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Tapioca Starch Based Antimicrobial Food Packaging Material

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***Abstract*— In the food packaging sector, starch-based material has received great attention owing to its biodegradability, wide availability as agricultural surplus raw material, abundant, can be produced at low cost and at large scale, nonallergic, easy to use and thermoprocessable. Through this project we are trying to develop a starch based film packaging material with antimicrobial activity. Antimicrobial packaging has been touted as a major focus in the next generation of 'active' packaging. Antimicrobial packaging is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods. The new antimicrobial function can be achieved by adding antimicrobial agents in the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements. The base for the film is made of starch derived from tapioca. As a plasticizer we incorporated glycerol. The antimicrobial activity was achieved by adding medicinal plant extracts. The extracts of common medicinal plants such as neem ,betle leaves,guava leaves,rhizomes of lotus and turmeric, and flowers of hibiscus were used. The extracts were prepared using ethanol extraction method. The effectiveness of extracts were tested by using agar diffusion method. Five different bacterial strains such as *E.coli, Pseudomonas, S. aureus, B. cereus* and *Klebsiella* were used for the study. Minimum inhibitory concentration and minimum bactericidal concentration values of extracts were also found out. We also conducted model food studies for the film using raw carrot and chicken.**

Keywords*— *Antimicrobial packaging, Medicinal plant extracts, Starch based film, Food pathogens

I. INTRODUCTION

Avoidance of pathogenic and spoilage microorganisms in food is usually achieved by using chemical preservatives. These chemicals act as antimicrobial compounds which inhibit the growth of undesirable microorganisms. There is a currently strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic attributes as well as residual toxicity. For these situations, consumers have a propensity to be suspicious of chemical additives and thus the exploration of naturally occurring antimicrobials for food preservations receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives. That has led to search for novel antimicrobial compounds from natural sources.

Naturally derived compounds and other natural products may have applications in controlling bacteria in foods. The primary incentive for indentifying effective antimicrobials among naturally occurring compounds is to expand the spectrum of antimicrobial activity over that of the regulatory-approved substances.

Traditional antimicrobials are generally limited to high acid, low fat food products because of interactions with pH and food components. Interest in natural antimicrobials is also driven by the fact that international regulatory agencies are generally very strict as to requirements for toxicological evaluation of novel direct food antimicrobial. One group of naturally derived antimicrobial compounds is medicinal plants and their essential oils. These compounds have been safe, have been shown to have varying degree of antimicrobial activity, and could provide another hurdle to growth of food borne pathogens and spoilage bacteria, thereby improving the shelf-life of food products. Numerous studies have reported that medicinal plants produce a large number of secondary metabolites with antimicrobial effects on pathogens. Medicinal plant extracts, therefore, for the control of the growth of food borne pathogens and food spoilage bacteria are emerging as alternatives to conventional natural preservatives as they are generally safe to humans, and environmentally friendly. However, natural antimicrobial activity of medicinal plants and their essential oils is often variable. Most research on medicinal plants as natural antimicrobial has been conducted in vitro in microbiological media.

II. METHODS

Stock culture preparation

All the test organisms were maintained in nutrient broth at 4°C. Preparation of working stock culture, test organisms were thawed at 4°C and grown separately in 100 ml nutrient broth on an orbital shaker at 37°C for 24 hours. Cultures were stroke out on nutrient agar and incubated at 37°C for 18-24 hours. All the cultures were maintained on nutrient agar at 4°C and sub cultured to maintain the viability.

Preparation of medicinal plants

Fresh and dried samples used in this study were collected from the herbal garden. All samples were transferred to the laboratory and started to be processed. They were washed in 50µg/ml hypochlorite solution, sliced and sun dried. The dried samples were ground to powder using a mechanical grinder and kept separately in dry condition until use. We also used some raw plant parts for comparison studies of extraction methods.

