NAE, ICAR sponsored Virtual Training on "Bioprospecting of Natural Resources for the Production of Biopesticide"

15-19th March, 2021

e-Manual

Editors:
Aditi Kundu, Supradip Saha & Anupama Singh

Division of Agricultural Chemicals
ICAR-Indian Agricultural Research Institute
New Delhi-110 012

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Compiled by:

Dr. Aditi Kundu
Dr. Supradip Saha
Dr. Anupama Singh

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Division of Agricultural Chemicals

ICAR-Indian Agricultural Research Institute, New Delhi-110012

Dr. Anupama Singh
Head and Principal scientist
E mail: head_chem@iari.res.in

Integrated crop management (ICM) is the only feasible approach to climate resilient agriculture in present day context. Crop protection is key component of ICM. Overuse and misuse of pesticides has led to general awareness towards integrated pest management, an approach adopted by India in cardinal principle. Biopesticides offer a promising alternative to synthetic pesticides, to be used solely or in combination with synthetic chemicals. India's rich diversity of both flora and fauna presents furthermore opportunities in biopesticide R&D and commercial sectors. Govt of India's emphasis towards sustainable farming, green farming, organic farming, Make in India, Vocal for Local, natural farming etc., necessitates the capacity building of the stakeholders in NAREES system, that would strengthen the biopesticide oriented green farming mission of country. There is a need to create exposure platform for the academia and students of the SAUs, research institutes and ATARIs to learn methodologies of extraction, chemical profiling and characterization of natural products and their transformation into ready to use formulations.

Division of Agricultural Chemicals, ICAR-IARI, New Delhi has been serving as seat of excellence in basic and applied research of national importance in multifarious domains of development, formulation and safety aspects of agrochemicals such botanicals and biopesticides, bioformulations and adjuvants. Division has been hosting capacity building trainings in the past and it is a matter of pleasure that the present training entitled, "Bioprospecting of natural resources for the production of biopesticides" is being organised under the umbrella of ICAR Niche Area of Excellence capacity Building scheme. " Plant source based crop protection and production te technologies: Development and capacity building. it is hoped that even though the training is in online mode, the lecture topics and hands on practicals in virtual mode and a comprehensive chapter wise e-manual prepared will be able to satisfy the trainees' quest to learn the techniques and the underlying principles of biopesticides. I wish them wonderful learning experience throughout training period.

(ANUPAMA SINGH)

HeadD, Agril. Chem. & PI, NAE

CONTENT

Botanical Pesticides: Opportunities in Present Day Context

Suresh Walia
Consultant, All India Network Project on Pesticide Residues
Division of Agricultural Chemicals
ICAR-Indian Agricultural Research Institute, New Delhi-110 012
Email: s_walia@Yahoo.com

Pest management relying heavily on synthetic pesticides has serious drawbacks in terms of handling hazards, high persistence, insect resistance and threat to human health and the environment. Such drawbacks have brought up new challenges for developing safer pesticides. As defined by the Environment Protection Agency (EPA), safer pesticides are those which are less persistence in the environment, less likely to cause toxicity to non-target organisms, lesser chance to contaminate soil and groundwater, allow lesser exposure to humans and the environment, and can be rid of with more practical and affordable disposal technology. In this respect botanical pesticides are considered as environment friendly safe option to synthetic pesticides as they are renewable, biodegradable, least persistent in the environment and exhibit low mammalian toxicity and phytotoxicity.

The commercial use of plant extracts as pesticides began in the nineteenth century. Nicotine, rotenone, pyrethrum and sabadilla alkaloids were among the first-generation botanical pesticides, but use of nicotine, rotenone and sabadilla was subsequently restricted due to their high mammalian toxicity, fish toxicity and negative impact on human health. Pyrethrum (*Tanacetum cinerariaefolium*) comprising of six bioactive esters (pyrethrins, cinerinsI and jasmolins) continued to be widely used due to its use in gardens and other indoor application. Several botanical pesticides like azadirachtin, rocaglamides, annonins, isobutylamides etc. were later developed/commercialized and many more promising plants are currently being explored for pest control properties. Rocaglamides and methyl rocaglate from *Aglaia odorata* (Family Meliaceae) and other *Aglaia* species were found to be as effective as azadirachtin. Pesticidal properties of acetogenins isolated from Annona (custard apple) were attributed to asimicin and other acetogenins. A number of unsaturated isobutylamides have been reported from a number of piper species. Ryanoids from *Ryania speciosa*, capsaicinoids from chilli, and insecticidal naphthoquinones from *Calceolaria andina*, were other botanicals effective against various insect pests. The SAR studies on the *C. andina* constituent (1,1-dimethylprop-2-enly-3-hydroxy-1,4-naphthoquinone) have resulted in several semi-synthetic derivatives with outstanding activity. Structurally related Kanemite@ (3-dodecyl-1,4-dihydro-1,4-dioxo-2-naphthyl acetate) based on the natural naphthoquinone developed in Japan is an effective acaricides.

In India efforts have been made to develop botanical pesticides, plant growth stimulants and seed coats based on *Azadirachta indica* (neem), *Madhuca indica* (Mahua), *Sapindus*

mukorossi (soapnut), *Curcuma longa* (turmeric), *Pongamia glabra* (karanja), *Anethum sowa*, *Eupatorium adenophorum*, *Tagetes erecta* (marigold), *Curcuma longa* (turmeric), ginger (*Zingiber officinale*), *Rheum emodi* (Himalayan Rhubarb), and many other plants. Several formulations based on neem oil, azadirachtin, and reduced azadirachtin have been patented and their technology transferred to industries for commercialization. Some essential oil bearing plants and their constituents such as carvone, citral, citronellal, pulegone and fenchone etc. have also been reported to exhibited significant insecticidal, larvicidal, antifungal and nematicidal properties. Efforts have been made to further improve their bioefficacy by chemical modification, structure optimization and SAR studies. Natural waxes, policosanols, sucrose esters have also been investigated for their pest control and plant growth stimulant activity. Natural insecticide synergists and stabilizers have been used to enhance efficacy, prolong shelf life and counter resistance in insect pests. For example, dillapiole, the chief constituent of Indian dill (*Anethum sowa*) seed oil, dihydrodillapiole (5-n-propyl 6,7-dimethoxy-1,3-benzodioxole), furapiole, nonanoyl/nonenyl dihydrodillapiole, and the semi-synthetic piperonal oxime N-O-alkyl ethers exhibited superior synergistic activity than the commercial synergist PBO. Synergistic combination of botanical ingredients, synergists, stabilizers and other adjuvants have yielded more stable and effective products. Nanotechnological interventions have led to products with enhanced pest control properties.

At present only a few botanical pesticides like neem oil, azadirachtin, pyrethrum, citronella oil, triacontanol etc. are registered with the CIB&RC, and some are included in the Insecticide Schedule. This is largely because of lesser acceptability by the farmers due to their moderate or less efficacy as compared to synthetic pesticides under field conditions. More botanical pesticides will emerge if shortcomings and impediments such as insufficient availability of the raw material, inadequate stability, poor shelf life, diminished residual toxicity, non-availability of reliable standards, problems in large-scale production, lack of effective delivery systems, and difficulty in getting regulatory approvals are addressed and effective, stable and safer formulations are made available to the farmers.

New Paradigms in Biopesticide Formulations

Anupama Singh
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute
New Delhi
Email: anupamanil2000@gmail.com

Since 1985, India has adopted Integrated Pest Management (IPM) as cardinal principle and main plank of plant protection in the overall crop production programme. Biopesticides based on microorganisms specifically offer an alternative path in crop protection because of their safety to humans and non-target organisms, both in individual applications and within integrated pest management (IPM). IPM involves minimum use of chemical pesticides to maintain crop health and biopesticides have an important place in this strategy. Biopesticides comprise important tools in integrated pest management (IPM) programs by reducing the risk of resistance to chemical pesticides, improving worker safety through short restricted entry intervals, conserving natural enemies, and maintaining environmental health. Biopesticides have long been used in combination with synthetic chemistries to provide the basis for excellent control programs that effectively manage resistance. Additionally, they typically have modes of action that are different from synthetic pesticides and do not rely on a single target site for efficacy. There are different kinds of biopesticide active ingredients (living/nonliving) and each one has distinct and peculiar properties and thus have to be formulated in different product forms. The properly developed as well as applied, the biopesticidal products have the potential to extend the effective field life of all products by curtailing the development of resistant pest populations. Biopesticidal products though are highly target-specific, both the development of up scalable knowhows and the acceptable formulations are very difficult. The reason for this is that, apart from the required good physical properties and convenience in use, the formulated product must also keep its biological agent/ bioactive chemical constituent functional throughout storage and during application. The technologies of biopesticide manufacture should be economical to produce at mass scale, product should have persistent storage stability, high residual activity, be easy to handle, mix and apply, and provide consistently effective control of target pests. Different formulations of biopesticides are being introduced to overcome problems relating to their efficacy and degradation and to be convenient during handling and application. Biopesticides are usually formulated as either dry formulations for direct application such as dusts (DP), powders for seed dressing (DS), granules (GR), micro granules (MG), water dispersible granules (WG), and wettable powders (WP) or liquid formulations for dilution in water suchas emulsions, suspension concentrates (SC), oil dispersions (OD), suspo-emulsions (SE), capsule suspensions (CS), ultralow volume formulations. Through this technical lecture, it is envisaged to provide to the trainees, an overview of the innovations in different categories of biopesticide formulations. It is likely to buildup their capacity to decide and attempt the required type of formulation as suited for the bioactive/bioagent and the intended situation of application.

