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in vitro Analysis of Citric Acid Crosslinked and *Juglans regia* Extract Loaded Hydrogel for Biomedical Applications

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Herbal medicines are a familiar source of therapeutic agents for the medicaments of infections in various countries these days. In this work, biological study of citric acid crosslinked hydrogel loaded with *Juglans regia* leaves extract against 5 standard bacterial and fungal strains. The FTIR and FESEM results exhibited that leaves extract stick on the surface of hydrogel and it does not reacted with the functional groups of used polymers. The obtained results of MTT assay against L929 mouse fibroblasts cell line showed that hydrogel was biocompatible. Highest minimum inhibitory concentration was found in case of *S. typhi*, *E. coli* and *C. albicans*. *P. aeruginosa* and *C. albicans* were the most susceptible strains, while *E. coli* and *S. typhi* were the most resistant strains.

Keywords: Chitosan, Poly(vinyl alcohol), *Juglans regia*, Hydrogel, Antimicrobial studies.

INTRODUCTION

Herbal medicines ability to control the inflammation and infection makes it a suitable candidate for wound or skin infection. They, broadly used in oriental medicine, are a popular source of bioactive molecules since the medieval time to cure injuries. Their use as curing agents may be a fascinating aspect these days. Herbal plant has found applications as antiulcer, anti-inflammatory, antiepileptic, antidiabetic, antibacterial, and antifungal drugs [1]. Plants, *Salix alba*, *Azardica indica*, *Moringa oleifera*, *Kigelia Africana*, *Juglans regia*, *etc*. with antioxidant, anti-proliferative, antibacterial and antifungal features have been used in the treatment of infections and are usually applied as an ointment or crude extract on the infected area [2]. A temperate region plant, *Juglans regia* L. of family *Juglandaceae*, is one of the medicinal plants like other herbal medicines that are associated with analgesic, antibacterial and antifungal, associated with analgesic, antibacterial and antifungal effects [3,4] due to the bioactive compounds [5]. Its' leaves are used to treat rheumatic pains, fever, diabetes, skin diseases, *etc*. [6-9].

Hydrogels are 3D, hydrophilic, crosslinked networks of polymer which imbibe large amount of water or biological fluids without de-morphing its shape [10,11]. This is owing to the -OH, -$CONH_2$ and -SO_3H, groups [12]. Their hydration depends upon pH, temperature, radiations, *etc*. [13]. They have a soft regularity close to living tissues. The biocompatibility and flexibility in methods of synthesis make them favourable for a broad range applications like drug delivery systems, pharmaceutics, agriculture, food, biomedicals, diapers and personal care products [14,15]. Biocompatible hydrogels can be used as a base material for herbal medicine to possess useful strategy for the enhancement of the drug availability. Owing to the greater biomedical pertinence, there is a high interest to synthesize antimicrobial hydrogels [16].

Generally, hydrogels are synthesized from natural as well as from synthetic polymers such as chitosan, hyaluronic acid, poly(ethylene glycol), poly(vinyl alcohol), *etc*. [17]. Chitosan is a biopolymer of chitin and it is cationic, and biodegradable polysaccharide consists of 2-amino-2-deoxy-H-D-glucan with glycosidic linkage. It is one of the most research materials in recent years. Chitosan hydrogels possess poor mechanical properties and an uncontrolled dissolution rate [18] that's why they need to mix with synthetic polymer to increase the strength. Poly(vinyl alcohol) widely used synthetic polymer in hydrogels, which is hydrophilic, chemical stabile, low toxic and biocompatible [19]. Hydrogels of it has a high swelling and good

compressive and elastic strengths [20]. These hydrogels are synthesized by physical or chemical crosslinking. Various crosslinker like glutaraldehyde, glyoxal, epichlorohydrin, genipin and citric acid used to develop hydrogels. Though, they are in numbers but toxic in some extent [21] and low price citric acid has received considerable attention. It forms ester linkage between the polymer chains by anhydride formation [22,23]. Free -COOH and -OH groups help to balance the hydrophilicity, give hydrogen bonding and more binding sites to control the delivery of weakly basic drugs [24]. In several occasions, the mixing of two or more polymers with crosslinker leads to the formation of hydrogels with desired properties.

