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SYNERGISTIC EFFECT OF GELATIN FROM TILAPIA FISH (*Oreochromis niloticus*) AND CLOVE EXTRACT (*Syzygium aromaticum*) TO BE USED FOR PRESERVATION

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ABSTRACT : The purpose of this study was to investigate the potential preservation benefits of combining clove (*Syzygium aromaticum*) extract with Tilapia (*Oreochromis niloticus*) fish gelatin. The study revealed the capability of gelatin to act as a binding agent and the clove extract to inhibit the microbial growth thus extending the shelf life of products. Gelatin was extracted using alkali treatment method. Presence of gelatin protein was confirmed through gelatinase test and biuret test. The estimation of protein in the gelatin was done using Lowry's method. The clove extract was extracted using soxhlet extraction and the compound present in them were identified using GC-MS analysis. The combined preservative effect of gelatin and clove against food borne pathogens, was studied using the agar well diffusion method.

Keywords *: Syzygium aromaticum, Oreochromis niloticus, gelatin, Tilapia fish, Preservation.*

INTRODUCTION

The most common protein in animal skin, bones, tendons, and ligaments is collagen, which is hydrolytically broken down to produce gelatin, a naturally occurring polymer (L.S. Kumosa *et al.*, 2018, G. Bou-Gharios *et al.*, 2020). Gelatin has a high protein content, low energy content, and is free of fat and cholesterol. It is frequently used as a gelling agent, foam stabilizer, and structural enhancer to foods, medications, cosmetics, photographic films, and other products including paints, matches, and fertilizers(Gudmundsson *et al.*, 2002). Gelatin is frequently used in food products because it can enhance the consistency, suppleness, and stability of the final product.

The main components of gelatin, which comprises of different types of complex amino acids, are 57% glycine, proline, and hydroxyproline, and the remaining 43% are members of other amino acid families such glutamic acid, alanine, arginine, and aspartic acid(Sultana *et al.*, 2018). In addition to having very good physical qualities including jelly force, affinity, high dispersibility, low viscosity characteristics, dispersion stability, and water retention, gelatin has a considerably higher gel strength. Fish, cow bones, and pig skin are the main sources of gelatin. Based on the source, there are two distinct types of mammalian gelatins: type B gelatin from cows and type A gelatin from pigs. The components of both types of gelatin have molecular weights ranging from 10 to 400 kDa. Gelatin can be found in good quantities in the heads, feet, and bones of poultry animals. The molecular weight, secondary structure, and amino acids of these animal tissues are comparable to those of mammalian gelatin. Proline or hydroxyproline, alanine, and glycine are the primary amino acids found in fish gelatin, which is transparent to slightly translucent, colorless to yellowish, and tasteless. Scales, bones, and skins are the byproducts of the fish processing industry that can be used to make gelatin. The Cichlidae family of fish includes tilapia, which are primarily freshwater fish that live in lakes, rivers, and ponds. They are also occasionally found in brackish water. Their long dorsal fins allow them to be deeply cooked and squeezed laterally.

Clove is a member of the Myrtaceae family, which includes between 1200 and 1800 species of flowering plants. These plants are commonly found in tropical and subtropical regions of Madagascar, Asia, Africa, and the Pacific ocean (Cock,Cheesman., 2018). Because of their antibacterial and antioxidant qualities, they have been used for generations as a food preservative and medicine. They have a rich brown color and a strong, aromatic smell. Eugenol is the primary bioactive component of cloves, and they are

also an essential source of phenolic compounds such hydroxyphenyl propenes, hydroxycinnamic acids, hydroxybenzoic acids, and flavonoids. The aerial sections of cloves contain a significant amount of essential oil(Mittal *et.al.*, 2014).

MATERIALS AND METHODS

1. COLLECTION OF FISH SCALES

The fish sample for the study was collected from the local fisherman. It was stored in cold until use.

2. RECOVERY OF SCALES

Scalpels were used to remove the fish's scales. After that, it was cleaned for two or three hours under running tap water. The scales were then allowed to dry in the shade. The scales were weighed once they had dried, then placed under an airtight cover and kept at 4°C. Later, the scales were retrieved in order to begin the extraction process.

