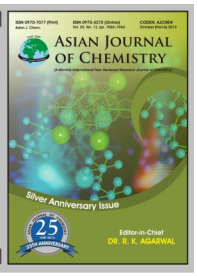
Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10470-10474

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15717



Preparation, Characterization and *in vitro* Antimicrobial Activities of Chinese Herbs Incorporated Edible Starch Films

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(*Received*: 2 May 2013; *Accepted*: 20 November 2013) AJC-14429

Films made of corn starch and Chinese medicinal herbs with antibacterial activity on *Escherichia coli* were developed. Influences of concentration of Chinese medicinal herbs on performances of the films were investigated based on indicators of antibacterial effects, absorbance, extensibility and moisture resistance. Moreover, the optimal concentration was determined. *Scutellaria baicalensis* and *Schisandra chinensis* stood out in the antibacterial test, with the MIC values of 0.7813 and 1.5625 mg/mL respectively. The addition of herbs increased antibacterial effectiveness and absorbance, meanwhile decreased extensibility and moisture resistance. To ensure the antibacterial effects and mechanical properties, concentration of *Scutellaria baicalensis* can be added in the film with MIC~4MIC (including MIC and 4MIC) and *Schisandra chinensis* with MIC~2MIC (not including MIC and 2MIC).

Key Words: Starch films, Chinese medicinal herbs, Antibacterial effects.

INTRODUCTION

Edible packaging materials are made from natural edible substances offering advantages such as edibility, barrier properties, being non-toxic, non-polluting and low cost[1,2]. In recent years, a number of detailed and meticulous research on edible packaging films has been conducted by scholars at home and abroad[3-6]. At present, plant or animal polysaccharide based edible packaging films mainly include starch films[7], modified cellulose films[8], protein films[9] and chitosan films[10]. Starch based films have been particularly considered for the reason that they have such advantages *i.e.*, transparent, odorless, tasteless, good extensibility and moisture resistance.

Foodborne disease is one of the most widely distributed and common diseases in the world today, including a variety of diseases caused by peroral infection by microorganisms and chemical substances[11]. Bacteria are a broad class of microorganisms, most of the diseases of humans and animals, approximately 85-90 %, are due to eating food contaminated by bacteria, especially *E. coli. E. coli* infection will lead to urinary tract infections, acute diarrhea and other inflammation[12,13]. At the same time, microbial contamination is also an important factor affecting the shelf life of food[14]. As a result, the study of antibacterial packaging films is of great significance.

As packaging material for food preservation, antibacterial properties in addition to some general packaging performances are required to protect food against contamination. However, there is little research on antimicrobial edible starch film currently, the antibacterial agents mainly used were chitosan[15,16], potassium sorbate[17], garlic oil[18,19], *etc*.

Chinese herbal medicines were selected as the antibacterial agent in this experiment for their excellent antibacterial effects[20], low cost and other advantages. The best type and optimum concentration of Chinese medicinal herbs added were determined on the basis of previous research.

EXPERIMENTAL

Eight kinds of Chinese medicinal herbs of *Schisandra chinensis, Scutellaria baicalensis, Syzygium aromaticum, Polygonum cuspidatum, Chrysanthemum indicum, Rheum officinale, Coptis chinensis* Franch and *Lonicera confusa* and corn starch were bought from a local supermarket (Harbin, Heilongjiang). Glycerine and sodium carboxymethyl cellulose (CMC) were purchased from Jindong Tianzheng fine chemical reagent factory (Tianjin, China). *Escherichia coli* were provided by microbiology laboratory of Northeast Forestry University. Beef extract-peptone medium: nutrient agar is produced by Tianjin Fuchen Reagent Company (Tianjin, China).

Preparation of herbal ethanol extracts: These 8 kinds of herbs were immersed in 60 % ethanol with liquid/solid ratio of 1:20 at 45 ºC for 24 h. The insoluble fractions were separated by centrifugation and filtration. The filtrates were concentrated by rotary evaporator and then diluted to the final concentration of 100 mg/mL. The obtained samples were stored at 4 ºC for further experiment.

