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Milking method' - Novel Technology for Venom Collection from Aculeate Hymenoptera and used for Screening of *In- Vitro* Antimicrobial Activity Against Pathogens

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Abstract

Wasps were collected live from the field by using a hand net/sweep net and were maintained in the laboratory under caged condition by providing sugar solution (1:1) along with proteinex. Venom was collected from wasps by 'milking'. Initially wasps were forced to sting on potato blocks with the help of forceps but the wasps failed to sting on potato blocks. In later trials the wasps were made to sting on sterile paper disc, the wasps were not able to sting on paper disc and did not appear to release the venom either. The wasps were then offered freshly prepared pre-sterilized two per cent agar blocks of approximately 1 cm^3. The wasps were able to sting the agar block during this 'milking' process and the venom containing agar block was stored in the eppendorf tube containing 50 per cent acetonitrile (ACN) with 0.1 per cent trifluoroacetic acid (TFA). Venom from the selected wasps was collected by 'milking' at regular intervals.Then, extract was further concentrated and lyophilized and checked for the biological activity.

Highlights

'Miking method' developed for collection of venom from wasps easily on agar block under laboratory condition and its simple method everybody can use it for venom collection and this can be utilized for conducting different bioassay studies against pathogenic micro organisms.

Keywords: *Wasps, Venom, Milking, Agar, Acetonitrile*

Antimicrobial peptides from insects are increasingly being explored as potential therapeutic agents to manage diseases caused by antibiotic resistant pathogens. The continuous use of antibiotics in human medicine, agriculture and animal husbandry has resulted in multi-drug resistant bacterial strains all over the world. Further, hospitals have become breeding grounds for these multi-drug resistant human-associated microorganisms (Mainous and Pomeroy, 2001). Nevertheless, the same time-bomb effect is slowly developing with animal-associated pathogens in commercially driven activities, such as aquaculture and confined poultry breeding, where the indiscriminate use of antibiotics is perceived as essential for industries survival. Infections caused by pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* spp, *Enterococcus* spp, *Legionella* sppand *Aspergillus* spp. and a dozen other infectious diseases are becoming a major health concern. The major contributors to the evolution of

antibiotic resistance are over-prescription and misuse of antibiotic drugs. Another potential reason for the situation is the massive use of preventive antibiotics in animal food. Because of a fast growth rate, the frequencies of genetic mutations, selections and the ability of bacteria to rapidly exchange genes, bacterial resistance to antibiotics seems to favour a swift evolution of resistance in bacteria.

Among all multi-cellular organisms encountering microbe laden-environment insects show considerable success in eliminating the primary infections caused by the intruder organisms. Insects alone comprise about 55 per cent of the total biodiversity and more than 80 per cent of estimated number of species on earth. Many insects are nutritional used as food due to high protein content (46-96%) with good digestibility between 76 to 96 per cent (Ramos-Onsins *et al*., 1998). They are about 16 times as many insect species as there are plant species, yet plant chemistry has been studied, 7000 times more intensively than insect chemistry on research per species basis. Surprisingly no efforts have been made to utilize till recently.

The species with a solitary life history surprisingly evolved their venoms to be used as paralytic tools in order to keep their prey alive for feeding and reproduction. The many wasp species taking this evolutionary path include the solitary aculeate wasps belonging to the superfamilies Bethyloidea, Scolioidea, Pompiloidea, Sphecoidea and Vespoidea. The last superfamily is considered as a single family, the Vespidae and contains the solitary subfamilies Massarinae and Eumeninae as well as the social Vespinae. Members of this group of solitary wasps are seasonal even in the tropics and little is known about their ecology and life-history. In the temperate regions they spend the cooler periods as diapausing larvae in nests provided by the mother-wasp. In most cases, the food provided consists of arthropod prey paralyzed by injection of venom into their bodies. The prey, immobilized by stinging in a specific location, is carried to the nest, where the eggs are laid on the prey and the larval development takes place (Piek and Spanjer, 1986). The constituents of these venoms are low-molecular-mass neurotoxins, such as polyamines, a cocktail of neurotransmitters and a few peptides (Konno *et al.*, 2002). Another group of solitary wasps, which include the superfamilies like Ichneumoidea, Cynipoidea, and Chalcidoidea evolved in the direction of parasitic behavior*i.e.*, their venom evolved to promote short or long-lasting transient paralysis of the prey in order to permit egg-laying on or within the prey body.

Benton *et al.*, (1963) reported that pure bee venom was collected by the electric shock method. Bee venom was dissolved in 0.5 ml distilled water at different concentrations and centrifuged at 12,000 rpm for 10 min to remove insoluble materials. The resulting solutions were administered in various schemes to male and female CBA mice.

