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Synthesis, Characterization, Swelling Properties, and Biological Applications of pH-Sensitive Polymeric Hydrogels based on Citric Acid, Triethanolamine, and 2-Furoic Acid

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KEYWORDS
Hydrogels, Swelling equilibrium, pH sensitive, Antioxidant activity, Antibacterial and Antifungal properties, Cytotoxic studies.

ABSTRACT:

Introduction: The objective of this endeavor is to create novel hydrogels utilizing three monomers namely Citric acid (CA), Triethanolamine (TEA), and 2-Furoic acid (FA). Citric acid (CA), a crosslinking agent that can overcome the toxicity and connects with other crosslinking substances. FTIR analysis shows that the synthesis of hydrogels based on triethanolamine and citric acid leads to the esterification reaction of carboxylic acid of citric acid with hydroxyl group of triethanolamine to the formation of pre-polyester. The pre-polyester combines with 2-Furoic acid results in hydrogel formation.H^1-NMR and C^{13}NMR spectroscopy of the hydrogels have been examined which conforms the ester bond formation. Investigations on swelling and swelling equilibrium were carried out at various pH ranging from 2.0 to 11.0. The swelling e quilibrium was improved by increased CA composition in hydrogels for pH levels of 2.0, 4.0, 7.0, 9.0, and 11.0 than acidic media. The percentage of swelling is larger in neutral (pH 7.0) and alkaline (pH 9.0) medium. With increasing anionic charge, the ionic strength of the medium and the degree of crosslinking are increased and the swelling capacity consequently decreases in pH 11. The hydrogel has shown strong antibacterial activity against *Escherichia coli* (gram negative) and moderate inhibition in *Staphylococcus aureus* and *Bacillus subtilis*(gram positive) bacteria. The antifungal studies were carried out with *C.albicans* and *A.niger*results into inferior antifungal action with respect to the standard clotrimazole 20 mg/well. Further, cytotoxic studies have also been carried out. The results reveal moderate activity with respect to Ascorbic acid. In overall aspects, the hydrogels of present investigation may be recommended for biomedical applications.

Objectives: To synthesis CTF based biopolymeric hydrogel which is having considerable antibacterial and antioxidant property for biomedical recommendations.

Methods: The monomer, 0.020 mol citric acid (3.842 g) was dissolved in ethanol and placed in a round-bottomed flask equipped with a mechanical stirrer. Triethanolamine [0.020 mol (2.640 g)] dissolved in ethanol was added drop wise using a dropping funnel. The mixture was stirred for one hour at 140 °C. The completion of the pre-polyester reaction was indicated by the formation of a sticky white gel-like compound Citric acid-Triethanolamine (CT). Subsequently, 2-furoic acid (0.020 mol, i.e., 2.241 g) dissolved in ethanol was added to the pre-polyester CT at 140 °C and stirred constantly for 2 hours. The formation of a glassy brown gel (CTF) indicated the completion of the reaction.

Results: The pre-polyester combines with 2-Furoic acid results in hydrogel formation.H^1-NMR and C^{13}NMR spectroscopy of the hydrogels have been examined which conforms the ester bond formation. Investigations on swelling and swelling equilibrium were carried out at various pH ranging from 2.0 to 11.0. The swelling e quilibrium was improved by increased CA composition in hydrogels for pH levels of 2.0, 4.0, 7.0, 9.0, and 11.0 than acidic media. The percentage of swelling is larger in neutral (pH 7.0) and alkaline (pH 9.0) medium. With increasing anionic charge, the ionic

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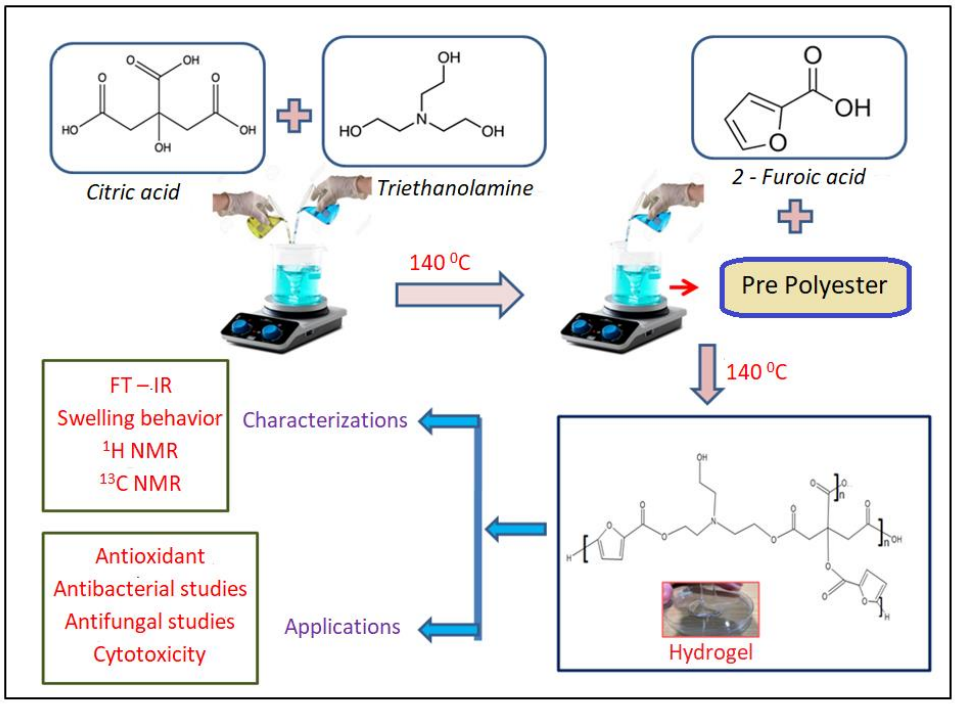
strength of the medium and the degree of crosslinking are increased and the swelling capacity consequently decreases in pH 11. The hydrogel has shown strong antibacterial activity against *Escherichia coli* (gram negative) and moderate inhibition in *Staphylococcus aureus* and *Bacillus subtilis*(gram positive) bacteria. The antifungal studies were carried out with *C.albicans* and *A.niger*results into inferior antifungal action with respect to the standard clotrimazole 20 mg/well. Further, cytotoxic studies have also been carried out. The results reveal moderate activity with respect to Ascorbic acid.

