African Journal of Microbiology Research Vol. 5(18), pp. 2765-2772, 16 September, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR11.593
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Antimicrobial activity of crude venom extracts in honeybees (*Apis cerana, Apis dorsata, Apis florea)* tested against selected pathogens

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Accepted 15 July, 2011

Antimicrobial activity of crude venom extracts from different honeybee species was tested against selected pathogens. Toxicity of bee venom is known to man since ages, which varies from mild inflammations to death. Bee venom is synthesized in the venom glands of worker bees and queen and is stored in the venom sac. In the present study the toxic potentialities of honeybee venom pertaining to different honeybee species *Apis cerana, Apis dorsata* and *Apis florea* was carried out *in vitro* on selected species of bacteria and fungi. The selected bacteria and fungal species were *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Xanthomonas subtilis, Proteus vulgaris, Salmonella typhimurium* and *Candida albicans*. They were collected from St. John Medical College, Bangalore. The antimicrobial activity of different species of *Apis* bee venom (ABV) was studied by Disc Diffusion Assay. Minimal inhibitory concentration (MIC) was determined using Broth dilution method at lowest dilution (0.5 mg/ml). Bacterial growth was assessed by the measurement of inhibitory zone. The order of susceptibility of the pathogens against the ABV recorded was: *A. cerana > Apis dorsata> Apis florea.* The results showed that, ABV has significant antimicrobial effects and could be a potential alternative antibiotic.

Key words: Bee venom, *Apis* species, broth dilution, inhibitory zone, bacteria, fungi.

INTRODUCTION

Honeybees are the earliest known social insects to man. They have survived alongside their ever-changing environment for 120 million years. They are recognized and appreciated as the single most important insect pollinators and thus, increase the productivity of food plants on earth (Free, 1993; Jyothi, 1994; Chaudary et al., 2001; Sharma and Gupta, 2001). Besides pollination, honeybees provide honey, bee wax, royal jelly, pollen, venom and propolis. The venom gland of worker bee is located in posterior portion of the abdomen, between the worker's rectum and ovaries (Owen and Bridges, 1984).

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The two glands (Dufours and Venom gland) associated with sting apparatus of the worker produce venom. The venom gland is a thin long, distally bifurcated integumentary gland with cuticular lining. It consists of a secretary filamentous region, connected to a reservoir at its proximal portion, in which the venom is stored (Kerr and Lello, 1962). The small flat cells also bearing canaliculi form the distal region of the reservoir where their products contributes to venom composition (Lello, 1971). The workers sting only once, which leads to their death.

Venom contains 88% water. At least 18 pharmacologically active components have been described so far; including various enzymes, peptides and amines (Dotimas and Hider, 1987). The glucose, fructose and

phospholipids contents of venom are similar to those in bee's blood. Venom from *Apis* species is similar, but even the venoms from various races within each species are slightly different from each other. Bee venom is haemorrhagic and contains apamine, melittin, phospholipase, hyaluronidase. These oppose the inhibiting action of the nervous system and also stimulate the heart and adrenal glands. Sulphur is the main element in inducing the release of cortisol from the adrenal glands which protects the body against infections. The venom also contains mineral substances, volatile-organic acids, formic acid and some antibiotics. Venom is one of the products of honeybee, which is an important component in the pharmaceutical industry. Use of naturally available substances as medicines, in Asia represents a long history of human interactions with the environment. The medicinal value of these substances lies in some chemicals that produce a definite physiological action on the human body. The venom production is usually complete within two weeks and then glands start to degenerate in the adults. Not only has the age affected the venom composition but also seasonal factors like availability of food sources etc. A newly emerged bee has very little venom content, but the amount gradually accumulates with age, to about 0.3 mg in a 15 day old *A. mellifera* worker bee (Owen and Bridges, 1976), after the age of 18 days no additional venom is produced. Subsequently, the weight of the venom in the venom sac remains unchanged (Cruz- landim et al., 1966, 1967).

The study of social Hymenoptera (bees, wasps, and ants) venom proteins is of great interest, since these venoms can trigger serious allergenic reactions in humans. The allergenic reactions of Hymenoptera venoms are caused mostly by low molecular weight compounds, which can result in pain, local inflammation (Hider, 1981, 1988; Hoffman, 1977), itching, and irritation as immediate responses that after some hours are attenuated (Golden et al., 1989). Melittin is the main compound responsible for most of these reactions, and it is present in several bee venoms.

