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In vitro screening of medicinal plant leaf extracts against fungal pathogen Beauveria bassiana (Bals) Vuillemin on silkworm Bombyx mori L

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Abstract
The silk industry is one of the biggest and oldest textile industries in the world. The silk industry has been a vital source of income for many rural communities, particularly in silk-producing regions. It provides employment opportunities for sericulturists, weavers, and artisans involved in the various stages of silk production. Silkworm diseases have a significant impact on the silk industry, affecting silk production, quality, and overall profitability. There are several common silkworm diseases caused by different pathological micro-organisms. Muscardine is a severe disease that is highly contagious. The estimated loss due to muscardine is around 10–40% of the total loss caused by the different diseases.

The use of chemical fungicides can lead to the temporary suppression of disease outbreaks caused by fungus. However, once the fungicide loses its effectiveness due to resistance or other factors, fungal infections may resurge, posing a challenge to disease management. Due to these limitations, there is increasing interest in developing alternative and more sustainable approaches to managing fungal diseases in silkworm rearing. Medicinal plant products can be used as an alternative to fungicides and reduce the impact of synthetic chemicals on the environment.

In view of the above, the study was carried out with the objective of screening medicinal plant extracts against the fungal pathogen Beauveria bassiana in the silkworm Bombyx mori. For the investigation, 46 medicinal plants were selected based on their antifungal activity and medicinal properties. Among the plants selected for the study, nine plant products have shown effectiveness in inhibiting the growth and development of the fungal pathogen Beauveria bassiana in different solvents.

Keywords: Medicinal plant extracts, in vitro screening, Bombyx mori L., Beauveria bassiana (Bals) Vuill

I. INTRODUCTION

Agriculture has been the backbone of the rural economy of India, and around 70% of the total population of India lives in villages and depends on agriculture and allied sectors. Sericulture is an agro-based industry and a good source for the economic upliftment of rural people in India, especially in south India. It plays a significant role in the economies of many countries. Sericulture refers to the cultivation of silkworms and the production of silk fibres from their cocoons. Silk is an esteemed and valuable commodity with a lot of domestic demand as well as export potential. The industry has vast employment potential and is an important instrument for the reconstruction of rural economies. Sericulture is often practiced in rural areas where alternative livelihood opportunities might be limited. It can contribute to rural development by providing frequent income and employment opportunities. The major constraint on the sericulture industry is the outbreak of silkworm diseases. The silkworm is a delicate little insect that has been tamed for centuries. Due to continuous taming, the silkworms lost their wild characters and became sensitive, which leads to the breakout of diseases and cocoon crop losses for the sericulture farmers. Silkworm diseases have a significant impact on the silk industry, affecting silk production, quality, and overall profitability. Cocoon crop loss due to disease occurrence is around 30–40%. In total loss, 10–40% of the loss is accounted for muscardine disease. Frequent cocoon crop loss due to the breakout of diseases affects the yield parameters of cocoon crops and disheartens the farmer's community. Jayaramaiah (1986) reported that, though India occupies 2nd place in the world, it has a comparatively low silk yield per 100 dfls, and affects qualitatively and quantitative parameters of cocoons and silk. It is due to crop losses on account of the incidence of disease and pests. Silkworms are prone to several diseases due to biotic and abiotic factors that take a considerable toll. Silkworms are affected by many diseases, namely Pebrine, a protozoan disease in silkworms caused by *Nosema bombycis;* Grasserie, a viral disease caused by *Bombyx mori* nuclear polyhedrosis virus, a baculovirus; Flacherie, a bacterial disease caused by densonucleosis virus; muscardian disease is a fungal disease caused by *Beauveria bassiana*. Muscardine disease is the most contagious of all silkworm diseases. Sources of infection include dead silkworms and contaminated mulberry leaves. This spreads rapidly among the larval colony and from one place to another by contact and by air. The larvae lose their appetite, become inactive, and on death, gradually become harder and finally mummify. The predisposing factors for muscardian disease are low temperatures and high humidity. Several researchers examined the effect of different chemical fungicides to prevent or treat the white muscardine in the silkworm *Bombyx mori.* The use of chemical fungicides leads to the temporary suppression of fungus outbreaks. However, once the fungicide loses its efficiency due to resistance or other factors, fungal infections may resurge, posing a challenge to disease management. Due to these disadvantages, there is increasing interest in developing alternative and more sustainable approaches to manage fungal diseases in silkworm rearing.