Morse and Benton (1964) modified their venom collector and used it for collecting venom from *Apis cerana indica* but removed the sting of *A. dorsata* and *A. florea* to collect its venom. However, Marz (1983) collected venom of *A. mellifera* on glass and membrane using electric shock. The venom collected from the membrane was cleaner than collected on glass even though the bees died. Later, Fakimzadeh (1990) designed a box type venom collector using electric shock, which is not so commonly used for venom collection.

The stinging organ was carefully removed from bees sedated with co_2 without disturbing their acid and alkaline gland. Venom was obtained by two methods; bee venom was taken as a freshly discharged liquid from the sting lancet tip and from the poison sac only. Both venoms were allowed to dry separately on clean glass slides wherein all volatile fractions were lost into atmosphere, it was taken as above, from the sting tip and poison sac under water, wherein the otherwise volatile fractions went into solution to mingle and chemically transfigure the venom crystals during the slow evaporative process (Pence, 1981).

Material and Methods

The material used and methods employed for exploring venom extracts of selected wasps for antimicrobial activity during 2010-11. All the laboratory experiments were carried out at Department of Agricultural Entomology and School of Ecology and Conservation Lab, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore (12º 57½ N 72º 35½ E, 916 m MSL), Karnataka, India. The method used for collection of venom is 'Milking' method.

Collection of insects

Wasps were collected live from the field by using a hand net/sweep net and were maintained in the laboratory under caged condition by providing sugar solution (1:1) along with proteinex (Plate1). The procedure and extraction is explained in results and discussion.

Plate 1: Wasps were maintained under caged condition

Test organisms for bioassay

Anti-microbial assays were carried out by Kirby-Baer disk diffusion assay against standard bacterial strains Gram-positive bacteria, *Staphylococcus aureus* (ATCC12600), Gram-negative bacteria, *Escherichia coli* (ATCC 12435) and fungal pathogen, *Candida albicans* (ATCC 10231) obtained from The Institute of microbial technology (IMTECH), Chandigarh, India. These cultures were maintained on slant culture using nutrient agar and Martin Rose Bengal Agar respectively and were used in the study.

Antimicrobial Assay/ Bioassay

Antimicrobial assay was performed using the standard Kirby-Bauer, zone of inhibition method. A pure culture of standard microorganisms were grown overnight in broth medium (in case of fungi potato dextrose broth and nutrient broth for bacteria was used) and then serially diluted to a concentration of 1 x 10^6 CFU/ml (approximately 100 folds). A 25ìl of prepared inoculum was dropped over a presterilized 90 mm Petri dish, on which 20 ml of freshly prepared nutrient broth containing 1.5 per cent agar (In case of fungi Martin Rose Bengal agar containing 2% agar) was poured and allowed to solidify. After the media hardened, 20 ìl of test sample was taken up using a 6 mm sterile disk and placed onto the surface of the top agar. A 10 micrograms of Tetracycline for bacterial plates and 10 micrograms of Nystatin for fungal plates were used as positive control and the solvent used for reconstitution of crude extract was used as negative control. The petri dishes were incubated overnight at 37^{o}C for bacteria and in case of fungi; incubation was done at 27^{o}C for 28 hours. The zone of inhibition on the surface of the top agar was measured using a digital vernier caliper.

Results and Discussion

Venom was collected from wasps by 'milking.' Initially wasps were forced to sting on potato blocks with the help of forceps but the wasps failed to sting on potato blocks. In later trials wasps were made to sting on sterile paper disc, the wasps were not able to sting the paper disc and did not appear to release the venom either. The wasps were then offered freshly prepared pre-sterilized two per cent agar blocks of approximately 1 cm^3. The wasps were able to sting the agar block during this 'milking' process (Plate 2) and the venom containing agar block was stored in the eppendorf tube containing 50 per cent acetonitrile (ACN) with 0.1 per cent trifluoroacetic acid (TFA). Venom from the selected wasps was collected by 'milking' at regular intervals.The extract was further concentrated and lyophilized and checked for the biological activity. The wasps used for screening of antimicrobial activity against pathogens (Table 1).

Bee venom was milked from three bees at the same time by Herberger *et al.*(1961) by placing them on platinum hot seats and applying low voltage electric current. O'Connor (1963) also reported venom collections from hymenopterans, which was found to be adequate for obtaining pure venom from one to several hundred individual bees and wasps, one at a time. Benton *et al.* (1963); Morse and Benton (1964) designed the "Cornell venom collector" provided an electric shock and collected venom from several thousand honey bees at the same time, which can be fixed inside the hive. This method is quite commonly used for venom collection. Palmer (1961) also collected venom using electric shock with magazines fitted inside the hive.

