{50} 28.97±3.03 NS ΔΔΔ (8.755±0.91) +cotreatment IC_{50}	IC_{50} value in µM (µg/mL)	free quercetin IC_{50} 231.25±6.01**** (69.89±1.81) +cotreatment IC_{50}	Quercetin micelles IC_{50} 6.57±0.94**** ΔΔΔ (1.98±0.284) +cotreatment IC_{50}
Val	72.50±8.20*** (8.49±0.96)	14.60 ±2.40* ΔΔΔ (1.71±0.28) (chemosensitizing)	NI Ineffective	NI Ineffective	2.15 ±0.138*** ΔΔΔ (0.25±0.015) (chemosensitizing)	NI Ineffective	NI	57.07±10.38* ΔΔΔ (6.68±1.21) (chemosensitizing)	NI Ineffective
Leu	82.10±15.27** (10.76±2.00)	NI Ineffective	NI Ineffective	NI Ineffective	12.20±0.57** ΔΔΔ (1.60±0.07) (chemosensitizing)	NI Ineffective	NI	79.60±14.23 NS ΔΔΔ (10.44±1.86) (chemosensitizing)	NI Ineffective
Ile	319.90±8.06**** (41.96±1.06)	NI Ineffective	NI Ineffective	NI Ineffective	2.42±0.28** ΔΔΔ (0.31±0.03)	NI Ineffective	NI	29.57±3.47*** ΔΔΔ (3.87±0.45) (chemosensitizing)	NI Ineffective
Arg	NI	NI	NI Ineffective	682.05±113.49** (118.81±19.7)	0.82±0.12*** ΔΔΔ (0.14±0.02) (chemosensitizing)	NI Ineffective	NI	7.15±0.64**** ΔΔΔ (1.24±0.11) (chemosensitizing)	NI Ineffective
Cisplatin	**25.57±2.90 (7.67±0.87)**			**32.91±4.49 (9.87±1.35)**			**88.66±1.33 (26.60±0.40)**		

Results are mean ± SD (n = 3-4 independent replicates). IC_{50} values (concentration at which 50% inhibition of cell proliferation took place in comparIlen to non-induced basal 72 h incubations) were calculated within 0.1-400 µg/mL range. NI is a lack of cytotoxicity within the tested 0.1-200 µg/mL concentration range. P-value calculated by unpaired t-test between test compound IC_{50} values and cisplatin›s (µM) using Graph Pad Prism software version 8.0.1. * When P value<0.05 and ** when P value<0.01 or 0.001, *** when P value< 0.001or 0.0001, **** when P value<0.0001, NS: not significantly different from reference agent. **Bolded** numerals that stand out as the least IC_{50} values (most active) among others enlisted in the same tested cell line. ΔWhen P value<0.05 and ΔΔ when P value<0.01 or 0.001, ΔΔΔ when P value< 0.001or 0.0001, ΔΔΔΔwhen P value<0.0001, NS: not significantly different from free quercetin

Table 3.Cytotoxicity (as of %Control) IC_{50} value in µM (µg/mL) of quercetin, quercetin loaded nanomicelles in cotreatment to IC_{50} of the selected BCAAs, vs. reference drug in 6 cancer cell lines

