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PREPARATION AND POTENTIAL OF CONJUGATED ELLAGIC ACID-INULIN AS A NATURAL ANTIMICROBIAL AND ANTIOXIDANT AGENT IN FOOD PRODUCTS

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Abstract

Despite their exceptional antimicrobial performance, natural antimicrobial effectiveness suffers from loss of food processing and long storage periods. The combination of different types of individual natural compounds has gained significant attention as a new trend of natural antimicrobial agent for food-safe applications due to their mutual performance in enhancing food quality. In this study, different types of natural compounds of acid and polysaccharide, Ellagic acid (EA) and Inulin (IN), were successfully conjugated via a simple, direct, and eco-friendly method. The structure of conjugated samples was investigated by physiochemical characterisations of Fourier transform infrared, nuclear magnetic resonance, UV-Vis spectroscopic, and differential scanning calorimetry. An efficient concentration and potential promising composition of EA conjugated IN were chosen based on the dispersion stability and antimicrobial and antioxidant activities. The results revealed significant antimicrobial activity of the conjugated sample of 75% EA:25% IN at 0.02 mg/mL against *E. coli* and *S. aureus* bacteria with inhibition zones of about 15 and 17 mm, respectively. Additionally, the radical scavenging activity exhibited a remarkable improvement in the IN-antioxidant capability with a high EA quantity of 75%, from 4.04% to 52.75%. These findings indicate that the exceptional performance of conjugated EA with IN, even at low concentrations, has promising potential as a natural antibacterial, antioxidant, and cost-effective agent for food preservative application.

Keywords: Antimicrobial and antioxidant properties, Conjugation, Ellagic acid, Inulin, Natural agent.

1. Introduction

Natural antimicrobials have been employed in food preservation as an effective alternative agent to conventional chemical additives and a new approach to containing foodborne illness in the food industry. Conventional food preservative techniques have already been implemented, such as freezing, declining water content, altered packaging conditions, acidification, fermentation, and further physical remedies to contain food illness pathogens in the food industry [1].

However, the employment of these methods was sluggish and inefficient to hinder the microbial growth in the food products due to their restrictions by pH and food component interactions. Consequently, several methods of chemical and developed preservatives have been introduced to overcome food spoilage by microbial pathogens. Despite a considerable pharmacological and biological performance, these chemicals have detrimental effects on human health because they cause liver damage, asthma, a range of allergic reactions, and even cancer when used in excess [2, 3].

Recently, food scientists and many researchers have been motivated to find an effective and promising antimicrobial extracted from natural ingredients sources for food preservation. The main sources of natural antimicrobials can be derived from plants, herbs, peels, seeds, animals, and compounds from microorganisms [4]. Even with proper performance, the antimicrobial efficiency of the chemical compounds in the foodstuffs remains a major concern during high thermal processing like sterilisation, pasteurisation, dehydration, and long-term storage [3].

Nowadays, functionalised/modified polysaccharides with natural chemical compounds have attracted the tremendous interest of food researchers as novel antimicrobial agents with potential functions to improve the safety and quality of food products [5]. However, Inulin is one of the cheapest, nontoxic, biocompatible, and naturally available polysaccharides. Further, it has exceptional physiological functions, compared with well-developed polysaccharides, for instance, chitosan and cellulose. Although Inulin has many health benefits in the food industry, it is classified as an unabsorbed polysaccharide in the human digestive system, specifically in the stomach and small intestine [6]. Besides, Inulin has limitations for long-term stability at storage food preservation, especially in acidic food, leading to the loss of its antimicrobial activity against food pathogens.

Natural acids such as ellagic acid, gallic acid, caffeic acid, ascorbic acid, and tannic acid have been extracted from the low-grade plant and fruit waste to improve polysaccharide stability and biological performance to alter the natural inhibition impact and restrict the pathogenic microorganisms [7]. Among natural acids, ellagic acid has been widely employed for food preservation due to its significant antioxidant properties and antibacterial potential against microorganisms that cause food pathogenicity and spoilage [7]. Additionally, it is one of the abundant and cost-effective natural wastes obtained from discarded pomegranate peels during consumable food production [8].

In this respect, several researchers have investigated the employment of functionalised or encapsulated chemical compounds as an alternate approach for stabilising and enhancing the effectiveness of natural antimicrobial agents under extreme conditions [9, 10]. Different techniques have developed a combination of natural chemical compounds extracted from various natural sources to produce

multifunctional antimicrobials with desirable properties for multidisciplinary health applications [5].