Bee venom has interesting pharmacological properties (Dong. et al., 2007) and is used in the treatment of various health conditions such as arthritis, rheumatism (Putz et al., 2006), pain (Kim et al., 2003), cancerous tumors (Russell et al., 2004) and skin diseases.

MATERIALS AND METHODS

Collection of bee venom

Venom, from forager honeybees of three *Apis* species descended from naturally mated queens were used to study its antimicrobial activity. These bees, derived from a pool of 3 colonies were collected from the Apiary of the Centre for Apiculture Studies, Department of Zoology, Bangalore University, Jnana Bharathi Campus, Bangalore. They were immobilized by quick freezing at -20°C. The venom reservoirs were extracted at 4°C by dissecting the stinging apparatus and stored at -20°C until required. Venom sacs were re-suspended in deionized water (MilliQ) and extracts of whole bee venom (WBV) were made by reservoir disruption under rapid defrosting and light pressure by a glass rod. These samples were centrifuged at 10,000 g at 4°C for 5 min, and the supernatants were used as protein and enzyme sources and then lyophilized (Biotran, Speed vaccum concentrator, Model: Ecospin 3180C). Lyophilized Bee venom was dissolved in MilliQ water and filtered using 0.22 mμ syringe filter to further estimations.

Collection of bacterial isolates

The test clinical control isolates used in the present study were collected from Research Laboratories of St. John's Medical College and Hospital, Bangalore. These clinical isolates were identified based on the standard microbiological techniques (Chees brough, 1998) and drug susceptibility test for each clinical isolate was done following the standard agar Disc Diffusion method (Bauer et al., 1966). The microbial strains, *P. aeruginosa, C. albicans, E. coli, K. pneumoniae, X. subtilis, P. vulgaris and S. typhimurium* were used to determine the antimicrobial activity of venom from different *Apis* species.

Pure culturing of microbial strains

The collected clinical control microbial strains were maintained in the laboratory on Nutrient Agar (Hi-Media) by Slant–Streak technique for further pure cultures (Mackie Mc Cartney, 1999).

The nutrient Agar Hi-Medium composed of 5 g peptone, 2 g Beef extract, 5 g Sodium chloride and 20 g Agar-Agar was dissolved in one liter of double distilled water and pH was maintained at 7.0 ± 0.2. The mixture of contents were later transferred into a sterile conical flask and plugged with cotton for air tightening. The conical flask with contents was autoclaved and the flasks were cooled and stored at 5 to 10°C.

Under sterile conditions, the contents when needed were dissolved on heating mantle and 10 ml of medium was poured into sterile test tubes and cooled in Laminar Air Flow by placing in slanting position.

The solidified medium was streaked with specific bacterial strains using sterile inoculation loop. The slants with strains were incubated in Bacterial incubator at 35 to 37°C for a period of 24 to 48 h. The slants with strains were stored at 4°C.

Maintenance of pure bacterial culture suspension in Nutrient Broth

Under aseptic conditions, pure colonies of Bacterial isolates from slants were picked with an inoculating loop and suspended in 3 to 4 ml of nutrient broth in sterile test tubes and incubated for 24 h at 37°C. The contents were transferred into sterile conical flask and plugged with cotton (Andargrchewmulu et al., 2004).

Investigation of antibacterial potency of venom

The nutrient agar Disc Diffusion method (Molan, 1992; Sommeijer and Francke, 1995; Perumalsamy et al., 2006) was employed to test the antibacterial activity of venom of different species. The sterile control bacterial nutrient broth was further used to prepare the culture suspension in order to set an inoculum's density of 100 µl: $0.1A_{600}$ culture containing 3.2×10^8 colony forming unit (cfu /ml). Further, 20 µl was spread on to 20 ml of sterile agar plates by using a sterile cotton swab. The surface of the medium was allowed to

