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Activity of extracts of *Synzium aromaticum* against microbes of veterinary importance

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ABSTRACT

The study was conducted with the objective to evaluate the antibacterial as well as antiviral activity of the aqueous and alcoholic extracts and oil of the flower buds of clove against the microbes responsible for causing diseases in poultry and livestock. The aqueous and alcoholic extracts of dried flower buds of clove were obtained by extraction in soxhlet apparatus using water and ethanol (95%) as solvents respectively. Oil was obtained from the clove flower buds by steam distillation. Both the extracts and oil were assessed for their antibacterial activity against *Streptococcus zooepidemicus, Salmonella* Gallinarum, *Escherichia coli, Staphylococcus aureus* and *Pasteurella multocida* while antiviral activity was tested against equine herpes virus – I (EHV I) and infectious bursal disease (IBD) virus. The extracts as well as oil were effective against the bacteria tested with zone of inhibition ranging from 11.67±0.33 to 24.00±0.00 mm. The Minimum inhibitory concentration (MIC) values for the extracts ranged from 0.75 to 4.00 mg/ml. Clove was also found to have potent antiviral activity against EHV–1 up to $10^4 TCID_{50}$/ml, while IBD virus was found to be resistant to the clove extracts.

Key words: Antimicrobial activity, *Escherichia coli, Pasteurella multocida, Salmonella* gallinarum, *Staphylococcus aureus, Streptococcus zooepidemicus* and *Synzium aromaticum*

Indians have been traditional users of plant derived medicines both directly and as an integral constituent of plethora of packages and practices of indigenous medicine. These plants and their extracts are being used in the pharmaceutical preparations of modern medicine, veterinary and in agriculture (Iyengar 1985, Chopra *et al.* 1992, Zafar 1999). The antimicrobials obtained from plants are of much therapeutic potential and are effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Kokoska *et al.* 2002).

Synzium aromaticum (clove, family *Myrtaceae*) is an aromatic plant, grown extensively in India and is known for its antiseptic, carminative, stimulant, analgesic, antispasmodic properties (Prajapati *et al.* 2003). Clove is being used by the Indian Ayurvedic healers since ancient times to treat various ailments. To have suitable therapeutic control of the diseases of known importance of the livestock, there is a need of developing some plant based derivatives which protect the poultry and livestock against the diseases as well as maintain or enhance their production performance. With this aim, we selected *Synzium aromaticum* to assess its antibacterial and antiviral activity against pathogens of animal health importance. The pathogens selected for the present study included bacteria, viz. *Streptococcus zooepidemicus*, *Salmonella* Gallinarum, *Escherichia coli*, *Staphylococcus aureus* and *Pasteurella multocida* and virus (Equine Herpes Virus 1 and infectious bursal disease virus) which are of veterinary importance as they cause huge economic losses to the country due to morbidity and mortality in livestock as well as poultry and thereby leading to decrease in productivity.

MATERIALS AND METHODS

Plant material and preparation of extracts

The dried flower buds of clove were purchased from the local market.The dried flower buds were ground to powder form, soaked for 48 hr in distilled water and 95% (v/v) ethanol for aqueous extraction and alcoholic extraction, respectively and extracted as per procedure of Virmani *et al.* (2008). Clove oil was obtained from the clove buds by steam distillation. Per cent yield of various extracts from flower buds of clove

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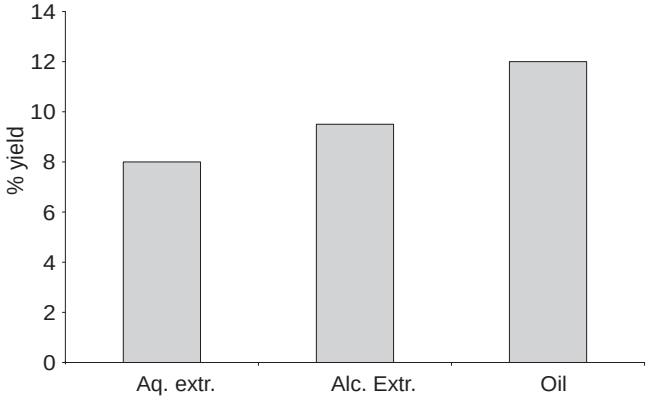

Fig. 1 Per cent yield of extractive from *Synzium aromaticum* flower bud.

is given in Fig. 1.