Chromatographic techniques for characterization of organic molecule

Aditi Kundu and Supradip Saha
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi 110012
Email: chem.aditi@gmail.com

Chromatography is a technique in which the components of a mixture are separated based on the rates at which they are carried through a stationary phase by a gaseous or liquid mobile phase. Components of a mixture are carried through the stationary phase by the flow of a gaseous or liquid mobile phase, separations being based on differences in migration rates among the sample components. Chromatography involves a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible stationary phase. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

The chromatography techniques available are a) Column chromatography b) High performance liquid chromatography (HPLC) c) Gas chromatography (GC) d) Ion-exchange chromatography e) Size exclusion chromatography. f) Thin layer chromatography (TLC) g) High performance thin layer chromatography (HTLC) h) Paper chromatography.

Affinity chromatography

Chromatographic technique was developed long ago and the simple thin layer chromatography has been greatly modified and developed to meet the growing demands of scientific research and analysis. The modifications have led to improved efficiency in the separation and also the quantification. Further the range of and type of substances which could be analysed has been greatly increased due to advancement in chromatography techniques. Chromatographic methods are of two types. In column chromatography, the stationary phase is held in a narrow tube and the mobile phase is forced through the tube under pressure or by gravity. In planar chromatography, the stationary phase is supported on a flat plate or in the pores of a paper. The mobile phase moves through the stationary phase by capillary action or under the influence of gravity Techniques such as HPLC (High Performance Liquid Chromatography) and GLC. (Gas Liquid Chromatography) use columns - narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called elution. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase. Chromatographic methods fall into three categories based on the nature of

the mobile phase. The three types of phases include liquids, gases, and supercritical fluids. There are five types of liquid chromatography and three types of gas chromatography that differ in the nature of the stationary phase and the types of equilibrium between phases.

Paper Chromatography

In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action. Components of the mixture are carried along with the solvent up the paper to varying degrees, depending on the compound's preference to be adsorbed onto the paper versus being carried along with the solvent. The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually consisting of a mixture of water and an organic liquid. The paper is called the stationary phase while the solvent is referred to as the mobile phase. Performing a chromatographic experiment is basically a three-step process: 1)application of the sample, 2) "developing" the chromatogram by allowing the mobile phase to move up the paper, and 3) calculating Rf values and making conclusions. Affinity chromatography Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure.

Ion Exchange Chromatography

The most popular method for the purification of proteins and other charged molecules is ion exchange chromatography. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. To optimize binding of all charged molecules, the mobile phase is generally a low to medium conductivity i.e., low to medium salt concentration) solution. The adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support. The strength of the interaction is determined by the number and location of the charges on the molecule and on the functional group. By increasing the salt concentration (generally by using a linear salt gradient) the molecules with the weakest ionic interactions start to elute from the column first. Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient. The binding capacities of ion exchange resins are generally quite high.

Size exclusion chromatography

The principle of size exclusion chromatography is that molecules move through the column under the influence of mobile phase based on their relative physical size or mass. Those with

smaller size or mass move through the all possible pores due to their small size and travel a longer path and get eluted last. But large molecules travel only through few possible pores due to their size and pass out of the column first as they travel shorter path. The technique is also called as molecular sieve filtration, gel filtration, exclusion chromatography.

Column Chromatography

In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column. The mobile phase, a liquid, is added to the top and flows down through the column by either gravity or external pressure. Column chromatography is generally used as a purification technique: it isolates desired compounds from a mixture.The mixture to be analyzed by column chromatrography is placed inside the top of the column. The liquid solvent (theeluent) is passed through the column by gravity or by the application of air pressure. An equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as the solvent drips from the bottom of the column.

Gas Liquid Chromatography

Gas liquid chromatography involves a sample being vapourised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependant upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities. For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapour - slow injection of large samples causes band broadening and loss of resolution. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around 15 | P a g e 10-3 mL. For capillary GC, split/splitless injection is used. Have a look at this diagram of a split/splitless injector; The injector can be used in one of two modes; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column. There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase. Most packed

columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm.Capillary columns have an internal diameter of a few tenths of a millimeter.

High-performance liquid chromatography

HPLC is a separation technique that can be used for the analysis of organic molecules and ions. HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid 17 | P a g e mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases. HPLC can be used to assess the purity and/or determine the content of many pharmaceutical substances. It can also be used to determine enantiomeric composition, using suitably modified mobile phases or chiral stationary phases. Individual separation mechanisms of adsorption, partition and ion exchange rarely occur in isolation since several principles act to a certain degree simultaneously. The apparatus consists of a pumping system, an injector, a chromatographic column, stationary and mobile phases, connecting tubing and fittings, a detector and a data collection device (computer, integrator or recorder). Most separations are based on partition mechanisms using chemically modified silica as the stationary phase and polar solvents as the mobile phase (reversed-phase HPLC). The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Ultraviolet/visible (UV/vis) and PDA absorption spectrophotometers are commonly used detectors in HPLC. In specific cases fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors, evaporative light-scattering detectors (ELSD), charged aerosol detectors (CAD), mass spectrometers (MS) or other special detectors may be used.

Fourier transform infrared (FT-IR) spectroscopy for characterization of organic compounds

Rajesh Kumar

Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi – 110 012
Email: rkb1973@gmail.com

The interaction of electromagnetic radiation (light) with matter (organic compounds) is known as spectroscopy. Molecules undergo different processes like rotation, vibration, electronic transitions, and nuclear transitions. Spectral properties are helpful for characterization of organic compounds.

Infrared (IR) spectroscopy

The energies associated with molecular vibrations fall in the infrared region of electromagnetic spectrum. IR spectroscopy deals with the infrared region of the electromagnetic spectrum and interaction between matter and infrared radiation (electromagnetic radiation with a longer wavelength than visible light). IR spectroscopy is used to probe the vibrations in molecules and is also therefore known as vibrational spectroscopy. Infrared spectroscopy is a very useful technique for functional group identification in organic compounds.

Infrared (IR) region is a small part of the electromagnetic spectrum and lies between visible and microwave regions. Infrared region is usually divided into three regions: near infrared, mid-infrared, and far infrared.

Region	**Wavelength range** (nm)	**Wave number range** (cm^{-1})
Near infrared	780 – 2500	12800 - 4000
Middle infrared	2500 – 50000	4000 - 200
Far infrared	50000 -1000000	200 – 10

Mid-IR region between $\lambda = 2.5\text{-}25\ \mu m$; $\bar{\nu} = 4000 - 400\ cm^{-1}$ is most frequently used for studying molecular vibrations. Wavenumbers ($\bar{\nu}$) are used for representing the IR spectra.

Absorption of infrared radiation

IR light passes the sample material. Depending on its energy, this trigger the vibrations of specific molecular bonds (absorption). The absorbed energy is consumed and thus "missing" from the original IR beam.

Fourier transform spectrometer

A Fourier transform spectrometer uses an interferometer. An interference of polychromatic radiation is generated using an interferometer, usually a Michelson interferometer. Absorption of any particular wavelength will bring a change in the interferogram which gets detected. An

interferogram is a time domain signal and is converted to frequency domain signal though Fourier Transformation.

Advantages of FTIR spectrometers

- Better speed as it detects absorption of all the frequencies simultaneously.
- Better sensitivity because of their speed of data acquisition.
- More radiation energy as these spectrometers do not need slits for filtering of radiation.
- Simple design as FTIR spectrometers do not require dispersion and filtering of the radiation.

Functional group region and fingerprint region

The absorption bands in the 4000 – 1500 cm^{-1} region help in the identification of functional groups; this region therefore is also termed the functional group region of the IR spectrum. The lower energy portion of the mid-IR region (1500 – 400 cm^{-1}) usually contains a very complicated set of peaks arising due to complex vibrations involving several atoms. This region is unique to a particular compound and therefore is known as the fingerprint region of the IR spectrum. Though it is difficult to assign the vibrational modes to these peaks, these are useful to identify a compound if the spectrum of the compound is already known.

Typical vibrational frequencies of functional groups

Bond	**Compound**	**Wavenumber (cm^{-1})**
C=O	Aldehydes, ketones, esters, carboxylic acids, amides	1750 – 1630
C–O	Alcohols, ethers, esters, carboxylic acids, etc.	1300 – 1000
N–H (Stretching)	Amines and amides	3500 – 3100
–N–H (Bending)	Amines and amides	1640 – 1550
O–H	Alcohols	3650 – 3200
C–N	Amines	1350 – 1000

FT-IR spectroscopy is mainly used for

- Identification of functional groups of organic compounds as different functional groups vibrate at different frequencies allowing their identification. The frequency of vibration also depends on other factors such as delocalization of electrons, H-bonding, and substitutions at the nearby groups.

- Identification of compounds by comparing the IR spectra in the fingerprint region with IR spectrum in the fingerprint region of the known compound.

Nuclear Magnetic Resonance Spectroscopy: A Brief Introduction

Abhishek Mandal
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi – 110 012
Email:abhishekmandal@gmail.com

Nuclear magnetic resonance (NMR) is a spectroscopic method that is even more important to the organic chemist than infrared spectroscopy. Many nuclei may be studied by NMR techniques, but hydrogen and carbon are most commonly available. Whereas infrared (IR) spectroscopy reveals the types of functional groups present in a molecule, NMR gives information about the number of magnetically distinct atoms of the type being studied. When hydrogen nuclei (protons) are studied, for instance, one can determine the number of each of the distinct types of hydrogen nuclei as well as obtain information regarding the nature of the immediate environment of each type. Similar information can be determined for the carbon nuclei. The combination of IR and NMR data is often sufficient to determine completely the structure of an unknown molecule.