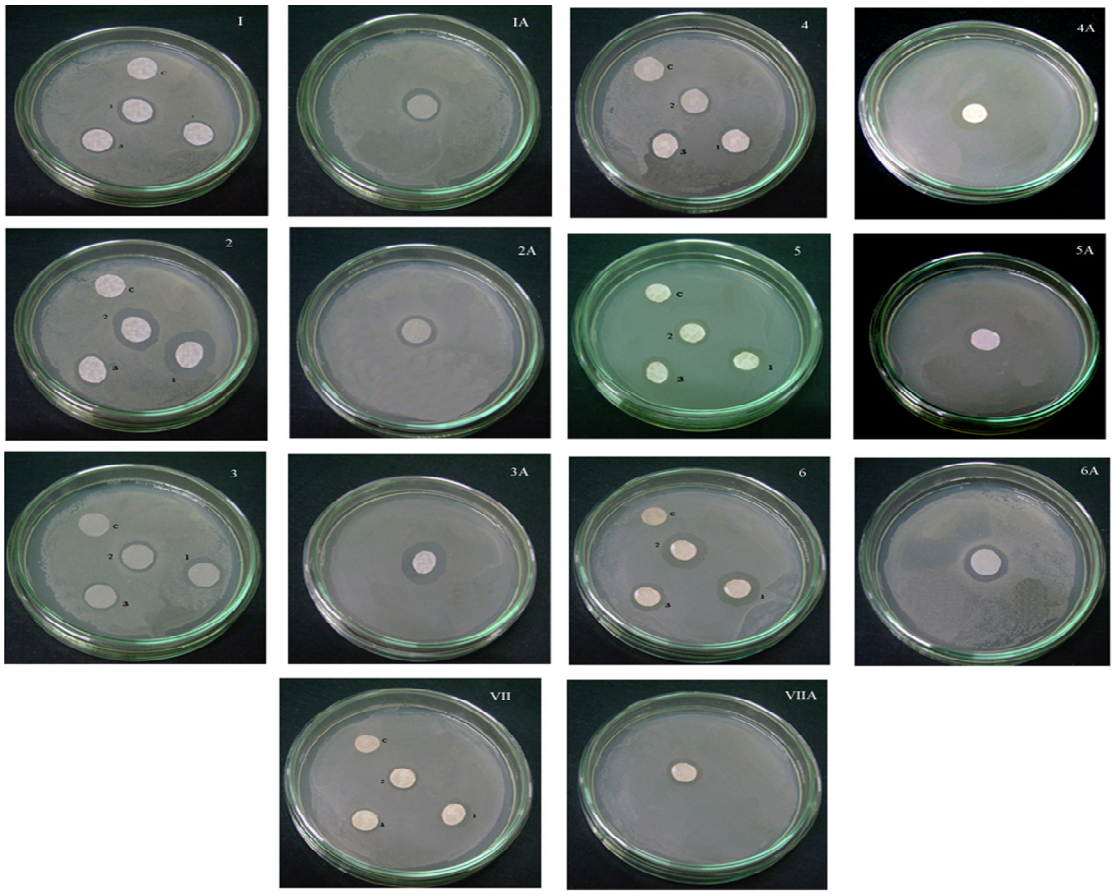

Figure 1. Inhibition zones of venom from different *Apis* species (1-3) with Control (C) against selected microbial strains (I-VII) along with their Ampicillin Standards (IA-VIIA). C: Control, 1: *Apis cerana*, 2: *Apis dorsata*, 3: *Apis florea*.
I and IA: *Escherichia coli*; II and IIA: *Pseudomonas aeruginosa*; III and IIIA: *Klebsiella pneumonia*; IV and IVA: *Proteas vulgaris*; V and VA: *Xanthomonas camperstris*; VI and VIA: *Solmonella typhimurium*; VII and VIIA: *Candida albicans.*

dry for about 3 min and sterile paper discs (10 mm in diameter) were placed on them. 20 µl of various concentration of proteins that is, 10, 20, 30 and 25 µg of venom sample was added per disc. Disc containing 20 µl of MilliQ water served as control. The bacterial plates were incubated at 37°C for 24 h and fungal plate at 37°C for 48 h. The diameters of inhibition zones were measured. The results were compared with standard Ampicillin. Inhibition effects were measured in terms of zone of inhibition around the paper disc with scale. The zone of inhibition was measured from the centre of the disc till the edge of the inhibition zone. This was repeated at four directions in the inhibition zone and the mean radius was calculated. The area of zone of inhibition was calculated using the formula:

$A_{in} = \pi R^2 - \pi r^2$

Where A_{in} = inhibition zone
π = 22/7, R = radius of the inhibition zone and r = radius of the paper disc.
πr^2 in this case was the area of the paper disc that was deducted from the area of the zone of inhibition to obtain the mean area of inhibition produced due to the action of bee venom against respective pathogens. Various clear zones, more than 10 mm in diameter, that were observed at different concentrations of venom for varied bacterial strains were measured in millimeter and the average of the inhibition zones were recorded. In this experiment as a positive control, we used standard antibiotic Ampicillin disc (10 µg/ml each).