Economically, the food industry has sought to implement a cost-effective antimicrobial agent with a synergic performance at a low concentration to apply as an individual or combination natural antimicrobial component for food preservation [5].

A few researchers have focused on developing strategies to functionalise Inulin with hydrophilic and hydrophobic chemical moieties/polysaccharides using purified water as the base solvent by aiding different catalysts [11]. Chen et al. [6] reported the chemical modification of Inulin via various approaches for improving the antifungal and antibacterial activities. The analysis results revealed the remarkable antifungal activity with 6-amino-(N-4-chlorobenzylidene), 6-deoxy-3, 4-di-O-acetyl Inulin (4CBSAIL), and 6-amino-(N-3,4-dichlorobenzylidene)-6-deoxy-3,4-di-O-acetylinulin (3,4DCBSAIL), that have the potential to entirely inhibit the growth of the test fungi at 1.0 mg/mL at room temperature.

Moreover, the ability of Inulin to enhance the antimicrobial of biofilm via conjugation with natural polysaccharide chitosan has been examined by Zhang et al. [12], who stated that conjugation considerably increases the bacterial activities of biofilm and planktonic against S. aureus., with good solubility and nontoxic properties.

Although the promising results of inulin modification/conjugation in the previous literature have improved antimicrobial performance but are still suffering from the antibacterial activity and economic limitations at high concentrations. Furthermore, the mechanism of conjugation between the natural compounds has not been discussed yet. According to the recent literature, there is a shortage of experimental research evaluating conjugated insulin with natural organic acid for food preservation.

Herein, the major contribution of the current study is the development of conjugation natural compounds as a new, cost-effective, and efficient natural agent with promising biological activities under long-term storage. The physicochemical and antimicrobial characteristics of ellagic acid-modified Inulin were evaluated and their performance was compared to individual compounds at a minimum inhibition concentration for long-term storage.

2. Materials and Methods

2.1. Materials

The individual native materials have been utilised in this study for the conjugation of two different natural compounds. High-purity inulin powder (food grade) was purchased from a local supplier. Absolute ethanol and methanol were employed during the peel extraction process and obtained from Merck Sdn Bhd, Malaysia. All the chemical materials were used in this research immediately without any further purification.

2.2. Pomegranate peel extraction

The matured red pomegranate fruits have been selected and collected from the local market in Iraq. Initially, pomegranate peels were separated manually and then washed with tap water to remove the contamination. Next, the peels were exposed

to air-dry and cut into small chips to dry in the controlled air oven at 50 ºC for 48 hours. After that, the dried peels were grounded and sieved to acquire the fine powder, with 25 µm diameter and kept inside glass vials for further process. The procedure of pomegranate peel extraction was earlier performed in the previous research study by Lacivita et al. [8].

2.3. Conjugation process

The extraction process of ellagic acid was initially inspired by an earlier reported procedure by Kaderides et al. [13]. In this study, the conjugation process will be carried out by dissolving a suitable amount of inulin powder in 100 mL of 80:20 vol.% methanol and warm deionised water under a stirrer until the transparent solution is at room temperature [14]. After that, the pomegranate peel powder will be added to the inulin solution with various weight ratios during the extraction process of ellagic acid via the ultrasonication process, as illustrated in Table 1.

The mixture solution was transferred to a probe sonicator for 2 hours at maximum amplitude, as recommended by Balaban et al. [15], to extract ellagic acid from pomegranate peel powder, as shown in Fig. 1. The suspension solution will relocate to mix with acetic acid overnight at ambient temperature to obtain a homogenous solution of conjugated natural compounds.

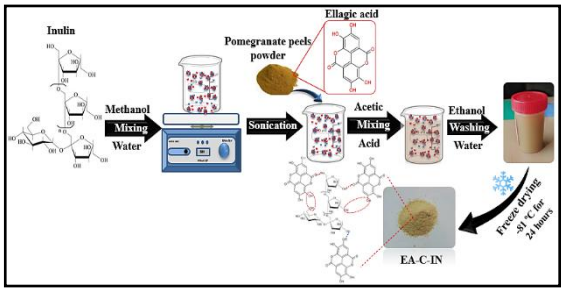

Fig. 1. Schematic illustrates the procedure of conjugated Inulin with ellagic acid via the ultrasonication process.

