In-vitro antimicrobial efficacy of certain herbal seeds essential oils against important poultry microbes

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ABSTRACT

Two in-vitro experiments were conducted to evaluate the antibacterial, antifungal and antiviral properties of essential oil of herbal seeds. In-vitro antimicrobial properties of essential oils of phytobiotics was determined by disc diffusion method against Escherichia coli, Staphylococcus aureus, Pasteurella multocida and Salmonella typhi and two fungi namely Aspergillus fumigatus and Candida albicans. Thyme oil had statistically similar or significantly (P<0.05) higher inhibition zone against all the bacterial and fungal species compared to standard antibiotic, chloramphenicol or antifungal drug, nystatin. Fenugreek oil was not found to be active against microbes compared to control. Fennel and cumin oils had statistically similar or significantly (P<0.05) higher inhibition zone against all microbes except Staphylococcus aureus and Salmonella typhi compared to control. Antiviral activity of essential oils was determined by anti-NDV assay against New Castle disease virus (LaSota). The results indicated that essential oils of herbal seeds do not possess antiviral activity against NDV LaSota virus at 1mg/ml concentration.

Key words: Cumin, Essential oils, Fennel, Fenugreek, In-vitro antimicrobial, Thyme.

INTRODUCTION

Bacterial resistance to antimicrobial drugs has become an issue of increased public concern and scientific interest during the last couple of decades. This resulted from a growing concern that the use of antimicrobial drugs in veterinary medicine and animal husbandry may compromise human health if resistant bacteria develop in animals and are transferred to humans via the food chain or the environment. Antimicrobials are being used in poultry production to treat or prevent diseases and also to promote growth in livestock and poultry grown for meat.

Antibiotic as growth promoter in poultry industry has been seriously criticized by governmental policy makers and consumers as a potential harm to human health (William and Losa, 2001). It has been reported that the use of antibiotics as a growth promoter in chicken has caused some unwanted results in humans (Botsoglou and Flotours, 2001). Therefore, most antibacterial performance promoters have been banned due not only to cross-resistance but also to multiple resistances (Neu, 1992). Recognizing this, the European Union has totally banned the use of growth promoting antibiotics in animal feed. This has consequences in the international trade of poultry meat as well, because many countries adopt stringent Sanitary and Phyto-sanitary measures on imports of animal and plant products.

Herbs or products including plant extracts, essential oils or the components of the essential oils hold promise as alternatives to antibiotics (Ocak et al., 2008). Substantial attention has been paid to medicinal herbs as replacements for antibiotic growth promoters (Ibrahim et al., 2005). There is evidence suggesting that herbs, spices, and various plant extracts have appetizing, digestion-stimulating and antimicrobial properties (Sharifi et al., 2013).

Phytobiotics are the preparations of vegetative origin containing chemical constituents, which favourably affect the microflora of the digestive system. Several studies indicated that these feed additives could be used in poultry ration as antifungal, antibacterial and antioxidant compounds (Khosravi et al., 2008). Even though phytobiotics provides potential alternative to the antibiotic usage, more research is needed to understand their mechanism of action. The aim of this study is to shed light on the antimicrobial properties of essential oils of thyme, fenugreek, fennel and cumin by conducting in-vitro studies.
**MATERIALS AND METHODS**

*In-vitro* antimicrobial laboratory work was performed using bacterial strains of *Escherichia coli* (MTCC 10239), *Staphylococcus aureus* (MTCC 3160), *Pasteurella multocida* (MTCC 1160) and *Salmonella typhi* (MTCC 3224), and fungal strains of *Aspergillus fumigatus* (MTCC 6482) and *Candida albicans* (MTCC 3018) obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The essential oils of thyme (*Thymus vulgaris*), fenugreek (*Trigonella foenum graecum*), fennel (*Foeniculum vulgare*) and cumin (*Cuminum cyminum*) of commercial origin were used in the experiment to test antimicrobial properties.

