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Comparison of the Effects of *Juglans nigra* Green Husk and Clotrimazole on *Candida albicans* in Rats

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Abstract

Background: Black walnut (*Juglans nigra*) is known to have antimicrobial and antifungal effects *in vitro*.
Objectives: This study aimed to compare the effects of extracts derived from green-husked walnut with clotrimazole against *Candida albicans* in female rats.
Methods: In this study, 35 female Wistar rats were randomly assigned to 5 groups. A total of 4 groups of rats were infected vaginally with *C. albicans* and 1 group was not infected (negative control). The 4 infected groups received the following treatments: 2 groups received vaginal creams containing 2% or 4% of *J. nigra* extracts, respectively, 1 group received 1% clotrimazole, and 1 group did not receive any treatment (positive control). All rats received treatments for 2 weeks.
Results: The mean number of colony forming unit (CFUs) before intervention was 308.2 $\pm$ 8.73 and 219 $\pm$ 13.4 in the 2% and 4% *J. nigra* group, 312.7 $\pm$ 28.32 in the clotrimazole group, 233.85 $\pm$ 8.15 in the positive control, and 0 in the negative control group ($P < 0.001$). After 1 week, the number of CFUs significantly decreased to 71.1 $\pm$ 3.9 in the 2% *J. nigra*, 0 in both 4% *J. nigra* and clotrimazole groups, 230.7 $\pm$ 9.86 in the positive control, and 0 in the negative control group ($P < 0.001$). After 2 weeks, no CFUs were detected in any of the infected groups except for the positive control group.
Conclusions: Vaginal creams containing 4% *J. nigra* significantly eliminated *C. albicans* in female rats after 1 week and its effect was similar to that of clotrimazole.

Keywords: Clotrimazole, Vaginal Cream, *Candida albicans*, *Juglans nigra*

1. Background

Candida albicans is a type of fungi that causes candidiasis in humans (1). In recent years, the prevalence of infections caused by *C. albicans* has increased due to antimicrobial resistance and limitations in the number of antifungal agents (2). *Candida* yeast may cause complications such as dysphagia, esophagitis, and urinary tract candidiasis, which can spread to other organs (3). *Candida* is a part of the normal vaginal flora in 20-50% of women, and therefore they do not display any signs or symptoms (4).

The prevalence of vaginal candidiasis has been reported differently in different parts of the world. A study in the United States showed that *C. albicans* is the most prevalent fungal species, and there is a relationship among immunosuppression, antibiotics use, and emerging *C. albicans* infection (5). Anoter study in Iran showed that the prevalence of vaginal candidiasis among women of the reproductive age was 67%, including symptomatic and asymptomatic women (6). Furthermore, Fatahinia et al., in Ahvaz, showed that 32% of married women had a Candida strain in direct microscopic examination and culture medium and *C. albicans* was the most common species (7).

Various medications such as echinocandins, caspofungin, micafungin, anidulafungin, and fluconazole are recommended for the treatment of vaginal candidiasis (8). Antifungal medications may cause some side effects in the body, including abdominal pain, diarrhea, headache, rash, indigestion, allergic reaction, and severe skin reaction (9). Some natural products are commonly used to treat *C. albicans*. Probiotic supplements (10), organic apple cider vinegar (11) and organic garlic (12) are examples of natural products used to prevent and treat *Candida* vaginitis.

Juglans nigra (black walnut) is a deciduous tree that

is also referred to as eastern black walnut. This tree is cultivated in places such as the South Dakota in North America. The active component in black walnut is juglone, which can inhibit specific enzymes that are required for metabolism. Juglone is toxic to many insect herbivores and has antioxidant effects (13). The anti-cancer, anti-bacterial, antiviral, and antiparasitic effects of juglone have been previously demonstrated (14). Juglone can also tighten the skin, relieve irritation (15), and improve cardiovascular status (16). It also has antifungal and antimicrobial activity (17). More recently, Wang et al., further demonstrated the antimicrobial effects of juglone (18). Other studies have found positive effects of juglone on *Pseudomonas*, *Burkholderia cepacia*, *Staphylococcus aureus*, and *Bacillus subtilis* (19, 20). Juglone also showed antifungal activity comparable to that of other medications such as griseofulvin, clotrimazole, and tolnaftate (19). Furthermore, the antifungal activity of juglone has been demonstrated in other studies (21, 22).