A substantial amount of work is reported in the literature concerning the antimicrobial applications of extract loaded hydrogels. Haq *et al*. [25] synthesized the hydrogel of poly-(vinyl pyrrolidone) and hydroxypropyl methylcellulose loaded with thymoquinone investigated for *S. aureus* infection on skin wound. In other work, *Moringa oleifera* leaf extract used to dressings for wound infections [26]. Eakwaropas *et al*. [27] studied the effect of *Ipomoea pescaprae* extract loaded hydrogel on infected wounds. The purpose of this study is to develop and characterize a biocompatible hydrogel that has the prospects to release antimicrobial agent directly at the targeted site that may be helpful for the care and management of different microbial infections. To achieve this objective, methanolic extract loaded in to the chitosan-*co*-poly(vinyl alcohol) (CS-*co*-PVA) hydrogel crosslinked with citric acid by using solvent casting method. Characterization done by phytochemical analysis, GC-MS, FTIR, FESEM, MIC and antimicrobial assays performed against different clinical strains like *S. typhi*, *S. aureus*, *E. coli*, *P. aeruginosa* and *C.* albicans. To our best of knowledge, this is the first time that *Juglans regia* extract loaded hydrogel synthesized for *in vitro* study. This work seems to demonstrate that developed hydrogel is effective in the control of microbial infections on wounds or injuries.

EXPERIMENTAL

Chitosan middle-viscous (high m.w. >75% deacetylated) was purchased from Sigma-Aldrich, India. Acetic acid (glacial, 99-100%), poly(vinyl alcohol) (m.w. 125,000 and degree of acetylation, 19.5-22.7%), citric acid and glycerol were purchased from Merck (Mumbai, India). Mineral salt broth and nutrient agar purchased from Himedia. Bacterial strains *viz. E. coli* SN-1224, *S. typhi* SN-0464, *S. aureus* SN-1175, *P. aeruginosa* SN-1184 were arranged from Holy Family Hospital, New Delhi, India, while the fungal strain (*C. albicans* SN-2320) was arranged from Department of Microbiology, Vardhaman Mahavir Medical College, New Delhi, India. Leaves of *Juglans regia* were collected from Jammu & Kashmir, India.

Preparation of CS-*co*-PVA hydrogel: The 2% solutions of chitosan (CS), poly(vinyl alcohol) and citric acid were prepared separately in doubled distilled water. Different quantities of PVA and chitosan blended into the chitosan/poly(vinyl alcohol) molar ratios of (0:5), (1:4), (1:3), (1:1), (3:1) (4:1) and (5:0). The blend was kept under stirring for one hour at 25 ºC until the PVA and CS formed a clear solution. Citric acid, as a cross linker, slowly added by pippet under continuous stirring. The last amount of citric acid in gel was 10% v/v of polymeric solution. Further in this process, the solution was poured into pettry dishes and let them be there to dry for 72-120 h at 25 ºC. After peeling it off from pettry dish, transferred it into the desiccators followed into refrigerator.

Swelling study: For swelling study, all the dried chitosan/poly(vinyl alcohol) (CP) hydrogels of molar ratios 1:4, 1:3, 1:1, 3:1 and 4:1 were weighed (W_d) before being dipped in PBS of different pH 4.4, 7.4 and 9.4 at 37 ºC. Hydrogels were prudently taken off from the PBS after immersion for different time. After wiping off extra water from the surface, determine the wet weight (W_s) as a function of the immersion time. The swelling kinetics of hydrogels was calculated by using eqn. 1:

$$\text{Swelling (\%)} = \frac{W_s - W_d}{W_d} \times 100$$

where W_s and W_d are the weight of the wet film and dry film, respectively.

Preparation of methanolic *Juglans regia* leaves extract: Bioactive compounds from lyophilized leaves of *Juglans regia* (1 g) were extracted by rotating with 25 mL of methanol (25 ºC at 150 rpm) for 1 h and eventually filtered through Whatman filter paper No. 4. The residue was then extracted with 25 mL of methanol. The remaining solvent from extracts was evaporated at 40 ºC by rotary evaporator [28].