NUCLEAR MAGNETIC MOMENTS

Spin states are not of equivalent energy in an applied magnetic field because the nucleus is a charged particle, and any moving charge generates a magnetic field of its own. Thus, the nucleus has a magnetic moment m generated by its charge and spin. A hydrogen nucleus may have a clockwise or counterclockwise spin, and the nuclear magnetic moments in the two cases are pointed in opposite directions. In an applied magnetic field, all protons have their magnetic moments either aligned with the field or opposed to it.

ABSORPTION OF ENERGY

The nuclear magnetic resonance phenomenon occurs when nuclei aligned with an applied field are induced to absorb energy and change their spin orientation with respect to the applied field. The energy absorption is a quantized process, and the energy absorbed must equal the energy difference between the two states involved.

THE MECHANISM OF ABSORPTION (RESONANCE)

To understand the nature of a nuclear spin transition, the analogy of a child's spinning top is useful. Protons absorb energy because they begin to precess in an applied magnetic field. The phenomenon of precession is similar to that of a spinning top. When the magnetic field is applied, the nucleus begins to precess about its own axis of spin with angular frequency ω, which is sometimes called its Larmor frequency. The frequency at which a proton precesses is directly proportional to the strength of the applied magnetic field; the stronger the applied field, the higher the rate (angular frequency ω) of precession. For a proton, if the applied field is 1.41 Tesla (14,100 Gauss), the frequency

of precession is approximately 60 MHz. Since the nucleus has a charge, the precession generates an oscillating electric field of the same frequency.

If radiofrequency waves of this frequency are supplied to the precessing proton, the energy can be absorbed. That is, when the frequency of the oscillating electric field component of the incoming radiation just matches the frequency of the electric field generated by the precessing nu -cleus, the two fields can couple, and energy can be transferred from the incoming radiation to the nucleus, thus causing a spin change. This condition is called resonance, and the nucleus is said to have resonance with the incoming electromagnetic wave.

THE CHEMICAL SHIFT AND SHIELDING

Nuclear magnetic resonance has great utility because not all protons in a molecule have resonance at exactly the same frequency. This variability is due to the fact that the protons in a molecule are surrounded by electrons and exist in slightly different electronic (magnetic) environments from one another. The valence-shell electron densities vary from one proton to another. The protons are shielded by the electrons that surround them. In an applied magnetic field, the valence electrons of the protons are caused to circulate. This circulation, called a local diamagnetic current, generates a counter magnetic field that opposes the applied magnetic field. This effect is called diamagnetic shielding or diamagnetic anisotropy.

Circulation of electrons around a nucleus can be viewed as being similar to the flow of an electric current in an electric wire. From physics, we know that the flow of a current through a wire induces a magnetic field. In an atom, the local diamagnetic current generates a secondary, induced magnetic field that has a direction opposite that of the applied magnetic field.

As a result of diamagnetic anisotropy, each proton in a molecule is shielded from the applied magnetic field to an extent that depends on the electron density surrounding it. The greater the electron density around a nucleus, the greater the induced counter field that opposes the applied field. The counter field that shields a nucleus diminishes the net applied magnetic field that the nucleus experiences. As a result, the nucleus precesses at a lower frequency. This means that it also absorbs radiofrequency radiation at this lower frequency. Each proton in a molecule is in a slightly different chemical environment and consequently has a slightly different amount of electronic shielding, which results in a slightly different resonance frequency. In this manner, a field-independent measure called the chemical shift (δ) is obtained

$$\delta = (\text{Shift in Hz}) / (\text{spectrophotometer frequency in MHz})$$

MAGNETIC ANISOTROPY

There are some types of protons with chemical shifts that are not easily explained by simple considerations of the electronegativity of the attached groups. For instance, consider the protons of benzene and other aromatic systems. Aryl protons generally have a chemical shift as large as that of the proton of chloroform! Alkenes, alkynes, and aldehydes also have protons with resonance values

that are not in line with the expected magnitudes of any electron-withdrawing or hybridization effects. In each of these cases, the anomalous shift is due to the presence of an unsaturated system (one with π electrons) in the vicinity of the proton in question. Take benzene, for example. When it is placed in a magnetic field, the p electrons in the aromatic ring system are induced to circulate around the ring. This circulation is called a ring current. The moving electrons generate a magnetic field much like that generated in a loop of wire through which a current is induced to flow.

SPIN–SPIN SPLITTING (n + 1) RULE

We have discussed the manner in which the chemical shift and the integral (peak area) can give information about the number and types of hydrogens contained in a molecule. A third type of information to be found in the NMR spectrum is that derived from the spin–spin splitting phenomenon. This phenomenon, called spin–spin splitting, can be explained empirically by the so-called n + 1 Rule. Each type of proton "senses" the number of equivalent protons (n) on the carbon atom(s) next to the one to which it is bonded, and its resonance peak is split into (n + 1) components.

THE COUPLING CONSTANT

In the previous section we discussed the splitting pattern of the ethyl group and the intensity ratios of the multiplet components but did not address the quantitative amount by which the peaks were split. The distance be-tween the peaks in a simple multiplet is called the coupling constant J. The coupling constant is a mea-sure of how strongly a nucleus is affected by the spin states of its neighbor. The spacing between the multiplet peaks is measured on the same scale as the chemical shift, and the coupling constant is always expressed in Hertz (Hz). In ethyl iodide, for instance, the coupling constant J is 7.5 Hz.

Natural nematicides of plant origin

Matiyar Rahaman Khan
Division of Nematology, ICAR-Indian Agricultural Research Institute,
New Delhi-110012
email: mrkhan@iari.res.in

Nematodes are relatively hardy animals; they can be killed with some insecticides at relatively higher than insecticidal doses. Therefore, nematode control using currently available nematicides involves high costs and environmental risks. With growing environmental concerns and the non-availability of effective nematicides, botanical products such as neem, karanja, castor, brassicas etc. could be an alternative and safer approach. Nematode management in crops is based on the principle of reduction of initial population densities using low-cost inputs, suppression of nematode reproduction, and preventing crop damage. There are good agricultural practices through which the nematode population could be maintained at levels that do not cause economic losses to crops. The current focus is on the search for green chemicals that protect crops from pests with minimum hazards to human beings and the earth's biodiversity. Many plants in nature possess nematicidal and nematostatic properties in their different parts; their extracts, essential oil, oilseed cakes, and other plant-based products that have proven nematicidal against many plant parasitic nematodes. Different groups of naturally occurring chemicals (metabolites) also exert nematicidal or nematostatic actions.

Nematicidal plants include marigold (*Tagetes* spp.), neem (*Azadirachta indica*), karanja (*Pongamia pinnata*), mustard (*Brassica* spp.), sudan grass (*Sorghum sudanense*), etc. Different parts of plants such as leaves of Karanja, *Cassia fistula* (5% w/w), *Crotolaria juncea* (5% w/w), *Sesbania aculeate* (5-10% w/w), subabool (40g/kg), *Argemone mexicana*, castor (40-60g/kg) etc. are proved to be effective for the control root knot nematode (*Meloidogyne* spp.) infecting vegetable crops. Dried leaf powder of *Vitex trifolii* is toxic to *M. incognita* infecting tomato; *Phlogacanthus thyrsiflorus, Parkia javanica, Calotropis procera, Prospis juliflora, Datura stramonium,* and *Crotolaria juncea* are toxic to lesion nematode (*Pratylenchus coffeae*) infecting banana and coffee. Shoots of some plants of Compositae and latex bearing plants, *Spilanthus acmella*, and *Costus speciosus* are nematicidal to *M. incognita, Rotylenchulus reniformis* and *Tylenchorhynchus brassicae*. Leaf extracts of *Datura metel*, *Cannabis sativa, Eclipta alba*, *Mentha piperita*, *Ocimum sanctum*, *Bhangra*, *Argimone mexicana,Tagetes lucida*, *Erythrina indica*, *Lantana camara*, *Calotropis gigentea*, etc. exhibited nematicidal efficacy against *M. incognita*. Similarly, leaf extracts of *Piper betel, Kalachoe pinnata, Glyricidia machalata, Ricinus communis, Crotolaria juncea* were found toxic to burrowing nematode (*Radopholus similis*) while *Cassia fistula*, *Solanum torvum, Acalypha indica* to *P. coffeae*. Extracts of seeds of *Embelia ribes, Vernonia cinerea, Areca catechu, Citrullus lanatus,* and *Tagetes lucida* are toxic to *M. incognita*. Extracts of roots and shoots of *Rauwolfia serpentine, Tagetes lucida, Aloe barbadense, Ammi majus, Artemisia pallens, Lactuca sativa, Gloriosa superb, Scillia indica, Tagetes erecta, Zinnia*

elegans were found toxic to *M. incognita* while *Indigofera tinctoria* and *Solanum indicum* to *R. similis*. The whole plants of *Myristica fragrans, Solanum surrattense, Theretia peruviana, Calotropis procera, Thuja siensis, Parthenium hysterophorus* when incorporated in soil exhibited killing effects on *M. incognita* and *Rotylenchulus reniformis*.