Table 1: List of traditional medicinal plants used in the antimicrobial assay

Sl.no	Botanical species	Family	Plant part
1	*Curcuma longa*	Zingiberacceae	Rhizome
2	*Azadirachta indica*	Meliaceae	Leaf
3	*Piper betle*	Piperaceae	Leaf
4	*Psidium guajava*	Myrtaceae	Leaf
5	*Hibiscus rosa-sinensis*	Malvaceae	Flower
6	*Nelumbo nucifera*	Nymphaeaceae	Rhizome

Preparation of crude extracts

15g of the dried powder was extracted using 100 ml of 50% ethanol for 24 hours at 4°C. The ethanolic extracts were centrifuged at 8000rpm for 30 minutes and filtered using Whatmann No: 1 filter paper for getting clear extracts. Remaining ethanol in the crude extract was removed by conducting the distillation at 78^0 C until 90% of the ethanol was removed. The ethanolic extracts were stored at 4^0C in a sterile reagent bottle.

Antimicrobial studies of extracts

Agar diffusion method

Antibacterial assay of ethanolic extracts were determined by using agar diffusion method on nutrient agar medium. Inoculums of bacteria were spread on the solid plates with a sterile swab moistened with bacterial suspension. Two wells are made in nutrient agar plates using micropipette tips and solvent, ethanol was added to one of the wells as a control. Then 40 µl of ethanolic extract were placed in the well made in inoculated plates. The agar plate was incubated for 24 hrs at 37°C and zone of inhibition around the wells were measured in millimeter.

Determination of minimum inhibitory concentration (MIC)

For determining the minimum inhibitory concentration of the ethanolic extract, different concentrations (0%, 20%, 40%, 60%, 80% & 100%) of the extracts were prepared. To each of the test tubes 2ml of nutrient broth and extract with different concentration were added. 10µl of the test organism was added to these test tubes. Three growth controls were adopted, one containing 2ml of sterile distilled water and 2ml of extracts, the second one contain 2ml of nutrient broth and 10µl of bacterial suspension and the third one contain 2ml of nutrient broth alone. These test tubes were incubated at 37°C for 24 hours. And the absorbance was taken at 520nm. This procedure was repeated for all the test organisms and all the extracts. Growth of the test organisms resulted in a visible clouding of the culture medium. The lowest concentration of each extract that inhibited visible growth was determined as the Minimum Inhibitory Concentration (MIC).

Determination of the minimum bactericidal concentration (MBC)

Referring to the results of the MIC assay, all tubes showing complete absence of growth were identified. 100µl of solution from each of these tubes were transferred on to a nutrient agar plate and performed spread plating. All the plates were incubated at 37^0C for 24hrs.The lowest concentration of the extract with complete absence of growth was considered as the Minimum Bactericidal Concentration (MBC).

Film production and optimization

The ingredient used for the production of film includes tapioca starch, sodium alginate, sodium EDTA and glycerol.

Starch preparation

Tapioca was peeled and washed well. It was then grated and mixed properly with water. The mixture was filtered using filter cloth and the filtrate was allowed to settle. After settling the starch was thoroughly washed and again kept for settling. Dewatering was carried out by pressing in a clean bag. It was then dried and powdered.

Preparation of alginate-tapioca based film

Tapioca starch was modified by autoclaving at 130°C for 30 minutes. This modified tapioca starch and sodium alginate were diluted in distilled water. Na-EDTA was added as additive, and glycerol was added as plasticizer. In the gelatinization process, the film based solution was heated and continuously stirred at 85°C for 45 minutes. The incorporated film solution was cast by pipetting the solution into sterile plastic plate. The solutions were dried for approximately 24 hours at 70°C, after which the film were peeled from the plastic plates and store at room temperature until use. The concentrations of each of the components were to be optimized to obtain a film with desired properties. So we experimented with different concentrations as tabulated below.

