Microbiostatic and Synergistic Actions of Extract of Green Walnut Peel

Li Yang, Yao Gu, Rusi Wen and Lizhu Zhou*

Guangxi Key Laboratory of Special Non-wood Forest Cultivation and Utilization, Guangxi Zhuang Autonomous Region Forestry Research Institute, Nanning 530002, China
Email: 125103877@qq.com

Abstract. The content of juglone in green walnut peel reach the highest in the period of July to August. Juglone, extracts of green walnut peel and tee tree oil were tested in antimicrobial experiments against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. The results demonstrated that extract from green walnut peel, and juglone were microbiostatic for all the tested strains. Distinct synergic effects were observed with mixtures of tee tree oil and extract of green walnut peel. Microbiostatic effects on the four microorganisms was highest with a 1 to 10 ratio of tee tree oil and green walnut peel extracts. This mixture was essentially nontoxic and showed good biocompatibility. There were no irritating reactions observed in the repeated dermal irritation test in rabbits, no allergic reactions in guinea pigs and a very low irritating effect in the vaginal mucosa of rabbits. The mixture is promising for use in medical antibacterial products.

Highlights
• Content of juglone in green walnut peel reach the highest in the period of July to August
• Green walnut peel extract was more microbiostatic than juglone.
• Tee tree oil and green walnut peel extract had synergic microbiostatic effects.
• The mixture of tee tree oil and green walnut peel extract was not toxic to animals.
• This mixture is promising for antibacterial products.

1. Introduction
Green walnut peel is the external layer, a thick green peel, of the immature walnut (Juglans sigillata Dode). As the walnut gradually matures, the green peel becomes black and, finally, sheds naturally[1][2]. The green peel contains many types of secondary metabolites with antibacterial activities[3]. Juglone is the primary toxic substance in green walnut peel[4] and walnut peel pigment is a stable dyeing agent[5]. Produce walnut 1 kg will remain walnut peel residue 1 kg. Most walnut peel residue is discarded as waste, walnut peel pigment can be dissolved in water and take a long time to be decomposed naturally. Juglone and walnut peel pigment can seriously pollute the environment by penetrating into ground water system[6]. Studies showed that juglone had significant antimicrobial[7][8], antitumor, antihypertensive[9][10] and enzyme inhibitory activities[11][12]. Tee tree oil were extracted from Melaleuca ahemifolia, has showed significant antimicrobial ability. The variation of the content of juglone in green walnut peel was studied. It was showed that the content of juglone reach the highest in the period of July to August. Microbiostatic and synergistic actions of extract of green walnut peel, juglone and mixture of tee tree oil and green walnut peel extract were detected. Distinct synergic effects were observed with mixtures of tee tree oil and extract of green walnut peel. The study
provided a theoretical basis for applications of green walnut peel extracts in antibacterial products.

2. Materials and Methods

2.1. Materials and Reagents
Green walnut peel of *Juglans sigillata* Dode was provided by Purapharm Co., Ltd. Tee tree oil (≥98%) was provided by Nanning Wan Yao Tang Co., Ltd. PBS containing 0.5% lecithin, 0.5% histidine and 1.0% Tween-80 was used as neutralizer and obtained from Shanghai ML Biotechnology Co., Ltd. Seventh to ninth generation *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* were from CGMCC Mice and white rabbits were obtained from Guangxi Medical University Laboratory Animal Center and white guinea pigs from Kunming Medical University Laboratory Animal Center. Juglone standards and other materials and reagents were purchased from Nanning Lantian Experiment Equipment Co., Ltd.

2.2. Equipment
HH-B11-600-S-II Electrothermal thermostatic incubator was from Shanghai Longyue Equipment Co., Ltd., LRH-250A biochemical incubator was from Shaoguan Taihong Medical Equipment Co., Ltd, JY10001 electronic scales were from Shanghai Shuangxu Electronics Co., Ltd. and HNW-S-6 thermostatic water bath from Shanghai Hannuo Instrument Co., Ltd.