Many nematicidal compounds found in plants: **aldehydes and ketones** (aldehyde furfurale in *Melia azedarach*; *Eucalyptus meliodora*; *Ailanthus altissima*), **alkaloids** (*Loncocarpus, Derris, Tithonia diversifolia, Chromolaena odorata, Crotalaria, Ageratum houstonianum, Borago officinalis, Senecio bicolor, Symphytum officinalis*, etc.), **glycosides** (cyanogenic glycosides in Sudangrass cv. Trudan 8, cassava roots, *Arisaema erubescens*), **glucosinolates and isothiocyanates** (nematicidal isothiocyanates in *Brassica* and *Sinapis* sp.; seed meal of *Brassica juncea* 'Pacific Gold', *B. napus* 'Dwarf Essex' and 'Sunrise', and *Sinapis alba* 'IdaGold', defatted seed meal of *Raphanus sativus* ssp.*oleiformis* and *Eruca sativa* ssp. *oleiformis*), **limonoids quassinoids, and saponins** (azdirachtin in *Azadirachta indica* and furfurale in *Melia azedarach*), **organic Acids** (*Lantana camara* var. *aculeate*, *Mucuna* spp.), **phenolics, flavonoids, and quinines** (*Lantana camara*, *Viola betonicifolia*, the flower of *Tagetes patula*, branches of *Magnolia tripetala*, *Chromolaena odorata*, *Acacia gummifera*; *Psiadia punctulata*, *Nothofagus alessandri* and *N. pumilio*; *Gochnatia barrosii*), **piperamides** (capsaicin in *Capsicum frutescens*), **polyacetylenes and polythienyls** (*Tagetes* spp.) and **Terpenes** (*Carum carvi*, *Foeniculum vulgare, Mentha rotundifolia*, *Mentha spicata*; *Mentha arvensis*, *Mentha piperita*, and *Origanum vulgare*, *O. syriacum*, *Coridothymus capitatus*; *Eucalyptus citriodora, Eucalyptus hybrida*, *Ocimum basilicum*, *Pelargonium graveolens*, *Cymbopogon martini*, etc.).

Among the natural nematicidal plants, a few of them have been studied for their potential benefits. Neem (azadirachtin), castor (ricin), brassicas (allylisothiocyanate), marigold (alpha-terthienyl), and karanja (karanjin) are the most studied plants for nematicidal properties. Commercial formulations (such as seed powder, seed kernel powder, seed cake powder, dry leaf powder, and aqueous neem extracts) of neem contain the most nematotoxic/repellent compound, azdirachtin (available formulation, for example, NemaAzal contains 10, 000 to 50,000ppm Aza). Several formulations (oil - or kernel-based) of neem are available in the market for the control of insects (antifeedant) and nematodes (nematotoxic/repellent). They are known to exhibit good efficacy against root-knot nematodes and a few other nematodes as well. An oil-based formulation of *Pongamia pinnata* (karanja) is commercially available as nomite for the control of mite and nematodes. Most of the studies conducted are under *in vitro* conditions and incorporation of whole plant or parts (phytotherapeutic treatment), and plant-derived products (as soil amendments) for field treatments. The pyrrolizidine alkaloids producing plants are mostly various species of *Crotalaria* (*Crotalaria juncea, C. retusa, C. spectabilis* etc.); they have been used as a pre-plant cover or green manure crops for nematode management.

There are several benefits of using nematicidal products from plant origin: they are eco-friendly, may evolve as low-cost input technology, inexpensive, easy to prepare and apply, available in bulk, fewer chances of developing resistance in nematodes, biodegradable, selective toxicity to target nematodes, safe to non-target nematodes and environment, improve soil health structurally and nutritionally, antagonistic/repellent towards nematodes, produce toxic materials killing (nematicidal) or paralyzing (nematostatic), antifungal, and antibacterial properties. Abundant natural chemicals (phytoanticipins/phytoalexins) are available in the plants for their defense. The commercial exploitation of natural compounds helps to overcome the overdependence on synthetic chemicals, which pose threat to the environment and human health. The wealth of current knowledge on the anti-nematode activity of natural products of plant origin could be explored further for commercial exploitation as potential biopesticides. Further screening of myriad diversity of plants and their effects on nematodes could reveal many more promising candidates for effective control of nematodes.

Sustainable management of soil-borne diseases of horticultural crops employing a microbial consortia

V. Shanmugam
Division of Plant Pathology, ICAR-Indian Agricultural Research Institute,
New Delhi-110012
E-Mail: shanpatho@yahoo.com

Plant diseases are one of the major concerns in cultivation world wide resulting in loss of billions of dollars. There is a pressing need to control the diseases to ensure a steady and constant supply of marketable produce for the ever increasing world population. Lack of knowledge on the etiology of crop diseases is one of the constraints in developing suitable control measures. In disease management, the increased reliance on chemical solutions has compromised environmental quality, endangered a negative impact with consumers and resulted in a rise of fungicide resistant microorganisms. A variety of alternative approaches have also been considered which include the use of pathogen-resistant crop cultivars and biocontrol agents (BCAs). Developing resistant varieties can be difficult in the absence of dominant genes and development of new races of the pathogen overcoming host resistance.

Biological control (abbreviated synonym biocontrol) by use of antagonistic microbes represents a potentially attractive alternative disease management approach since these are known for growth promotion and disease reduction in crops. In plant pathology, the term "biocontrol" applies to use of antagonistic microbes to suppress plant diseases besides use of host-specific pathogens to control weed populations. Biocontrol of plant pathogens using antagonistic microbes is usually achieved by manipulation of resident (autochthonous microbes) microbial communities in suppressive soils to enhance natural disease suppression by different mechanisms or by introduction (allochthonous microbes) of specific microbes. Accordingly, biocontrol refers to the purposeful utilisation of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens. The agents which because biocontrol is called biocontrol agents (BCAs), which can be bacteria, fungi, or nematodes. These BCAs are native to the soil and the environment and are not pathogenic to birds, mammals including humans, and fish. They are also not genetically modified with short re-entry and days to harvest intervals. The biocontrol agents also gain more significance for products that need to be certified organic and for diseases where no control is available. They display a range of mechanisms such as competition with the pathogen for space and nutrients, parasitism or predation, inducing the plant's natural defense system, and/or production of allelochemicals such as lytic enzymes (chitinases and glucanases), hydrogen cyanide, volatile compounds, antibiotics-and detoxification and degradation of virulence factors

(Aggarwal et al., 2016). Several BCAs act as biofertilisers, either directly by providing nutrients to the host plant, or indirectly by influencing root growth and morphology or by aiding other beneficial symbiotic relationships. Enhanced growth of plants indirectly helps in control of pathogens. The BCAs in general display more than one mechanisms, which function together to exert their potential.

Successful biocontrol of soil-borne diseases depends on manipulation of microbial communities in the rhizosphere to enhance natural disease suppression (Shanmugam et al., 2011a). Often the usage of introduced biocontrol microbes is not adequate to cause disease suppressiveness in the soil since the introduced organisms need to compete with existing microbes for nutrition and also may not thrive under inadequate soil conditions. General disease suppression that results from a high diversity of residential or autochthonous microbes may therefore be a potential alternative to the uncertainties encountered in specific suppression of plant diseases by the introduced microbes. To understand the ecology and diversity of microbes in plant disease suppressiveness, we employed molecular profiling of rhizosphere microbial communities of ginger as a biocontrol strategy. The studies elucidated the role of microbial communities associated with healthy rhizospheres or roots of ginger in fact promotes disease (scab and rhizome rot, respectively) suppression, respectively or whether their presence is simply a direct consequence of the absence of the pathogens.

In bio-management of plant diseases, the application of a single biocontrol agent often results in inconsistent field performance because it is less likely to be active in different soil environments, and agricultural ecosystems. Combinations of biocontrol agents reduces the inconsistency from using a single biocontrol agent as the combination can protect the plants at different times or under different conditions and occupy different or complementary niches. Development of stable formulations of biocontrol agents is of great importance for subsistence farming of crops grown with limited rotation, where soilborne diseases are the main problem and where fungicide treatments are unaffordable. Though many products of biocontrol agents are increasing, yet they represent only a small volume in the sales of agro-chemicals. The excessive specificity of biocontrol agents in most cases, and biosafety or environmental concerns in others are major limiting factors. The effect of formulation constituents and production process dictated by commercialisation couldn't be negated for the limited representation of biocontrol agents. Also, the disease problems chosen by researchers for bioproduct development are for financially small markets to justify developmental costs or much expansive to define product effectiveness for making them commercially acceptable. In pursuit of the goal for translational research in agriculture for crop improvement, we developed carrier based formulations of biocontrol strain mixtures for seed and soil application for control of soil-borne diseases in gladiolus (Shanmugam et al., 2011b) ginger (Shanmugam et al., 2012, 2013) and tomato (Shanmugam and Kanoujia, 2011) under field conditions and elucidated their mechanisms of disease suppression. The

ability of a biocontrol strain mixture in controlling vascular wilt of tomato both locally and systemically has also been demonstrated. The benefit cost ratio of the products was worked out to be >1.9 and showed no loss of microbial activity during storage. Besides reducing the disease incidence, the products also promoted the plant growth. These carrier based formulations are of great importance for subsistence farming and crops grown with limited rotation, where soilborne diseases are the main problem and where fungicide treatments are unaffordable. The formulation of strain mixture has also the potential as a preferred input in integrated disease management systems once commercialized.