Table 2: Film optimization

Trial no	Starch (g)	Sodium alginate(g)	Sodium EDTA (g)	Glycerol (ml)
1	4	1	0.1	40
2	5	2	0.1	40
3	6	3	0.1	20
4	6	3	0.1	15
5	6	3	0.1	16
6	6	2	0.1	18
7	4	1	0.1	0.63
8	4	1	0.1	1
9	4	1	0.1	2
10	4	1	0.1	4
11	4	1	0.1	3
12	4	1	0.1	2
13	4	1	0.1	1.5
14	4	1	0.1	1

Antimicrobial film production

Preparation of film with antimicrobial extract

The antimicrobial film based solution was prepared by adding ethanolic extracts of medicinal plants into the gelatinized film based solution with concentrations of 20.0%v/v, 15.0%v/v,10%v/v and 5%v/v. After the gelatinization process the antimicrobial extract was added to the film based solution. The incorporated film solution was cast by pipetting the solution into sterile plastic plate. The solutions were dried for approximately 24 hours at 70°C, after which the film were peeled from the plastic plates and store at room temperature until use.

Determination of the antimicrobial activity of the extract incorporated film

The antimicrobial test on films was carried out using the agar diffusion test method. The zone of inhibition assay on solid media was used for the determination of antimicrobial effects of the films against the test organism *E.coli.* The films were cut into 2x2cm and then placed on nutrient agar plates which had been previously inoculated. The plates were incubated for 24 hours. After that plates were examined for zone of inhibition around the films.

Model food system study

The antimicrobial properties of alginate tapioca based films incorporated medicinal plant extract was studied in a model food system. The experiments were performed on the normal foods, and those covered using film without extract and foods covered with antimicrobial films. The numbers of bacterial survivors were determined after different storage times at room temperature.

Preparation of model food system

Carrot and chicken were selected as model food system for studying the antimicrobial activity of Alginate-Tapioca based films incorporated ethanolic extracts of medicinal plants. The carrots were washed and peeled off, and cut into circle pieces of approximately 10g per piece. Chicken meat was also cut in to pieces of approximately 10g each and prepared according the same method as described for carrot.

Application of antimicrobial films on the model food system

Alginate–Tapioca based antimicrobial films with 20% v/v ethanolic extract of all selected medicinal plants were used for the test. The food materials were covered with antimicrobial film. Each sample was placed in a sterile petri dish. The petri dishes were placed in an open condition. The samples without application of the antimicrobial films and samples with alginate –tapioca films without medicinal plant extracts served as controls. The number of bacterial survivors was monitored after 0, 24 and 48hours storage times. After the storage times the sample was homogenized with 90ml of normal saline solution. The solution was serially diluted and using the appropriate dilution pour plating was done and kept for incubation at 37^0C for 24 hours. When the incubation time was over the plates were enumerated in a bacterial colony counter. This procedure was repeated for all the food samples at different storage time.

III. RESULS AND DISCUSSIONS

Comparative studies of extracts from different plants

As per our literature and experimental studies, ethanolic extraction was proved to be the best choice. To recover the crude extract from the organic solvent- ethanol, distillation was adopted. From the comparative study of ethanolic extracts prepared from all the plant parts, neem leaves showed better activity and later studies were carried out using the same.

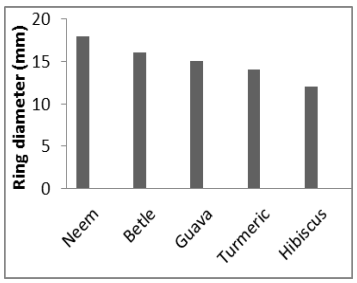

Figure1. Comparison studies of extracts

Antimicrobial studies of extracts

Agar diffusion method

With the incorporation of antimicrobial extract into the well, it diffused through agar gel and furthermore resulted in clear zone of inhibition. Comparing all the ethanolic extracts, neem was found to be the best. Lowest zone of inhibition was observed around the hibiscus extract. In order to prove that the antimicrobial activity was not due to the presence of organic solvent-ethanol, it was added to the control well. No zone of inhibition was observed around the control well. To find more susceptible test organism, agar diffusion of neem extract was done.