2. Objectives

There is not enough information regarding the effectiveness of *J. nigra* green extract on *C. albicans*. Therefore, the main objective of this study was to compare the effects of *J. nigra* to those of clotrimazole on *C. albicans* in rats.

3. Methods

3.1. Ethics Statement

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (Ref. No.: IR.AJUMS. REC.1394.616). We carried out a double-blind randomized controlled trial to compare the effects of *J. nigra* to those of clotrimazole on *C. albicans* in female rats.

3.2. Preparation of C. albicans Suspension

Candida albicans was isolated from a patient with *Candida* vaginitis (according to the DNA Data Bank of Japan, Accession number: LC201976) and used to infect rats. The cells were cultured in yeast extract peptone dextrose (YEPD) medium at 28 - 30°C. After 48 hours, the culture was centrifuged and the yeast cells were washed 3 times with sterile phosphate-buffered saline (PBS). The OD was measured[H1] using a spectrophotometer and a suspension of 1×10^8 cells/mL was prepared (1×10^7 cells in 100 μL).

3.3. Creating Vaginitis in Rats

Female Wistar rats (n = 35), with the weight of 150 - 200 grams, were purchased from the animal center of Ahvaz Jundishapur University of Medical Sciences, and were randomly divided into 5 groups (7 per group). Each group was housed in a separate cage with a sterile straw under standard temperature and light (12 hours light-dark cycle, temperature 20 - 25, and humidity 50% - 60%) for 1 week. Vaginal sample were obtained using sterile swabs dipped in PBS to ensure the absence of infection. Samples were cultured on Sabouraud dextrose agar, with and without chloramphenicol, to ensure that there was no primary infection in the rats. *Candida albicans* were cultured in YEPD medium. The cultured yeasts were washed with PBS 3 times after 18 hours. This mixture was centrifuged and 5×10^7 - 1×10^8 yeast cells were suspended in PBS. To suppress the immune systems of the rats, 2 doses of estradiol benzoate were injected (0.5 - 0.6 mg) subcutaneously in the lower abdominal peritoneum over 3 days. Immediately after the second injection of estradiol, 100 μL of yeast suspension was injected into the vagina and the rats were kept upside down for approximately 1 minute to ensure the inoculum.

3.4. Drug Development

Fresh Iranian walnuts with green husks (Herbarium code: JPS017113) were gathered (this type of walnut with husk is available during the fall). Green walnut husks were shade- dried and then transferred to the pharmacognosy lab at the Pharmacy School of Ahvaz Jundishapur University of Medical Sciences. The dried green husks were powdered and an extract was prepared using the maceration method. The powdered green husk was combined with 80% ethanol and the resulting solution was maintained at room temperature (27 - 32°C) for 72 hours with occasional stirring. The mixture was filtered through Whatman paper using a vacuum flask. The extract was dried using a vacuum rotary evaporator at 45°C then freeze-dried to a fine bulk powder. The powder was kept refrigerated below 4°C.

Juglans nigra green husk cream was prepared by adding the extract to a suitable cream base (emulsion of water in oil) at concentrations of 2% and 4% (w/w). In the pharmacy, 1% of clotrimazole cream was prepared using the same base. All final products had the same appearance and an approved color was used to maintain uniformity. Microbiological and physicochemical tests (uniformity, physical stability, distribution, and acidity) were performed on the prepared formulations. 2% and 4% *J. nigra* and clotrimazole creams were placed in similar canisters and were encoded by a blinded third party. All creams had the same appearance and texture. Creams were applied and samples were prepared by a blinded researcher.

3.5. Grouping

Rats were randomized into 5 groups:

1- A control group infected with *C. albicans* but not treated (positive control)

2- An un-infected control group, also not given treatment (negative control)

3- An infected group that received treatment with 2% *J. nigra* extract.

4- An infected group that received treatment with 4% *J. nigra* extract.

5- An infected group that received treatment with 1% clotrimazole.