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Essential oils for the control of agricultural pests

Aditi Kundu* and Supradip Saha
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi 110012
Email: chem.aditi@gmail.com

Essential oils are defined as any volatile oil(s) that have strong aromatic components and that give distinctive odour, flavour or scent to a plant. These are the by-products of plant metabolism and are commonly referred to as volatile plant secondary metabolites. Essential oils are found in glandular hairs or secretory cavities of plant-cell wall and are present as droplets of fluid in the leaves, stems, bark, flowers, roots and/or fruits in different plants. Essential oils obtained from the medicinal and aromatic plants, are volatile in nature and a complex mixture of monoterpenes and sesquiterpenes besides hydrocarbons and also derivatives of aliphatic and aromatic compounds. This complex mixture of natural volatile compounds may even have composed of 50 to 250 compounds, of which some of them are in abundant and other in minor amounts. Typically, these oils are liquid at room temperature and get easily transformed from a liquid to a gaseous state at room or slightly higher temperature without undergoing decomposition. The amount of essential oil found in most plants is 1 to 2%, but can contain amounts ranging from 0.01 to 10%. Orange tree produce different composition of oils in their blossoms, citrus fruits, and/or leaves. In certain plants, one main essential oil constituent may predominate while in others it is a cocktail of various terpenes. In Ocimum basilicum (basil), for example, methyl chavicol makes up 75% of the oil, β-asarone amounts to 70–80% in Acorus calamus rhizomes, linalool, in the range of 50– 60%, occurs in coriander seed and leaf oils procured from different locations at different time intervals.

Aromatic plants have been used for centuries as spices, and condiments to confer aroma and flavour to beverages and food. The aroma and flavours in the different plant species and their parts are due to presence of essential oils or volatile oils. Thus, these aromatic plants are considered to be rich sources of essential oils of commercial values. Major and minor compounds present in the essential the oils together are responsible for characteristic fragrance, flavor and therapeutic effect. Essential oils are mainly used in the food industries as flavouring agents, in the perfume and cosmetic industries as fragrance and pharmaceutical industries add them for taste or aroma and or suppressing undesirable medicine's taste, as cooling agent and for scenting incense and household cleaning products. Essential oils and their chemical compounds are also widely used in aromatherapy, reliving of pain, skin diseases and as promising antimicrobial and pesticidal agents.

The gas chromatographic method is used almost exclusively for the qualitative analysis of the volatiles. Retention times were utilised as primary criterion for the peak identification. Without preliminary data regarding the compounds retention times, the identification is practically impossible. Even if the retention time for a certain compound is well known, it is

possible that it can elute at the same time with other compounds in the sample. The mass spectrometer used as chromatographic detector offers additional data for the identification of the separated compounds. As gas chromatography-mass spectrometry (GC-MS) availability has increased, a large number of scientists have turned to mass spectra (MS) for identification of peaks. The most frequent identification method is the comparison of the recorded spectra with an MS library. The computer decreases dramatically the time that is necessary for the comparison, even if the library contains hundreds of thousands of spectra. The wide variety of MS in the libraries, recorded in different conditions, can make it impossible to predict with sufficient precision, the identity of certain peaks of isomers or of similar substances, from the chemical structure point of view.

Analysis of an EO usually involves the separation, identification and quantitative determination of its components. The volatility and polarity of EO components make capillary gas chromatography the technique of election for their analysis, because EOs in general are complex mixtures of components with similar physicochemical characteristics. An exhaustive EO separation can preferably be obtained by combining two different-polarity stationary phases. The most used a polar stationary phases in EO routine analysis are in general those based on methyl polysiloxanes (SE30, OV-1, OV 101, DB-1, HP-1, PS 347.5, etc.) and methylphenyl-polysiloxanes (SE-52, SE-54, DB-5, HP-5, PS-086, etc.) and polyethylene glycol (PEG-20M, CW-20M, DB-Wax, etc.) as the polar phase. Identification is generally carried out either by chromatographic data (Kováts indices, linear retention indices, relative retention time, retention time locking), measurable with a universal detector such as FID or TCD, or by spectral data, mainly by mass spectrometry (GC–MS) or, better, by their combination.

Plant essential oils are produced commercially from several botanical sources, many of which are members of the mint family (Lamiaceae). The oils are generally composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes. Essential oil constituents are primarily lipophilic compounds that act as toxins, feeding deterrents and oviposition deterrents to a wide variety of insect pests. Insecticidal properties of several monoterpenoids to the housefly, red flour beetle and southern corn root-worm have been reported. Although many monoterpenoids have insecticidal properties, the degree of toxicity of different compounds to one species differs considerably. The rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine by some oils and with GABA-gated chloride channels by others. Evaluation of toxicity of monoterpenoids against *Coptotermes formosanus* (a subterranean termite) of which eugenol was found most effective as termiticide. It was also effective as a fumigant and as feeding deterrent. Monoterpenes being volatile are more useful as insect fumigants. Several studies have been undertaken in the past to explore the potential of essential oils and their constituents as insect fumigants. Pulegone, linalool and limonene are known effective fumigants against rice weevil, *Sitophilus oryzae*. While Mentha citrata oil

containing linalool and linalyl acetate exhibit significant fumigant toxicity to these rice weevils. Antifeedant chemicals may be defined as being either repellent without making direct contact to insect, or suppressant or deterrent from feeding once contact has been made with insects. Essential oil constituents such as thymol, citronellal and α-terpineol are effective as feeding deterrent against tobacco cutworm, *S. litura* and synergism or additive effects of combination of monoterpenoids from essential oils have been reported against *S. litua* larvae.

Volatile oils of herbs have been documented for possessing significant nematicidal activity against *M. incognita*. Volatiles isothiocyanates and aromatic aldehydes such as phthalaldehyde, salicylaldehyde, and cinnamic aldehyde have been reported to kill root-knot nematodes. However, mode of action of EOs has rarely been thoroughly investigated. Aromatic and medicinal plant essential oils and their components demonstrate antibacterial, antifungal, and food preservative activities against a wide range of microbial pathogens. Moreover, curiosity about essential oil applications that can act as antimicrobial agents is growing because of the broad range of activities, natural origins, and generally recognized as safe (GRAS) status of essential oils. Currently, essential oils are frequently studied for their antimicrobial, antifungal, antiulcer), antihelminthic, antioxidant, anti-inflammatory, repellent, insecticidal, antifeedant, cytotoxic, antiviral, ovicidal, anesthetic, molluscicidal, immunomodulatory, antinociceptive, and larvicidal properties

Eco-benign pesticides derived from plant essential oils do have several important benefits. Due to their volatile nature, there is a much lower level of risk to the environment than with current synthetic pesticides. Predator, parasitoid and pollinator insect populations will be less impacted because of the minimal residual activity, making essential-oil-based pesticides compatible with integrated pest management programs. It is also obvious that resistance will develop more slowly to essential-oilbased pesticides owing to the complex mixtures of constituents that characterize many of these oils.

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Molecular Docking & Virtual Screening: Tools for Drug Design

Abhishek Mandal * and Randeep Kumar
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi 110012
*email: abhishekmandal@iari.res.in

The research phase can be broken down into two main tasks for those involved in drug design, such as medicinal and computational chemists: identification of new compounds that show some activity against a target biological receptor, and progressive optimization of these leads to a compound with improved potency and physicochemical properties in-vitro, and, eventually, improved potency and physicochemical properties in-vivo. Random screening or a guided design approach are used to find leads, and both methods have historically been equally important, depending on the issue at hand. For medicinal chemists and molecular modeling scientists to use the guided approach, they need a logical starting point. Analogs of a drug known to be active against a target receptor and mimics of an enzyme's natural substrate are two examples. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are increasingly revealing the three-dimensional structure of many biological targets, paving the way for the creation of novel molecules that directly exploit the receptor-binding site's structural characteristics. This structure-based design approach has had a significant effect on the rational design and optimization of new lead compounds in cases where the receptor structure is well defined in recent years. Screening, or the method of analyzing a large number of molecules for action in a model system that is predictive of a human disease, is well-known in the pharmaceutical industry. As part of the latest drug development process, high throughput screening technology enables thousands to millions of molecules to be tested for action against a new target system. Virtual screening, also known as *in-silico* screening, is a modern branch of medicinal chemistry that provides a fast and cost-effective way to computationally scan databases for novel drug leads. Digital screening can be traced back to structure-based drug design and molecular modeling.

Concept of Virtual Screening

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Structure Based Virtual Screening (Docking)

Docking and scoring strategies for high throughput screening can be used to computationally scan a database of hundreds of thousands of compounds against a target of proteins. Molecular docking approaches are computational methods that predict the three-dimensional structure of a protein-ligand complex.

Since the protein ligand docking events are entirely independent of one another, virtual screening as a computational task can be easily completed using parallel computing. While docking was originally developed as a specialized modeling tool for use on a computer workstation, it is now possible to use distributed computing over networked PCs for virtual screening in expensive Linux clusters. This boosts the *in-silico* throughput to 100,000 compounds per day on Linux clusters, bringing it up to speed with today's high-throughput screening. When calculating the binding free energy between protein and ligand, energy functions will use heuristic terminology. As a result, such functions are referred to as scoring functions. Protein structure preparation, ligand database preparation, docking measurement, and post processing are all necessary steps. Unless various protein conformations are considered, the protein only needs to be prepared once for the virtual screening experiment. Charges must be assigned and the receptor site must be determined. Modeling the protein sites as precisely as possible is needed. Determining protein surface atoms and site points, as well as assigning interaction data like labeling hydrogen-bond donors/acceptors and so on, is sometimes done internally in docking software (e.g., FlexX) (Schneider et al., 2013) and sometimes done separately.