Antibacterial sensitivity assay and determination of minimum inhibitory concentration (*MIC*)

An inoculum of each of the bacterial strains (single colony) was suspended in 5 ml of broth (tryptose soy broth for *S. zooepidemicus*) and incubated at 37°C for 18 hr. The antibacterial activity was tested by the disc diffusion assay (Bauer *et al.* 1966). 0.1 ml of inoculum (10^5 CFU/ml) was spread on sterile Mueller Hinton plates (blood agar for *S. zooepidemicus* and *P. multocida*) and sterile paper discs were placed on the inoculated surface. The discs were impregnated with 15µl of each of the extract at 10% concentration, kept at room temperature for absorption of extract in the medium and then incubated at 37°C in the incubator for 24 hr. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone as per the procedure described by Kim *et al.*, 1995. The experiment was carried out in triplicate and the mean of the diameter of inhibition zones was calculated. Four commercially available antibiotic drugs, viz. gentamycin, ciprofloxacin, cephotaxime and cefixime were used simultaneously as control.

For determination of MIC, 1 ml of broth medium was taken into 10 test tubes for each bacteria. Different concentrations of plant extracts ranging from 0.25 to 10.00 mg/ml concentration were incorporated into the broth and the tubes were then inoculated with 0.1 ml of inoculum of respective bacteria (10^5 CFU/ml) and kept at 37°C for 24 hr. The test tube containing the lowest concentration of extract which showed reduction in turbidity, when compared with control was regarded as MIC of that extract. The optical density of each tube was measured in the spectrophotometer (at 620 nm).

Antiviral activity

Propagation of equine herpes virus 1 in RK–13 monolayer: RK–13 cells, grown as monolayer in MEM (minimum essential medium) containing 10% foetal calf serum, were infected with the virus ($10^{4.2}$ $TCID_{50}$/ml) at 1: 1000 multiplicity of infection (m.o.i) and maintained in MEM containing 2% foetal calf serum and incubated at 37°C with 5% CO_2 tension in an incubator (Virmani *et al.* 2004)

Cytotoxicity assay: Each extract was separately diluted 1: 10 with the maintenance medium and filter sterilized through 0.22µ millipore filter. Further dilutions (1: 20, 1: 40, 1: 60, 1: 80 and 1: 160) were made from the stock. The cytotoxicity assays were carried out using 100µl of cell suspension containing 10^4 cells in each well of the microtitre plate. The highest concentration of each extract found to be non-toxic to the cells was recorded and used further for antiviral assay.

Antiviral assay: The non-toxic concentrations of the extracts were checked for antiviral property by cytopathic effect inhibition assay in cell monolayers infected with serial dilutions of virus (10^1 to 10^4 $TCID_{50}$/ml). The cultures were overlaid with maintenance medium containing plant extracts at dilutions mentioned in Table 2 and incubated at 37°C for 5 days. Every 24 hr, the observations were made and cytopathic effects recorded. The antiviral activity was determined by the inhibition of CPE when compared with controls.

For IBD virus, Chicken Embryo fibroblast (CEF) cells were freshly prepared and used for propagation of the virus, cytotoxicity assay and antiviral assay. The assays were performed as per the protocol mentioned for Equine Herpes Virus 1. However, the medium used for CEF was M 199 and the extracts were diluted in ratio of 1: 50, 1: 100, 1: 150 and 1: 200. Rest of the procedure was similar as for EHV 1.