Conclusions: The percentage of swelling was significantly higher at alkaline pH (9.0) compared to acidic pH.The swelling equilibrium of the polymeric hydrogels has shown increased water absorbency with increasing citric acid concentration. The CA_4 hydrogels showed mild inhibition in gram negative bacteria *Escherichia coli*. Antioxidant studies have shown that lower concentrations of both furoic acid and citric acid exhibit better activity compared to higher concentrations. The present Cytotoxic studies exhibits the IC_{50} in 80 µL in Ethanol for TEA_1 and CA_1 hydrogels, then remaining hydrogels shows IC_{50} in 100 µL in Ethanol. Lower the concentration of FA, TEA and CA, higher the toxicity and higher the concentration of FA, TEA and CA lower the toxicity is studied.

Graphical Abstract



1. Introduction

Hydrogels are the crosslinked networks of hydrophilic water-soluble polymers. They tend to absorb enormous amounts of water and swell[1]. They are gifted for absorbing large quantities of water, saline, or physiological fluid without being dissolved[2-3]. Hydrogels behave similarly to natural tissue due to their soft and fragile nature. Excellent hydrophilicity and biocompatibility of hydrogels contributed to the

Journal of Chemical Health Risks
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fast development of materials for various biomedical applications[4]. The acidic or basic pendant functional group present on the polymer backbone viz, -COOH, -SO_3H, -OH, -$CONH_2$, etc., is responsible for volume or phase changes such as in the pH, electric field, or ionic strength of the swelling agent, temperature[5]. The preparation of polymeric hydrogel has found a wide range of biomedical applications, including controlled drug delivery systems, blood replacement, wound dressing, coatings for biosensors, contact lenses, dye removal, etc.,[6]

The biocompatible nature and versatile chemistry of citric acid (CA) have drawn the attention of researchers across different fields. CA can actively engage in hydrogen bonding interactions with other polymer networks, improving their properties due to its three carboxylic (COOH) groups and single hydroxyl (–OH) group [7-9]. It is commonly used as a poly-functional modifier to convert OH polymers into reactive functional polymers, referred to as citrate-based biomaterials, through cross-linking reactions [10,11]. Citric acid has gained significant attention in various biomedical and eco-friendly applications. It is a cost-effective, multifunctional monomer primarily used in ester crosslinking formation. Additionally, it offers hydrogen bonding and other binding sites for bioconjugation[12]. CA is readily available, non-toxic, renewable, and inexpensive. It is a valuable monomer for its modifying ability and non-toxic nature. Our research group has recently synthesized biocompatible hydrogels of citric acid capable of demonstrating pH-dependent swelling behavior [4,13-18]. To the best of our knowledge, there is no literature available on the tricomponent synergic combination of monomers resulted into biopolymeric hydrogels. Hence, the present investigation aims to synthesis the tricomponentpolyesterification reaction using citric acid, triethanolamine and 2-furoic acid and to find out the biomedical application like antimicrobial, cytotoxic and antioxidant studies of these hydrogels.

2. Objectives

To synthesis CTF based biopolymeric hydrogels with 2:1:1 mole ratio.

To notice the formation of glassy brown geliation as the completion of hydrogel.

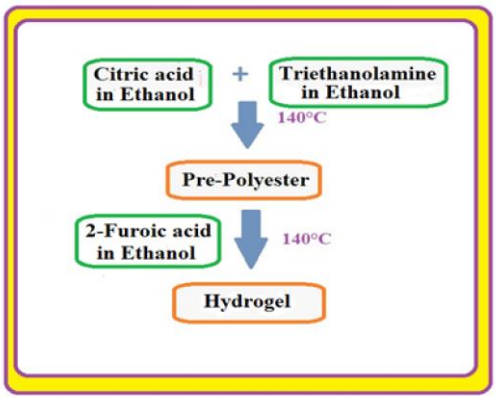
To characterize CTF hydrogel using spectral studies like FT-IR, ^{1}H NMR and ^{13}C NMR.