Protein estimation

The protein content in the honeybee venom samples was estimated by using Bovin serum albumin as standard at 610 nm (Lowrys et al., 1951).

Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE) was performed using 12% polyacrylamide at 120 V and 20 mA. Venom samples were dissolved in 20 µl of doubled distilled water, 5 µl of sample buffer (0.001% mercaptoethanol, 75% of 0.313 M Tris- HCl and 10% glycerol) and 0.001% bromophenol blue (pH 6.8). The samples were boiled for two minutes, shaken in vortex for 30 s and loaded onto the gel. The gels were stained in 0.25% Coomassie Brilliant Blue R-250 solution and destained with 30% methanol and 10% acetic acid to reveal proteins.

Table 1. Antimicrobial activity of crude honeybee venom extract from different species of honeybee workers tested against the selected microorganisms showing zone of inhibition in diameter (mm^2) N=5.

Concentration (µg)	*Escherichia coli* (mm^2)	*Pseudomonas aeruginosa* (mm^2)	*Klebsiella pneumoniae* (mm^2)	*Proteus vulgaris* (mm^2)	*Xanthomonas subtilis* (mm^2)	*Salmonella typhimurium* (mm^2)	*Candida albicans* (mm^2)
			Apis cerana				
10	10.50 ± 0.25	12.50 ± 0.25	19.00 ± 0.25	12.00 ± 0.50	14.00 ± 0.25	18.00 ± 0.25	13.00 ± 0.25
20	12.00 ± 0.20	13.00 ± 0.20	20.00 ± 0.14	13.50 ± 0.10	16.00 ± 0.32	19.65 ± 0.25	14.00 ± 0.20
25	12.50 ± 0.20	14.00 ± 0.29	21.00 ± .20	12.75 ± 0.20	17.50 ± 0.12	20.25 ± 0.10	15.50 ± 0.20
30	13.50 ± 0.12	15.00 ± 0.16	22.50 ± 0.18	15.00 ± 0.12	18.50 ± 0.12	21.30 ± 0.10	17.00 ± 0.31
			Apis dorsata				
10	11.50 ± 0.21	10.50 ± 0.20	16.00 ± 0.18	10.75 ± 0.16	13.00 ± 0.29	17.00 ± 0.20	10.50 ± 0.20
20	12.00 ± 0.33	11.00 ± 0.25	17.00 ± 0.43	11.50 ± 0.15	14.00 ± 0.25	18.50 ± 0.25	11.50 ± 0.13
25	13.00 ± 0.25	12.00 ± 0.02	18.00 ± 0.15	12.50 ± 0.25	14.50 ± 0.15	19.5 ± 0.25	13.50 ± 0.25
30	14.00 ± 0.25	13.50 ± 0.25	19.00 ± 0.25	13.50 ± 0.25	16.50 ± 0.15	21.50 ± 0.25	14.00 ± 0.14
			Apis florae				
10	11.00 ± 0.25	0.00 ± 0.00	13.50 ± 0.15	0.00 ± 0.00	11.00 ± 0.05	14.25 ± 0.25	0.00 ± 0.00
20	11.50 ± 0.13	10.50 ± 0.14	14.00 ± 0.25	13.50 ± 0.15	13.00 ± 0.25	16.00 ± 0.25	11.00 ± 0.07
25	12.00 ± 0.23	11.00 ± 0.03	15.00 ± 0.12	14.25 ± 0.00	14.00 ± 0.25	15.25 ± 0.10	11.50 ± 0.16
30	13.00 ± 0.25	12.00 ± 0.18	16.00 ± 0.25	15.00 ± 0.25	16.00 ± 0.25	17.75 ± 0.25	12.00 ± 0.25

Two molecular weights were estimated using standard molecular. Weight marker (1KDa) was obtained from Genei pvt Limited, Bangalore, India.