**Experiment 1. In-vitro antibacterial and antifungal assays**

Antimicrobial susceptibility test was carried out by Kirby Bauer disc-diffusion method (Daouk et al., 1995). Briefly, bacteria and fungi were cultured overnight in nutrient broth (Hi-media) and in Sabouraud Dextrose Broth (SDB) (Hi-media) respectively at 37±0.1°C for 24h and then adjusted with sterile saline to a concentration of 1.0×10⁶CFU/ml. The essential oils of thyme, fenugreek, fennel and cumin were individually applied onto empty sterilized discs of 6mm diameter at the rate of 10µl per disc. In addition, chloramphenicol discs as standard antibiotic and nystatin discs as standard antifungal agent were used as positive control. Fifteen ml of Mueller Hinton Agar (MHA-Hi-media) and Sabouraud Dextrose Agar (SDA-Himedia) (sterilized in a flask and cooled to 45 - 50°C) were homogenously distributed onto the sterilized petri dishes. Then the sterile discs with essential oils were placed on agar plates which had previously been inoculated with the above organisms. The agar plates were left at 4°C for 2h and then incubated at 37±0.1°C for 24h. After incubation, the diameters of inhibition zones were measured in mm by using Hi-media calliper. All assays were applied in duplicates.

**Experiment 2. In-vitro antiviral assay**

The concentration of thyme, fenugreek, fennel and cumin oils were adjusted to 1mg/ml in sterile phosphate buffered saline. The virus strain used in the assay was NDV LaSota. Viral dilutions of 10⁴ to 10⁹ were used with three eggs per treatment and pure virus was used as a control. One milliliter of each compound was combined with 1ml of virus at each dilution, vortexed and allowed to sit for 10 min. After the waiting period, 0.2ml of each virus-compound solution was injected into the chorio-allantoic sac of embryos. The eggs were candled after 24 hours and mortality was recorded. After 48 hours, the eggs were placed at 4°C overnight to kill the remaining live embryos for haemagglutination analysis. Allantoic fluid was harvested from embryonated eggs that survived 48 hours. A few drops of fluid was mixed with chicken red blood cells and evaluated for the presence of haemagglutination indicating viral infection. (Reed and Muench, 1938). After incubation, reading was taken as the reciprocal of highest dilution showing haemagglutination. The virus titre was then calculated to determine log₁₀ reduction of virus within the embryos.

**Statistical Analysis**

All the statistical analyses were performed using SPSS software (version 7.5) as per Snedecor and Cochran (1994). The data is presented as mean ± SE and the observed differences were considered statistically significant (P < 0.05 or P < 0.01) or not significant (P > 0.05). Duncan’s post-hoc analysis was performed to test the homogeneity of mean values.

**RESULTS AND DISCUSSION**

*In vitro* antibacterial and antifungal activities of essential oils: Antibacterial activity of essential oils of herbal seeds in comparison to chloramphenicol and antifungal activity of these oils in comparison to nystatin as assessed by zone of inhibition are presented in Table 1. Significant difference in antibacterial property among different essential oils and chloramphenicol was observed against *Escherichia coli* (P<0.05) *Salmonella typhimurium* (P<0.01), *Staphylococcus aureus* (P<0.01) and *Pasteurella multocida* (P<0.05) and antifungal property among different essential oils and nystatin was evident against *Aspergillus fumigatus* (P<0.01) and *Candida albicans* (P<0.01). Thyme oil has shown

**Table 1:** Antimicrobial activity of four essential oils of phytobiotics-containing herbal seeds along with an antibiotic and an antifungal agent by disc diffusion method (mean ±SE.).

<table>
<thead>
<tr>
<th>Tests</th>
<th>Zone of Inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Thyme</td>
<td>25.0±7.0</td>
</tr>
<tr>
<td>Fenugreek</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Fennel</td>
<td>13.0±3.0</td>
</tr>
<tr>
<td>Cumin</td>
<td>10.0±0.0</td>
</tr>
<tr>
<td>Chloramphenicol (antibiotic control)</td>
<td>22.5±0.5</td>
</tr>
<tr>
<td>Nystatin (anti-fungal control)</td>
<td>-</td>
</tr>
<tr>
<td>F value</td>
<td>8.717*</td>
</tr>
</tbody>
</table>

1 Mean values within the same column sharing a common superscript letter are not statistically different:

Non-significant (P > 0.05), * P < 0.05, ** P < 0.01, *' Not tested.
statistically similar inhibition zone against *Escherichia coli* and *Salmonella typhimurium* compared to control but significantly (P<0.05) higher inhibition zone against *Staphylococcus aureus*, *Pasteurella multocida*, *Aspergillus fumigatus* and *Candida albicans* compared to their respective controls. Fenugreek oil was found to have no activity against the microbes compared to control. Fennel and cumin oils had comparable results to that of chloramphenicol against *Escherichia coli* and *Pasteurella multocida*. Cumin oil was found to show significantly (P<0.05) higher inhibition zones against *Aspergillus fumigatus* and *Candida albicans*; while, fennel against *Aspergillus fumigatus* only.