Gunnison (1966) by introducing a cooling system to the "Cornell venom collector" devised a method for collecting liquid fraction of venom. Similarly, Benton and Morse (1966) modified the electric device designed by them to collect liquid fraction of venom. Mello (1970) used venom sacs

Plate 2: Venom collection on agar block by "Milking method"

of *Apis mellifera* for venom collection. Peiren *et al.* (2003) ruptured the venom sac and used for studying the venom proteins. However, O'Connor *et al.* (1963) and Hoffman (1977) determined that proteins from venom sac and venom proteins discharged from sting were not entirely similar.

Protocol adopted for extraction of venom-by 'Milking' method

Collection of wasps

Live wasps were maintained under laboratory

Preparation of agar blocks (2%)

At regular intervals milking of venom was done by forcing the wasp to sting on agar block

Agar blocks containing venom was homogenized with 50% ACN (v/v) containing 0.1% TFA

Centrifuged at 10, 000 rpm for 15 minutes

Collect the supernatant

Repeat the above steps for 2 to 3 times and collect the supernatant in each stem

Pool all the supernatant collected and excess acetonitrile was removed by vacuum evaporator

The extract was further concentrated and lyophilized and checked for the biological activity

Mello (1970) used venom sacs of *Apis mellifera* for venom collection. Peiren *et al.,* (2003) ruptured the venom sac and used for studying the venom proteins. However, O'Connor *et al.,* (1963) and Hoffman (1977) determined that proteins from venom sac and venom proteins discharged from sting were not entirely similar. Mohamed Ali (2012) used collection of venom through electrical methods work to an extent with other stinging insects this method appears to be viable method mainly for Studying bee venom and its medical uses. The standard electro-shock method cannot be recommended for venom collection from Africanized honeybees or the more defensive races. Venoms of *Vespa magnifica* were collected by electronic stimulation. The adult *Vespa* wasps were collected and subjected to electronic stimulation (3-6 volts). Approximately 0.1 mg of venom can be obtained from one adult worker wasp. After electronic stimulation and venom collection, wasps were released. In total, 5 g of venom (wet weight) was obtained from about 50, 000 worker wasp (An *et al.,* 2012; Zhou *et al.,* 2006; Xua *et al.,* 2006)

The stinging organ was carefully removed from bees sedated with co_2 without disturbing their acid and alkaline gland. Venom was obtained by two methods; bee venom was taken as a freshly discharged liquid from the sting lancet tip and from the poison sac only. Both venoms were allowed to dry separately on clean glass slides wherein all volatile fractions were lost into atmosphere, it was taken as above, from the sting tip and poison sac under water, wherein the otherwise volatile fractions went into solution to mingle and chemically transfigure the venom crystals during the slow evaporative process (Pence, 1981).

Oukkache *et al.,* 2013, reported that two methods used successfully in a large-scale program for the collection of scorpion venoms, namely the milking of adult scorpions *via* manual and electrical stimulation and finally concluded that to produce high quality antivenom with specific antibodies, it is necessary to collect venom by the gentler electrical stimulation method.

Table 1: List of wasps collected for screening of *in vitro* antimicrobial assay

Sl. No	Wasps species	Family	Location
1.	*Sphex argentatus* Fabricius	Sphecidae	GKVK, Banglore
2.	*Delta conoideum* Gmelin	Vespidae	GKVK, Banglore
3.	*Rhynchium brunneum* Fabricius	Vespidae	GKVK, Banglore
4.	*Sphex madraspatanum* Fabricius	Sphecidae	GKVK, Banglore
5.	*Sphex sericeus* Fabricius	Sphecidae	GKVK, Banglore
6.	*Ammophila atripes* F.Smith	Sphecidae	GKVK, Banglore
7.	*Chalybion bengalense* Dalhbom	Sphecidae	GKVK, Banglore
8.	*Ropalidia marginata* Lepeletier	Vespidae	GKVK, Banglore
9.	*Vespa tropica* Linnaeus	Vespidae	GKVK, Banglore

Conclusion

The species with a solitary life history surprisingly evolved their venoms to be used as paralytic tools in order to keep their prey alive for feeding, reproduction and in case of colonies venom used as defensive purposes. The many wasp and species taking this evolutionary path their survival and defend themselves against predators and parasites. By looking into evolutionary history of these wasps and bees venom contains antimicrobial peptides. These antimicrobial peptides having property of antifungal or antibacterial activity against human pathogens. So, there by exploring of these activity against human pathogens 'Miking method' has been developed for frequent collection of venom from wasps easily on agar block under laboratory condition maintained under caged condition and its simple method everybody can make use of it for utilising venom for conducting different bioassay studies against pathogenic micro organisms.

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