RESULTS AND DISCUSSION

In the present investigation six Gram-negative bacteria and fungal strains were used to investigate the antimicrobial properties of venom from different species of honeybee by using Disc Diffusion Assay. The sensitivity of seven microbial strains was tested against the venom of three *Apis* species (*A. cerana, Apis dorsata, Apis florea*). Venom of all three species showed antimicrobial activity against selected microbial strains (Figure 1). The antimicrobial spectrum of venom of three *Apis* species in terms of inhibition zone (IZ) is shown in Table 1. The deionised water (MilliQ) had no inhibitory effect on tested microbial strains.

The venom from *A. cerana* showed high antimicrobial activity and exhibited larger zone of inhibition among three species (Table 1, Figure 2). The venom concentration of 30 µg, showed highest inhibition zone against *K. pneumoniae* (22.50 ± 0.18) followed by *S. typhimurium* (21.30 ± 0.10), *X. subtilis* (18.50 ± 0.12), *C. albicans* (17.00 ± 0.31), *P. aeruginosa* (15.00 ± 0.16), *P. vulgaris* (15.00 ± 0.12) and *E. coli* (13.50 ± 0.12).

The venom of *A. dorsata* showed at the 30 µg concentration of venom showed highest inhibition zone against *S. typhimurium* (21.50 ± 0.25) followed by *K. pneumoniae* (19.00 ± 0.25), *X. subtilis* (16.50 ± 0.15), *E. coli* (14.00 ± 0.25), *C. albicans* (14.00 ± 0.14), *P. aeruginosa* (13.50 ± 0.25) and *P. vulgaris* (13.50 ± 0.25). *P. aeruginosa*, *P. vulgaris, C. albicans* and *E.*

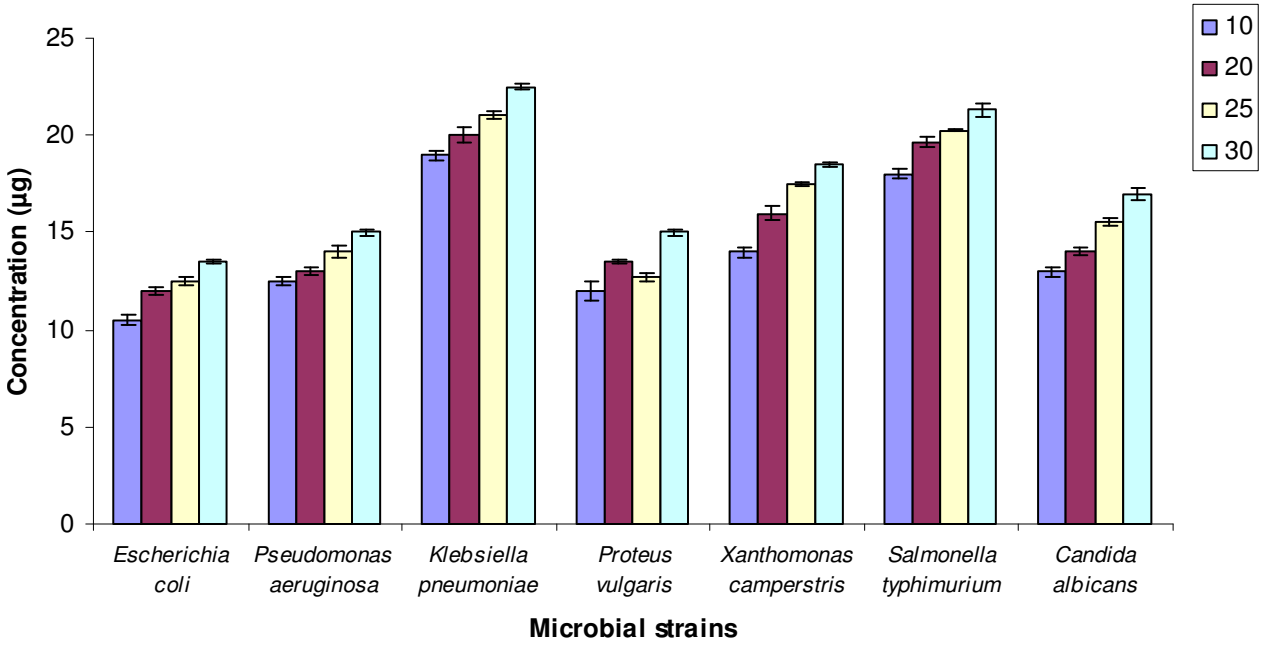

Figure 2. Inhibitory zones at different venom concentration of *Apis cerana* tested against the selected microbial strains.