Because of the large number of molecules, manual steps in the ligand database preparation must obviously be avoided. Bond types must be tested, protonation states must be determined, charges must be allocated, and solvent molecules must be extracted, usually

starting with a 2D structure. A software like ChemDraw can be used to create 3D coordination. Any docking procedure must evaluate and rank the configurations produced by the search process, which is referred to as "scoring". The most closely related scoring scheme to experiment, the “ab initio” estimate of the binding free energies, is not easily computed. The docking and design scoring is basically made up of three different aspects:

1. The ranking of the conformations produced by a docking quest for a single ligand interacting with a given protein; this aspect is critical for identifying the binding mode that best approximates the experimentally observed situation.

2. Ranking various ligands in terms of how well they bind to a single protein, i.e., prioritizing ligands based on their affinity; this is an important feature of virtual screening

3. Ranking one or more ligands based on their binding affinity to various targets; this factor is critical in determining ligand selectivity and specificity.

Different types of docking

There have been various forms of docking recorded:

1. Flexible docking of proteins and ligands

For the development of new drugs, flexible protein-ligand docking is important. The new programs and algorithms are capable of predicting the action of chemical compounds and protein molecules through the exhaustive integration and fine tuning of various variables, allowing researchers to find more effective drug leads. This approach greatly reduces the required cost money and time spent, as well as reducing non-specific drug molecule protein interactions; this aspect is critical when considering selectivity and specificity.

2. Protein-protein docking that is flexible

Protein-protein interactions are also crucial because they are responsible for a number of critical biological functions. The ability to predict such experiences is critical to a full understanding of human physiology. A basic biological phenomenon and an unresolved theoretical issue is the association of two biological macromolecules. Several groups have developed tools in recent years to try to solve the so-called protein-protein docking problem, which is the prediction of a complex's geometry from the atom coordinates of its uncompleted constituents.

3. Docking with a hydrophobic surface

Since hydrophobic groups are more common at contact sites, they contribute to more intermolecular atom-atom contacts per unit area for accurate matches than for false positive fits. The hydrophobic groups are also present. At first glance, it seems to be less versatile. As a result, compared to complete representation, a partial representation of the molecules based on hydrophobic groups could increase the consistency of the results in finding molecular

recognition sites.

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Extraction of volatile components from plants and their characterization by GC-MS

Aditi Kundu* and Supradip Saha
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi
*Email: chem.aditi@gmail.com

Essential oils are used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. The world production and consumption of essential oils and perfumes are increasing very fast. Production technology is an essential element to improve the overall yield and quality of essential oil. The traditional technologies pertaining to essential oil processing are of great significance and are still being used in many parts of the globe. Water distillation, water and steam distillation, steam distillation are the most traditional and commonly used methods.

Essential oils are generally derived from one or more plant parts, such as flowers (e.g. rose, jasmine, carnation, clove, mimosa, rosemary, lavander), leaves (e.g. eucaly ptus, mint, *ocimum* spp., lemongrass), leaves and stems (e.g. geranium, patchouli, petitgrain, verbena, cinnamon), bark (e.g. cinnamon, cassia, canella), wood (e.g. cedar, sandal, pine), roots (e.g. angelica, sassafras, vetiver, valerian), seeds (e.g fennel, coriander, caraway, dill, nutmeg), fruits (bergamot, orange, lemon, juniper) and rhizomes (e.g. ginger, curcuma) etc. In order to isolate essential oils by hydrodistillation, the aromatic plant material is packed in a glass container and a sufficient quantity of water is added and brought to a boil. Due to the influence of hot water, the essential oil is freed from the oil glands in the plant tissue. The vapour mixture of water and oil is condensed by condenser cooling with chilled water. From the condenser, distillate flows into a separator, where oil separates automatically from the distillate water.

GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect and detect the ionized molecules separately.

The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio. Use of GC-MS together, allow a much finer degree of substance identification than either unit used separately. It is not possible

to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a pure sample while gas chromatography using a traditional detector (e.g. FID) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (*i.e.* have the same retention time) which results in two or more molecules to co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically lends to increased certainty that the analyte of interest is in the sample.

Chemicals and glasswares

Clevenger's apparatus, condenser, RB flask, heating mantle

Procedure

Take the weight of the fresh sample (leaves/flower/rhizome/root etc.). Cut the sample in small pieces and keep in the round bottom flask. Add distil water to the flask. Assemble the distillation assembly. Keep the flask on heating mantle and join Clevenger's apparatus. Put the condenser upon Clevenger's apparatus with all the cold water connecting pipes. Boil the mixture on heating mantle. The water vapour containing essential oil will condense in the condenser and collect over the water layer in the Clevenger's apparatus. Continue the distillation process for 4-5 hours to achieve maximum oil. Taken out the oil from the apparatus and passed through anhydrous sodium sulphate to remove traces of water, if any. Keep the oil inside refrigerator for further use.

Essential oils are concentrated hydrophobic liquid containing volatile aroma compounds from plants. GC-MS analysis was carried out using 7890A GC (Agilent Technologies) with equipped with flame ionization detector (FID), directly coupled to the mass spectrometer system and HP-5MS column (30 m × 0.25 mm; 0.25 μm, Agilent Co., USA) which was directly connected to a triple axis HED-EM 5975C mass spectrometer (Agilent Co., USA). The injection volume was 1 μL. Temperature programming was done 60-125ºC at 2ºC/min hold time 2 min., 125-160 ºC at 0.5 ºC /min hold time 5 min. and 160-240ºC at 5ºC/min. Injector and detector temperatures were maintained at 220ºC and 290ºC, respectively. Helium was used as carrier gas with a flow rate of 1 mL/min. Ionization potential was 70 eV and ionization current, 2 A. Ion source temperature was 270ºC and mass transfer line 250 ºC with split ratio 1: 20, mass range 50-500 and resolution 1000. Identification of the constituents of essential oil was performed by comparison of their retention times and comparison of mass spectral fragmentation pattern with those reported in the literature and stored in the MS library.

Results:

Yield (v/w) :

% Yield (fresh weight basis):

Total Ion Chromatogram

Chemical composition identified in GC-MS

Results:

- Interpretation of Total Ion Chromatogram (TIC) of GC-MS
- Determination of volatile constituents of essential oil

HPLC analysis of phytochemicals

Anirban Dutta* and Supradip Saha
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi 110012
*Email: anirban.iari@yahoo.com

Upon growing interest on agrochemicals and natural antioxidants, quantification of active principle with high accuracy and precision is utmost important. Among the various available techniques, high-performance liquid chromatography (HPLC) can be accurate, however, it is laborious, requires skilled labor and costly solvents. Estimation of polar, thermo labile compounds which is not possible by other analytical methods solely depends on HPLC analysis.

Spectrophotometric methods, although easier than HPLC, also require time-consuming extractions and may not be as accurate as HPLC. HPLC, on the other hand, potentially affords separation of bioactive compounds well as their quantitation. Existence of an extensive polyene chromophore makes the carotenoids highly sensitive to heat, oxygen, light, and, in some cases, acids and alkalis. This means that the precautions taken with other natural products have to be stretched to the maximum when working with carotenoid pigments. Whenever possible, a quick and careful manipulation will minimize possible losses from destruction and the appearance of artifacts. Similarly, the sample should not be subjected to excessive heat, so that the use of solvents with high boiling point is generally unadvisable when evaporation is envisaged.

Andrographolide is one of the major constituents of *Andrographis paniculata*, commonly called Kalmegh. It is grown widely in India and known for its wide biological properties. Traditionally the plant has been used for treating viral, microbial dis eases. Andrographolide and its terpenoidal derivatives have been characterized using HPLC and required sensitive and reliable method for its quantitation in extracts, for monitoring its efficacy, stability and toxicity. Reverse phase liquid chromatography is used for separation and quantitation of andrographolide in the *Andrographis* extracts.

Sample preparation: Standard solution of andrographolide (100 μg mL^{-1}) was prepared by dissolving 10 mg of the compound in 100 ml of HPLC-grade acetonitrile. Serial dilutions were made in the range of 10-100 μg mL^{-1} to plot the calibration curve. The standard solutions were stored at –20°C.

Chromatographic conditions

The samples were filtered through a 0.25 um membrane filter before injection and the retention time (R_t) for each compound was measured. The sample (20 □l) was injected into the HPLC for analysis.

HPLC conditions:

1. Detector: UV

2. Stationary phase: C_{18} column (250 X 4.6 mm; 4 □m) or phenyl column (250 X 4.6 mm; 4 µm)
3. Mobile phase: acetonitrile: water (40:60 v/v) flow rate of
4. Flow rate: 0.75 mL min^{-1}
5. $\lambda_{max:}$ 225 nm
6. Run time: 10 min

Quantification of andrographolide:

Andrographolide in the samples was quantified by employing standard Andrographolide sample (95% pure). The value of Andrographolide content was calculated based on calibration and expressed as ppm (mg/g of the kernel weight).

Andrographolide content= (A_1/A_2) (m_1/m_2) x P

Where, A_1= peak area of Andrographolide in sample

A_2= peak area of Andrographolide in reference standard

m_1=mass, in grams, of the test sample.

m_2=mass, in grams, of the reference standard.