Figure 2: Agar Diffusion Study

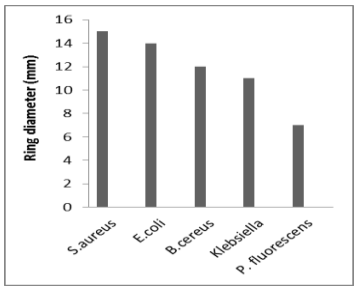

Figure 3: Microbial Susceptibility study

E.coli and *S.aureus* were found to be more susceptible to antimicrobial extract. We also tried with mixed culture of microorganisms and the ring diameters were narrow.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

MIC and MBC of five medicinal plants were determined by a broth dilution susceptibility assay. The MIC could not be determined because of the interaction between extracts and culture media. The solution became turbid appearance. As a result the test tubes with no visible bacterial growth were selected for the MBC test.

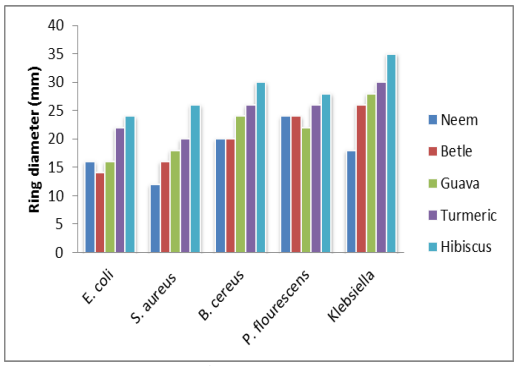

Figure 4: Determination of Minimum bactericidal concentrations

Film Preparation and optimization

The starch based film was prepared with the additions of sodium EDTA, sodium alginate and plasticizer, glycerol. The concentrations of each of these components were to be optimized to obtain a film with desired properties. Glycerol was the key component in determining the film properties. Hence glycerol concentration remained the crucial component to be optimized in the film preparation. Thus we performed series of experiments and standardized film composition. The optimized compositions are starch-4g, sodium alginate-1g, sodium EDTA-0.1g and glycerol-1ml. The film thus obtained was found to be less sticky, more flexible, and easy to handle. This optimized film as shown in figure 5 was used for our further studies.

Figure 5: Optimized Film

Antimicrobial film production

Films were prepared with different volumes of extract comprising 5%, 10%, 15% and 20% v/v. Agar diffusion tests were performed with these films to determine the minimum volume of extract to be added to obtain a film with best antimicrobial activity. The film to which 20% v/v of the extract was added showed the maximum zone of inhibition against *S.aureus* and *E.coli.* Using these standardized volumes of extracts, antimicrobial films of all the extracts were prepared. The results can be plotted graphically as given in figure 6. Of all the films, film with neem extract showed maximum area of inhibition followed by betle, guava, turmeric and hibiscus extracts. All the films were devoid of underneath bacterial growth.

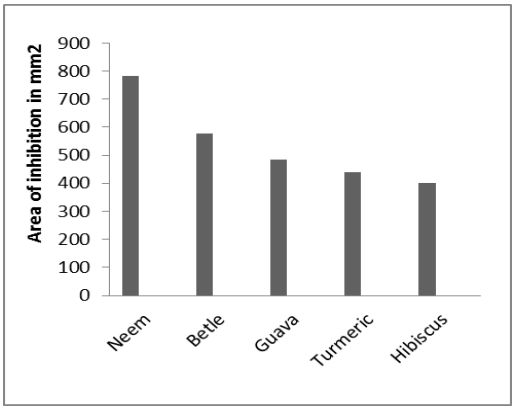

Figure 6: Determination of Antimicrobial activity of films

Of all the films, film with neem extract showed maximum area of inhibition followed by betle, guava, turmeric and hibiscus extracts. All the films were devoid of underneath bacterial growth.