Phytochemical analysis: The qualitative phytochemical analysis of the alcoholic extract of flower bud of clove as well as oil of clove bud was undertaken using standard qualitative methods as described by other workers (Vogel 1958, Kapoor *et al.* 1969, Scalbert 1991 and Chukwurah 1997). The plant extracts were screened for the presence of biologically active compounds including glycosides, phenolics, alkaloids, tannins, flavonoids and saponins.`

RESULTS AND DISCUSSION

The extracts of *Synzium aromaticum* (clove) were found to be active against the bacteria tested (Table 1). The aqueous and alcoholic extracts of dried flower buds gave the inhibition zones of diameter ranging from 11.67±0.33 mm to 21.00±0.57 mm, while clove oil showed inhibition zones up to 24.00±0.00 mm for *S. zooepidemicus*. Clove oil was observed to have most potent antibacterial activity with MIC of 0.75 to 2.00 mg/ml against different bacteria (Fig. 2). Our findings corroborate with the findings of other workers who also reported clove to have high antimicrobial activity against different bacteria (Deans *et al.* 1995, Ahmad *et al.* 1999 and Nascimento Gislene *et al.* 2000). Arora and Kaur (1999) tested the spices against human pathogenic bacteria and yeasts and found that aqueous extract of clove possesses antimicrobial activity against *Shigella flexnerii* and *Candida*

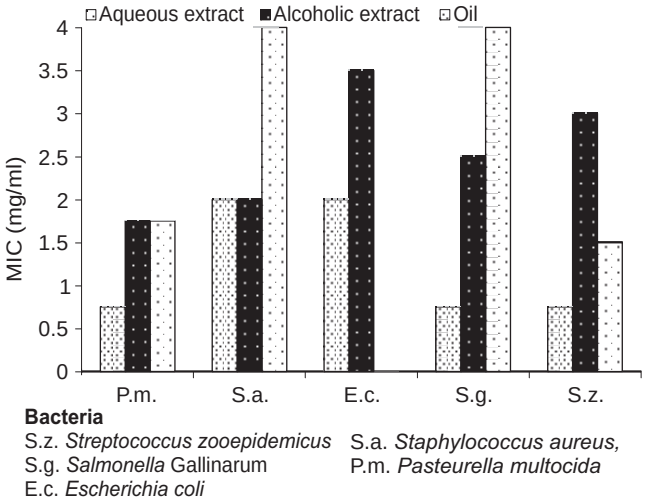

Fig. 2. Minimum inhibitory concentration (MIC) of the extracts (mg/ml) against bacteria.

species resistant to the antibiotics. Dormans and Deans (2000) observed that the volatile oils of clove exhibited inhibitory effects against all the organisms under test mainly *Escherichia coli, Salmonella pullorum, Staphylococcus aureus, Bacillus subtilis, Clostridium sporogenes,* etc. and were reported to be equally effective against Gram + and Gram – organisms. Kim *et al.* (1995) studied the antibacterial activity of some essential oil components including eugenol from clove oil against the food borne pathogens at 5, 10, 15 and 20% levels using paper disc method and observed MIC for eugenol to be 1000 µg/ml for all the bacteria tested. Eugenol showed dose related increase in the zone of inhibition against all pathogens. Suresh *et al.* (1992) found eugenol to be sensitive at a concentration of 5 g/assay against *E. coli, Enterobacter sakazaki* and *Klebsiella pneumoniae* which were resistant to antibiotics like ampicillin, erythromycin and sulfamethizole. The antibacterial activities of the standard antibiotics, viz. gentamycin, ciprofloxacin, cephotaxime and cefixime are mentioned in Table 1.

The extracts were screened for antiviral activity against EHV 1 and IBD virus as well. The cytotoxic concentration of all the 3 extracts (aqueous, alcoholic and oil) was assayed and their cytotoxic levels were recorded. Alcoholic and aqueous extracts of *Synzium aromaticum* were found to be cytotoxic for both RK–13 cells and Chicken embryo fibroblasts at the concentration of 5 mg/ml while the oil extract was found to be cytotoxic at 1250 mg/ml (Table 2). The extracts of clove (both aqueous and alcoholic) as well as clove oil showed potent antiviral activity against EHV 1 up to the level of $10^4 TCID_{50}$/ml, while none of the extracts had any antiviral activity against any of the dilutions of IBD virus. Benecia and Courreges (2000) reported Eugenol, an active component of clove oil to possess antiviral activity against Herpes Simplex virus 1 (HSV 1) and Herpes Simplex virus 2 (HSV 2) viruses and showed inhibitory concentration 50% values for the anti HSV effect to be 25.6 µg/ml and 16.2 µg/ml respectively for HSV 1 and HSV 2. The higher dose requirement in present study might be due to the crude preparations used as against the purified eugenol used by Benecia and Courreges (2000). Vijayan *et al.* (2004) observed different parts of medicinal plants of Nilgiris and found *Hypericums myorense*, *Hypericums. hookerianum* and *Usnea complanta* to be having antiviral activity against HSV 1 which is a virus belonging to Alpha herpes virinae similar to Equine Herpes Virus 1.