The resultant suspension will rinse and filtrate with 100 mL of absolute ethanol and deionised water (DW) to discard the residual peels; meanwhile, acquire the conjugated solution by using syringe filtration (0.25 µm, Whatman). The supernatant of an ellagic acid conjugation inulin (EA-C-IN) product marked by the mass-weight ratio of EA to IN natural compounds will then dry in the oven at 50 ºC for 48 hours.

Table 1. The developed of combination natural compounds at a specific weight ratio.

Sample No.	Total weight g/100 mL		
	Ellagic Acid (EA)	Inulin (IN)	Acetic Acid
1	100	0	0.5
2	75	25	0.5
3	50	50	0.5
4	25	75	0.5
5	0	100	0.5

2.4. Physiochemical characteristics

The chemical bonding of modified IN with EC and the interaction between the natural compounds were identified by Fourier transform infrared (FTIR) (Perkin Elmer, USA). Further, the chemical structure natural components of EA and IN before and after the conjugation process were characterised by a nuclear magnetic resonance (NMR) spectrometer (Bruker BioSpin GmbH, Germany), the ^{1}H-NMR experiment was conducted with dimethyl sulfoxide (DMSO) as the solvent and tetramethyl silane (TMS) as the internal standard. The chemical shift values were measured in the spectrum scan range between 0-16 ppm at ambient temperature. Scanning Electron Microscope (SEM, Zeiss EVOLS15, Germany) was utilised to evaluate the morphologies of individual pure and conjugation samples. The spectral absorbance of UV-Vis spectroscopy analysis was performed to evaluate the storage stability of natural individual chemical compounds and their conjugation via using (UV-Vis-1900 Shimadzu, Japan). The photometric repeatability accuracy is ± 0.0002 of maximum Abs.

2.5. Antimicrobial assay

The antimicrobial activity of EC-C-IN samples was evaluated against one Gram-positive bacteria (*Staphylococcus aureus*) and one Gram-negative bacteria (*Escherichia coli*). All bacterium types were obtained from food illness isolates in a private medical centre. The bacterial suspensions were grown in nutritional agar; subcultures were prepared monthly and stored at 4 °C until necessary usage. The remaining bacterial cultures were dispersed on petri dishes and incubated at 37 ºC for 24 hours. The conjugated samples of (EA-C-IN) with low concentrations of 0.001, 0.01, and 0.02 mg/mL were placed into the Petri dishes of different microorganisms to determine the minimal inhibition concentration against bacterial growth. Following incubation, inhibitory zones for microorganisms were measured in a few millimetres for each sample through optical microscope images.

2.6. Antioxidant assay

The antioxidant potential of the developed samples was determined by DPPH radical scavenging activity. In brief, 10 ml of the sample at a concentration of 0.02 mg/ml was mixed with 1.0 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution at 0.135 mM in methanol. A standard antioxidant, 0.02 mg/ml of ascorbic acid, was utilised as a positive control sample. The mixture was gently stirred and then left in a dark place at room temperature for 30 minutes. The UV-Vis spectrophotometry was used to measure the absorbance of the test samples at a specific wavelength of 517 nm, with precision ± 0.0002. All measurements were repeated in triplicate for two independent experiments. Finally, the capacity to scavenge DPPH radicals was estimated based on the familiar equation, as clarified in [16, 17].

3. Results and Discussion

3.1. FTIR spectroscopy

The chemical structure of individual and conjugated IN with EC samples was identified through FTIR spectroscopy in the transmittance wavelength range between 400-4000 cm^{-1}, as shown in Fig. 2. The FTIR characteristic peaks of Inulin revealed the broad and strong absorption peak of the stretching hydroxyl (O-H)

band at 3,352.3 cm^{-1} [18]. Besides, the two remarkable peaks at 2,935.7 and 1662.4 cm^{-1} are attributed to symmetric stretching (C-H) and stretching (C=O) in the backbone of an inulin polysaccharide [19]. The analysis spectra have detected a series of weak peaks at the wavelength range of 1469 - 605 cm^{-1}, related to the stretching vibration of C-O-C, C-O, C-C, and C-H bands, respectively [20]. This IR result is well consistent with FTIR analyses of [19, 21].