**Anti-NDV assay:** As shown in Table 2, thyme essential oil alone showed numerical reduction of viral titre compared to control. But inoculation of essential oils of fenugreek, fennel and cumin in embryonated chicken eggs resulted into death of embryos within 48 hours.

**Table 2:** Virus titre results for four essential oils and control from anti-NDV assay

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Virus Titre</th>
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<tbody>
<tr>
<td>Control</td>
<td>10^7.00/ml</td>
</tr>
<tr>
<td>Thyme</td>
<td>10^7.2/ml</td>
</tr>
<tr>
<td>Fenugreek</td>
<td>D</td>
</tr>
<tr>
<td>Fennel</td>
<td>D</td>
</tr>
<tr>
<td>Cumin</td>
<td>D</td>
</tr>
</tbody>
</table>

D – indicates death of embryos

Among the essential oils of herbal seeds used as phytobiotics in the study, thyme revealed highest antibacterial activity with the inhibition zone matching that of the standard antibiotic, chloramphenicol against *Escherichia coli* and *Salmonella typhimurium* but had significantly wider inhibition zone against *Staphylococcus aureus* and *Pasteurella multocida*. In terms of antifungal property, in addition to thyme, cumin also found to exhibit significantly higher inhibition zone than the standard antifungal agent, nystatin against *Aspergillus fumigatus* and *Candida albicans*; while, fennel oil showed significantly wider inhibition zone only against *Aspergillus fumigatus*. The results indicated that the wide spectrum antimicrobial activity of thyme could be due to the presence of thymol and carvacrol in it which are the main antimicrobial compounds as earlier reported (Nickavar *et al.*, 2005). A comparison of our results on thyme against *Escherichia coli* with the published reports (Singh *et al.*, 2007; Kazemi *et al.*, 2012) showed favorable agreement. Our results indicate that fenugreek possesses very poor antibacterial and antifungal properties with zero resistance against all the microorganisms except an insignificantly low resistance against *Staphylococcus aureus*. The fennel and cumin oils revealed comparable or slightly lower antibacterial activity against the pathogens studied. Results on antibacterial activity of fennel against *Escherichia coli* in the current study disagreed with the results of Khafagi *et al.* (2000). On comparison, the present findings revealed high level of antifungal activity of fennel against *Aspergillus fumigatus*, whereas, the literature showed both positive (Barkat and Bouguerra, 2012) and negative (Abed, 2007; Bansod and Rai, 2008) results. Cumin revealed similar or slightly lower anti-bacterial property against various bacteria but possessed very strong antifungal property when compared with chloramphenicol and nystatin respectively. A comparison of present findings with the published reports revealed both kinds of positive (Toroglu, 2011) and negative (Rahman *et al.*, 2010) antibacterial properties against *Escherichia coli* and low antifungal activity against *Aspergillus fumigatus* (Bansod and Rai, 2008). Our study provides new information on antimicrobial properties of phytobiotic containing seeds as this study was extended to bacterial and fungal species other than much studied *Escherichia coli* and *Aspergillus fumigatus*.

Antiviral effects of essential oils in embryonated chicken eggs were inconclusive, although greater than 2 log<sub>10</sub> reduction in virus was seen for 1mg/ml thyme essential oil the difference between test and positive control was not statistically significant. On the basis of the results, it could be assumed that the phytobiotic containing seeds cannot be used to control New Castle disease viral multiplication. The death of embryos occurred in all other treatment groups could be due to physical injury or irritant action of essential oils. Pennesi (2010) also reported that at lowest virus dilutions (10<sup>-4</sup> and 10<sup>-5</sup>) all of the fluids were positive for hemagglutination.

**CONCLUSION**

The results of *in-vitro* studies lead the authors to hypothesize that the antibacterial and antifungal properties of essential oils of herbal seeds when used as phytobiotics can benefit the poultry in maintaining gut health for better growth rate. In summary, among all essential oils as phytobiotics, thyme demonstrated overall good antibacterial, antifungal and antiviral properties followed by cumin and fennel. This study reveals the possibility to replace the antibiotic growth promoters with herbal seed essential oils as phytobiotics in poultry farming, especially that of thyme and cumin.

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