To charaterize hydrogel using swelling studies and swelling equiliburm at varying pH from 2.0 to 11.0.

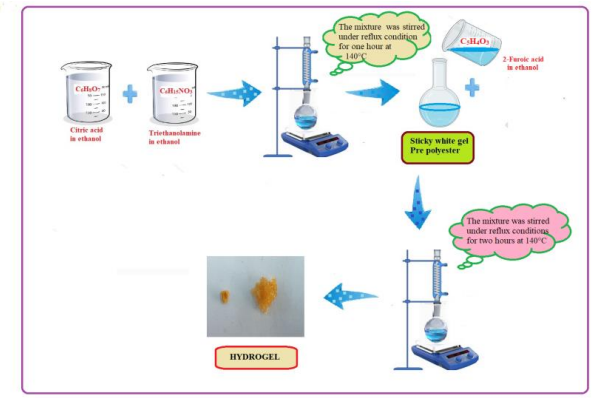
To find out the antibacterial efficacy using gram postive and gram negetive pathogens.

To evaluvate the antioxidant and cytotoxic activities using DPPH radical scavening method and MTT assay respectively.

3. Methods



Scheme 1 : Synthesis of biopolymeric hydrogel



Scheme 2 : Synthesis of CTF glassy brown biopolymeric hydrogel

Scheme.1 & 2 illustrate the monomer, 0.020 mol citric acid (3.842 g) was dissolved in ethanol and placed in a round-bottomed flask equipped with a mechanical stirrer. Triethanolamine [0.020 mol (2.640 g)] dissolved in ethanol was added drop wise using a dropping funnel. The mixture was stirred for one hour at 140 °C. The

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completion of the pre-polyester reaction was indicated by the formation of a sticky white gel-like compound Citric acid-Triethanolamine (CT). Subsequently, 2-furoic acid (0.020 mol, i.e., 2.241 g) dissolved in ethanol was added to the pre-polyester CT at 140 °C and stirred constantly for 2 hours. The formation of a glassy brown gel (CTF) indicated the completion of the reaction. The resulting gel was soaked in purified ethanol for 24 hours to remove unreacted monomers and then dried in a vacuum oven at lukewarm conditions for 24 hours. Similar methodology have been adopted by varying the composition of monomers.The Polymeric reaction yields 13 g of Hydrogel compound**.** The experimental parameters for the series of synthesized polymeric hydrogels are listed in Table 1.

Table 1. Physical Parameter series of Polymeric Hydrogels based on CA, TEA and FA

S.No	Sample	Composition (mole)			Description
		CA	TEA	FA	CTF
1	CTF	0.020	0.020	0.020	Brown glassy gel , insoluble in water
2	FA_1	0.020	0.020	**0.01**	Brown glassy gel , insoluble in water
3	FA_2	0.020	0.020	**0.02**	Brown glassy gel , insoluble in water
4	FA_3	0.020	0.020	**0.03**	Brown glassy gel , insoluble in water
5	FA_4	0.020	0.020	**0.04**	Brown glassy gel , insoluble in water
6	TEA_1	0.020	**0.01**	0.020	Brown glassy gel , insoluble in water
7	TEA_2	0.020	**0.02**	0.020	Brown glassy gel , insoluble in water
8	TEA_3	0.020	**0.03**	0.020	Brown glassy gel , insoluble in water
9	TEA_4	0.020	**0.04**	0.020	Brown glassy gel , insoluble in water
10	CA_1	**0.01**	0.020	0.020	Brown glassy gel , insoluble in water
11	CA_2	**0.02**	0.020	0.020	Brown glassy gel , insoluble in water
12	CA_3	**0.03**	0.020	0.020	Brown glassy gel , insoluble in water
13	CA_4	**0.04**	0.020	0.020	Brown glassy gel , insoluble in water

4. Results and Discussion

FT-IR studies characteristics of (CTF) hydrogel

FT-IR Spectroscopy analyses of CTF hydrogel have been shown in **Fig 1**. The CA_4 hydrogel exhibited various absorption peaks in its infrared spectrum. A peak was observed at 3413 cm^{-1}, indicating the presence of a hydrogen-bonded OH group[5]. A peak at 2987 cm^{-1}corresponded to aromatic C-H stretching, while the aliphatic -C-H stretching vibration was noticed at 2904 cm^{-1}. Additionally, an absorption peak at 1675 $cm^{-1}$was ascribed to the ester group band C=O stretching. New absorption peaks were observed at 1440 cm^{-1}related to $-COO^-$ stretching frequency, and for the ester at 1318 cm^{-1} indicating C-O stretching of the ester group[4]. The peak at 1015 cm^{-1} indicates C-N stretching of the triethanolamine. These absorption peaks are consistent with those observed in all series of hydrogels, indicating similar chemical properties.