Treatment	A375			A549			PC3		
	IC_{50} value in µM (µg/mL)	**free quercetin IC_{50}** 49.65±6.32** (15.00±1.91) **+cotreatment IC_{50}**	**Quercetin micelles IC_{50}** 6.20±1.20** ΔΔΔ (1.87±0.36) **+cotreatment IC_{50}**	**IC_{50} value in µM (µg/mL)**	**free quercetin IC_{50}** 148.40±2.02*** (44.85±0.61) **+cotreatment IC_{50}**	**Quercetin micelles IC_{50}** 9.83±1.17*** ΔΔΔ (2.97±0.35) **+cotreatment IC_{50}**	**IC_{50} value in µM (µg/mL)**	**free quercetin IC_{50}** 45.67±5.71** (13.80±1.72) **+cotreatment IC_{50}**	**Quercetin micelles IC_{50}** 9.50±0.85**** ΔΔΔ (2.87±0.26) **+cotreatment IC_{50}**
Val	NI Ineffective	338.67±57.59*** ΔΔΔ (39.67±6.74) (chemosensitizing)	465.75±38.54**** (54.56±4.51) (chemosensitizing)	NI Ineffective	NI Ineffective	NI Ineffective	492.20±83.72** (57.66±9.80)	392.00±75.44* ΔΔΔ (45.92±8.83) (chemosensitizing)	61.93±10.86* (7.25±1.27) (chemosensitizing)
Leu	NI Ineffective	331.60±38.61*** ΔΔΔ (43.49±5.06) (chemosensitizing)	547.28±81.17*** (71.78±10.64) (chemosensitizing)	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	556.50±102.53** ΔΔΔ (72.99±13.44) (chemosensitizing)	68.03±1.87*** (8.92±0.24) (chemosensitizing)
Ile	350.20±4.95**** (45.90±0.65)	368.60±47.72*** ΔΔΔ (48.3±6.25) Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	118.80±4.53* ΔΔΔ (15.58±0.59) (chemosensitizing)	84.40±1.84** (11.07±0.24) (chemosensitizing)
Arg	696.15±108.68*** (121.26±18.93)	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	NI Ineffective	293.15±36.56** ΔΔΔ (51.06±6.36) (chemosensitizing)	44.02±5.22*** (7.68±0.90) (chemosensitizing)
Cisplatin	**23.83±4.00(7.15±1.20)**			**35.21±0.03(10.56±0.01)**			**101.70±4.10(30.51±1.23)**		

Results are mean ± SD (n = 3-4 independent replicates). IC_{50} values (concentration at which 50% inhibition of cell proliferation took place in comparllen to non-induced basal 72 h incubations) were calculated within 0.1-400 µg/mL range. NI is a lack of cytotoxicity within the tested 0.1-200 µg/mL concentration range. P-value calculated by unpaired t-test between test compound IC_{50} values and cisplatin›s (µM) using Graph Pad Prism software version 8.0.1. * When P value<0.05 and ** when P value<0.01 or 0.001, *** when P value< 0.001or 0.0001, **** when P value<0.0001, NS: not significantly different from reference agent. **Bolded** numerals that stand out as the least IC_{50} values (most active) among others enlisted in the same tested cell line.Δ When P value<0.05 and ΔΔ when P value<0.01 or 0.001, ΔΔΔ when P value< 0.001or 0.0001, ΔΔΔΔwhen P value<0.0001, NS: not significantly different from free quercetin

Quercetin cotreated CACO2 wells of Arg exhibited significantly chemosensitizing antiproliferation affinities of IC_{50} values from 682 µM to 82 nM (P value<0.01-0.001; vs. cisplatin's 32.9). Principally in quercetin cotreatment; ineffective Val and Ile as well as Leu had chemosensitivity of growth inhibition to respective IC_{50} values 2.3-12.2 µM (P value<0.01-0.001; vs. cisplatin's 32.9). As of mammary MCF7 monolayers cotreated with quercetin; ineffective amino acids were in ascending order of viability reduction IC_{50} values (µM) Arg<Ile< Val<Leu (7.2<30<57<80; P value<0.05-0.001 and NS (not significant) vs. cisplain's 88.7). Surprisingly; although quercetin loaded nanomicelles could chemosensitize quecetin cytotoxicity in all 6 cancer adherent monolayers; quercetin loaded nanomicelles failed to perform similarly in aminoacids co-incubations (except for PC3 cancer cells). All 4 aminoacids ranked as markedly less potent to mostly ineffective in comparison to cisplatin's antiproliferation capacities in A375, A549 and PC3 monolayers incubations (vs. cisplatins' IC_{50} values (µM) 23.8, 35.2 and 101.7 respectively). Remarkably bioactive flavonoid quercetin loaded nanomicelles were proved of significantly more potent antineoplastic bioactivity with micromolar affinities in skin A375, lung A549 and prostate PC3 cancer cell lines (IC_{50} values (µM) of 6.2, 9.8, and 9.5 respectively) vs. both free quercetin's and cisplatin's. However both free quercetin and quercetin loaded nanomicelles pronouncedly lacked comparable antiproliferation chemosensitizing effectiveness with aminoacids in both skin A375, lung A549 cancer cells. Exquisitely in PC3 monolayers; free quercetin was more potent than cisplatin (IC_{50} values (µM) 46 vs. 102; P value<0.01) and chemosensitized cotreated less potent aminoacids (IC_{50} values (µM) 119-557); quercetin loaded nanomicelles posed substantially greater synergy in growth suppression potencies of cotreating aminoacids vs. both free quercetin and cisplatin's (IC_{50} values (µM) <50-100 vs. cisplatin's 102 and free quercetin; P value<0.05-0.001) thus reducing their doses used against PC3 tumor cells.