Loading of extract into the optimized hydrogel: From the above experiment the optimized CP3 ratio (1:3) at 10% v/v citric acid solution, which we have discussed in our previous paper [29], loaded with 300 mg of extract in to 30 mL polymeric solution. Extract loading was optimized by MIC of methanolic *J. regia* leaves extract as 10 mg/mL. The extract loaded hydrogel is denoted by CPW.

Phytochemical analysis: The methanolic *J. regia* leaves extract was put through to phytochemical analysis of major constituents like flavonoids, glycosides, saponins, tannins, steroids and terpenoids.

Characterization: For the identification of metabolites in the extract showing antimicrobial activity, the samples were subjected to GC-MS analysis. The FTIR spectrums of the samples were obtained by using a BIORAD-FTS-7PC type FTIR spectrophotometer. The solubility of CS-*co*-PVA with *J. regia* extract was characterized by using FESEM. The specimens observed by using an accelerating voltage of 15 kV to observe the structure of CS-co-PVA hydrogel as control and with extract. The cell viability analysis was conducted according to GB/T 16886.5-2003 (ISO 10993-5: 1999) [30]. MIC and antimicrobial studies were performed on obtained strains. The procedure of antimicrobial studies already discussed in literature [31].

RESULTS AND DISCUSSION

Swelling study: From Table-1, fixed amount of 2% citric acid to different ratios of 2% chitosan and 2% PVA mixture were added. By measuring the swelling of each ratio, it is found that 1:3 was the optimized ratio at which hydrogel did not break apart. From Table-2, the optimized hydrogel (CP3) treated by high (15%v/v) and low (5%v/v) amount of crosslinker citric acid at 37 ºC and at pH 7.0. At 15% v/v of polymer mixture,

TABLE-1
TREATMENT OF DIFFERENT CP RATIOS WITH FIXED AMOUNT OF CROSS LINKER

Ratio of blends (CS/PVA)	% of Citric acid (v/v)	Swelling at pH 7.4 (in %)
0:5 (CP1)	10%	600
1:4 (CP2)	10%	400
1:3 (CP3)	10%	320
1:1 (CP4)	10%	175
3:1 (CP5)	10%	110
4:1 (CP6)	10%	210
5:0 (CP7)	10%	500

TABLE-2
TREATMENT OF CP3 WITH DIFFERENT AMOUNT OF CITRIC ACID

Swelling at 15% of citric acid	Swelling at 5% of citric acid
200%	550%

the swelling % of CP3 decreased and at 5% v/v swelling % of CP3 increased after 24 h [32].

For brief discussion on optimization of hydrogel, the concentration of PVA in CP3 hydrogel affects the swelling. On increasing amount of PVA hydrophilicity and swelling % of CP3 increased because swelling is directly related to the hydrophilicity of the polymer. But when chitosan content increased, swelling % was decreased sinice chitosan is a less hydrophilic [33]. So, both CP1 and CP7 hydrogel becomes gel or fragile and thus did not picked for further progression. Optimum swelling of CP3 observed at pH 7.4 but swelling of the same sample decreased at pH 9.4. Maximum swelling percent was 330 g/g at pH 7.4. The sample CP3 showed that hydrogel film becomes gel at pH 4.4. The NH_2 group of chitosan protonated to ammonium group (NH_3^+) at pH 4.4 resulted in to strong electrostatic repulsion in the chains of polymer. Due to these electrostatic repulsions polymer networks becomes gel [34]. At pH 7.4 and 9.4, deprotonation of NH_2 of chitosan resulted in decreased electrostatic repulsion in the polymer chains. Now, the cross linked swollen network CP3 was stabilized through hydrogen bonding in -NH_2 and -OH groups of chitosan, -OH group of PVA and -COOH group of citric acid.

Phytochemistry and GC-MS analysis: Table-3 exhibited the qualitative phytochemical screening of methanolic *J. regia* leaves extracts. It was found that most of the biologically active and therapeutically potent phytochemical compounds were present in the extract. It revealed that tannins, flavonoids, sterols and saponins were found in the extract. Glycosides were not present in extracts used for phytochemical analysis [35,36]. The GC-MS study revealed the presence of compounds from the methanolic leaf extract of *J. regia* [5]. The major constituents along with other minor constituents were present which are shown in Table-4.