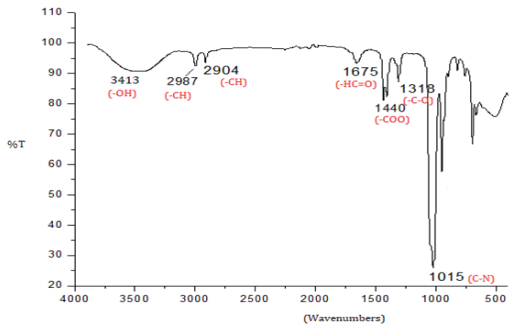

Fig 1 : FT-IR Spectra of CA_4 hydrogel

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Swelling Equilibrium Studies of CA_4 Hydrogel

The polymeric hydrogels were taken in 13 different series and swelling studies were carried out to all the different series in different pH ratios. The swelling equilibrium studies have been performed to CA_4 series of hydrogels in various buffers with pH value ranging from 2.0, 4.0, 7.0, 9.0 and 11.0 at room temperature. The dry CA_4 hydrogel of 0.200 g was weighed which results in exhibiting rapid absorption of the solution after immersion in different pH value, results in increase in the volume of the sample have also been observed. After immersion of 24 hrs in different pH 2.0, 4.0, 7.0, 9.0 and 11.0 were found to be increase in its swelling equilibrium percentage of 100, 150, 170, 260 and 200%. The S% was significantly higher at alkaline pH 9.0 compared to acidic pH. Gradual decrease in the alkaline medium of pH 11 is observed due to the increasing anionic charge, the ionic strength of the medium and the degree of cross linking are increased and the swelling capacity consequently decreases in pH 11. The citric acid (CA) plays the major role in the absorption of water, the low CA shows lesser absorption where as high CA shows greater absorption. The water absorbency of polymeric hydrogels with a pH >4 was clearly much higher. The pH medium increased from 2.0 to 11.0, which led to a greater number of carboxylate ions forming because many hydrogen bonds were broken and the anionic groups electrostatic repulsion increased profoundly in the hydrogels polymer backbones, which in turn caused an increase in swelling[19,20].Table. 2 shows the Swelling Equilibrium (%) of 2-Furoic acid-based on Citric acid containing polymeric hydrogels.

Table 2 :Seq% Values of 2-Furoic acid Based Citric Acid Containing Polymeric Hydrogels

S.No	CA + TEA + FA (in grams)	Sample	Swelling Equilibrum (%) pH 2	4	7	9.0	11
1	0.200 + 0.200 +0.200	CTF	20	25	100	20	20
2	0.200 + 0.200 +**0.100**	FA_1	20	25	35	40	30
3	0.200 + 0.200 +**0.200**	FA_2	25	30	40	50	40
4	0.200 + 0.200 +**0.300**	FA_3	30	35	44	65	50
5	0.200 + 0.200 +**0.400**	FA_4	50	55	63	87	75
6	0.200 + **0.100** +0.200	TEA_1	25	25	30	46	29
7	0.200 + **0.200**+0.200	TEA_2	25	30	43	59	39
8	0.200 + **0.300**+0.200	TEA_3	30	39	45	70	62
9	0.200 + **0.400** +0.200	TEA_4	52	59	69	85	70
10	**0.100** + 0.200 +0.200	CA_1	2	3	3	7	10
11	**0.200**+ 0.200 +0.200	CA_2	90	115	135	140	130
12	**0.300** + 0.200 +0.200	CA_3	90	140	155	175	140
13	**0.400** + 0.200 +0.200	CA_4	100	150	170	260	200

The hydrogels CTF, FA_2, TEA_2, and CA_2 have equimolar compositions with the same molar ratios but different names, indicating variations in monomer addition during hydrogel Synthesis. Seq% Values of CTF polymeric hydrogels comparative graphical representation with 2-Furoic acid, Triethanolamine and Citric acid series was shown in the **Fig 2.**

Journal of Chemical Health Risks
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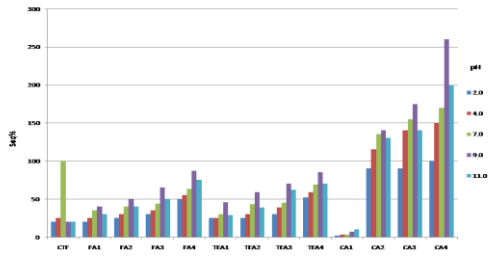

Fig 2: The Seq% Values of CTF polymeric hydrogels

^{1}H NMR :

In **Fig 3,** CA_4 polymeric hydrogel shows multiple peaks at 2.640 – 2.901 ppm and 4.002 – 4.094 ppm were related to methylene protons present in citric acid (2H, m) and the –OH group of citric acid. The peak at 2.55(2H, t) and 3.304 (2H,t) combined to get multiplet could be due to the proton signal of -OCH_2CH_2- from triethanolamine. The signal 3.6 ppm indicates N attached methyl group in TEA.The aromatic protons of 2-furoic acid establish signal at 7 to 8 ppm multiplet clearly pointed out that the incorporation of 2-furoic acid unit in the polyesterification process. Further, it is evident that the disappearance of the signal at 11 ppm indicates that the protons of the all three carboxylic units of citric acid's proton as well as carboxylic proton of furoic acid were consumed during the poly esterification reaction with trientholamine. Similar to the findings reported by Franklin,D.S.,Guhanathanet. al., we also observed the disappearance of carboxylic proton for our tricomponent hydrogels [5].