3. EXTRACTION OF GELATIN FROM THE SCALES

Around 50g of scales were weighed. Gelatin-Type B was extracted by alkali treatment method. Initially, dried scales were agitated for 30 minutes at room temperature using a 5% sodium chloride (NaCl) solution (1/10,w/v). After doing this twice, the second step was finished by agitating the scales for 60 minutes with 0.4% sodium hydroxide (NaOH) (1/10, w/v) in order to extract the non-collagenous proteins. Every 30 minutes, the alkali solution was changed, and the third stage involved removing the lipids from the scales using 10% isobutyl alcohol (1/4 w/v). For thirty minutes, this process was carried out three times in a digital linear shaker. Demineralization using 0.5N ethylenediamine tetraacetic acid (EDTA) solutions at an intrinsic pH of 7.66 for four distinct shaking times—12 hours, 2 hours, 2 hours, and 1 hour—was the last step. In every step scales were obtained by passing them through a sieve and then washing them with distilled water to get rid of any leftover material. For three hours, recovered scales were immersed in a 0.05 M acetic acid solution. Following filtration, 1/3 (w/v) water was added, and the mixture was baked for an entire night at 60 °C. This was filtered, and the filtrate was removed for additional research.(Merina Paul Das *et al.*, 2017).

4. GELATINASE TEST

The sterilized and prepared media needed for the gelatinase test was used. Four test tubes, one for each commercial and fish gelatin control and test, each received approximately 10 milliliters of media. All test tubes except the controls received an inoculation with the *Pseudomonas* sp. culture. Following a full day of incubation at 37°c, the tubes were placed in a cold environment, and the outcomes were noted. (Edison and Martin., 2012).

5. BIURET TEST

Presence of protein in the sample was identified by noting the colour change during the addition of biuret reagent. 1 ml of biuret reagent was added to 1 ml of the sample.

6. ESTIMATION OF PROTEIN

Using Lowry's method, the protein concentration in the gelatin sample was calculated. The color blue that results from the reduction of phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the protein's tryptophan and tyrosine amino acids, as well as the color that results from the protein's reaction with the alkaline cupric tartarate in the biuret are measured with a colorimeter at 660 nm.

7. SDS-PAGE

Using APS and TEMED, the gel polymerization of acrylamide and bisacrylamide was allowed to finish for roughly 15 to 30 minutes. Meanwhile, preparations were made for the sample. After adding the test to the sample buffer in a 3:1 ratio, it was brought to boil for five to ten minutes. After the polymerization process was completed, the bottom spacer was taken out, the comb was carefully removed, and the wells were cleaned with tank buffer. The vertical gel electrophoresis device was used to clamp and tighten the gel plate. Each well was filled with the material using a micropipette. Tank buffer was gradually added to the top and bottom reservoirs as well as the top chamber. After the power supply and tank were connected, the electricity was turned on. The mode of constant current (100Mv) was selected for the power supply. Once the marker dye had reached the bottom of the gel, the machine was stopped and the power source was split off. With caution, the gel from the glass plate was removed and placed in a plastic tray. After filling the gel tray with the CBB G-250 staining solution, it was set on a rocker to sit overnight. Using destaining solution, the excess stain was eliminated following an overnight incubation period. The protein bands were visible once the gel was placed onto the transilluminator.

8. CLOVE EXTRACT EXTRACTION USING SOXHLET APPARATUS

Clove extract was extracted using the soxhlet extraction technique. For solvent extraction, 20g of the sample (clove powder) was weighed and transferred to filter paper extraction thimble and placed in the reflux flask followed by extraction with organic

solvent, the n-hexane. After completion of the extraction process for 3-4 hours, the obtained extracts were concentrated by keeping them in a water bath at a temperature of 70°c.

9. GC-MS ANALYSIS OF CLOVE EXTRACT

Gas chromatography-mass spectrometry analysis of clove extract was done to identify and quantify the chemical compounds present in the extract. The analysis allows for the separation of compounds based on their physical and chemical properties, followed by identification using mass spectrometry.

10. ANTIBACTERIAL STUDIES OF CLOVE AND GELATIN

The antimicrobial studies of clove and gelatin was conducted against food borne pathogens *Listeria monocytogenes* and *Shewanella putrefaciens* using agar well diffusion methods by taking 50µl and 100µl concentrations. The combination effect of both were also studied using the same method.

