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Antioxidant activity of gallic acid carbon-based nanomaterials

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ABSTRACT

Reactive oxygen species (ROS) play a role in multiple physiological processes. In contrast, an excessive generation of ROS can harm various biological components, including proteins, lipids, and DNA, leading to accelerated aging, illness, and inflammatory disorders. Carbon-based nanomaterials (CNMs) are well-known nanomaterials widely developed for antioxidant activity because of their great biocompatibility, low toxicity, easy synthesis, unique physicochemical properties, and especially great ROS scavenging ability. The most general method to prepare CNMs is bottom-up synthesis because it is more environmentally friendly and economical than top-down methods. In this study, the antioxidant activity of CNMs was evaluated. A microwave-assisted pyrolysis method was applied at 200°C for 20 min to prepare gallic acid carbon-based nanomaterials (GACNMs) by using 2.5 mg/mL of gallic acid as a carbon source according to the previous research. The morphology, cytotoxicity on normal human fibroblast cell lines by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay, and antioxidant activity using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay were elucidated. The results revealed that the GACNMs were spherical, with a particle size of 104.43±0.43 nm, and their structure was in line with the previous study. The GACNMs exhibited higher antioxidant activity (50% scavenging capacity (SC_{50}) of 0.99±0.07 µg/mL) than the gallic acid solution (SC_{50} of 1.25±0.03 µg/mL). The GACNMs were non-toxic to fibroblast cells. Therefore, the GACNMs could be a promising nanomaterial for skin antioxidants.

Keywords: antioxidants; carbon-based nanomaterials; gallic acid; reactive oxygen species

1. INTRODUCTION

Skin is the largest organ of the human body. It serves to envelop and shield its entirety, playing a pivotal role in separating and safeguarding the inner body from the external environment. Additionally, it serves sensory functions, aids in regulating body temperature, and facilitates the elimination of waste substances (Walker, 2022). Skin is predominantly influenced by its interaction with the environment rather than genetic predisposition (Ng and Chew, 2022). Hence, skin aging is closely associated with individual lifestyle choices in humans. For instance, factors such as smoking, excessive sun exposure, and low air humidity contribute to the development of wrinkles. Furthermore, inadequate dietary habits, excessive alcohol consumption, and certain medical

conditions like diabetes mellitus can markedly accelerate premature skin aging (Lidfeldt et al., 2005; Shi et al., 2013). The observed causal relationship between oxidative stress and various degenerative processes has sparked interest in utilizing various antioxidants for skin applications.

In recent years, antioxidant activity has been an effective measure against oxidative damage caused by free radicals. Antioxidants can interfere with the oxidation process by interacting with free radicals, chelating catalytic metals, and acting as oxygen scavengers (Choe and Min, 2009). Reactive oxygen species (ROS), including both non-free radical and free radical forms and various active oxygen species, play essential roles in diverse physicochemical processes within the body and contribute to aging (Abdal Dayem et al., 2017). The body relies on antioxidants to neutralize generated ROS. Nonetheless, excessive ROS production or insufficient antioxidant defense can lead to oxidative damage of various biomolecules composed of proteins, lipids, lipoproteins, and DNA (Vona et al., 2021). This oxidative damage serves as a significant underlying cause in numerous chronic human conditions such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and contributes to the aging process (Chen et al., 2012; Kumar et al., 2020). This can lead to the degradation of structural proteins, cellular changes, and the appearance of blemishes, marks, wrinkles, and other signs associated with both intrinsic and extrinsic skin aging processes. Antioxidants are chemical compounds frequently applied topically in clinical settings, playing a role in combating radical species accountable for numerous forms of skin damage (Calabrese et al., 2024).

Normally, ROS levels will be elevated in the regions where the antioxidant system has failed to demonstrate efficacy. In response to a sudden surge in external ROS levels, additional antioxidants can be administered as supplements—including vitamins A, C, E, lycopene and lutein. After the emergence of nanotechnology, numerous ROS-scavenging agents have been developed (Md Jaffri, 2023; Padmanaban et al., 2023). Furthermore, the understanding and utilization of these promising antioxidant properties to alleviate oxidative stresses have spurred numerous researchers to seek potent and economically viable antioxidants (enzymatic and nonenzymatic) derived from nanotechnology (Chaudhary et al., 2023; Eftekhari et al., 2018; Lü et al., 2010). Because of the limitations of conventional antioxidants, including low permeability, poor water solubility, instability during storage, and susceptibility to degradation in the digestive tract, nanotechnology has emerged as a promising candidate known as nano-antioxidants, representing a convergence of material sciences and nanotechnology, offering new avenues for antioxidant delivery and efficacy enhancement (Shah et al., 2022).