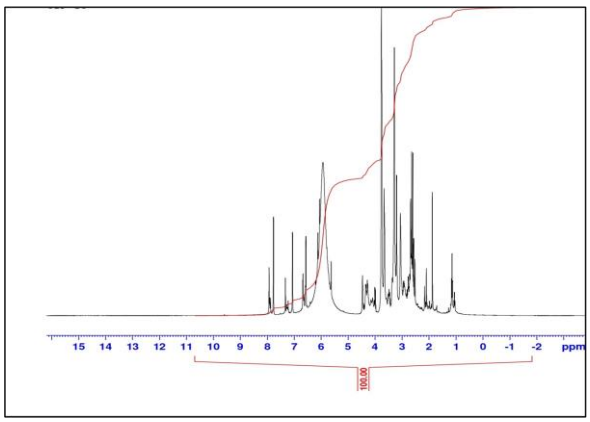

Fig 3 : ^{1}H NMR of CA_4 biopolymeric hydrogel

^{13}C NMR :

The C^{13} NMR signal from the CA_4 hydrogel shows peaks at 55 and 61 ppm, indicating the presence of methylene carbon of triethanolamine and methylene carbon units of citric acid were noticed at 44.6 ppm . The peak at 63.1 ppm was attributed to a carbon of triethanolamine (-O-CH_2- CH_2-) group involved in the polyester network. The signals at 110 to 120 ppm were related to the aromatic carbon of furoic acid and additionally, the C-O-C ring present in the furoic acid in the polyesterification have cited signal at 146 ppm. The signals at 78 ppm have been related to the tetrahedral carbon (sp^3) of citric acid. The peak at 158.35 ppm suggests the formation of an ester carbonyl of furoic acid and the three consecutive signals at 172.7 ppm 174.5 ppm and 177.5ppm corresponds to the ester carbonyls of citric acid units responsible for the formation of polyester network as shown in **Fig 4**. Similar finding of the polyester formation have also been notified by the researcher, Chitra, et.al(2017).,For their citric acid based hydrogel network [21].

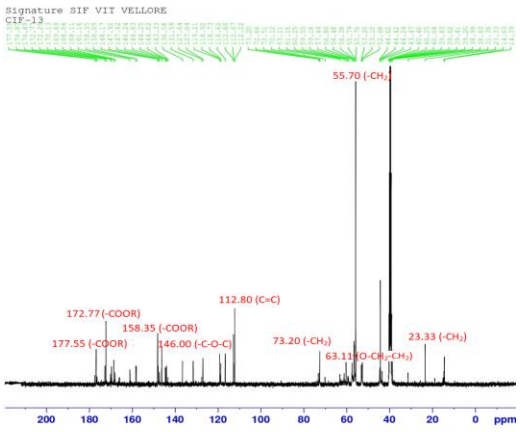

Fig 4 : C^{13} NMR of CA_4 hydrogel

Antioxidant activity :

Table.3 summarizes the antioxidant activity of tri-component polymeric hydrogels. The antioxidant activities have been carried out for the present investigation of selected compound on the basis of better swelling established by the composition of FA_1, FA_4, TEA_1, TEA_4, CA_1, & CA_4. Among these compositions, FA_1 refers to lower concentration of 2-furoic acid and other two ingredients are equal molar concentration

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15(2), 211-222 | ISSN:2251-6727

whereas FA_4 refers to higher concentration of 2-furoic acid and other two are same molar concentrations. Similar trend have been planned for TEA_1 and TEA_4 as well as CA_1 and CA_4 respectively. In each cases, the concentration have been varied from 25 to 500 µg/ml. The DPPH radical scavenging activity have been adopted for antioxidant activity with respect to Ascorbic acid as standard and its concentration have been ranged from 5 to 50 µg/ml**(Table.4)**. The results of the investigation found to be 48.7 to 95.12 % radical scavenging activity have been observed for the standard ascorbic acid. With respect to the ascorbic acid, the tri-component hydrogels of present investigation found to be inferior in radical scavenging activity even at its higher concentration of 500 µg/ml. However, among the selected composition, the lower concentration of both furoic acid and citric acid having better activity than their concern higher concentration whereas radical scavenging activity reciprocal with respect to the lower and higher concentration of TEA. This may be due to the presence of increased concentration of acidic nature responsible for the decreased inhibition towards antioxidant activity. But in the case of TEA the alkaline nature increases thereby decreasing the acidic nature of the polyester network. Henceforth, the antioxidant activity increased in basic nature [22].