P =Purity of the reference standard sample

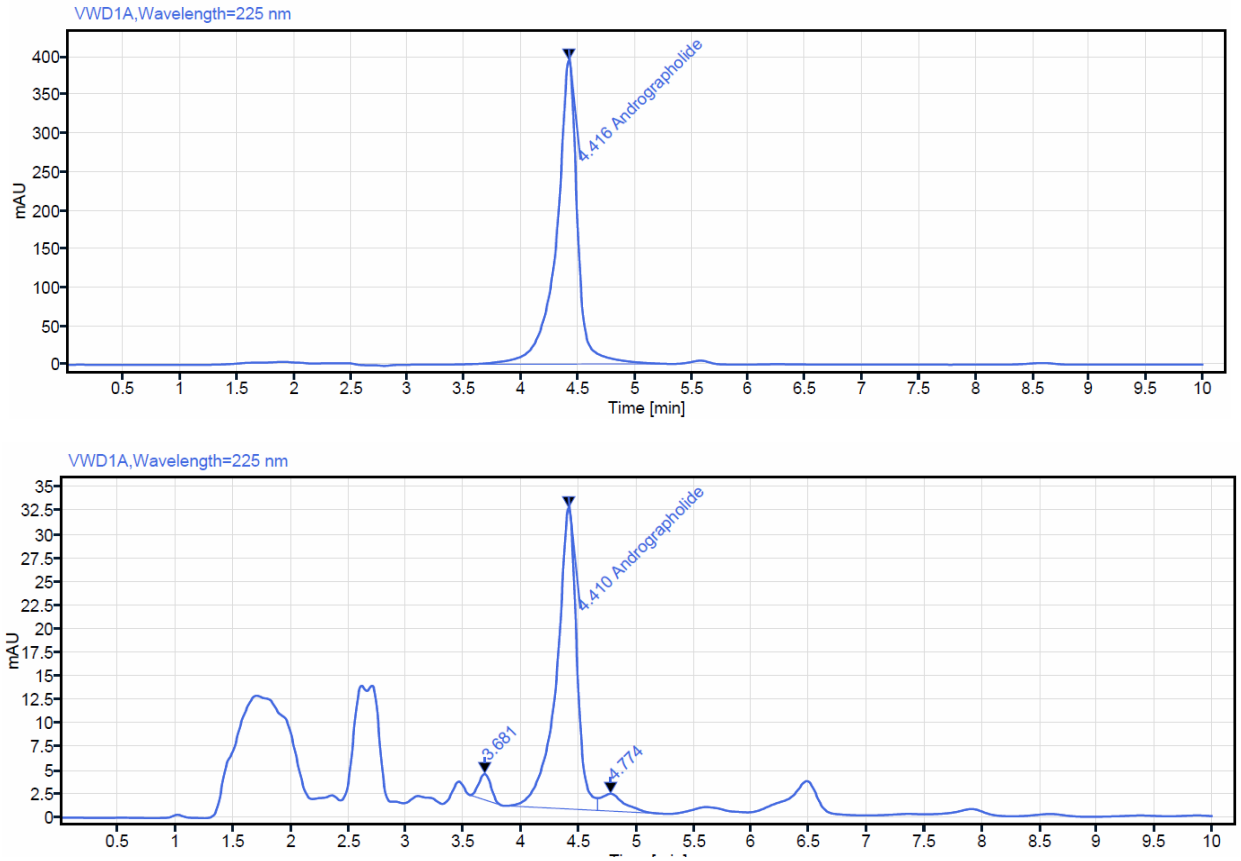


Fig. HPLC Chromatogram of methanol extract of *Andrographis sp*

Extraction of bioactive compounds and their characterization by HRMS

Supradip Saha* and Aditi Kundu
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute
New Delhi-110012
*Email: s_supradip@yahoo.com

Problems caused by chemical pesticides and their residues have resulted in upsurge of need for effective biodegradable pesticides with greater selectivity. Source Alternative strategies have included the search for new types of pesticides which are often effective against a limited number of specific target species, are biodegradable into nontoxic products and are suitable for use in integrated pest management programs. The natural plant products derived from plants effectively meet this criterion and have enormous potential to influence modern agrochemical research. When extracted from plants, these chemicals are referred to as botanicals. The use of botanical pesticides is now emerging as one of the prime means to protect crops and their products and the environment from pesticide pollution. Botanicals degrade more rapidly than most chemical pesticides, and are, therefore, considered relatively environment friendly and less likely to kill beneficial pests than synthetic pesticides.

Neem is an important source of botanicals. Azadirachtin-A as well as other derivatives of azadirachtin are the most important active ingredient in neem. Azadirachtin's use as a pesticide has been commercialized and a number of formulations have been introduced worldwide. However, application of azadirachtin as a pest control agent requires a sensitive and reliable method for its quantitation in neem extracts, for monitoring its efficacy, stability and toxicity. The most acceptable method of analysis of azadirachtin is the use of HPLC. Reverse phase liquid chromatography is used for separation and quantitation of azadirachtin.

Chemicals and reagents

Analytical grade solvents for analysis. Laboratory grade solvents for extraction purpose.

Procedure

Extraction

Neem seed was defatted by hexane and repeated thrice. Hexane extract was concentrated to get neem oil. Defatted seed kernel powder was extracted with methanol for azadirachtin. Methanol extract was purified by two steps, first by partitioning between water and ethyl acetate and secondly by precipitation in hexane. Azadirachtin rich powder was obtained after precipitation. Then the sample was analysed for it's constituents.

Analysis by QTOF

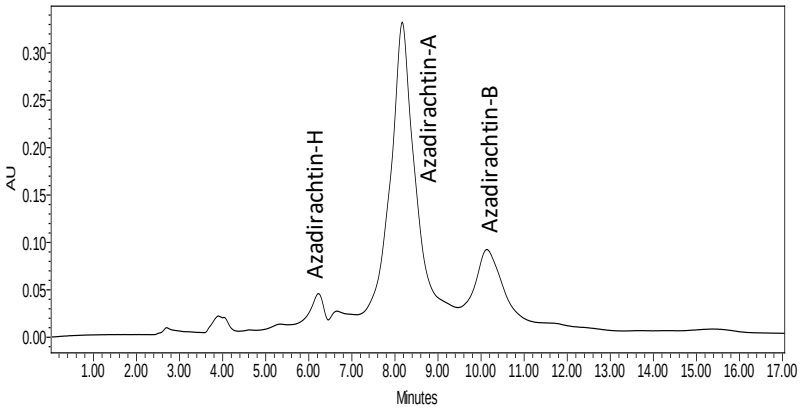
The measurements were carried out by LC-MS/Q-ToF (Xevo G2XS, Waters) system equipped with an ESI source (LockSpray, Waters). The system was coupled to an UPLC (H Class

Aquity, Waters), equipped with a quaternary pump, an thermostated autosampler at 10 °C and a column compartment thermostated at 20 °C.

The parameters used were the following: [ESI(+)]: polarity ES+, analyzer resolution mode, capillary (kV) 2.5, sampling cone 40, source temperature (°C) 150, source offset 80, desolvation temperature (°C) 400, cone gas flow (L/h) 50, desolvation gas flow (L/h) 1000. The column used was an RP-C18 (Waters C18 Column, 2.1 mm × 100 mm × 1.6 µm).

***Quantification of azadirachtin A*:**

Azadirachtin in the samples was quantified by employing standard azadirachtin sample (95% pure). The value of azadirachtin content was calculated based on calibration and expressed as ppm (mg/g of the kernel weight).



Azadirachtin content= (A_1/ A_2) (m_1/m_2) x P

Where, A_1= peak area of azadirachtin in sample

A_2= peak area of azadirachtin in reference standard

m_1=mass, in grams, of the test sample.

m_2=mass, in grams, of the reference standard.

P =Purity of the reference standard sample

Characterization by FT-IR and NMR based techniques

Rajesh Kumar
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi – 110 012
Email: rkb1973@gmail.com

Spectroscopy is the study of the absorption, emission, or scattering of electromagnetic radiation by chemical species. Infrared (IR) and Nuclear Magnetic Resonance (NMR) spectroscopic techniques are being employed for the characterization of organic compounds.
Aim: To characterize the protons and functional groups and ^{1}HNMR in FTIR spectroscopy.
Procedure:

FT-IR spectroscopy

Apparatus required

- FT-IR spectrometer, hydraulic press, die set

Reagents required

- Potassium bromide (KBr) powder (spectroscopic grade purity)

Procedure

- Clean the agate pestle, agate mortar and all the parts of die thoroughly to get rid of any dirt and dry them.
- Place a small amount of sample (1-2 mg) followed by the addition of about 200 mg of dry potassium bromide (spectroscopic grade purity) powder in an agate mortar.
- Grind the mixture using agate pestle and agate mortar to get a fine homogenous powder.
- Assemble the top and bottom guide of die set and insert the die into the cavity.
- Transfer the ground sample mixture into the cavity and spread evenly.
- Insert the plunger and place the die assembly into a hydraulic press.
- Move the wheel of hydraulic press and secure it tightly.
- Close the valve of the hydraulic press and apply pressure (normally 8-10 tons) using pump handle.
- Release the pressure and remove the die from the press.
- Dismantle the die, and transfer the KBr disk to a spectrometer disk holder. Do not touch the faces of the disk.
- Mount the disk holder in the FT-IR spectrometer.
- Record the FT-IR spectrum using FT-IR spectrometer software for data acquisition.

NMR spectroscopy

Apparatus required

- NMR spectrometer, NMR tubes

Reagents required

- Deuterated solvents (spectroscopic grade purity)

Procedure

- Take clean NMR tubes along-with their clean caps. NMR tubes should not have any scratch or glass defects.
- Take 5-25 mg of compound in clean sample tube. Dissolve it in 0.6-0.7 ml of suitable deuterated solvent in which it is sufficiently soluble. Transfer the solution to the NMR tube.
- Commonly used deuterated solvents for NMR Spectroscopy are acetone-d_6, benzene-d_6, chloroform-d, deuterium oxide (D_2O), dimethyl sulfoxide-d_6, ethanol-d_6, and methanol-d_4.
- Place the NMR tube containing the sample into the NMR spectrometer. Record the NMR ($^1H/^{13}C$ etc.) spectrum using NMR spectrometer software for data acquisition.