The utilization of nanomaterials has seen a notable increase. They can be applied to biomedical activity fields, including anticancer, antibacterial, and antifungal, particularly in their application for antioxidant activity (Kahkeshani et al., 2019; Yang et al., 2020). Many nanomaterials with potent antioxidant activity have been used due to their excellent nano size, which can improve surface area and lead to increased permeability, solubility, and stability. Moreover, they can design a functional group on the surface to contain the hydroxyl or carboxyl group for application as one potential mechanism for the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH), which could involve the quenching of DPPH through hydrogen atom transfer. Carbon-based nanomaterials (CNMs) have reported excellent biocompatibility among nanomaterials (Nandhini et al., 2024; Omran and Baek, 2021). In addition, previous research has reported on the dual nature of CNMs, wherein they can function as emitters of radical species and as scavengers of radical species, functioning as oxidants and antioxidants, respectively. This characteristic is not unexpected, as it is observed in numerous carbon-based nanomaterials. This may be due to the progress in enzymology targeting the reduction of abnormal ROS levels to levels compatible with cellular biological functions (Padmanaban et al., 2023). Moreover, nanomaterials utilized as antioxidants offer several advantages over conventional antioxidants, including enhanced bioavailability, controlled release, and targeted delivery to the intended site of action (Khalil et al., 2020).

Nanomaterials can be designed to act as antioxidant molecules in several ways, such as surface modification, catalytic activity, free radical scavenging, and nanomaterial composites. For surface modification, antioxidant molecules or compounds can be attached or incorporated onto the nanomaterial surface through functionalization. For catalytic activity, nanomaterials, like cerium oxide nanomaterials, exhibit inherent catalytic activity, allowing them to function as antioxidants. For free radical scavenging, nanomaterials can scavenge free radicals directly. They can effectively accept and stabilize unpaired electrons, neutralizing ROS and mitigating their harmful effects. Moreover, various factors influence the antioxidant effectiveness of nano antioxidants, including their particle size, shape, surface charge, surface-to-volume ratio, agglomeration, crystallinity, surface coating, dissolution, tunable configuration, and physicochemical properties (Omran and Baek, 2021). Finally, nanomaterials can be integrated into composite materials for nanomaterial composites, serving as antioxidants in the process. Hence, nanomaterials exhibit both pro- and antioxidant characteristics, offering the potential for addressing oxidative stress-related disorders (Padmanaban et al., 2023).

CNMs have emerged as a novel nanotechnology class with exceptional biocompatibility, low toxicity, high stability, and easy synthesis (Patel et al., 2019). In addition, all these benefits make them highly promising for diverse applications within the pharmaceutical industry. CNMs have reported particle sizes from 1 to 100 nm (Jeevanandam et al., 2018). In recent years, there have been many methodologies for synthesizing CNMs. All these methodologies can be divided into 2 groups following technology composed of top-down (ultrasound, electrochemistry, and laser ablation) and bottom-up technology (thermal decomposition, ultrasound, microwave digestion, and hydrothermal method) (Khan et al., 2022; Li et al., 2023; Manzoor et al., 2023; Wang et al., 2017). The most general technology to synthesize carbon-based nanomaterials is bottom-up technology because it is economical, environmentally friendly, and easier to synthesize than others. Hence, bottom-up technology is more appealing and commonly preferred over other technologies (Cutrim et al., 2021; Duan et al., 2019; Ross et al., 2020). However, appropriate carbon sources are critical (Chu et al., 2019).

Carbon sources can be divided into 4 groups composed of bulk carbon materials, polymers (natural and synthetic polymers), biomass, and small molecules. For the control

functional group on the surface of nanomaterials, small molecules have been suggested for utilization as precursors because of their well-defined structure, which can accurately predict the function on the surface of nanomaterials. Moreover, small molecules can be synthesized more easily than large molecules (Speranza, 2021). CNMs that can apply antioxidant activity should have a carboxyl or hydroxyl group on the surface of the nanomaterial responsible for DPPH radical scavenging. Hence, the small molecules suggested for application as precursors include tannic acid, pyrogallol, and gallic acid (Espina et al., 2022). Gallic acid is outstanding as a highly potent small molecule characterized by its active polyhydroxy phenolic compound, which is a potential candidate for versatile applications in various biological fields composed of antidiabetic, anti-inflammatory, anticancer, gastroprotective, antibacterial, and antioxidant activity. Consequently, gallic acid emerged as a prime candidate for synthesizing CNMs due to its ability to enhance antioxidant activity, in line with previous research findings (Kahkeshani et al., 2019; Yang et al., 2020).