DISCUSSION

The escalating threats of resistance to cancers' medications designate fundamental requirements for the improvement of more effectual anticancer agents. Incrementally proven efficacies of herbal medication can offer very sound alternate to modern medicine against cancer.[20] Nanotechnology/ Nanomedicine has been recognized to dominantly improve the quality of health care strategies as drugs with high toxic potential, such as chemotherapeutic cancer drugs, can be administered with a better safety via different nanotechnology platforms.[13,21]

Antiinflammatory effects of tested compounds on LPS-triggered RAW264.7 cell line

Table 1 showed that BCAAs had anti-inflammation activity in different degree, while Ile had strong antiinflammation and the rest with a weak antiinflammation, Lee, et al.,[7] suggests that BCAAs can work as antiinflammatory agents or anticancer agents. The suggested mechanism of BCAAs is suppressing the NO synthase mRNA expression. This will decrease the mRNA expression of interleukin-6 and cyclooxygenase-2 which are proinflammatory mediators. Quercetin and quercetin loaded nano-micelles showed a strong antiinflammation activity.[5] Chen, et al.,[22] reported that quercetin inhibited LPS induced cytokines such as IL-1β, TNF-α productions through blocking NF-kB activation.

Antiproliferative activity

Table 2 showed that Val and Leu have an anticancer activity on PANC1 cell line. BCAAs were found of antiproliferative efficacies on PANC1 evidently in presence or absence of insulin but unbranched amino acid Arg lacked on antiproliferative efficacies in these cell lines.[8a-b] Likewise, quercetin has antioxidant, antiinflammtory and anticancer activity. It suppresses tumor growth of various cancer cell lines, including breast, colorectal, stomach, head and neck, lung, ovarian, melanoma and leukemia.[1] Low solubility and poor permeability across the cells are the major challenges in quercetin therapeutic protocols. Using novel and effective delivery systems that can ameliorate in the side effects of these components is the pivotal purposes in cancer treatment. Minaei, et al., [23] found that no cytotoxicity in mammry MCF7 cells when they applied quercetin up to 75 µM but when employed 75 µM nano-quercetin for enhancement of quercetin effect in sensitization of cancer cells to doxorubicin the result was that co-treatment of nano-quercetin and doxorubicin could be considered as a promising and an advatageous strategy for cancer therapy protocols. Quercetin; with IC_{50} value (36±1.9822 µM) after 72 h, was cytotoxic to PC3 cell line by inducing apoptosis. The anticancer mechanism of quercetin in PC3 was principally via reduction in antiapoptotic Bcl-2, increase in pro apoptotic Bax and inhibition PI3K. Another study showed that a quercetin's IC_{50} value (µM) in MCF7 after 72 h was 230 ± 4.144 µM whereas quercetin encapsulated in solid lipid nanoparticles IC_{50} value (µM) in MCF7 was 24.7 ± 2.7 µM.23 In our research quercetin nanomicelles in MCF7 was found to be 6.57±0.94 µM. Inadvertently the micelles encapsulated in P123 polymer had superior cytotoxicity than the querctin encapsulated in solid lipid nanoparticles.

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Structure-activity relationship of antiproliferative and antiinflammatory activity

Phenol group is known that it has antioxidant, free radical -scavenging effect as well as inducing apoptosis by stimulating caspases mediate enzyme these characteristics grant phenolic group works as anticancer activity.[24] Quercetin has a chelator groups and antioxidant group as shown in Figure 1.