Preparation of bacterial suspension: *E. coli* grown in agar slant was inoculated in the liquid culture medium and incubated at 37 ºC for 24 h in a shaking bath. The bacterial suspension was diluted to approximately 1×10^8 cfu/mL and stored in a refrigerator for subsequent experiments.

Determination of diameter of inhibition zone (DIZ): Filter paper method[21] was performed for determination of the diameter of inhibition zone value. 0.4 mL of bacterial *E. coli* suspension was spread evenly onto the surface of 9 agar plates. Four filter papers were soaked in each herbal extract for 2 min and air-dried at room temperature, then were sticked carefully on the surface of the plates equidistantly. The control group was prepared under the same conditions except soaking the filter papers in sterile water. The plates were incubated at 37 ºC for 24 h in the incubator. Antibacterial activity was evaluated by measuring the diameter of inhibition zone (in mm).

Determination of minimum inhibitory concentration (MIC): The MIC value was determined by double dilution method[22,23]. Twelve sterilized test tubes were prepared for each herb. Firstly, each tube was added 1 mL of Beef extract-peptone liquid mediums. In tube 1 was added 1 mL of herbal extracts, 1 mL of the mixture was removed to tube 2 after mixing thoroughly. Serially dilution was operated until tube 10 to create a 50.0000, 25.0000, 12.5000, 6.2500, 3.1250, 1.5625, 0.7813, 0.3906, 0.1953 and 0.0977 mg/mL herbal concentration range. Tube 11 was performed as a negative control by adding in no herbal extracts. Finally in tubes 1 to 11 were added 0.1 mL of bacterial suspensions respectively. While, tube 12 was performed as the positive control by adding 1 mL herbal extracts, discarded 1 mL after mixed well and then adding 0.1 mL sterile saline. These mixtures were then incubated at 37 °C for 24 h. After incubation, the results were observed. If liquids in the tube were cloudy, *E. coli* grew; if liquids in the tube were clear and transparent, *E. coli* didn't grow. The MIC was defined as the lowest concentration of the herb extract to inhibit the growth of *E. coli*.

Preparation of starch-based edible films: Starch-based films were prepared as follows. Starch slurry was first prepared from a dispersion of a certain amount of corn starch in 100 mL distilled water. The suspension was stirred for gelatinization at 100 °C for 5 min in water-bath. Glycerol was added and mixed well at 100 °C for 10 min and then sodium carboxymethyl cellulose (CMC) was added at the same temperature and continued to mix for 5 min. At last, some amounts of herbal extracts were added to obtain herbal concentration gradient of MIC, 2MIC, 3MIC, 4MIC, 5MIC in starch solution, A control group without herbal extracts instead of distilled water was set up. The mixture was degassed in vacuum, then cast on the plexiglass board and dried at 85 ºC for about 2 h to get the films[24]. Ratio of each component was made reference to previous research results. Starch: glycerin: CMC = 4.2:1.0: 0.25[25].

Antibacterial effects: The zone inhibition test was performed with a modified filter paper method. The films were made into a number of 6 mm diameter circular discs. Film discs were then placed on agar plates which had been seeded with 0.4 mL of bacterial *E. coli* suspensions. After 24 h incubation at 37 ºC, antibacterial activity was evaluated by measuring the diameter of inhibition zone.

Transparency: The transparency of the films was expressed indirectly by the absorbance. The films were cut into 12 mm × 60 mm rectangle and taped on the surface of the cuvettes (only one side) by scotch tape, then the absorbance of the film was measured by the spectrophotometer at 550 nm (722, Guangpu, Shanghai).

Extensibility: Smooth, clean and defect-free films were chose and cut into 50 mm × 10 mm bars to detect the extensibility. Elongation, which calculated as follows, was used to represent the extensibility.

$$E\ (\%) = \frac{G - G_0}{G_0} \times 100\ \%$$

where: G_0 - the distance between the original mark of the sample (mm); G - the distance between the mark of the sample when it fractured (mm).

Water vapour permeability: Water vapour permeability (WVP) of films was measured gravimetrically. 2 g silica gels were sealed up by the film and placed at room temperature. After 48 h, water vapour transmission rate was attained according to their weight changes. Water vapour transmission rate was calculated as follows:

$$W\ (\%) = \frac{W_2 - W_1}{W_1} \times 100\ \%$$

where: W_1 - the original weight (g); W_2 - the weight after 24 h (g).