Table 3: InhibitionActivity percentage of polymeric hydrogels

Tested	% of Inhibition					
Concentration (µg/mL)	**FA_1**	**FA_4**	**TEA_1**	**TEA_4**	**CA_1**	**CA_4**
500	25.8	16.46	5.72	33.58	73.64	16.38
250	19.71	15.54	4.56	28.94	42.17	12.56
100	15.7	14.23	2.94	22.89	24.17	10.68
50	12.04	11.99	1.62	17.81	14.37	8.57
25	9.38	11.29	0.71	11.86	10.77	6.43
Control	0	0	0	0	0	0

Table 4:InhibitionActivity percentage of Ascorbic acid

Tested concentration(µg/ml)	**% of Inhibition**
5	**48.70**
10	**88.52**
20	**90.62**
40	**93.95**
50	**95.12**
Control	**0.00**

Antimicrobial activity :

Fig.5 shows the antimicrobial activity of the present investigation. The gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis), and Gram negative bacteria (Escherichia coli)* have been selected for the study. In general, both low and high level concentration of the citric acid based hydrogels have been subjected to the bacterial pathogens using Agar well diffusion methodusing DMSO as positive control and Ciprofloxacin as standard antibacterial. The results of the investigation shows the high level concentration of CA found to have mild antibacterial activity against the pathogen of the present investigation than that of the lower concentration of CA. CA_4 hydrogelsexhibited the higher zone of inhibition against *Escherichia coli* (12 mm), *Staphylococcus aureus* (15 mm), *Bacillus subtilis*(0 mm) at 300 mg/well with respect to the Ciprofloxacin having *Escherichia coli* (30 mm), *Staphylococcus aureus* (20 mm), *Bacillus subtilis*(44 mm). Higher concentration of citric acid results in lesser activity which is found to be better in comparison with other component of the present investigation. The results summarizes as 40%, 75% and 0 % antibacterial action against *Escherichia coli, Staphylococcus aureus*and *Bacillus subtilis*in comparison with Standard Ciprofloxacin. The comparative study of lower concentration to higher concentration series of hydrogels is represented in the **Table 5**.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15(2), 211-222 | ISSN:2251-6727

Table 5 : Zone of inhibition (mm) in different series of hydrogels with pathogens

SL. NO	BACTERIA	POSTIVE CONTROL (Ciprofloxacin in mm)	ZONE OF INHIBITON (mm)					
			FA_1	FA_4	TEA_1	TEA_4	CA_1	CA_4
1	*Escherichia coli*	30	7	0	6	8	7	12
2	*Staphylococcus aureus*	20	0	0	0	8	12	15
3	*Bacillus subtilis*	44	11	11	13	0	8	0

Table 6 : Zone of inhibition (%) in different series of hydrogels with pathogens

SL. NO	BACTERIA	POSTIVE CONTROL (Ciprofloxacin in mm)	ZONE OF INHIBITON (%)					
			FA_1	FA_4	TEA_1	TEA_4	CA_1	CA_4
1	*Escherichia coli*	30	23	0	20	27	23	40
2	*Staphylococcus aureus*	20	0	0	0	40	60	75
3	*Bacillus subtilis*	44	25	25	30	0	18	0

high level of CA based hydrogel exhibited good antibacterial activity against pathogens compared with lower ratio of CA, where CA_4 has shown higher antibacterial activity. Similarly, our results also observedthe zone of inhibition in CA_4 hydrogel which were represented in **Table 5, 6 & Fig.5.** This results confirmed the moderate inhibition on grampositive bacteria (*Staphylococcus aureus and Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*) shown in Fig 5.

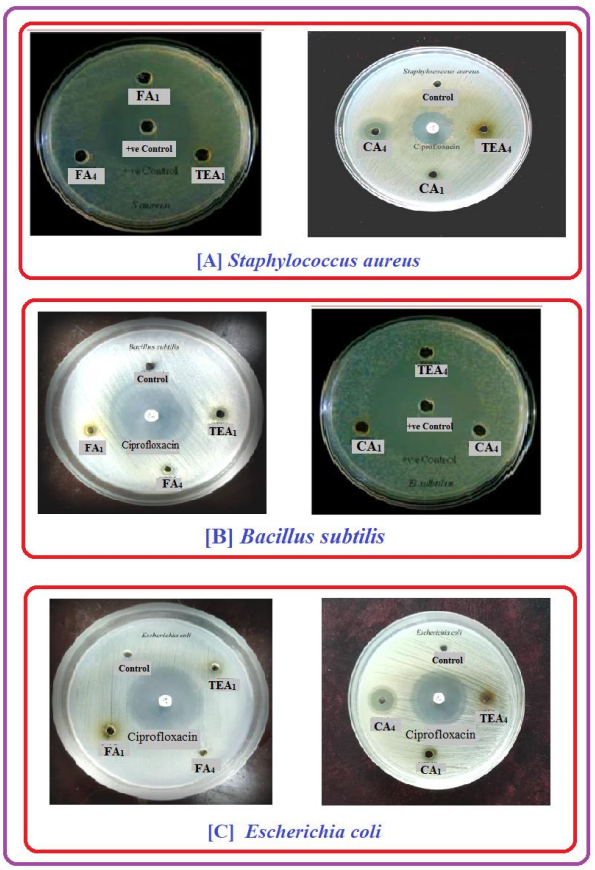

Fig 5: Antibacterial studies of CA_4 hydrogel

In **Fig.6** shows the antifungal activity of the present studies. The *Candida albicans* and *Aspergillusniger*were selected for the investigation.Both low and high-level concentrations of citric acid-based hydrogels were used in antifungal studies, with DMSO as the positive control and Clotrimazole as the standard. The results showed no antifungal activity against the pathogens. The zone of inhibition against Candida albicans and Aspergillusniger was 0 mm at 300 mg/well, with respect to the Clotrimazole having *Candida albicans* (26 mm) and *Aspergillusniger*(26mm). The results summarizes no activity were found to be better in comparison with Standard Clotrimazole.