RESULTS AND DISCUSSION

1. COLLECTION OF FISH SCALES

The fish sample was collected from the local fisherman and was taken for identification to ICAR-CENTRAL INLAND FISHERIES RESEARCH CENTRE, CMFRI Campus Ernakulam North P. O, Cochin, Kerala. Based on the mersitic and morphological characters, it was confirmed that the fish species was *Oreochromis niloticus.*

2. GELATIN YIELD

In the present study, gelatin was extracted from the Tilapia (*Oreochromis niloticus*) fish scales. Yield of gelatin was found to be 20%.

Yield of gelatin (%) = (Amount of gelatin produced (ml) ÷ Amount of fish scales used(g)) × 100

(10÷50)×100
=20%

The extraction yield of fish gelatin is less when compared to mammalian gelatin (Karim and Bhat.,2009).

CONFIRMATORY TEST

3. GELATINASE TEST

The gelatinase test performed using the extracted gelatin showed positive results. Complete liquefaction of the test tubes inoculated with the bacterial sample was observed after keeping them in cold conditions. The control tubes without bacterial species remained solidified even after exposure to the cold temperature.This indicates the presence of gelatin in the medium and also the ability of Pseudomonas to hydrolyze gelatin (Anchana Devi *et al.*, 2016).

4. ESTIMATION OF PROTEIN

In the present investigation, to identify and quantify the protein - Lowry's method was performed. The gelatin sample extracted from Tilapia fish scales were used for the study. The sample contained,

Test concentration = (Test optical density÷Standard optical density)×Standard concentration
=(0.80÷1.66)×600
=284µg
0.5 ml of sample contains = 284µg of protein
100 ml of sample contains = (284÷0.5)×100
=56,800 µg of protein
=56.8 mg of protein
1 ml of sample contains=0.568 mg of protein

5. BIURET TEST

The presence of protein in the gelatin sample was identified by the appearance of purple colour in them.

6. SDS PAGE

In the present investigation, SDS-PAGE was performed for the extracted fish gelatin.12% acrylamide gel was used and found that the bands of fish gelatin showed a molecular weight ranging from 120-250 kDa by comparing it with the protein size chart(Protein gel migration charts, Bio-Rad). Two protein markers of different molecular weights were used for the study. Three well defined bands observed for tilapia scale gelatin ranged in the molecular weight of approximately 202 kDa, 130 kDa and 119 kDa (Maria *et al.*,2018). Thus it can be inferred that the bands produced by the extracted fish gelatin falls in the molecular weight range of Tilapia scale gelatin.

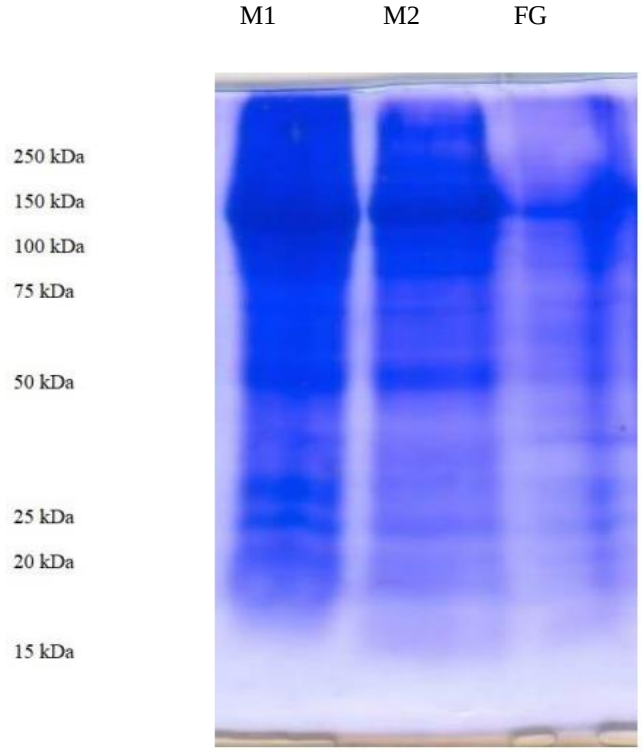

Fig. SDS-PAGE of extracted gelatin
(a) Protein marker 1 (M1)
(b) Protein marker 2 (M2)
(c) Fish gelatin (FG)

7. CLOVE EXTRACT EXTRACTION USING SOXHLET APPARATUS

Clove extract was obtained after extraction with the soxhlet apparatus. To concentrate the extract, it was kept in a boiling water bath at 70°c.