Statistical analysis: Experiments were conducted in triplicate and one-way analysis of variance was performed with SAS version 9.1. Significant differences were detected at $P < 0.05$.

RESULTS AND DISCUSSION

Chinese herbal medicine screening: Table-1 summarizes the results of the filter paper assay. There was a significant variation in the Chinese herbal medicine screening and MIC determining values of these eight kinds of Chinese herbal medicines ($P < 0.05$). Through statistical analysis, we found that there was a significant difference between 8 herb extracts and the control group ($P < 0.05$). As a result, compared with the control, they all showed various degree of inhibition against *E. coli*. At the same time, there was a significant difference in antibacterial effects among *Scutellaria baicalensis, Schisandra chinensis* and others. So effects of *Scutellaria baicalensis* and *Schisandra chinensis* were remarkable. The biggest inhibition zone was produced by *Scutellaria baicalensis* (diameter of inhibition zone = 14.6 mm), followed by *Schisandra chinensis* with the diameter of inhibition zone of 13.4 mm.

The inhibitory effects of *Scutellaria baicalensis* and *Schisandra chinensis* against test microorganisms in comparison with the control were shown in Fig. 1. It can be seen

TABLE-1
DIAMETER OF INHIBITION ZONE (DIZ) OF 8 KINDS OF HERBAL ETHANOL EXTRACTS UPON *E. coli*

Herb	A	B	C	D	E	F	G	H	I
DIZ (mm)	14.57±0.58[a]	12.88±0.21[b]	10.77±0.60[c]	10.33±0.25[cd]	10.27±0.47[cd]	10.03±0.40[cd]	9.50±0.40[d]	9.51±0.38[d]	8.21±0.39[e]

Note: A: *Scutellaria baicalensis*, B: *Schisandra chinensis*, C: *Syzygium aromaticum*, D: *Polygonum cuspidatum*, E: *Chrysanthemum indicum*, F: *Rheum officinale*, G: *Coptis chinensis* Franch, H: *Lonicera confuse*, I: The control group. Means followed by different letters are significantly different at $P < 0.05$.

that there is obvious diameter of inhibition zone in herbal extracts comparing to control, furthermore, diameter of inhibition zone of *Scutellaria baicalensis* on *E. coli* was larger than *Schisandra chinensis*. A possible explanation for the prominent inhibitory effect of *Scutellaria baicalensis* may lie in the existence of baicalin. Jiang *et al.*[26] extracted baicalin from *Scutellaria baicalensis* in their test and proved that it had a good bactericidal effect. An antibacterial test *in vitro* from Wang[27] revealed a supportive result that baicalin had a very high inhibitory effect, too. It was suggested by Yang and other researchers[28] that *Scutellaria baicalensis* showed its antibacterial effects through inhibiting biosynthesis *in vivo* of DNA, RNA and protein, which resulted in death of bacterial cells. Further mechanism has yet to be studied. *Schisandra chinensis* was just behind *Scutellaria baicalensis*. Liu *et al.*[29] summarized in her paper that the existing researches cannot confirm the antibacterial components of *Schisandra chinensis*. Moreover, its antibacterial mechanism was not clear.

Based on the *in vitro* assay findings above, *Scutellaria baicalensis* and *Schisandra chinensis* were selected as the antibacterial agent to be added in the films.

Minimum inhibitory concentration (MIC): Table-2 shows growth of *E. coli* under increasing concentration of Chinese herbal extracts incorporated to the starch films. It can be seen, the minimum amount of *Scutellaria baicalensis* that showed antibacterial effect was 0.7813 mg/mL. *Schisandra chinensis* was 1.5625 mg/mL. Their respective MIC value was regarded as a reference to be added to the films.

Antibacterial effects: Increasing levels of *Scutellaria baicalensis* or *Schisandra chinensis* extracts were incorporated to the films and were tested against *E. coli* for the zone of inhibition area (Fig. 2).