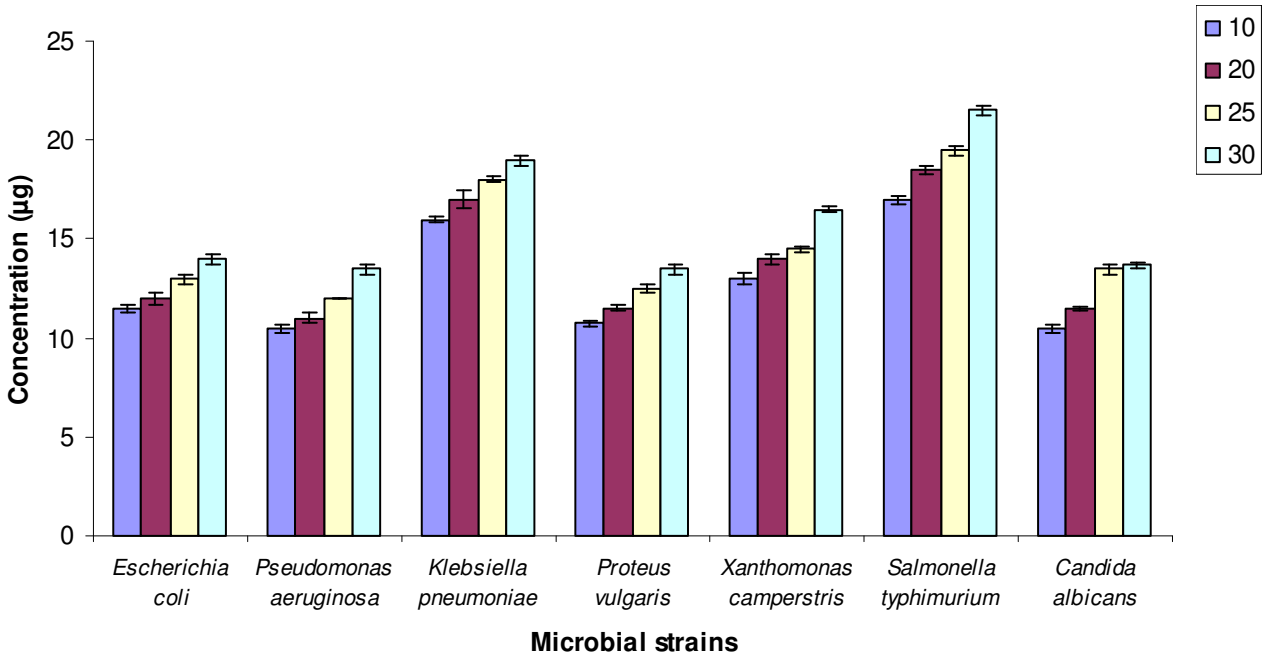

Figure 3. Inhibitory zones at different venom concentration of *Apis dorsata* tested against selected microbial strains.

coli showed almost similar effects at the same concentration (Table 1, Figure 3).

A. florea showed minimum antimicrobial activity when compared to *A. cerana* and *Apis dorsata*. The concentration of 30 µg showed highest inhibition zone against *S. typhimurium* (17.75 ± 0.25) followed by *X. subtilis* (16.00 ± 0.25), *K. pneumoniae* (16.00 ± 0.25), *E coli* (13.00 ± 0.25), *P. aeruginosa* (12.00 ± 0.18) and *C. albicans* (12.00 ± 0.25). *C. albicans, P. aeruginosa, K. pneumoniae,* and *X. subtilis* showed similar effects at 30 µg venom concentration (Table 1, Figure 4).