3.6. Assessment of Infection

Four days after the first inoculation, a vaginal sample was obtained from all rats. In addition to a direct smear, the samples were cultured on Sabouraud dextrose agar with chloramphenicol (SC) (QUELAB, UK) to check for the presence of infection. An additional dose of yeast was administered to the vagina if infection was not found. According to most references, the number of yeast present in the sample is determined by the criterion of 7 - 8 colonies per swab (23). In our study, the number of colonies grown at the plate surface was counted with a colony counter machine, and more colonies of yeast were observed in a direct sample.

3.7. Treatment

After ensuring that the rats were infected with *C. albicans*, treatment was initiated in the different groups. A total of 2 groups received either 2% or 4% of *J. nigra* extract, 1 group received clotrimazole cream, and 1 group remained without treatment. One week and 2 weeks after treatment, a vaginal sample from all 5 groups obtained using a sterile swab was cultured in SC medium at 35 - 37°C. The colonies formed were counted after 48 hours.

3.8. Statistical Analysis

ANOVA was used to compare means of CFUs of *C. albicans* before and after intervention, and LSD post-hoc tests were utilized for detecting differences between groups. All analyses were performed using SPSS version 22. $P < 0.05$ was considered to indicate a statistical significance.

4. Results

After exposure to *C. albicans* all rats, except for the negative control, showed colonies of *C. albicans*. The mean number of CFUs, before intervention, was 308.2 ± 8.73 in the 2% *J. nigra* group, 219 ± 13.4 in the 4% *J. nigra* group, 312.7 ± 28.32 in the clotrimazole group, 233.85 ± 8.15 in the positive control group, and 0 in the negative control group ($P < 0.001$). After 1 week, the mean number of colony forming units significantly decreased to 71.1 ± 3.9 in the 2% *J. nigra* group and 0 in both the 4% *J. nigra* as well as clotrimazole groups. The number in the positive control group remained unchanged (230.7 ± 9.86) and no CFUs were detected in the negative control group ($P < 0.001$). After 2 weeks, the mean number of colony forming units was 0 in all the groups, except in the positive control group ($P < 0.001$). The 4% *J. nigra* extract could eradicate colony forming unites after 1 week of treatment, whereas the 2% *J. nigra* extract significantly reduced the mean number of CFUs to 71.14 ± 3.97 ($P < 0.001$). There were no CFUs detected in either *J. nigra* extract treated groups after 2 weeks (Figure 1).

5. Discussion

The main objective of this study was to compare the effects of 2% and 4% *J. nigra* extract with those of clotrimazole on *C. albicans* in rats. Our results showed that the 4% *J. nigra* extract could eliminate *C. albicans* colony forming units after 1 week of treatment, which is similar to the effects of clotrimazole. The primary active component of walnut green husk is juglone. All parts of the walnut tree and its fruit have anti-oxidant effects, anticancer activity, and can boost the immune system (24). In this study we used the green husks of black walnuts.

Other studies have shown that juglone has antimicrobial and antifungal effects (25, 26). Grover et al., assessed the effect of juglone on planktonic and sessile *C. albicans* cells. Their results showed that at a concentration of 160 μg/mL of juglone could significantly reduce the virality of colonies of *C. albicans* as assessed by various tests such as XTT, AFM, confocal analysis, and SEM techniques (21). These results are in agreement with our findings. Moreover, in line with our study, Rodrigues et al., found that the green husk of *J. nigra* has an antifungal effect and can be used as an alternative for fluconazole against *C. krusei*, recurrent vaginal candidiasis, and dermatophyte infections of the nails as well as skin (27). Salamat et al., in their study, found that the extract of J. *regia* could prevent growth of *C. albicans* in fraction of 337 mg/mL (28), which is in line with our findings.

To the best of our knowledge, this is the first time that the effects of *J. nigra* green husk have been compared to the effects of clotrimazole on *C. albicans* in rats. This study has a limitation that should be mentioned. We tested clotrimazole against sensitive and resistance strains of *C. albicans* and then compared the activity of *J. nigra* extract on resistance and sensitive strains, which may have affected the results.