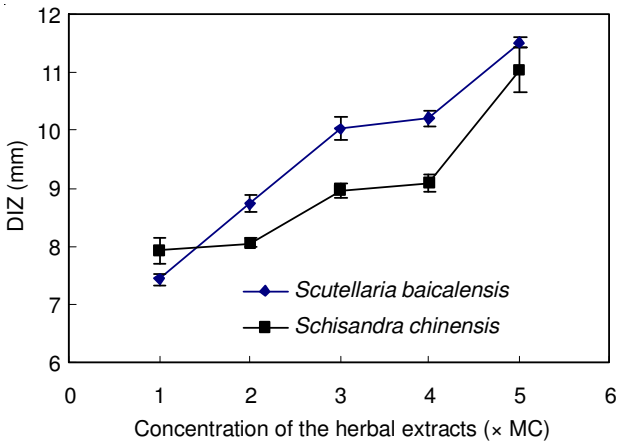


Fig. 2. Diameter of inhibition zone of films added increasing levels of *Scutellaria baicalensis* or *Schisandra chinensis* extracts; Note: for *Scutellaria baicalensis*, the MIC value is 0.7813 mg/mL, for *Schisandra chinensis*, the value is 1.5625 mg/mL. The diameter of inhibition zone value of the control group was 7.03 mm

As showed in Fig. 2, diameter of inhibition zone of films of *Scutellaria baicalensis* and *Schisandra chinensis* which represents antimicrobial activity showed a concentration dependent manner from the concentration MIC to 5MIC.

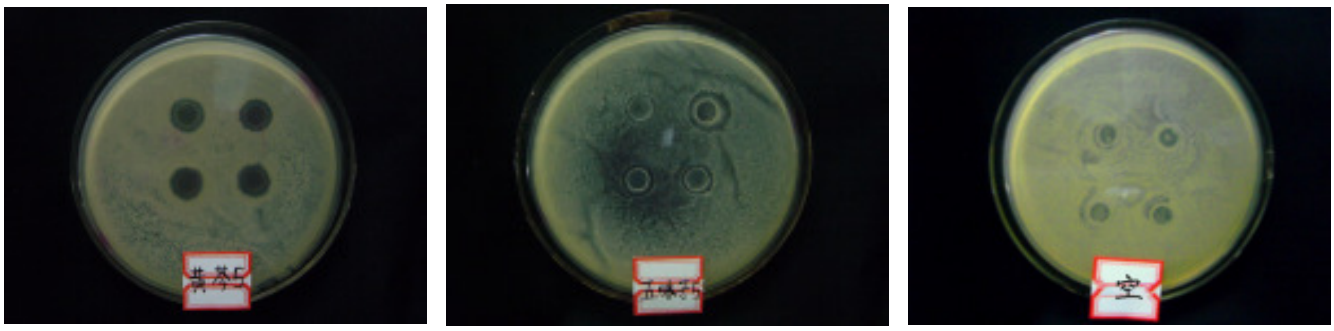
TABLE-2
GROWTH OF *E. coli* UNDER A CONCENTRATION GRADIENT OF CHINESE HERBAL MEDICINES

Concentration of herbs (mg/mL)	A	B	C	D	E	F	G	H	I	J	K	L
Scutellaria baicalensis	–	–	–	–	–	–	–	+	+	+	–	+
Schisandra chinensis	–	–	–	–	–	–	+	+	+	+	–	+

Note: '–': no bacterial growth, '+': bacterial growth. A: 50.0000, B: 25.0000, C: 12.5000, D: 6.2500, E: 3.1250, F: 1.5625, G: 0.7813, H: 0.3906, I: 0.1953, G: 0.0977, K: the positive control, L: the negative control.

1(1) 1(2) 1(3)

Fig. 1. Pictures of inhibitory zones of *Scutellaria baicalensis* and *Schisandra chinensis*; Note: 1 (1): *Scutellaria baicalensis*, 1 (2): *Schisandra chinensis*, 1 (3): The control group



(1) (2) (3)

Fig. 3. Representative pictures of inhibitory zones of films incorporated with *Scutellaria baicalensis* or *Schisandra chinensis* extracts on the concentration of 5MIC compared to control against *E. coli*; Note: (1) *Scutellaria baicalensis*, (2) *Schisandra chinensis*, (3) the control group

Scutellaria baicalensis and *Schisandra chinensis* both showed significant antibacterial effects significantly on the concentration above their own MIC value ($p < 0.05$).