FTIR and FESEM analysis: From Fig. 1a-b, there is no change in the FTIR spectrum of chitosan/PVA/methanolic *J. regia* leaves extract (CPW) hydrogel in comparison to control (CP3). It means that drug did not reacted with the hydrogel. Methanolic extract only embedded on to the surface of hydrogel not reacted to the polymers' group. The FTIR results were also confirmed by FESEM images. The surface area of CP3 and CPW hydrogel are shown by FESEM images. Fig. 2a exhibited no obvious phase separation of control hydrogel, which showed the smooth surface area. The FESEM images of Fig. 2b-c are of *J. regia* extract loaded hydrogel. This extract embedded into the hydrogel and distributed on to the whole surface. It embedded on to the surface, thus it does not show long range of antibacterial activity.

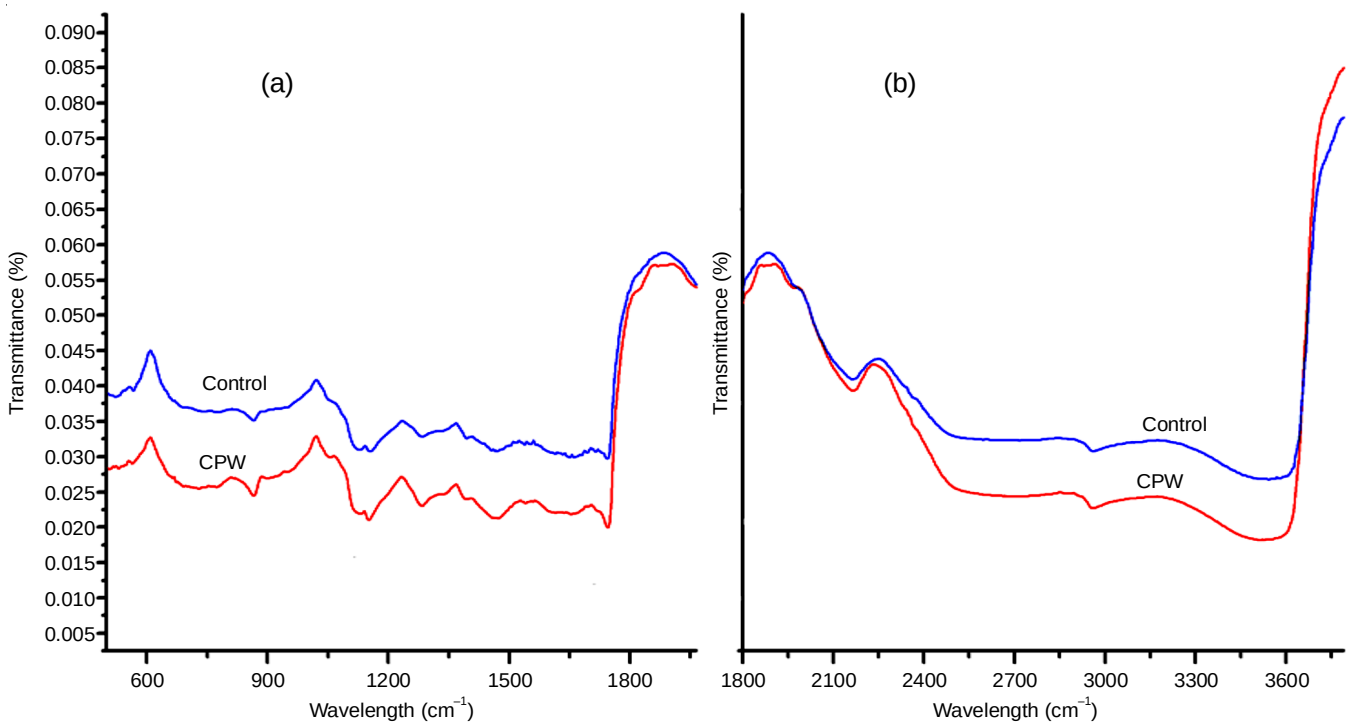

Fig. 1. FTIR spectra of CS-co-PVA hydrogel with and without aqueous *J. regia* leaves extract; (a & b) the peaks of control (CP3) and extract loaded hydrogel (CPW) showed no observable changes in peak positions except their intensity