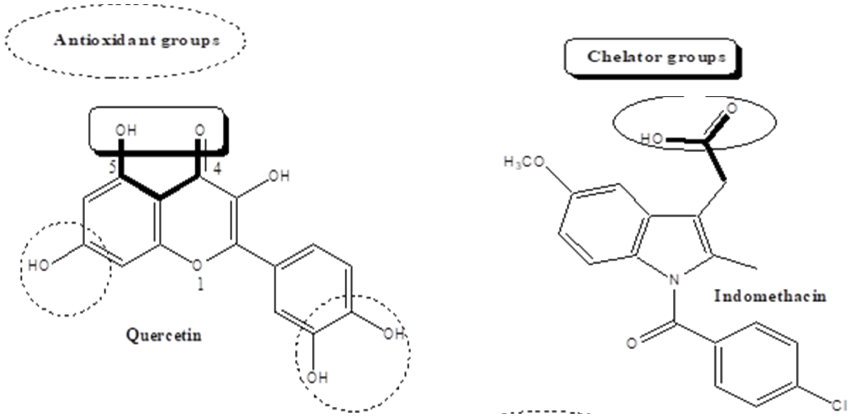
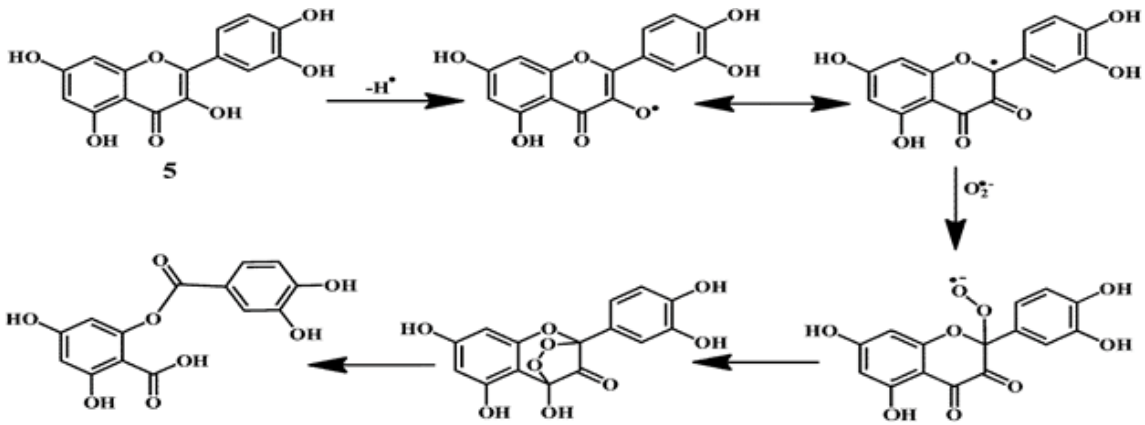
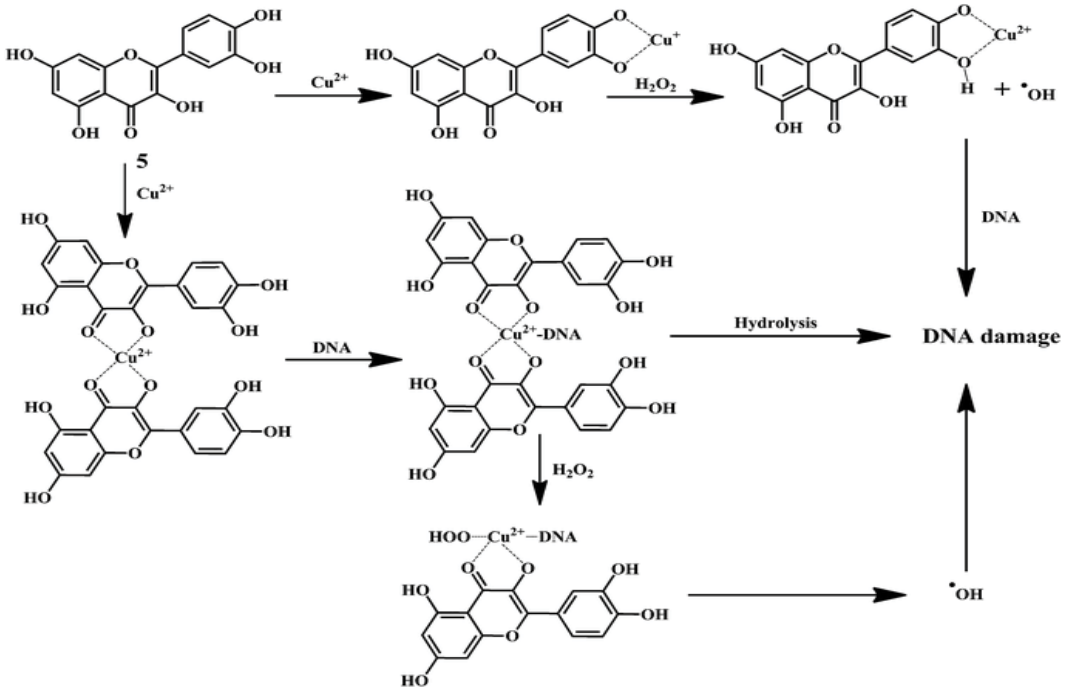