Model food system study

In both antimicrobial studies of extract and film, the film prepared using neem extract showed maximum zone of inhibition. Hence neem extract incorporated starch based film was used for studying in model food systems. After the storage times the carrot, chicken samples and the controls were taken and colony counting was carried out using Quebec colony counter. Colony forming units per ml were calculated during different time intervals using the formula given below.

$$\text{CFU/ml} = \frac{\text{Average no: of colonies per plate}}{\text{Dilution X Volume taken in ml}}$$

The results can be tabulated as follows:

Table 5: Results of model food system study in Carrot and Chicken

TIME (hrs)	CARROT (10 g)		CHICKEN (10g)	
	No: of Colonies	CFU/ml	No: of Colonies	CFU/ml
0	184	7.36×10^4	232	9.28×10^4
24 (Plain control)	202	8.08×10^4	263	10.52×10^4
24 (Control film)	171	6.84×10^4	248	9.92×10^4
24 (Test film)	61	2.44×10^4	161	6.44×10^4
48(Plain control)	257	10.28×10^4	294	11.76×10^4
48(Control film)	135	5.4×10^4	218	8.72×10^4
48(Test film)	33	1.32×10^4	87	3.48×10^4

The following graphs show the difference in the bacterial survivors between the controls and samples covered with antimicrobial film after 24 and 48 hours.

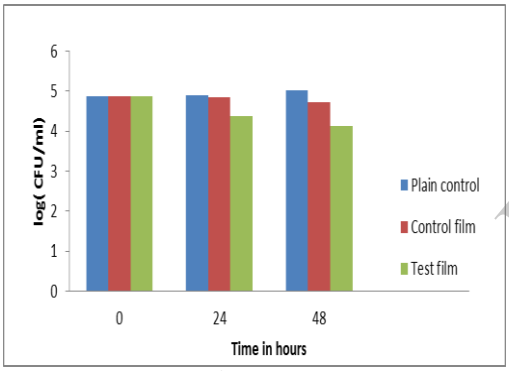

Figure 12: Model food system study in carrot

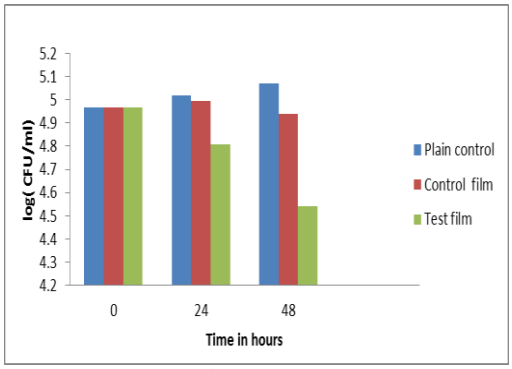

Figure 13: Model food system study in Chicken

Thus it is observed that the film with extract is found to have great impact on the growth of food borne pathogens. The decrease in number of colonies with time shows the antimicrobial activity of the film containing medicinal plant extract.

IV. CONCLUSION

The determination of the antimicrobial activity of medicinal plants included plant extracts of *Curcuma longa*, *Hibiscus rosa-sinensis*., *Nelumbo nucifera*., *Piper betle*, *Psidium guajava*, and *Azadirachta indica*. Based on the results of the broth dilution susceptibility assay to determine the MIC and MBC it can be concluded that: all the medicinal plant extracts tested showed antibacterial activity against the test organisms. Among all the medicinal plants used in this study, neem showed an outstanding antibacterial activity against the tested microorganisms. The most susceptible test organisms to the extracts were *E.coli* and S.aureus, while *Klebsiella* was the most resistant ones. As mentioned before the highest bactericidal activity was showed by neem followed by betle leaves, guava leaves, turmeric, and shoeflower. These results give additional evidence that those plant extracts are potential sources of biologically active compounds. And there is no antimicrobial activity for lotus rhizome. Due to the fact that the expected contact time in the intended field of application is relatively long during the storage time, all extracts can be recommended which inactivate the most test organisms within the longest contact time. Higher concentrations or longer contact times were necessary to reduce the test organisms. The finding of the present study demonstrate that the natural antimicrobial compounds of those medicinal plants can be successfully incorporated into Alginate-Tapioca starch based edible film and retain their inhibitory effect against microbial growth in model media based on the inhibitory zone and inhibitory underneath the films. The outstanding antimicrobial activity was obtained from Alginate-Tapioca starch based edible film incorporated with ethanolic extract of neem when studied in model food systems.

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