8. GC-MS ANALYSIS OF CLOVE

From the GC-MS analysis of clove extract it was found that there are about 40 compounds present in them. In this, the main constituents are caryophyllene oxide (1.65%), estragole (1.86%), humulene (2.57%), n-hexadecanoic acid (2.58%), anethole (13.44%), caryophyllene (14.64%), eugenol (26.44%).

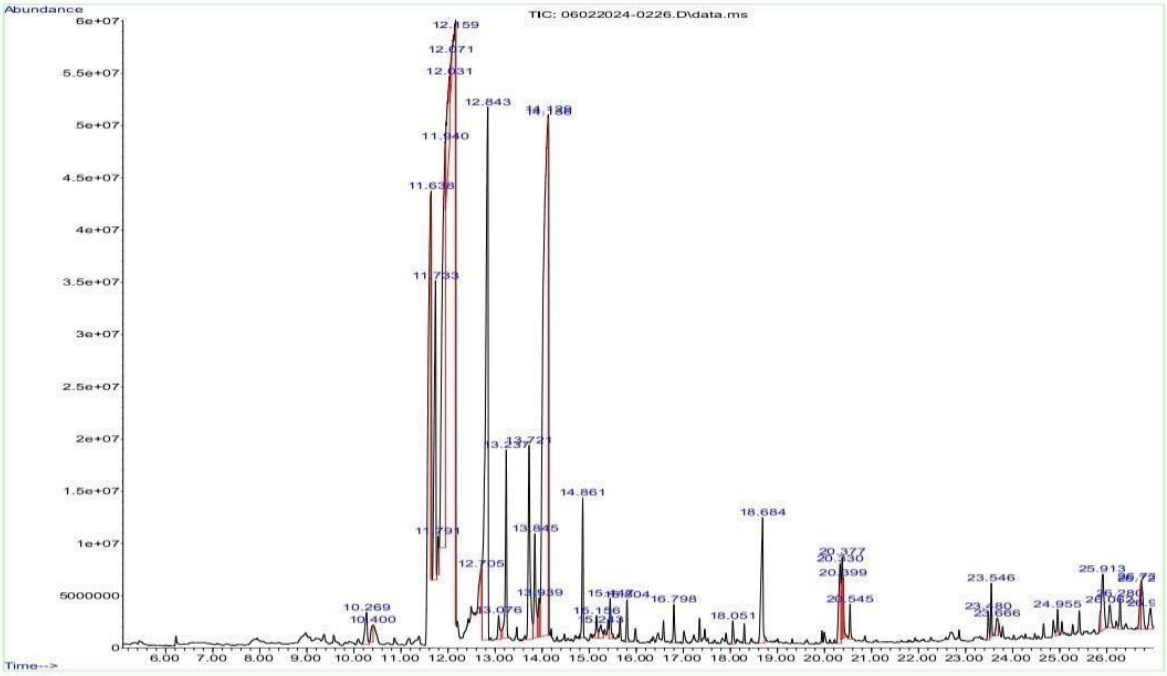

Fig. GC-MS analysis of clove extract

9. ANTIBACTERIAL STUDY OF CLOVE EXTRACT

The antibacterial study of clove extract was done using agar well diffusion method on MH agar (Mueller Hinton agar) plates. Two food borne pathogens, *Listeria monocytogenes* and *Shewanella putrefaciens* were used for the study and zone of inhibition was obtained. For *Listeria monocytogenes*, a zone of inhibition of 6mm and 8mm was obtained for 50 µl and 100 µl concentrations respectively. For *Shewanella putrefaciens*, a zone of inhibition of 7mm and 8mm was obtained for 50 µl and 100 µl concentrations respectively.

Fig. Antibacterial study of clove extract on MH agar plates
(a) *Listeria monocytogenes*
(b) *Shewanella putrefaciens*

10. ANTIBACTERIAL STUDY OF GELATIN

The antibacterial study of gelatin was done using agar well diffusion methods on MH agar (Mueller Hinton agar) plates. Two food borne pathogens, *Listeria monocytogenes* and *Shewanella putrefaciens* were used for the study and zone of inhibition was obtained. For *Listeria monocytogenes*, a zone of inhibition of 4mm and 6mm was obtained for 50 µl and 100 µl concentrations respectively. For *Shewanella putrefaciens*, a zone of inhibition of 5mm and 7mm was obtained for 50 µl and 100 µl concentrations respectively.