Many methods are used to investigate the antioxidant activity, such as altering the color change, which is either an increase or decrease in the absorbance of the probe at a specific wavelength. It is correlated to the concentration of antioxidants in the sample, including the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid assay (ABTS assay), DPPH assay, ferrous oxidation-xylenol orange assay (FOX assay), ferric thiocyanate assay (FTC assay), ferric reducing/antioxidant power assay (FRAP assay), potassium ferricyanide reducing power assay (PFRAP assay), and cupric reducing antioxidant power assay (CUPRAC assay) (Brand-Williams et al., 1995). DPPH assay is the simplest method that offers the first approach for assessing the antioxidant capacity of a compound, an extract, or other biological sources. DPPH is a stable free radical compound with a deep purple color in solution. When it reacts with antioxidants, it undergoes reduction, leading to a color change and reduced absorbance wavelength at 520 nm. This reduction occurs as antioxidants donate hydrogen atoms or electrons, converting DPPH into a non-radical form. The decrease in absorbance can be measured using UV spectrophotometry (Xiao et al., 2020). Hence, this colorimetric method can be an excellent representation of the reaction mechanism in hydrogen atom transfer (HAT) and single electron transfer (SET) reaction-based methods.

This work aimed to utilize gallic acid carbon-based nanomaterials (GACNMs) following previous protocol via microwave synthesis to enhance antioxidant activity by altering to nano-oxidation form. First, the GACNMs were prepared and investigated to confirm the characterization of their particle size and shape via dynamic light scattering (DLS) and scanning electron microscope (SEM), respectively. In addition, GACNMs were investigated for biocompatibility to confirm that they are non-toxic for skin application. Afterward, GACNMs were tested for antioxidant activity via DPPH free radical scavenging activity.

2. MATERIALS AND METHODS

The chemical reagents, including gallic acid monohydrate (assay > 98%, by HPLC), were purchased from Fluka Chemie AG (Buchs, Switzerland). DPPH and 3-(4,5-D=dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Normal human fibroblast (NHF) cell lines were used as a model for normal cells to assess the cytotoxicity. Cell culture media, including fetal bovine serum (FBS), penicillin-streptomycin, trypsin–EDTA, and Dulbecco's modified Eagle's medium (DMEM), were purchased from Gibco BRL (Rockville, MD, USA). The ultrapure water was prepared through a Milli-Q system (Millipore, USA). All other chemicals and solvents were used as obtained without purification.

2.1 Preparation of GACNMs

The GACNMs were prepared following a previous study (Dechsri et al., 2024). Briefly, the gallic acid solution was prepared at a 2.5 mg/mL concentration. After that, the solution was transferred to a microwave synthesis instrument for 20 min at 200°C (Discover SP, CEM Corporation, Matthews, NC, USA). When the reaction was complete, the dispersion of GACNMs was centrifuged at 12,000 rpm to discard large particles. The dispersion was then adjusted to pH 6.5 and stored in a refrigerator at 4°C for future utilization. To confirm the characterization, their morphologies were then investigated using an SEM (MIRA 3, Tescan, Brno, Czech Republic) and compared to a previous study. Before analysis, the samples were prepared by coating them with gold. In addition, DLS was utilized to detect the Brownian motion of macromolecules in solution and the corresponding particle sizes of the GACNMs.

2.2 Cell viability assessment

NHF cell lines were maintained in controlled incubators with 5% carbon dioxide at 37°C in a DMEM culture medium supplemented with 10% FBS and 1% penicillin-streptomycin. Observation of cell growth was performed using an inverted microscope (Nikon® T-DH, Nikon, Tokyo, Japan) until cells reached 70–80% confluency, before sub-culturing and seeding into 96-well plates of 1×10^4 cells per well for further MTT assay.

The MTT assay was examined according to ISO 10993-5: 2009. In brief, after seeding completely, the plate was placed in an incubator for 24 h. The medium was then substituted with GACNMs at various concentrations (0.025, 0.05, 0.1, and 0.2 mg/mL) and continuously incubated for 24 h. The viability of NHF cell lines was assessed by introducing 25 µL of MTT solution (5 mg/mL concentration) into each well and then incubating for 3 h at 37°C to allow formazan crystal formation in live cells. Subsequently, the medium in each well was replaced with 100 µL of dimethyl sulfoxide (DMSO) to dissolve the crystals, resulting in a bluish-violet solution. Finally, the absorbance (Abs.) signal at 550 nm was measured using a microplate reader to determine cell viability. Equation (1) was utilized to calculate cell viability. Untreated cells were utilized as a control group, and cells treated with PBS were utilized as a blank group.