TABLE-3
PHYTOCHEMISTRY OF METHANOLIC *J. regia* LEAVES EXTRACT

Phytochemicals	Name of the test	Colour observed	Colour intensity
Flavonoids	NaOH/ H_2SO_4	Color less	+
Glycosides	H_2SO_4	Dark brown	-
	Keller Kiliani	Brown ring	-
Sterols	Salkowski	Red color	+
Phenolics	NaOH	Deep yellow	+
Tannins	Braemer	Dark blue	+
	Iodine	Faint bluish	+
Alkaloids	2% H_2SO_4 and Dragencloffs reagent	Red Precipitate	+
Saponins	Frothing	Colorless	+

TABLE-4
LIST OF MAJOR COMPOUNDS EXTRACTED FROM METHANOLIC *J. regia* LEAVES EXTRACT

Name of compound	m.f.	m.w.	Retention time	Presence (%)
Vitamin E	$C_{29}H_{50}O_2$	430	29.61	15.53
Cholesterol base H	$C_{27}H_{46}O$	386	33.70	13.08
4-Cholesten-3-one	$C_{27}H_{44}O$	384	37.54	8.86
Heptacosanol	$C_{27}H_{56}O$	396	25.93	5.25
δ-Tocopherol	$C_{27}H_{46}O_2$	402	26.68	4.64
Methyl heptacosanoate	$C_{28}H_{56}O_2$	424	14.89	2.85
4-Camphenylbutan-2-one	$C_{14}H_{22}O$	206	12.19	2.33

MTT assay: According to GB/T 16886.5-2003 (ISO 10993-5: 1999), samples with cell viability larger than 75% can be considered as non-cytotoxic. The L929 mouse fibroblasts were incubated for 48 h. Data from MTT assays and the microscopic examinations (Fig. 3) exhibited that no difference was examined in the L929 mouse fibroblasts activity during the study with the negative control. The cells grew very well after 48 h of exposing time to the hydrogel extracts. Therefore, the hydrogels can be considered as biocompatible [30]. Additional compositions of CS-*co*-PVA were critic acid and plant extract. It is well-know that critic acid is a byproduct of Kreb cycle in human body [21] and plant extract is also non-toxic. So, the whole formulation is biocompatible.

Antimicrobial activity: The use of this extract in to CS-*co*-PVA hydrogel, as an active dressing for wounds, examined by observing antimicrobial activity (disc diffusion method) against bacteria and fungi isolates. For MIC, the activity range is from 125 to 4000 µg/mL of 10 mg/mL methanolic *J. regia* extract solution (Table-5). The highest MICs were found for *S. typhi*, *E. coli* and *C. albicans*.

TABLE-5
MIC AGAINST DIFFERENT CLINICAL STRAINS

Microorganism	Plant extract (µg/mL)
S. typhi	≥ 1000
S. aureus	≥ 500
E. coli	≥ 1000
P. aeruginosa	≥ 500
C. albicans	≥ 1000

The disc diffusion method used to know the zone of inhibition of different concentrated methanolic *J. regia* leaves extract discs and extract loaded hydrogel discs. The CS-*co*-PVA