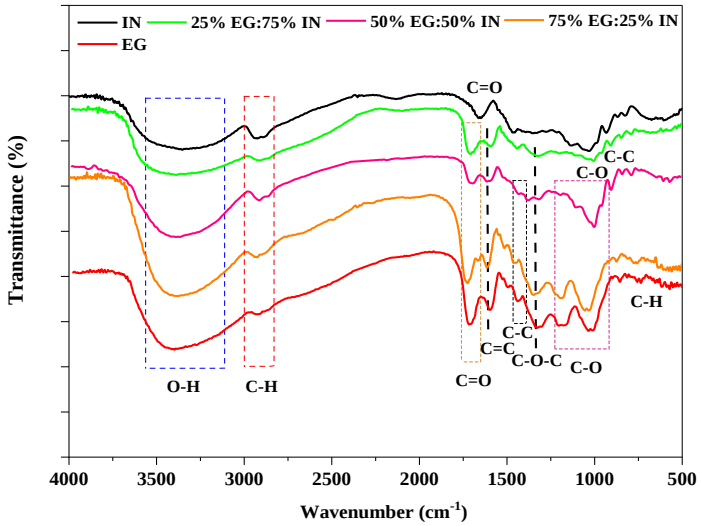

Fig. 2. FTIR spectra of Inulin, Ellagic acid, and conjugated EG-IN at various weight ratios.

On the other hand, the FTIR analysis of extracted ellagic acid exhibited intense peaks close to 3,433.3 and 1735.9 cm-1, ascribed to phenolic and carbonyl groups in the (O-H) stretching vibration bands [22]. Further, the distinctive bands of carbonyl (C=O) and aromatic ring (C=C) are observed at 1735.9 by Goriparti et al. [23] and 1616.4 cm-1 by Wei et al. [24]. The additional three absorption peaks at 1,354, 1,222.8, and 1030 cm-1 are correlated to the stretching of C-O-C and C-O ester linkage [22, 24]. However, the FTIR spectrum of all conjugated samples at different weight ratios displayed the major absorbance peaks of chemical groups in the structure of natural IN and EA. It can be noted that there is a shifting in the absorbance peaks of OH, CH, C=O, and C-O after the conjugation process. This moving in the sites of chemical bands is due to the hydrogen bonding among the chemical moieties in the structures of IN with EA [25]. Moreover, the appearance of the carbonyl band (C=O) with three conjugated samples referred to binding the EA functional groups with chemical moieties in the structure of IN through an esterification reaction Chehardoli et al. [26]. Hence, the observations of FTIR analysis results are affirmed to successfully functionalise IN with moieties of EA during the conjugation process. These findings are well in agreement with previous studies [26, 27].

3.2. ^{1}H-NMR spectroscopy

The chemical structure of natural components and their conjugated sample were further clarified through ^{1}H-NMR spectroscopy. Figure 3 shows the proton NMR

spectrum of (75% EA:25% IN) in comparison with pure IN and extracted EA. The proton signals of sucrose units in the inulin skeleton are observed in the ^{1}H-NMR spectra for all conjugated samples in the 3.0-5.4 ppm region [28]. Furthermore, the proton signals appeared at 7.47 and 7.51 ppm, which are attributed to the aromatic protons of the benzene ring in the EA structure [29]. In addition, the low-intensity absorption peaks at 0.83, 1.23, 1.96, and 2.94 ppm are assigned to ellagic acid chemical moieties [26].

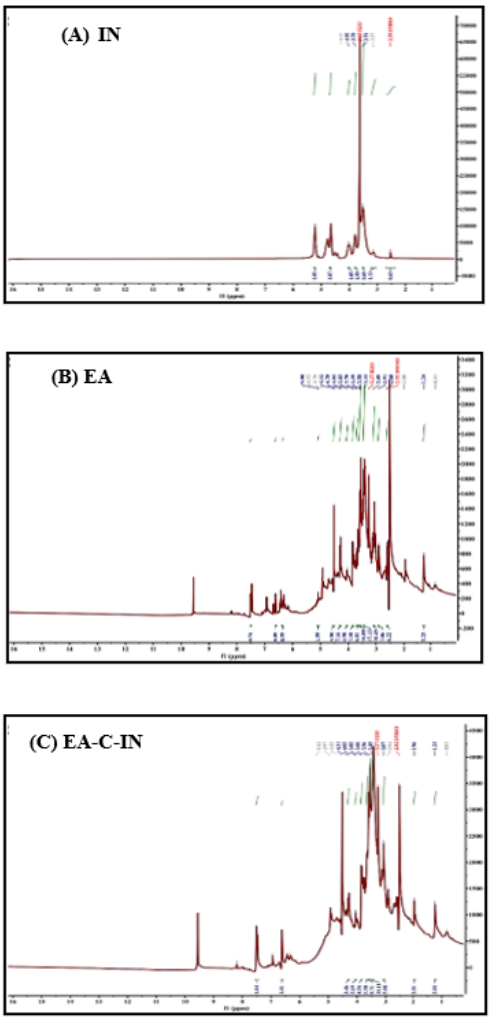

Fig. 3. ^{1}H-NMR spectrum analysis results.