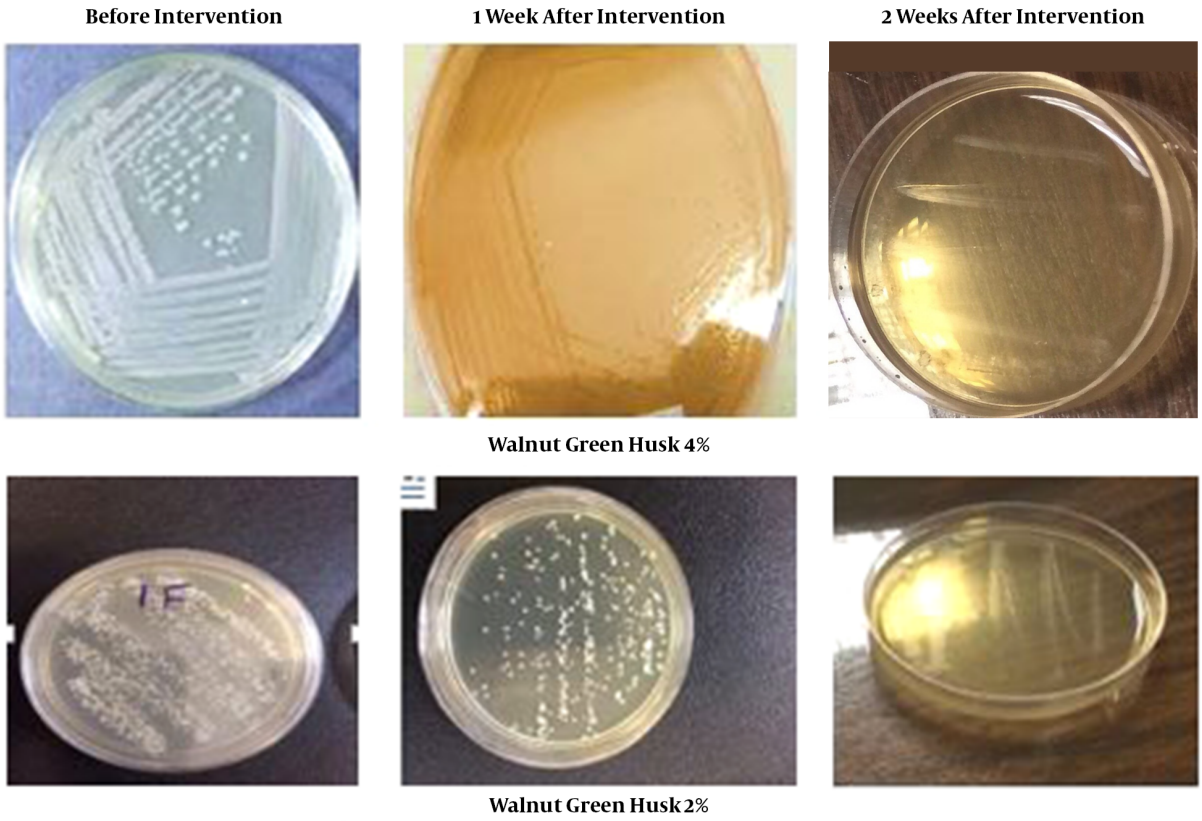

Figure 1. Comparison of *J. nigra* 2% and 4% After 1 Week and 2 Weeks

6. Conclusion

This study showed that *J. nigra* green husk in the form of a vaginal cream is effective against *C. albicans* in female rats. The 4% *J. nigra* extract was more effective than the 2% extract, and its effect was similar to that of clotrimazole. Based on our findings, further studies using *J. nigra* green husk vaginal creams in humans are recommended.

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Footnotes

Authors' Contribution: Masumeh Yaralizadeh, Parvin Abedi, and Foroogh Namjoyan were involved in the study design. Masumeh Yaralizadeh and Mahnaz Fatahinia were involved in data collection. Masumeh Yaralizadeh and Foroogh Namjoyan were responsible for drug development. Parvin Abedi and Masumeh Yaralizadeh were responsible for data interpretation. Parvin Abedi was responsible for writing and finalizing manuscript in English.

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