Journal of Chemical Health Risks
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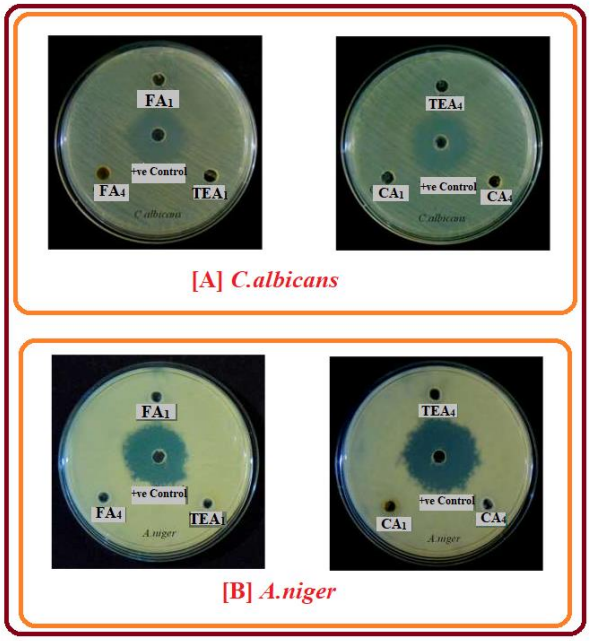

Fig 6: Antifungal studies of CA_4 hydrogel

Cytotoxic Studies :

Peripheral blood mononuclear cells (PBMC) were collected from fresh heparinized blood and separated by density gradient centrifugation (Ficoll) as described by Renno et al., (2008). Cells were cultured at 37 °C in a 5 % CO_2 humidified environment. Cell Proliferation Assay were carried out to investigate the cytotoxic potential of the isolated compounds, the MTT-**(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide)** colorimetric assay was performed . Briefly, 5 × 10^4 cells suspended in 100 µL of growth medium were seeded in 96-well plates (Hi media) containing 100 µL of medium in the presence of serial twofold dilutions of each tested compound previously dissolved in ethanol (1 % v/v, no adverse effects on cell growth were observed at this concentration). The hydrogels were evaluated at a final maximum concentration of 100 µM. After 72 hours, 20 µL of 5 mg/mL solution of MTT in sterile PBS was added to each well and further incubated for 4 hrs. Then, the supernatants were removed and replaced with 100 µL DMSO to solubilize the resulting purple formazan crystals produced from metabolically viable cells. Absorbance was measured with an ELISA micro-plate reader (Bio-Rad, USA) at 595 nm. Two wells were used for each concentration of the products assayed and three independent experiments were performed. Untreated and ethanol (1 %) treated cells were used as controls while Doxorubicin (DOX) (maximum tested concentration 69 µM) was used as reference.

The percentage of cytotoxic activity of the assayed compounds was determined by the following formula:

Cytotoxicity (%) = [1 −(optical density of treated cells − optical density DMSO)/(optical density of ethanol control cells − optical density DMSO)] × 100.

Medium inhibitory concentrations (IC_{50}) represent the concentrations of the tested hydrogels required to inhibit 50 % cell proliferation and were calculated from the mean values of data from wells.The present studies exhibits the IC_{50} in 80 µL in Ethanol for TEA_1 and CA_1 hydrogels , then remaining hydrogels shows IC_{50} in 100 µL in Ethanol as in **Table 7 & Fig 7**.

To confirm the effect of hydrogels on the proliferation of cell suspensions (5× 10^4 cells/well) were treated in culture medium with different concentrations of 1 (final volume of 200 µL) and incubated in triplicate for 24, 48, and 72 hrs. Two independent experiments were performed. The number of viable cells at the different time points was counted by the trypan blue dye exclusion method using a hemocytometer. The Cytotoxicity of medical devices is assessed by measuring cell viability versus toxicity. According to the research report on cytotoxicity, Cell viability is 100 % to 80 % after treatment is considered as non-toxic. Viability of 79 % to 60 % indicates mild toxicity, while 59 % to 40 % indicates moderate toxicity. Any viability below 39 % is considered strongly toxic[23,24]. Hydrogels at different concentrations ranging from 1 µg/mL to 60 µg/mL, no toxicity was observed. However, at 80 µg/mL and 100 µg/mL, weak toxicity was observed as shown in **Table 7**.