Meanwhile, the two new proton signals emerged at 5.42 and 9.59 ppm, which are related to the glucose anomeric protons and isoniazid aromatic rings, respectively [20]. The presence of these signals in the ^{1}H-NMR spectra of the conjugated sample implied that the interactions between the chemical molecules of EA and IN have occurred. However, the intensity and region of the absorption peaks of polysaccharide backbone and natural acid were remarkably varied after

the conjugation process. This variation indicates that the intermolecular interaction between the protons' functional groups of polyphenols originated via hydrogen bonding with oxygenated molecules in the IN structure during the combination of natural components [12]. It is worth mentioning that the variety of IN and EA content exhibited negligible shifts in the detected proton signals. Therefore, the detection of ^{1}H-NMR spectra supported the FTIR findings and confirmed the successful incorporation of EA chemical moieties with inulin molecules.

3.3. Morphological characterisation

The detailed surface morphologies of individual and conjugated samples of IN and EG at different weight ratios were characterised by analytical images of SEM investigation, as illustrated in Fig. 4. The morphological results of incorporated samples generally confirmed that the EA was effectively attached to the IN surface and the shape and size distribution varied with increasing EA addition.

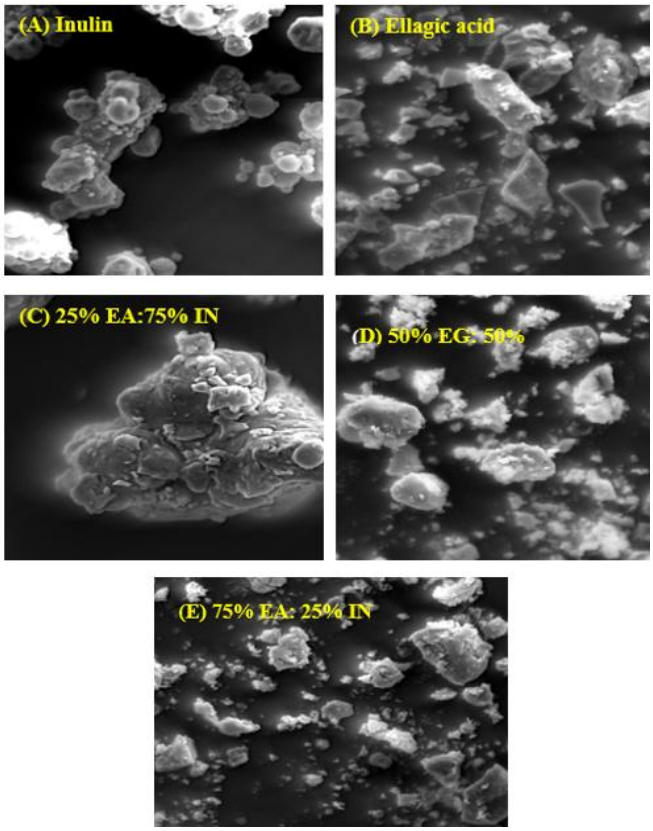

Fig. 4. The SEM microstructure images.

3.4. UV-Vis spectroscopy

The optical absorbance characteristic of the UV-Vis spectrum is a considerable indication to identify the binding among the functional groups of EA with IN after the combination process. Furthermore, it is utilised to evaluate the stability of

individual and conjugated samples in the storage environment. Figure 5 depicts the UV-Vis spectrometry of dispersed pure IN, extracted EA, and conjugated samples at various weight ratios in distiller water at low pH and ambient temperature. The UV absorbance of dispersed IN revealed a small absorbance peak at the wavelength 270 nm. Besides that, the UV-Vis spectroscopy profile of extracted ellagic exhibited three distinctive peaks at 254, 288, and 366 nm, respectively. The absorbance analysis peaks of the extracted EA are well in accordance with UV spectroscopy findings of the standard EA sample [30, 31]. At the same time, the optical absorbance analysis for all conjugated samples of (EA-C-IN) confirmed the physical interaction via π-π between the hydroxyl and polyphenols functional moieties of EA with IN during the sonication process. It can be noticed that the absorbance peak of incorporated samples increases with an increment in the weight additives of EA with IN.