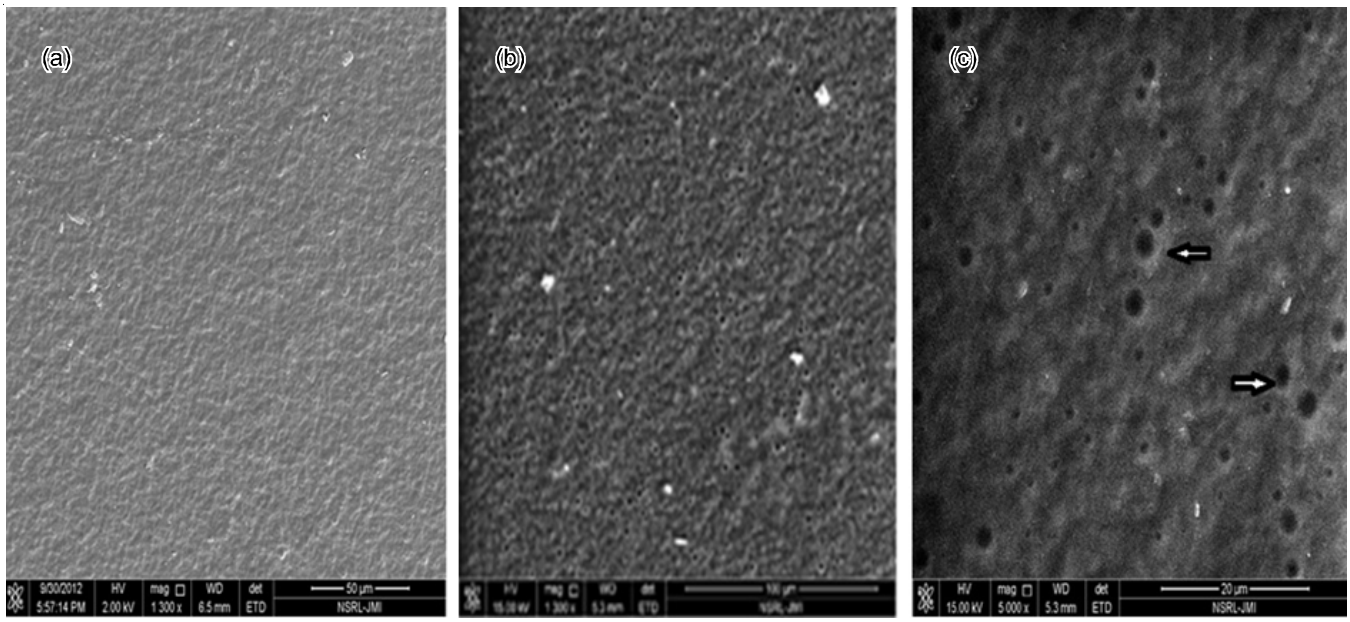

Fig. 2. FESEM images of dried CS-co-PVA hydrogel with and without extract. (a) control with no obvious phase separation; (b & c) extract loaded hydrogel on low and high resolution. Extract in circular form shown by white arrows