Preparation of Emulsifiable concentrate (EC) formulation of neem oil

Neeraj Patanjali* and Anupama Singh
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi 110012
*Email: neerajpatanjali@gmail.com

An EC formulation is an organic solvent-based liquid homogenous formulation, which need to be applied in the form of an emulsion after dilution in water. EC formulations are prepared from low melting point solids or oily active ingredient (*a.i.*) which are soluble in non-polar hydrocarbon solvents e.g. xylene, C-9, kerosene etc. Surfactant emulsifiers are added to these formulations to ensure spontaneous emulsification with good emulsion stability upon dilution. Emulsion droplets of size 0.1–5 µm are produced upon mixing of formulation with water. The total concentration of the emulsifier blend is usually 5–10% of the formulation. HLB stands for hydrophile-lipophile balance and the higher the HLB the more hydrophilic (water-soluble) is the surfactant. The HLB range 8–18 will normally provide good oil-in-water emulsions. The optimum ratio of anionic and non-ionic surfactants is determined experimentally to give spontaneous emulsification in water, and to give a stable emulsion with very little creaming and no oil droplet coalescence.

Aim: To prepare Emulsifiable concentrate of neem oil

The typical composition of an EC formulation is given below:

Component	**% (w/w)**
Active ingredient	20–70
Emulsifier blend	5–10
Solvent	to 100

Requirements
Neem oil, surfactant, solvent, shear mixer

Procedure

- Mix the active ingredient, emulsifiers and solvent with low shear mixing until a homogeneous solution is obtained.
- Check the stability and dispersibility at room temperature and elevated temperature as per the CIPAC methods.

Preparation of Suspension concentrate (SC) formulation of Tebuconazole

Neeraj Patanjali* and Anupama Singh
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi
*email: neerajpatanjali@gmail.com

Suspension concentrate (SC) formulation can be defined as a stable dispersion of one or more active ingredient (a.i.) in a fluid and required dilution with water before use. This system of SC consisting of finely divided solid particles dispersed in a liquid phase. The proportion of solid active ingredient usually ranged between 0–50%. Farmers generally prefer SC formulations over wettable powders because of their non-dusty nature, easy measurement and pouring into the spray tank. SC formulations are made by dispersing solid *a.i.* in an aqueous solution containing wetting and dispersing agent using a high shear mixer to give a concentrated premix. The obtained premix will then have subjected to wet grinding process in a bead mill to give a particle size distribution in the range 1–10 microns.

Aim: To prepare Suspension concentrate (SC) formulation of Tebuconazole

The typical composition of an EC formulation is given below:

Component	**% (w/w)**
Active ingredient	20–50
Wetting/dispersing agent	2–5
Antifreeze	5–10
Anti-settling agent (Rheology modifier)	0.2–2
Anti-foaming agent	0.01-0.5
Water to 100	Water to 100

Requirements

Tebuconazole, adjuvants, high shear mixer, bead mill

Procedure

- Mix all the adjuvants with low shear, then high shear (10,000 rpm, 10 min).
- Ensure complete homogenization of all the ingredients
- Mixture of a.i. and adjuvants will then subjected to bead mill for particle size reduction.
- Check for particle size it should range between 1-10 microns
- Check the stability and suspensibility at room temperature and elevated temperature as per the CIPAC methods.

Preparation of nano emulsion of essential oil

Anirban Dutta* and Abhishek Mandal

Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute,
New Delhi
*email: Anirban.iari@yahoo.com

Emulsion is a biphasic colloidal system of two immiscible liquids, where one liquid is being dispersed in another liquid (continuous phase). An oil-in-water emulsion is formed when a non-polar oil phase is dispersed in water. Emulsion of two immiscible liquids can only be formed in presence of surfactant(s). The amphiphilic surfactant molecules form micelles around oil droplets and helps in reducing the surface tension at the oil-water interphase. Thus, the surfactants act as emulsifier for the liquid-liquid dispersion. Application of high energy methods like ultrasonication, homogenization etc. during emulsification breaks the micelles into finer size within which small oil droplets get entrapped. Method can be optimized in such a way that the diameter of the oil loaded micelles falls in the nano range. Considering the droplet size distribution and thermodynamic stability of the system, a uniform and stable emulsion system with fine droplets can be prepared. However, the formation and stability of such system depends on the chemistry of surfactants, relative quantity of surfactants, oil:water ratio, pysico-chemical property of oil, and the external factors like temperature, salt concentration etc.

Aim

To prepare nano emulsion of essential oil by ultrasonication and its characterization by particle size analyzer

Requirements

Essential oil, surfactant, ultrasonicator, particle size analyzer

Procedure

- Dissolve required quantity of the surfactant in water (w/v) to prepare a surfactant solution of desired strength.
- Weigh exact amount of essential oil in a stoppered glass vial to prepare a primary stock emulsion of 1% concentration.
- Add required volume of the surfactant solution into it and vortex for few minutes. This forms the primary stock emulsion.
- To prepare nano emulsion by emulsion dilution technique, pipette out necessary volume of the primary stock emulsion and mix with the same surfactant solution.
- Keep the secondary emulsion in an ultrasonicator bath for a predetermined time.
- Remove the sample and analyze the size distribution of the droplets by a particle size analyzer.

In vitro bioassay of natural component/extracts for their antifungal and nematicidal efficacy

Aditi Kundu
Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi
*email: chem.aditi@gmail.com

Aim: i) To evaluate extracts/compounds for in vitro antifungal activity
ii) To evaluate extracts/compounds for in vitro nematicidal activity

Procedure of antifungal assay

A highly virulent *Fusarium oxysporum* f. sp. *lycopersici* (Fol) strain TOFU-IHBT available at the laboratory collections of the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi was used as the test pathogenic fungus [56]. The fungal culture was activated by sub-culturing on PDA medium and incubating at 27±1 °C temperature.

Poisoned food assay

Antifungal activity of MEO was tested against the pathogenic fungus by poisoned food technique as described earlier with slight modifications [57]. Briefly, PDA powder (39 g) was suspended in 1 L distilled water and autoclaved at 120 °C for 30 min. A stock emulsion of MEO (10000 μg mL^{-1}) was prepared in distilled water using Atlas G5002 surfactant (2%, w/v). Stock emulsion was aseptically mixed with a measured volume of PDA slurry to obtain desired test concentrations (1-1000 μg mL^{-1}). The medium was poured into sterile Petri plates (45 mm i.d.) and allowed to solidify. Mycelial discs (10 mm diameter) were punched from a 7-day-old culture of TOFU-IHBT and placed at the center of each Petri plate. Plates with only the PDA medium (without MEO) were also inoculated with the pathogen and kept as a negative control. While, Nativo 75 WG (25% trifloxystrobin + 50% tebuconazole; Bayer Crop Science Ltd., Mumbai, India) was used as a positive control. Three replicates were maintained for each treatment and the Petri plates were incubated at 27±1 °C. Mycelial growths were measured diametrically when the growth in the negative control plates reached full. Mycelial growth inhibition was calculated using the following formula [58],

$$\text{Inhibition of growth (\%)} = [(C - T)/C] \times 100$$

where, C is the average of three replicates of mycelial growth (diameter in mm) in the negative control and T is the average of three replicates of mycelial growth (diameter in mm) of treated plates.

Procedure

***In vitro* nematicidal assay**

Nematode cultures were collected from infested plants and soil. Egg masses were picked up from the galled roots of the infected seedlings using sterilized forceps. The egg masses were kept for hatching on a wet soft tissue paper supported by an aluminium wire gauze on a Petri plate containing fresh distilled water. The assembly was kept at 27±1 °C for 5 days for egg hatching. Freshly hatched second stage juveniles (J_2s) of the nematode wriggled through tissue paper into the clear water in the Petri plate. The suspension containing the J_2s was collected and their density was estimated using a stereo-microscope.

The test extracts/compounds in desired quantity were diluted in emulsifier water (0.5%) to prepared stock solution. Further dilution of stock solution resulted different test concentration.

The *in vitro* bioactivity of extracts/compound conducted following standard procedure where aliquots (1 mL) of nematode suspension containing ~120 J_2s were placed separately in Petri plates (40 mm i.d.). An equal volume (1 mL) of test solution of particular strength was dispensed in the individual Petri plate to achieve half of the test concentrations. Triton X-100 solution was used as negative control. The Petri plates were incubated at 27±1 °C and observations were recorded under a stereo-microscope at 2, 4, 6, 24, 48 and 72 h intervals. For each treatment and each time interval set, six replications were kept. The number of immobilized and freely moving juveniles was recorded at a specified time period (2, 4, 6, 24, 48 and 72 h). Revival test was conducted by transferring the immobilized nematodes with straightened bodies, to fresh Petri plates containing 1 mL of distilled water, after 24, 48 and 72 h of treatment exposure. Few drops of 1M sodium hydroxide (NaOH) solution were added to the Petri plates containing immobilized juveniles. The nematodes, that instantaneously responded to alkali exposure by changing from straight to curved or hook-shaped posture, were considered alive and the remaining ones that did not respond, were considered dead. Corrected mortality (%) of Mi J_2s was calculated as,

$$\text{Corrected mortality (\%)} = [(T - C)/(100 - C)] \times 100$$

where, T = average mortality (%) in treatment, C = average mortality (%) in negative control.