The present examination also showed that the

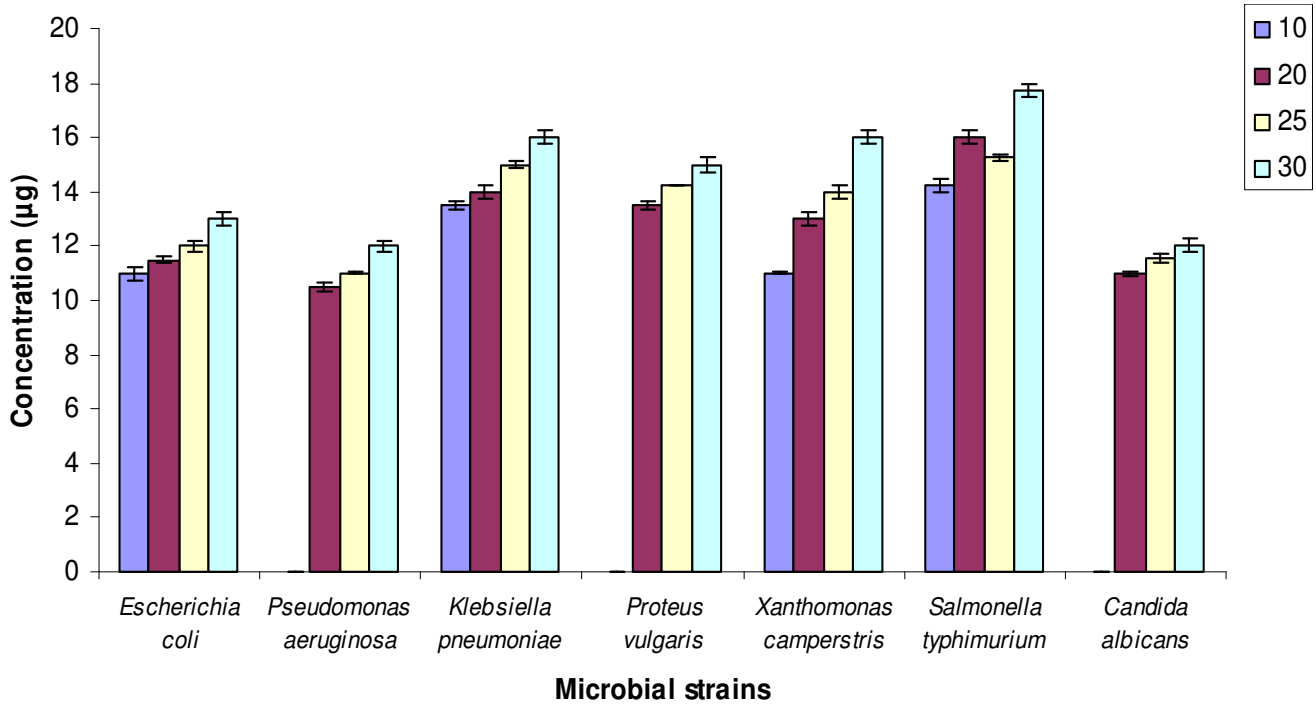

Figure 4. Inhibitory zones at different venom concentration of *Apis florea* tested against selected microbial strains.

Table 2. The zones of inhibition diameter (mm^2) of Ampicillin standard (10 µg/ml).

Names of microbial strains	Zone of inhibition (mm^2)
Pseudomonas aeruginosa	15.00
Klebsiella pneumoniae	22.00
Escherichia Coli	13.00
Xanthomonas subtilis	18.00
Proteus vulgaris	17.00
Salmonella typhimurium	19.00
Candida albicans	16.00

antimicrobial activity of venom of three *Apis* species at the concentration of 25µg was almost equal to the effect of Ampicillin at 10µg (Table 2, Figure 5). The SDS PAGE gel also confirmed the constituent proteins from the lyophilized crude venom such as phospholipase A2, Melittin and some of the small peptides with molecular weight ranges of 35, 34, 30, 27, 31, 16, 15, 11, 9, 8, 7, 6, 5 and 4 KDa were common in all the three *Apis* species (Figure 6).

The venom of *A. cerana* inhibits the growth of fungi; *C. albicans* as compared to venom of *A. dorsata* and *Apis florea*. Similarly, the venom of *A. cerana* inhibited the growth of bacteria such as *E coli, P. vulgaris, P. aeruginosa, X. subtilis, K. pneumoniae* and *S. typhimurium* to a maximum extent as compared to the venom of *A. dorsata* and *Apis florea.*

Since, reliable research information on the antimicrobial properties of Indian honeybee venom is not available; such a study has been carried out for the first time to know the functional status of bee venom especially on growth inhibition of fungi and bacteria. Benton et al. (1963) determined the inhibition rate on the spores of *Bacillus subtilis* from the venom of *A. mellifera*. Similarly Hancock et al. (1995) reported that Melittin in the venom of *A. mellifera* had wide spectrum of biological effects including antimicrobial activity. The toxicity of *A. cerana* venom has been reported to be twice as high has that of *Apis mellifera* (Benton and Morse, 1968). The non selective cytotoxic venom protein Melittin, was the most abundant polypeptide in the venom of *A. mellifera* (Habermann et al., 1967). This antimicrobial activity and protein profile of honeybee venoms reported herein will