2.3. Experimental Methods

2.3.1. Green Walnut Peel Extract Preparation. Walnuts of *Juglans sigillata* Dode were harvested every 15 days and the peel were separated by manual stripping in the period of July 1st to September 1st. Green walnut peel powder (100 g) was macerated with 250 mL 95% ethanol and stirred at 200 rpm at room temperature for 2 h. The extract was filtered and the residue re-extracted twice. The pooled extracts were stored in 4°C for content determination, and then concentrated under vacuum to 40 mL at 45 °C, then fully dried under vacuum to obtain 18 mg of an orange acicular crystal. The primary components of the crystals were juglone (5-hydroxy-1, 4-naphthalenedione), 4-naphthoquinone and 7-methoxy-3, 4-dihydro-l (2-H)–naphthalenone at relative contents of 96.420, 0.704 and 2.875 %, respectively.

2.3.2. Determination of Juglone Content by HPLC. Chromatography was performed on a C18 column (4.6 mm × 150 mm, 5 μm particle sizes) eluted with methanol-water (1:1) mobile phase, with the water phase adjusted to pH 4 with phosphoric acid. The flow rate was 0.8 mL/min, with UV detection at 250 nm and the column temperature was 30 °C [13] [14].

2.3.3. Preparation of Test Solutions. The juglone and green walnut peel extract were each diluted separately to 300 mg/L in 95% ethanol.

2.3.4. Preparation of Bacterial or Fungal Suspensions. The *E. coli*, *S. aureus*, and *P. aeruginosa* were activated on the slant side of bacterial medium at 30–35 °C for 24 h and *C. albicans* was activated on the slant side of Sabouraud’s agar medium at 20–25 °C for 48 h. Organisms were then diluted in normal saline to 105–110 cfu/mL (determined with a hemocytometer).

2.3.5. Determination of Microbial Growth Inhibition. Samples for testing were diluted with sterile water. Micro-organism suspensions (50 μL) (bacterial concentration at 1×10⁶ cfu/mL and fungal concentrations at 1×10⁵ cfu/mL) were spread onto each plate. A piece of filter paper was placed in the center of each plate and 30 μL sample solution was added. These plates were then cultured under the appropriate conditions (for bacteria, at 30–35 °C and for *C. albicans*, at 20–25 °C) and the diameters of the inhibition zones then measured. [15][16]

2.3.6. Determination of Microbicidal Actions. To each tube containing prepared sample, 2.5 mL in
medium, was added 0.5 mL microbial suspension (1×10^7 cfu/mL) and tubes were incubated for 24 h with shaking at 150 rpm at the appropriate culture temperatures. [17][18]The three bacteria were cultured at 30–35 °C and *C. albicans* at 20–25 °C. Next, 50-µL aliquots of incubation mixtures were spread onto individual plates. Plates were observed when microorganisms had fully grown in the control plates [19].

2.3.7. Determination of Biocompatibility. Acute oral toxicity, skin irritation, vaginal mucosa irritation and dermal allergic reaction tests were performed as described in ISO 10993-10 (Biological evaluation of medical devices. Part10: Tests for irritation and skin sensitization) and Technical Standard for Disinfection (2002) [20].

2.3.8. Effects of Mixtures of Green Walnut Peel with Tee Tree Oil. Tee tree oil was combined with extract from green walnut peel at ratios of 1:10, 1:20, 1:30, 1:40 and 1:50. Microbicidal effects were determined as described above for individual extracts.

2.3.9. Evaluation Criteria for Synergic Actions. CTC was calculated as described by Sun Yun-pei. The equation used was CTC = (ATI /TTI) *100. Synergic action is defined as when CTC>120, an additive effect when CTC is within the range of 80–120 and an antagonistic effect when CTC<80. [21]

3. Results and Discussion

3.1. Variation of Juglone from July 1st to September 1st

![Figure 1](image_url). Juglone content of Walnut varieties in sampling date

3.2. Microbiostatic and Microbicidal Effects of Green Walnut Peel Extract and Juglone

| Bacteria to be examined | Diameter of inhibition zone /mm |
|-------------------------|---------------------------------|
|                         | Control | extract of green walnut peel | juglone |
| *E. coli*               | 0.0     | 25.3±0.3                       | 20.5±0.2 |
| *P. aeruginosa*         | 0.0     | 20.3±0.2                       | 18.4±0.4 |
| *S. aureus*             | 0.0     | 25.1±0.4                       | 20.7±0.7 |
| *C. albicans*           | 0.0     | 19.3±0.5                       | 16.4±0.3 |