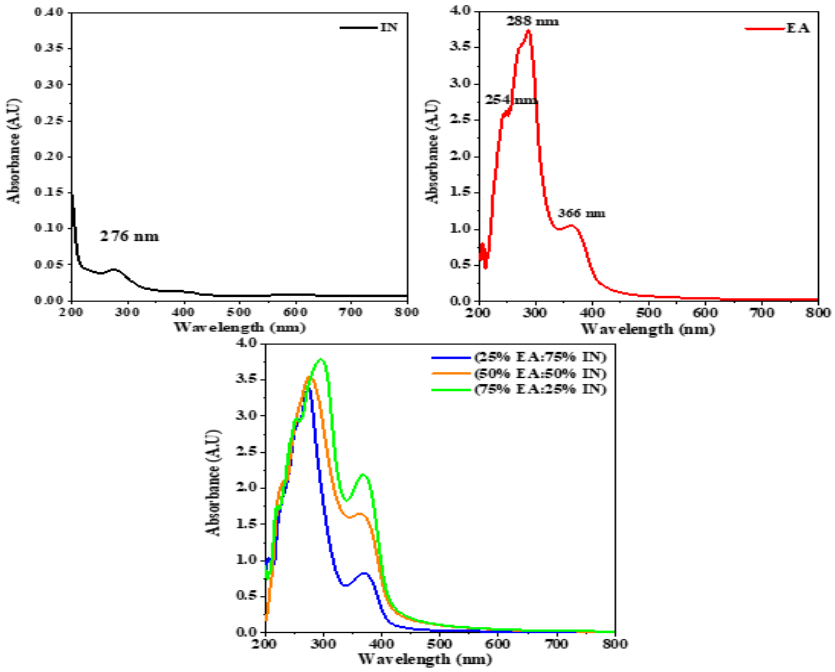

Fig. 5. UV-Vis's spectroscopy of IN, EA, and conjugated samples with different content of EA: IN.

Obviously, the storage stability of conjugated samples was remarkably enhanced after combination with functional groups of IN structure by comparing with EA alone. The highest stable sample was recorded with prolonged storage time over 10 days at 75% EA:25% IN)along the UV-Vis spectrum range, as depicted in Fig. 6. The degradation rates for all three conjugated samples after 10 days of storage at ambient temperature were 92, 95, and 100%, respectively. The low degradation rates for incorporation samples at different weight ratios, even with a low content of natural polysaccharide, confirmed that the functional groups of IN structure persisted in improving the solubility of dispersed EA in food products for a long storage time.

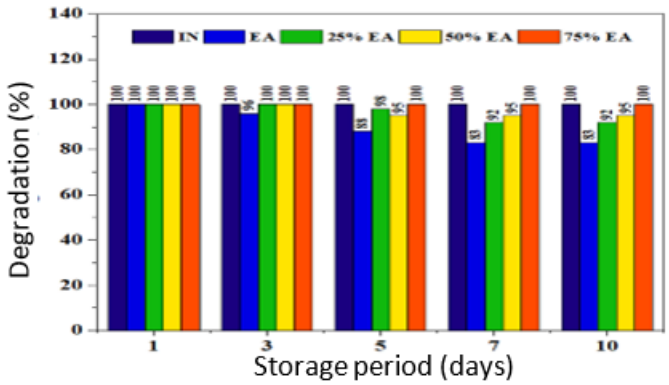

Fig. 6. Variation in the stability of individual and conjugated samples of EA with IN as a function of different storage periods at ambient temperature.

3.5. Antibacterial activity

The two most common pathogenic food bacterial, one-Gram positive (*E. coli)* and one-Gram negative (*S. aureus),* were utilised to investigate the antibacterial activity of suspended individual and conjugated samples of EA with IN at different concentrations of 0.001, 0.01, and 0.02 mg/mL, as illustrated in Fig. 7. It is worth noting that the pure IN has not shown any antibacterial potency to inhibit the selected bacterial growth at all concentrations.