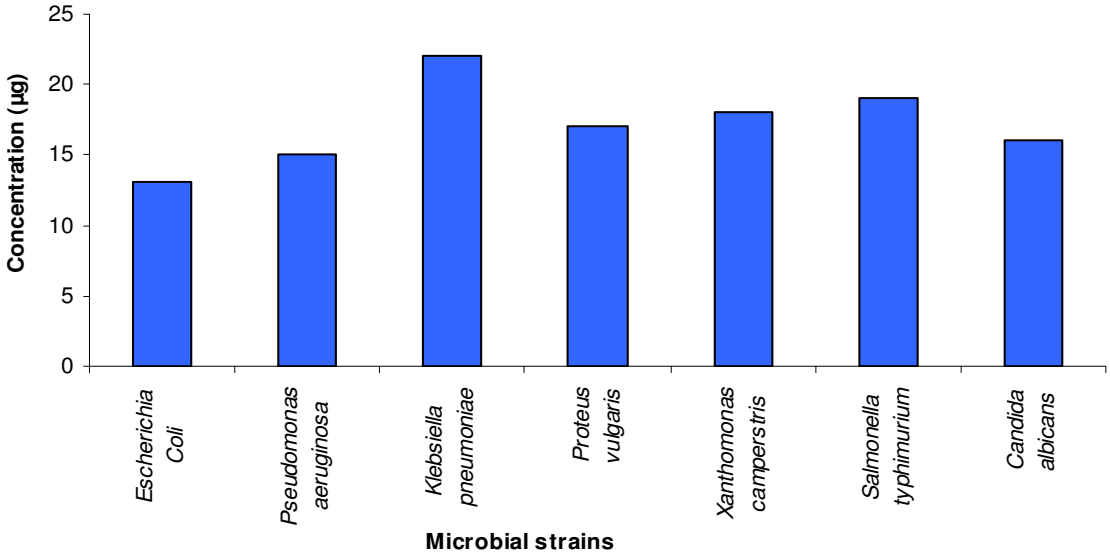

Figure 5. Zone of inhibition (diameter, mm^2) showed by Ampicillin standard against the selected microbial strains.

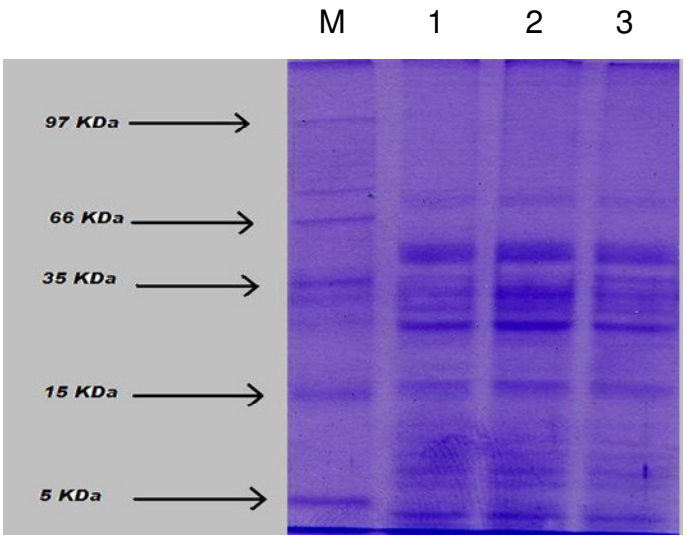

Figure 6. SDS-PAGE (12%) was performed with molecular marker to know the presence of different proteins and their molecular weight. M – 1 KDa standard marker, 1 - *Apis cerana*, 2 - *Apis dorsata*, 3 - *Apis florae.*

be useful in the search for potential antimicrobial agents against drug resistant microorganisms.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Jayaprakash, Professor and Chairman, Department of Zoology, Bangalore University, Bangalore for providing necessary research facilities. One of the authors (Surendra N.S) is grateful to University Grant Commission, New Delhi for financial assistance in the form of UGC Meritorious Research Fellowship (UGC - MRF).

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