Figure 1. Chemical classes of flavonoids and NSAIDs with antiproliferative, antiinflammatory and antioxidant activities

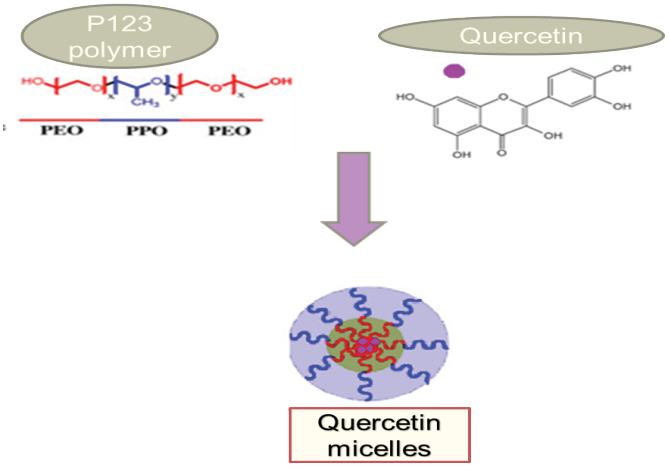


Scheme 1. Mechanism of superoxide anion radical scavenging activity of quercetin[25]



Scheme 2. Mechanism of DNA damage induced by quercetin copper complex[25]

This chelator group can bind to divalent and trivalent metals intra- and extracellularly. We hypothesize that this chelator qroup in quercetin does interact with DNA-Topoisomerase II complex through a metal, most probably a trivalent metal such iron or copper as shown in Scheme 1. This theory has some bases in literature with some flavonoids[2] may show multi functionalities. So quercetin is antiproliferative, antioxidant and antiinflammator. Scheme 1 shows the mechanism of quercetin in granting DNA protection from oxidative damage resulting from the attack of ˙OH, H2O2, and O2˙− on the DNA oligonucleotides and Scheme 2 shows how quercetin anion bind to Copper to damage the DNA in cancer cell.[25] P123 was one of most promising Pluronic polymers for targeting and controlling drug and gene delivery. It is interesting to note that P123 is used as pharmaceutical ingredients. Moreover, P123-conjugated polymers have shown a great potential as vectors for drug delivery. The hydroxyl terminal group of PEO-PPO-PEO block copolymer can be activated to couple new functional groups that endow it novel property. When quercetin encapsulated with P123 polymer quercetin micelles is formed. As shown in Scheme 3 the hydrophobic part of P123 will bind with the hydrophobic part of quercetin and hydrophilic part will be in the outer core of micelles. This micelles will facilities entry of cancer cell line and this shown in Table 2; Scheme 3. The major characteristics of quercetin micelles are more solubility and sustained release. The concentration of quercetin after dissolved from micelles only 5% in first 3 hours, while after 12 hour 80% released, and after 48 hour was 98% released.[26] So we suggest that quercetin micelles when put in MeOH in DPPH for 1 hour it was not enough to release all the concentration of quercetin in micelles for radical scavenging capacity.



Scheme 3. Graphical illustration of the preparation quercetin-loaded micelles

Concluding Remarks and Future Directives

Our study indicated that Quercetin loaded polymeric nanomicelles were a novel submicro-nanoagent of Quercetin with an enhanced antitumor activity, which could serve as a promising potential candidate for chemotherapy of a diversity of cancers.

- *Future* Studying in vivo antiproliferative effect of Quercetin nanomicelles using animal models of tumerogenesis.
- *Future* Studying quercetin nanomicelles in spleen cancer cell line and other cancer cell lines
- *Future* Clinical testing /toxicity studies of active hits.
- *Future* Studying quercetin nanomicelles in using other different antioxidant assays, antiinflammation assays and antiproliferative assays.

AUTHORS CONTRIBUTIONS

Authors contributed equally towards conceiving and study design, conducting research and providing research materials, along with data collection and organizing. Authors analyzed and interpreted data. All authors have contributed towards initial drafting, critical reviewing and approval of the final draft

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare none.

ETHICAL APPROVAL

This article does not contain any studies with animals performed by any of the authors.

DATA AVAILABILITY: The data presented in this study are available upon request from corresponding author.

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