$$Cell\ viability\ (\%) = \frac{Abs.\ of\ sample - Abs.\ of\ blank}{Abs.\ of\ control - Abs.\ of\ blank} \times 100\% \quad (1)$$

2.3 Evaluation of antioxidant activity

The antioxidant activity was evaluated via DPPH free radical scavenging activity using DPPH assay, which was

improved by the previous protocol (Aye et al., 2023). In brief, the GACNMs were prepared in ethanol at different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL). Then, each concentration of the GACNMs was mixed with freshly prepared DPPH ethanolic solution at the volume ratio of 1:1, leading to a final concentration of DPPH solution at about 0.004%. It was then incubated under dark conditions at room temperature for 30 min for the scavenging reaction. After the reaction was complete, the absorbance of the mixture solutions was evaluated using a multimode microplate reader at the wavelength of 520 nm (VICTOR Nivo™ Multimode Plate Reader, PerkinElmer, Germany). Vitamin C and DPPH solution without GACNMs were used as the positive and the negative control, respectively. The inhibition of DPPH radicals was computed in percentage following Equation (2). In addition, the SC_{50} values of the GACNMs were determined by linear regression analysis.

$$Inhibition\ (\%) = \frac{Abs.\ of\ control\ -\ Abs.\ of\ sample}{Abs.\ of\ control} \times 100\% \qquad (2)$$

where Abs. of control is the absorbance of the DPPH solution without the sample, and Abs. of sample is the absorbance of the DPPH solution with the sample.

2.4 Statistical analysis

The experiments were performed in triplicate, and the results were expressed as mean±SD. Statistical analyses were performed using the independent *t*-test and *F*-test in IBM SPSS Statistics version 28, with a confidence level of 95%. Significant differences were determined at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Preparation of GACNMs

GACNM dispersion was prepared from gallic acid using a microwave synthesis instrument. The gallic acid solution changed into brown dispersion after a 20-minute pass. A DLS particle size analyzer was utilized to investigate the particle size, the polydispersity index (PDI), and the zeta potential. As shown in Table 1, the particle size, PDI, and zeta potential were reported. The results confirmed that the GACNMs had particle sizes of 104.43±0.47 nm, which is in line with the previous study. In addition, SEM images confirmed that the GACNM particles were spherical in shape (Figure 1), as implied by the previous reports (Dechsri et al., 2024). The results of PDI was 0.47±0.02, indicating a narrow range of particle size distribution since the value was less than 0.5 (Biswas et al., 2021). In addition, the zeta potential was reported to be in negative charge. This result may be due to the hydroxyl group on the surface of GACNMs derived from gallic acid powder utilized as a precursor.

Table 1. The particle, PDI, and zeta potential of as-prepared GACNMs

Formulation	N	Particle size (nm)	PDI	Zeta potential (mV)
1	1	107.1	0.46	-27.6
	2	104.7	0.46	-24.4
	3	101.5	0.494	-23.0
Average	-	104.43	0.47	-25.0
SD	-	2.81	0.02	2.36

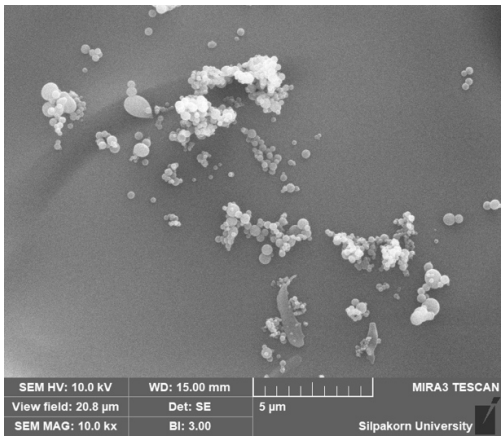

Figure 1. SEM image of GACNMs

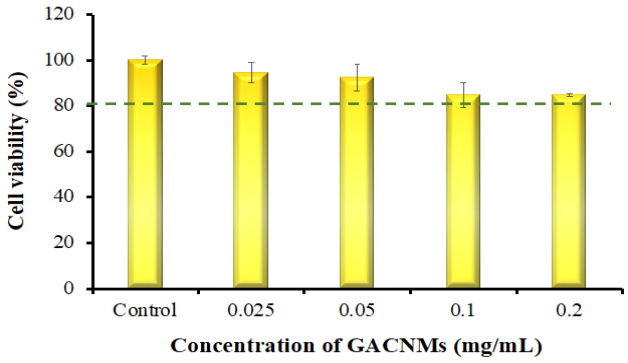

Figure 2. Cell viability after being treated for 24 h with GACNMs at different concentrations