Fig. 3 showed the inhibitory zones of films incorporated with *Scutellaria baicalensis* or *Schisandra chinensis* extracts on the concentration of 5MIC compared to control against *E. coli*. The results were consistent with the filter paper assay. Compared with the control group, the inhibition zone of films incorporated with *Scutellaria baicalensis* on *E. coli* was larger and more obvious. Whereas, diameter of inhibition zone of *Schisandra chinensis* was smaller than that of *Scutellaria baicalensis*, but was still larger then control.

Film transparency effected by concentration of herbal extracts: Fig. 4 shows the influence of concentration of *Scutellaria baicalensis, Schisandra chinensis* on the films' absorbance. Film transparency reduces as soon as the absorbance of the film increases. The result indicated that the absorbance of the film increased by adding Chinese herbal extracts in a concentration dependent manner.

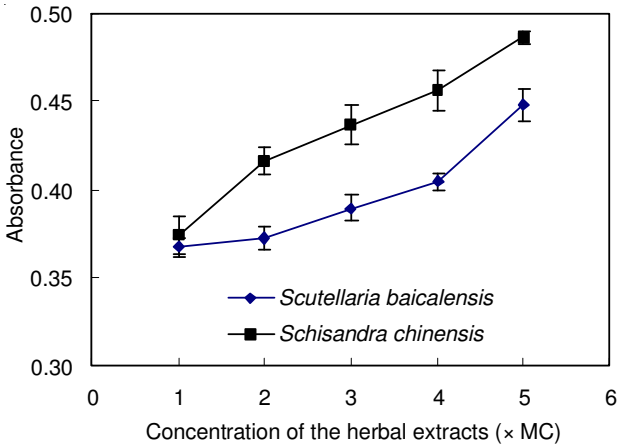


Fig. 4. The influence of concentration of *Scutellaria baicalensis*, *Schisandra chinensis* on the films' absorbance; Note: for *Scutellaria baicalensis*, the MIC value is 0.7813 mg/mL, for *Schisandra chinensis*, the value is 1.5625 mg/mL. The absorbance of the control group was 0.265

When concentration of *Scutellaria baicalensis* was up to the 5MIC value or concentration of *Schisandra chinensis* was increased to the 2MIC value, the absorbance of the films increased significantly, colour of the films deepened conspicuously. Significant decline in the transparency of the films would impact the sensory quality of the packaged foods. Decline in the transparency of the films containing *Schisandra chinensis* was due to the deeper colour of *Schisandra chinensis* extracts. As a result, on the premise of transparency of the films, concentration of *Scutellaria baicalensis* should below the 5MIC value, namely less then 3.9125 mg/mL; concentration of *Schisandra chinensis* should below the 2MIC value, which should be less then 3.1250 mg/mL.

Film extensibility: Fig. 5 shows the influence of concentration of *Scutellaria baicalensis, Schisandra chinensis* on the films' elongation. As shown in Fig. 5, as the concentration increased, elongation of the film decreased. The films containing *Scutellaria baicalensis* or *Schisandra chinensis* both showed this trend. When concentration of *Scutellaria baicalensis* was increased to the 4MIC value or concentration of *Schisandra chinensis* was increased to the 3MIC value, elongation of films showed an obvious downward trend. The may be because that association of hydrogen bonds between the gelatinized starch molecules was impeded by herbal extracts. Hydrogen bonds associated inadequately will make the mesh structure incomplete and untight, so that elongation of the films decreased. Therefore, in order to get good film extensibility concentration of *Scutellaria baicalensis* in the films should below 4MIC value and concentration of *Schisandra chinensis* in the films should below the 3MIC value.