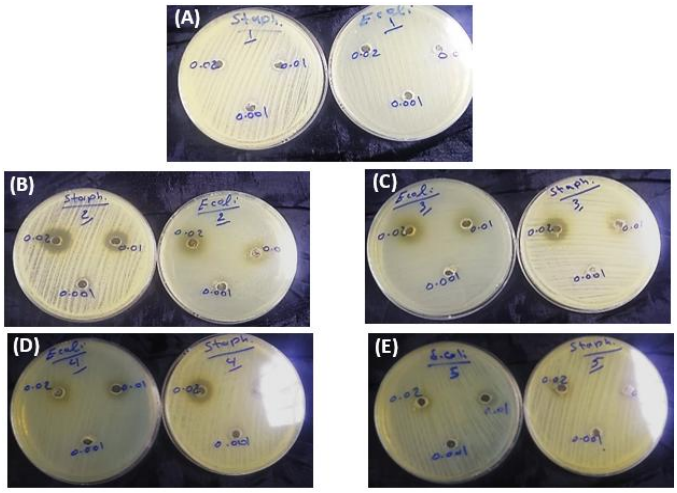

Fig. 7. Zone of inhibition against E. coli and S. aureus strains with different concentrations after 24 hrs incubation at 37 ºC, (A) IN, (B) EA, (C) 75%EA:25% IN, (D) 50%EA:50% IN, (E) 25% EA:75% IN.

Nevertheless, the insufficient antibacterial functionality of IN is due to the functional groups that constitute the structure of IN polysaccharides are not enough to weaken and break down the bacteria's cell membrane Chen et al. [6]. The antibacterial activity of IN is quite consistent with the reported results in the earlier research studies by Van den Abbeele et al. [32].

Interestingly, the inhibitory action of IN was reasonably improved when conjugated with EA at various weight ratios and became more sensitive against the bacterial strains, especially in samples with a high EA content. The quantitative results of inhibitory zones showed that the diameters of the zones increased steadily with increment in the weight content of EA to IN which is referred to actual evidence of successfully functionalised IN with EA chemical moieties for improving the antibacterial effectiveness of IN. Furthermore, the extracted EA and conjugated sample with high EA content of 75% EA: 25% IN at the concentration of 0.02 mg/mL have a great potential to inhibit the growth of food pathogenic bacteria, with maximum inhibition zones 16 and 20 mm, and 15 and 17 mm against E. coli and S. aureus ($P < 0.05$), respectively, as shown in Fig. 8.

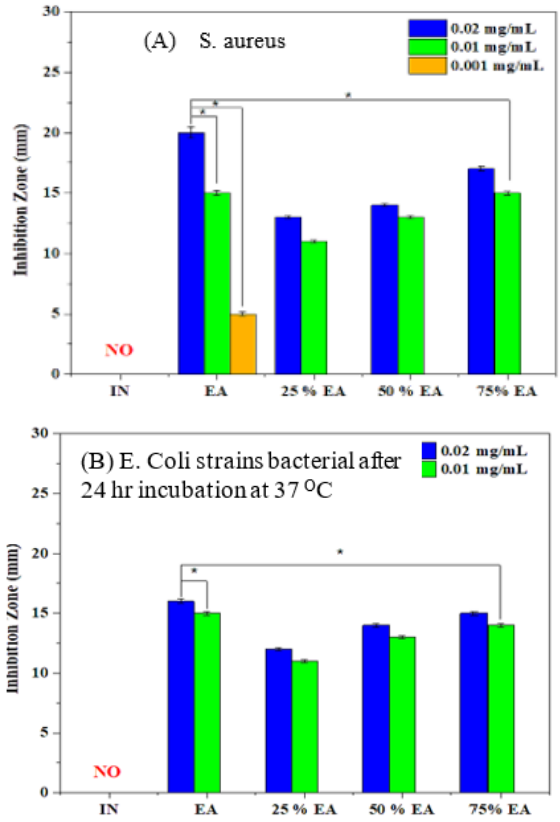

Fig. 8. Antibacterial activity is represented by the inhibition zone of individual and combined natural components at relatively low concentrations.

The data in Fig. 8 are of mean values with standard deviation ± SD; * indicating significant differences ($P < 0.05$), and ** insignificant. In this context, the extracted EA, either alone or in a conjugated system, exhibited remarkable antibacterial activity with a higher inhibitory effect toward gram-positive than gram-negative bacteria, even at low concentrations, because the intramembranous spaces and lipopolysaccharides layer in the Gram-negative bacteria cell could block the antibacterial functional groups from passing through

cell membranes [33]. The antibacterial findings inferred that the EA chemical moieties have a considerable prompt on improving the antimicrobial activity of EA-C-IN for suppressing foodborne bacterial strains at a low bactericidal concentration of 0.02 mg/mL in a short time. Hence, it can be concluded that the conjugated system of natural components (75% EA: 25% IN) at low concentrations with effective antibacterial activity might be employed as an inexpensive and efficient natural agent in food preservation.