3.2 Cell viability

The cell viability of NHF cells that were utilized as a model for normal cells after being treated with the GACNMs for 24 h was estimated using an MTT assay, as shown in Figure 2. It can be summarized that GACNMs were non-toxic to the NHF cells by exhibiting that all concentrations in the range of 0.025–0.2 mg/mL could maintain cell viability of more than 80%. In addition, the untreated cells as the control showed 100% cell viability. This result could be attributed to the biocompatibility of the GACNMs. It may be due to their nano size and spherical shape, which has been reported as nontoxic at lower concentrations (Egbuna et al., 2021). Therefore, the GACNMs were non-toxic to fibroblast cells and could be a promising nanomaterial for application on the skin.

3.3 Antioxidant activity

The antioxidant activity at different concentrations of GACNMs, gallic acid solution, and vitamin C are shown in Figure 3. The results indicate that both gallic acid solution and GACNMs represented higher or equivalent against DPPH radicals than vitamin C, the positive control, at all concentrations tested. In addition, the antioxidant activity of GACNMs was higher than that of a gallic acid solution. The results may be due to one potential mechanism for the reduction of DPPH by GACNMs, which could involve the quenching of DPPH through hydrogen atom transfer from functional groups: hydroxyl groups on the surface of GACNMs. In addition, as shown in Table 2, the SC_{50} values of antioxidant activity regarding GACNMs, gallic solution, and the positive control were reported. GACNMs represented a lower SC_{50} value than the gallic acid solution. The result may be due to an altered form of the gallic acid solution to gallic acid carbon-based nanomaterials (nano-antioxidant) that can improve the surface area, leading to increased permeability, solubility, and stability. Therefore, the nano-antioxidant form of GACNMs can exhibit excellent DPPH radical scavenging (Eftekhari et al., 2018). CNMs have been reported for their possible mechanism, which is responsible for DPPH radical scavenging, including three pathways: (1) the process of hydrogen transfer from surface functional groups such as hydroxyl group (-OH) or carboxyl group (-COOH); (2) the process of electron transfer mechanism (ETM) can be represented using a donator acceptor map (DAM), which is a molecule to either donate or accept electrons; and (3) the existence of regions enriched with sp^2 that distribute electrons across conjugated graphene-like regions effectively neutralizing the radicals through the formation of additional adducts (Innocenzi and Stagi, 2023).

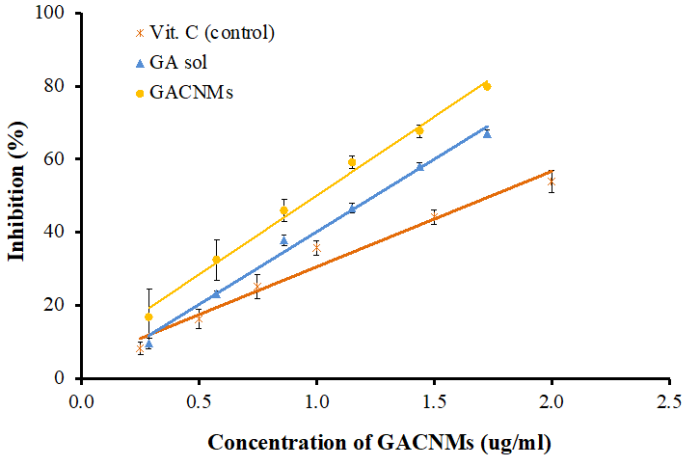

Figure 3. Percent inhibition of DPPH radicals in the presence of different concentrations of GACNMs, GA solution, and Vitamin C as the positive control at different concentrations

Table 2. The SC_{50} values of antioxidant activity of the samples

Sample	SC_{50} (µg/mL)	Strength category of antioxidant activity
Vitamin C (positive control)	1.74±0.05	Very strong
GA solution	1.25±0.03*	Very strong
GACNMs	0.99±0.07*,**	Very strong

Note: *Significant difference compared to the positive control, **Significant difference compared to the GA solution ($p<0.05$)

4. CONCLUSION

In summary, GACNMs were successfully prepared using a microwave-assisted pyrolysis method. The GACNMs presented biocompatibility with NHF cell lines at a concentration of up to 0.2 mg/mL. For antioxidant activity, GACNMs presented excellent antioxidant activity compared to the gallic acid solution because of their nano-antioxidant forms, which improve the surface area, leading to increased permeability, solubility, and stability, leading to enhanced DPPH radical scavenging. Therefore, by enhancing antioxidant activity, the GACNMs presented are promising candidates for nanomedical applications in the skin, yet further in vitro studies could be assessed.

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