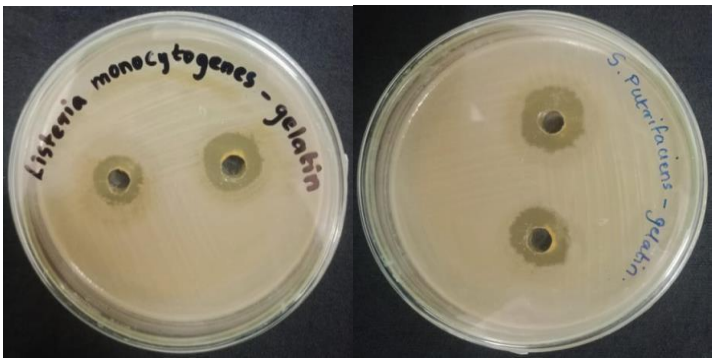

Fig. Antibacterial study of gelatin on MH agar plates
(a) *Listeria monocytogenes*
(b) *Shewanwella putrefaciens*

11. ANTIBACTERIAL EFFECT OF COMBINATION OF CLOVE AND GELATIN

The antibacterial study of the combination of clove extract and gelatin was done using agar well diffusion method on MH agar (Mueller Hinton agar) plates. Two food borne pathogens *Listeria monocytogenes* and *Shewanella putrefaciens* were used for the study and zone of inhibition was obtained. For *Listeria monocytogenes*, a zone of inhibition of 8mm and 9mm was obtained for 50 µl and 100 µl concentrations respectively. For *Shewanella putrefaciens*, a zone of inhibition of 9mm and 10mm was obtained for 50 µl and 100 µl concentrations respectively.

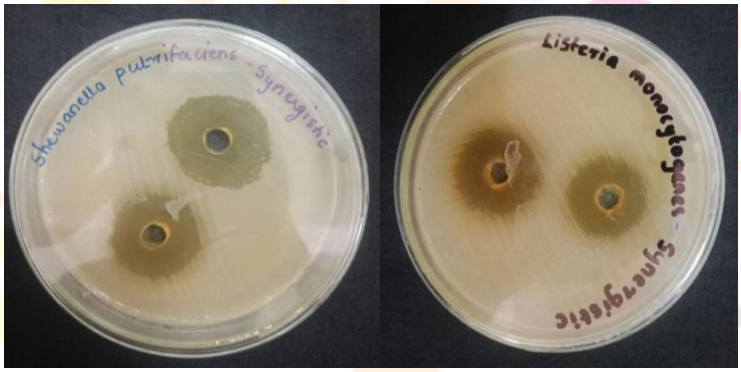

Fig. Antibacterial study of combined effect of clove and gelatin on MH agar plates
(a) *Shewanella putrefaciens*
(b) *Listeria monocytogenes*

CONCLUSION

Preservation is the method of maintaining the quality, freshness and safety of the food over time.It extends the shelf life of the food and reduces the risk of spoilage and food borne illnesses.The study revealed the potential of Tilapia fish scales as raw material for gelatin production.The gelatin obtained was analyzed, its antibacterial activities were studied and its capacity to act as a binding agent during preservation was determined.Raw material for gelatin, the fish scales were cost effective, environment friendly with various industrial applications.The clove extract obtained through soxhlet extraction contributes to food preservation by inhibiting microbial growth,enhancing flavor and preventing oxidation thereby extending the shelf life of products.The main compound present in the clove extract, the eugenol helps in inhibiting the growth of bacteria, fungi and other microorganisms which was proved through their antimicrobial studies.The combination of clove with the gelatin can be an innovative and environment friendly approach in preservation. Their combined effect showed great inhibitory activity against food borne pathogens.This combination can be used in the form of films and coatings thus increasing the shelf life of the products.They also enhance food safety by reducing the food borne diseases. They can be particularly applicable in preserving fruits, vegetables, meats and fishes providing protection from microbial growth and enhanced flavor.

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