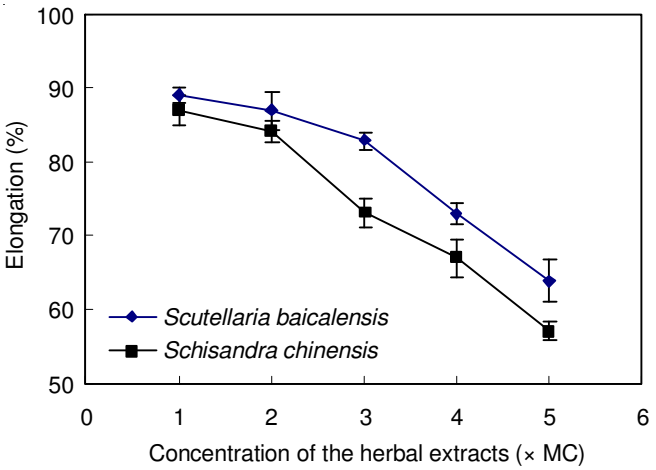


Fig. 5. The influence of concentration of *Scutellaria baicalensis*, *Schisandra chinensis* on the films' elongation; Note: for *Scutellaria baicalensis*, the MIC value is 0.7813 mg/mL, for *Schisandra chinensis*, the value is 1.5625 mg/mL. Elongation of the control group was 91 %

Moisture resistance of herbal incorporated films: Fig. 6 shows the influence of concentrations of *Scutellaria baicalensis* and *Schisandra chinensis* on the films' water vapour transmission rate. As the concentration of herbal extracts increased, water vapour transmission rate of the films increased, namely, moisture resistance of the films decreased. This trend was bound up with the change of elongation of the films. The reason was that, as the concentration increased, the association between the starch molecules declined, the spatial structure of the films became loose, which led to not only the decrease of elongation of the films, but also the increase of water vapour transmission rate. For films incorporated with *Scutellaria baicalensis* and *Schisandra chinensis*, water vapour transmission rates increased significantly when the concentration increased to the 4MIC value and 3MIC value respectively ($p < 0.05$). Therefore, for *Scutellaria baicalensis*, concentration in the films should below 4MIC value; for *Schisandra chinensis*, the reasonable value should below 3MIC value.

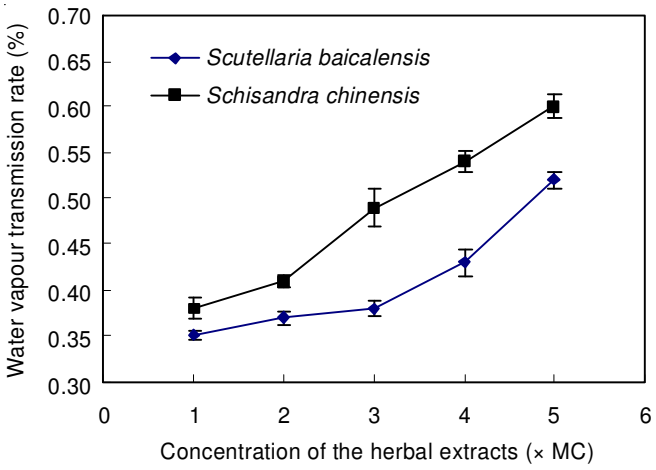


Fig. 6. The influence of concentration of *Scutellaria baicalensis*, *Schisandra chinensis* on the films' water vapour transmission rate; Note: for *Scutellaria baicalensis*, the MIC value is 0.7813 mg/mL, for *Schisandra chinensis*, the value is 1.5625 mg/mL. Water vapour transmission rate of the control group was 0.34 %

Conclusion

This study suggested that *Scutellaria baicalensis* and *Schisandra chinensis* were the two Chinese herbal medicines selected for the antibacterial test, whose antibacterial effects were notable. They all played inhibitory effects on *Escherichia coli* in a very low concentration. After adding such Chinese herbal medicines as bacteriostats, the starch films can not only play good antimicrobial effects, but also have few impacts on the mechanical properties of the films. In this test, MIC of herbal extracts was treated as a point of reference to be added in the films, thus further, good mechanical properties were ensured. The results showed encouraging prospect for the development of new edible antimicrobial films using natural Chinese medicine extract. Further studies will have to be performed in order to elucidate the mechanism of growth inhibition by films of *Scutellaria baicalensis* and *Schisandra chinensis*.

ACKNOWLEDGEMENTS

This work was supported by grant from the Fundamental Research Funds for the Central Universities (Grant No. HIT. NSRIF. 2013107). The authors acknowledged the financial support from the Fundamental Research Funds for the Central Universities (DL10BA16). Thanks are also due to professional teachers in College of Forestry for their valuable support.

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