Table 7: **Cytotoxic Studies of polymeric hydrogels**

Name of the compound	Percentage of viability in different concentration							
	1 µL in Ethanol	5 µL in Ethanol	10 µL in Ethanol	20 µL in Ethanol	40 µL in Ethanol	60 µL in Ethanol	80 µL in Ethanol	100 µL in Et

Journal of Chemical Health Risks

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JCHR (2025) 15(2), 211-222 | ISSN:2251-6727

								ha nol
FA_1	100	100	100	100	100	100	80	42
FA_4	100	100	100	100	100	100	60	44
TEA_1	100	100	100	100	100	100	42	38
TEA_4	100	100	100	100	100	100	70	42
CA_1	100	100	100	100	100	100	52	39
CA_4	100	100	100	100	100	100	80	52

IC_{50} for TEA_1, CA_1 is 80µl in Ethanol

For others 100 µL in Ethanol

[100 % means no cytotoxicity, 80 % means 20 % cytotoxicity)

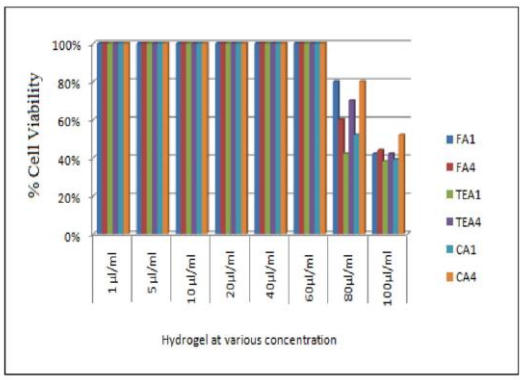

Fig 7 : Cytotoxic studies of CA_4 hydrogel

Based on the MTT assay among various percentage of viability in different concentration of ethanol with 1 µL, 5 µL, 10 µL, 20 µL, 40 µL, 60 µL found to be 100 % safe toward the human isolated peripheral blood mononuclear cells and such concentration are said to be non-toxic compounds. However, on increasing the concentration to 80 µL found to have 20-40 % toxic activity (mild toxic) of FA_1, FA_4, TEA_4 and CA_4 whereas TEA_1 and CA_1 shows 41-60 % (moderately toxic) activity towards peripheral blood mononuclear cells. Similarly, increasing the concentration to 100 µl found to have 48 % toxicity upon increase the concentration of citric acid. 58- 68 % of toxicity of FA_1, FA_4, TEA_4 and CA_4 whereas TEA_1 and CA_1shows below 40 % of cell viability is considered as strongly toxic to the cell (**Fig 7**). Viable cell counting under microscope using haemocytometer is shown in **Fig 8**. Based on the cell viability the lower concentrationsof citric acid (i.e., CA_1) found to be 61 % toxic than higher concentration of citric acid (i.e.,CA_4) found to be 48 %. Similarly, lower concentration of furoic acid (FA_1) has 58 % than higher concentration of furoic acid (FA_4) has 56 %. Similarly, triethanolamine (TEA_1) has 62 % and its higher concentration of TEA_4 has 58 % toxic. Hence lower the concentration of FA, TEA and CA higher the toxicity and higher the concentration of FA, TEA and CA found to be lower toxicity.

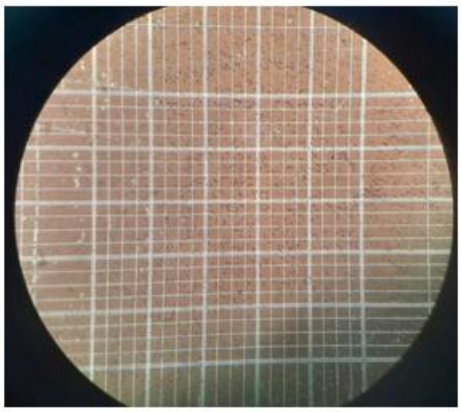

Fig 8: Viable cell counting of CA_4 hydrogel using haemocytometerConclusion

In this study, the synthesis of citric acid-based pH-responsive polymeric hydrogels was reported. The hydrogels was characterized using FT-IR spectroscopy,confirms stretching of the ester group and the structure of hydrogels.Additionally, ^{1}H-NMR and ^{13}C-NMR spectral analysis showed the group related to citric acid in the polyester network and the formation of an ester carbonyl of furoic acid.The percentage of swelling was significantly higher at alkaline pH (9.0) compared to acidic pH.The swelling equilibrium of the polymeric hydrogels has shown increased water absorbency with increasing citric acid concentration. The CA_4 hydrogels showed mild inhibition in gram negative bacteria *Escherichia coli*. Antioxidant studies have shown that lower concentrations of both furoic acid and citric acid exhibit better activity compared to higher

Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(2), 211-222 | ISSN:2251-6727

concentrations. The present Cytotoxic studies exhibits the IC_{50} in 80 µL in Ethanol for TEA_1 and CA_1 hydrogels, then remaining hydrogels shows IC_{50} in 100 µL in Ethanol. Lower the concentration of FA, TEA and CA, higher the toxicity and higher the concentration of FA, TEA and CA lower the toxicity is studied.

Conflict of Interest : On behalf of all authors the corresponding author states that there is no conflict of interest.

Data Availability Statement : Authors do not wish to share until it is published.

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