Microbiostatic and microbicidal effects of green walnut peel extract and juglone are shown in Table 1. The microbiostatic effects of the extract on the four microorganisms was superior to those of juglone. The effects of the two samples on *E. coli* and *S. aureus* were quite comparable.
Table 2. Lethal concentrations of two samples against various microorganisms

| Volume of sample /% | Extract of Green Walnut Peel | Juglone |
|---------------------|------------------------------|---------|
|                     | E.coli | P.aeruginosa | S.aureus | C.albicans | E.coli | P.aeruginosa | S.aureus | C.albicans |
| 50                  | -      | -            | -        | -          | -      | -            | -        | -          |
| 25                  | -      | -            | -        | -          | -      | -            | -        | -          |
| 10                  | -      | -            | -        | -          | -      | +            | -        | +          |
| 5                   | -      | -            | -        | -          | -      | +            | -        | +          |
| 1                   | +      | +            | +        | +          | +      | +            | +        | +          |

Note: In plating results, + means growing, - means non-growing.

Both the extract and juglone completely inhibited the growth of the four microorganisms at concentrations at or above 25% (Table 2). Moreover, the minimal bactericidal concentrations of the two samples against E. coli and S. aureus were 5%, indicating comparable bactericidal effects. The microbicidal effect of juglone against P. aeruginosa was greater than that against C. albicans. However, the minimal toxic concentration of juglone against these two organisms was higher than that of the extract.

3.3. Microbiostatic and Synergic Effects of Extract Mixtures

The microbiostatic effects produced by mixing extract of green walnut peel (A), tee tree oil (B) at various ratios are shown in Table 3. These data showed that synergic effects of the mixture of tee tree oil and green walnut peel extract were superior to those of tee tree oil and green walnut peel extract. Synergic effects were greatest for Coptidis rhizoma and green walnut peel extracts mixed at a ratio of 1 to 10. This mixture was used for subsequent biocompatibility testing.

Table 3. Inhibitory effects of various extract mixtures on four microorganisms

| Ratio of drugs | CTC (E. coli) | CTC (S. aureus) | CTC (P. aeruginosa) | CTC (C. albicans) |
|---------------|---------------|-----------------|---------------------|-------------------|
| B:A=1: 10     | 160.2546      | 181.4568        | 142.5423            | 175.4586          |
| B:A=1: 20     | 156.2456      | 177.1928        | 134.2165            | 173.5422          |
| B:A=1: 30     | 154.3784      | 172.4326        | 125.4982            | 168.5362          |
| B:A=1: 40     | 149.8546      | 168.843          | 122.2143            | 165.688           |
| B:A=1: 50     | 145.5684      | 162.4152        | 116.8406            | 160.268           |

3.4. Biocompatibility of the Mixture

3.4.1. Acute Oral Toxicity Test. There were no obvious toxic symptoms in test animals and no animals died after the mixture was administered. The acute oral ID50 of the mixture in mice was over 5000 mg/kg body weight. According to specifications for the acute evaluation of disinfectants, this essentially belongs to the “nontoxic” class. [20]

3.4.2. Skin Irritation Test. No irritating effects were observed with repeated skin irritation testing of the mixture in rabbits.

3.4.3. Vaginal Mucosa Irritation Test. In both treated and control groups, there was slight hyperemia observed in the vaginal mucosa of two rabbits. Mild edema was observed in the vaginal mucosa of two rabbits in the treated group. No edema was observed in the control group. The stimulation index of the vaginal mucosa was 0.33.
3.4.4. Dermal Allergic Reaction Test. As summarized in results of guinea pig skin allergic reaction test, no irritating reactions such as erythema or edema were observed in guinea pigs in the treated group. These were sensitized, then challenged by contact with the mixture for 24 or 48 h. Dermal sensitization in these animals was 0%. No abnormalities were observed on the skin of the guinea pigs in the negative control.

4. Conclusions

Microbiostatic effects on the four microorganisms reach the highest with a 1 to 10 ratio of tee tree oil and green walnut peel extracts. This mixture was essentially nontoxic and showed good biocompatibility. There were no irritating reactions observed in the repeated dermal irritation test in rabbits, no allergic reactions in guinea pigs and a very low irritating effect in the vaginal mucosa of rabbits. The mixture is promising for use in food preservation and medical antibacterial products.

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6. References

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