3.6. Antioxidant activity

The DPPH radical scavenging ability is widely utilised to evaluate the antioxidant activity of individual and combined natural constituents of EA and IN. The high antioxidant resistance of natural components with stable free radicals was assessed via the optical absorbance at a specific wavelength of 517 nm. Figure 9 shows the antioxidant potential of conjugated samples at various weight ratios compared with individual IN and EA for scavenging the DPPH radical. As expected, the combination sample with high EA content (75% EA: 25% IN) showed high DPPH-RS inhibition activity by 52.75% at 0.02 mg/mL compared to other conjugated samples.

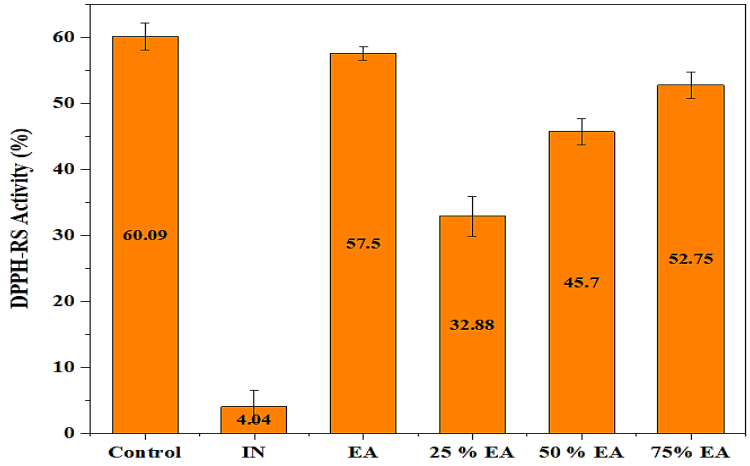

Fig. 9. DPPH free radical scavenging activity of conjugated samples at different weight ratios of EA to IN, compared with individual IN and EA at 0.02 mg/mL.

Noteworthy, the IN sample has a minimal suppression toward the DPPH radical by about 4.04%, which agrees with DPPH observations of IN in [34, 35]. However, even at a low quantity, the conjunction of EA with IN considerably improved the antioxidant activity; the physical and chemical interactions of EA chemical molecules along the backbone structure of IN have effectively aided in the antioxidant potential to inhibit the DPPH radical Arizmendi-Cotero et al. [27]. Likewise, IN acts as a carrier for the chemical components of EA that may increase its solubility in the aqueous medium, thus increasing the EA release rate to improve the antioxidant activity. These findings support the research hypothesis that successful conjugation of EA with IN and exceptional reciprocal performance at low IN dosages have a high potential to be a natural antioxidant and antibacterial agent at low concentrations.

4. Conclusions

The main objective of this research is to develop a hybrid system of conjugated natural components as an effective agent for food preservation that has been successfully prepared through a simple method. The results revealed the below significant findings.

- The physicochemical analyses of FTIR, ^{1}H-NMR spectrums, DSC thermal properties, and SEM morphological characteristics affirmed the functionalised IN backbone with chemical moieties of EA via the conjugation process.
- The combination of a good quantity of EA of 75% with IN enhanced their stability over a long period of time during the transport and storage conditions.
- The antibacterial effect showed that 0.02 mg/mL of 75% EA:25% IN has considerable inhibition against the growth of E. coli and S. aureus microorganisms than pure IN and other conjugated samples.
- The reciprocal performance has significantly improved the antioxidant activity of Inulin to 52.75% when combined with 75% of EA, compared to the negligible potential of IN by about 4.04% at a low concentration.

The conjugated samples, even with low concentrations could have promising potential as a novel agent for bioactivity and nutrition applications. Furthermore, the exceptional properties of natural acid-functionalised polysaccharides developed in this study might be suitable for employment in sectors other than the food industry, such as pharmaceutical, biomedical, agriculture, and engineering applications.

Nomenclatures

DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH-RS	DPPH radical scavenging
E- coli	Escherichia coli bacteria
S. aureus	Staphylococcus aureus
EA	Ellagic Acid
EA-C-IN	Ellagic acid conjugated IN
FTIR	Fourier Transform-Infrared
^{1}H-NMR	Proton Nuclear Magnetic Resonance
IN	Inulin
Nm	Nanometre
UV-Vis	Ultraviolet-Visible light
Std. Dev.	Standard Deviation
SEM	Scanning Electron Microscope

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