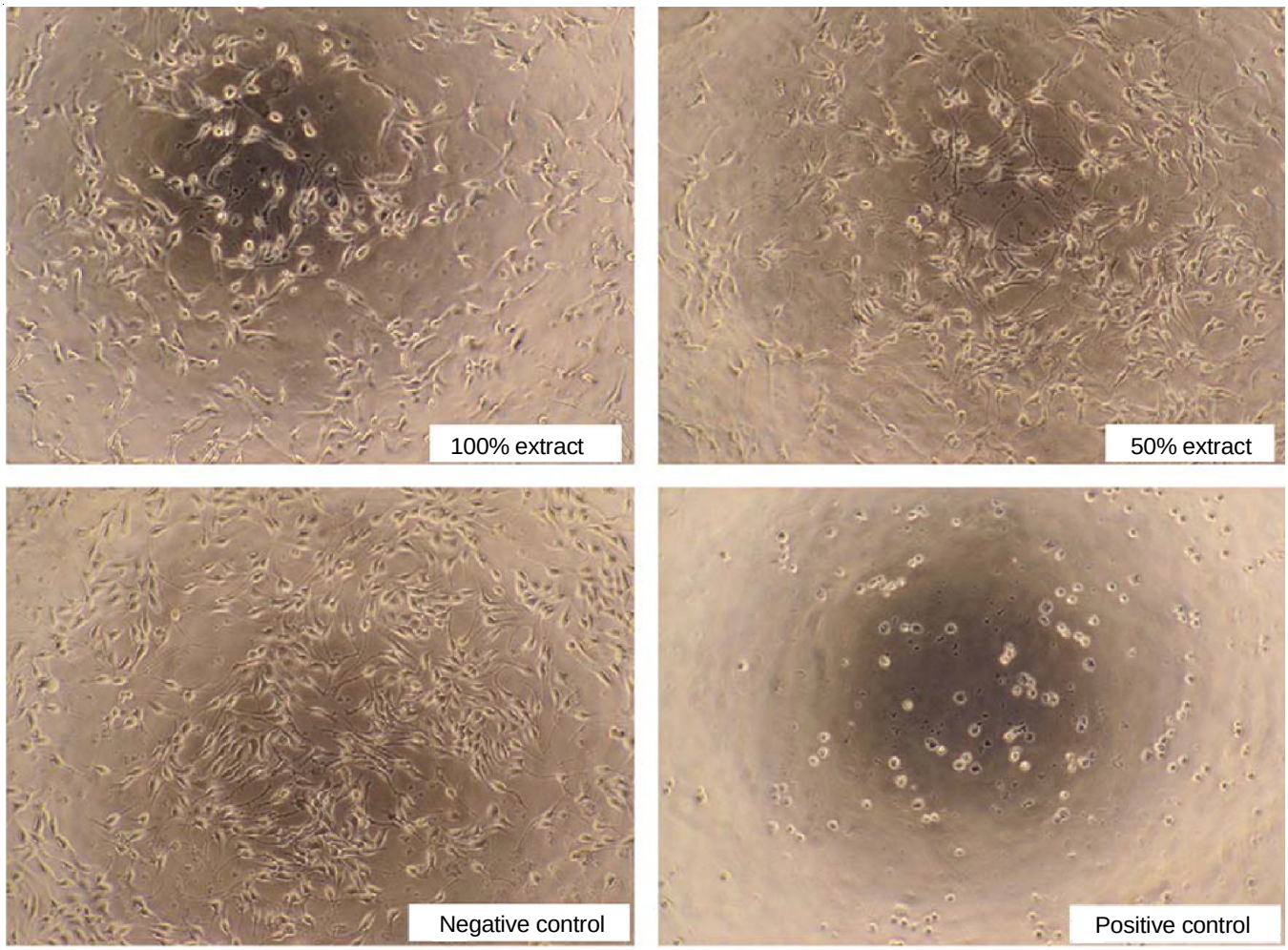

Fig. 3. Photomicrographs of L929 mouse fibroblasts cultured for 48 h, magnified 100 times. Obtained from reference No. [36]

hydrogel (30 mL) was encapsulated by 300 mg of methanolic extract in order to study the effect on the microbial strains by observing zone of inhibition. The sterile discs of 10, 15 and 20 mg/mL different concentrated extract solution were also in placed to see the effect of pure extract simultaneously. The results of inhibition effect by the extract on the growth of microbes exhibited good variations. The *E. coli* and *S. typhi* were the least affected strains, whereas *S. aureus* and *P. aeruginosa* exhibited the largest zone of inhibition with 15 mm at 20 mg/mL (Table-6). The *S. aureus* is the most susceptible bacteria for leaves extract of *J. regia* [37]. In case of fungus, the leaves extract also showed the effective on to *C. albicans* (12 mm) at 20 mg/mL. In case of CPW, *P. aeruginosa* (8 mm) and *C. albicans* (8 mm) were the most effective followed by *S. aureus* (6 mm), whereas CP3 as a control does not show inhibition zone [38].

TABLE-6
ZONE OF INHIBITION OF DIFFERENT CONCENTRATION DISCS OF ETHYL ACETATE *S. alba* LEAVES EXTRACT AND CP1A AGAINST DIFFERENT BACTERIAL STRAINS

Strains	10 mg/mL (mm)	15 mg/mL (mm)	20 mg/mL (mm)	CPW (mm)
S. typhi	00	00	05	04
S. aureus	00	05	15	06
E. coli	00	00	00	00
P. aeruginosa	08	10	15	08
C. albicans	00	08	12	08

Conclusion

Biocompatibility of extract loaded hydrogel makes them a suitable tool for biomedical applications. The performed *in vitro* analysis of hydrogel as wound dressings has been justified by this work, as it exhibited the antimicrobial activity against different strains. The methanolic *Juglans regia* leaves extract successfully attached on to the surface of hydrogel without making any change in the FTIR peaks of control. The major compounds of plant extract strongly affect the antimicrobial property of chitosan/PVA/methanolic *J. regia* leaves extract. This study facilitates the research in wound treatment by natural means.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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