Phytochemical analysis of the extracts depicted that the alcoholic extract of flower bud of clove was having glycosides, flavonoids, phenolics and tannins while only flavonoids and phenolics were present in oil of the clove bud. The results are in agreement with the reports of other workers (Chopra *et al.* 1992, Bruneton 1995 and Ahmad and Beg 2001). Sakagami *et al.* (1995) have suggested that a major part of antiviral activity in polyphenols probably derives from their direct inactivation of the virus and or from the inhibition of the virus binding to the cells. They also noted the inhibitory effect of polyphenols on the viral replication enzymes. Kurokawa *et al.* (1998) extracted the phenolic compound eugeniin (ellagitannin) from *Genum japonicum* and *Synzium aromaticum* and found it to be having anti-HSV activity. Kurokawa *et al.* (1998) and Liu *et al.* (1999) reported that one of the major target sites of inhibitory

Table 1. Inhibition zone diameter (mm) of extracts against bacteria (mean ± SE)

Plant extract/antibiotics	S.z.	S.g.	E.c.	S.a.	P.m.
Aqueous extract	19.00±0.57	21.00±0.57	–	11.67±0.33	20.67±0.67
Alcoholic extract	15.00±0.00	15.67±0.33	14.33±0.33	17.33±0.67	16.33±0.88
Oil	24.00±0.00	23.33±0.33	16.33±0.33	17.33±0.67	21.83±1.05
Gentamycin	12	24	30	22	24
Cephotaxime	26	22	32	31	26
Ciprofloxacin	21	36	30	32	30
Cefixime	16	24	26	12	24
- means no activity					

S.z., *Streptococcus zooepidemicus*; S.g., *Salmonella* Gallinarum; E.c., *Escherichia coli*; S.a. *Staphylococcus aureus*; P.m., *Pasteurella multocida*

Table 2. Cytotoxicity assay and antiviral activity of plant extracts against EHV-1 virus

Plant extract	Cytotoxicity (µg/ml)	Concentration of extract used for antiviral activity (µg/ml)	CPE inhibition assay			
			10 TCID50*	102 TCID50	103 TCID50	104 TCID50
Aqueous extract	5000	4000	3/3	3/3	3/3	3/3
Alcoholic extract	5000	4000	3/3	3/3	3/3	2/3
Oil	1250	1000	3/3	3/3	3/3	3/3

*Each dilution of virus was used in triplicate.Numerator, number of wells showing no CPE, Denominator, total number of wells per dilution of virus.

action of eugeniin is viral DNA synthesis. Meerbach *et al.* (2001) while working with 17 polyhydroxycarboxylates, a phenolic compound, against herpes simplex virus type 1 (HSV 1), HSV 2, thymidine kinase deficient HSV 1, human cytomegalovirus (HCMV) and HIV 1 and HIV 2 have attributed the antiviral properties of phenolic compounds to the presence of the carboxylic groups which cause inhibition of the virus adsorption. Phenolic compounds which possess C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils such as Eugenol and often cited as antimicrobials (Cowan 1999).

This study is an evaluation of the antibacterial and antiviral activity of *Synzium aromaticum* against some important selected pathogens (bacteria and viruses) of veterinary importance, which are known to cause huge losses to livestock and poultry industry. *Synzium aromaticum* was found to be effective against all the selected bacteria while antiviral properties were found against EHV 1 virus only. Active phytochemicals from *Synzium aromaticum* in alone and in synergism with drugs especially such as Acyclovir against Herpes virus need to be studied further along with the *in vivo* trials of the extracts of the plant.

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