Therapeutic usefulness of plants is decided by their chemical contents or phytochemical ingredients, which are present naturally in plants. Flavonoids, alkaloids, tannins, saponins, phenols, and glycosides are the major secondary metabolites found in plants that have antioxidant, anti-inflammatory, anticancer, and antimicrobial properties. Medicinal plants are nature's boon to mankind and the medicinal plant extracts are natural products with a potential source of therapeutics and can be used as an alternative to synthetic fungicides that reduce the impact on the environment. They are biodegradable and do not persist as harmful residues in the soil or water. Medicinal plant extracts often have lower toxicity to non-target organisms compared to chemical fungicides. Phyto-chemicals such as alkaloids, flavonoids, tannins, saponins, phenols, steroids, and glycosides found in plants have many therapeutic value.

In view of the above, the study was carried out with the objective of screening medicinal plants against the fungal pathogen *Beauveria bassiana* under *in vitro conditions.* For the investigation, 46 medicinal plants were selected based on the literature and their medicinal anti-fungal properties.

II. METHODOLOGY

The methodology of the present study comprises of three components. They are

- Collection of leaves of the medicinal plants and preparation of extracts
- Preparation of *Beauveria bassiana* culture
- *In vitro* screening through the agar-well diffusion method

Collection of medicinal plants and preparation of extracts

For the experimentation, as a first step, forty six medicinal plants were selected after a thorough scan of literature with medicinal and antifungal properties. Fresh leaves of the selected plants were collected in the early hours of the day from the medicinal garden of the Department of Bioscience and Sericulture, Sri Padmavati Mahila Visvavidyalayam (SPMVV), Tirupati and within the campus of Sri Padmavati Mahila Visvavidyalayam, (SPMVV),Tirupati, and identified with the help of experts and faculty of Botany, Department of Bioscience and Sericulture, Sri Padmavati Mahila Visvavidyalayam (SPMVV), Tirupati. The freshly collected leaves were taken to the microbiology laboratory and thoroughly cleaned under running tap water, then with distilled water. Then, the leaves were cut into pieces and dried in the shade at room temperature for two weeks. Then the dried leaves were powdered using an electric mixer. The leaf powder was stored in airtight containers in dark conditions until it was used. By using soxhlet method, plant extractions were prepared by using the solvents methanol, ethanol, and distilled water. The extract is stored in an amber-colored bottle at 4°C until it is used for experimentation.

Preparation of *Beauveria bassiana* Culture

Mummified cadavers of silkworms infected with *Beauveria bassiana were* collected, sterilized for 5 seconds by using 70% ethanol, and then rinsed thoroughly with sterile distilled water to eliminate traces of ethanol. The mummified cadavers were gently scratched with sterilized surgical blades and forceps, and the white powdery material was collected. The material was placed in a petridish containing potato dextrose agar media. 20 ml of the sterilized media was dispensed into each petri plate. The complete operation was carried out under aseptic conditions. The petri plates inoculated with fungal spores were incubated for 7 to 9 days at 25°C. The conidia from a single colony of *Beauveria bassiana*, the fungal pathogen, were collected and transferred to PDA slants, and the pure culture of *Beauveria bassiana was* maintained by repeated transfers for every 15 days.

***In vitro* screening through Agar-well diffusion method**

The sensitivity of *Beauveria bassiana* to medicinal plant extracts was determined on Potato Dextrose Agar (PDA) plates by following agar-well diffusion method. The experiment was carried out by using Potato Dextrose Agar (PDA) as the culture medium for the fungal pathogen *Beauveria bassiana.* Sterilized molten Potato Dextrose Agar (PDA) was poured into Petri plates and seeded with *Beauveria bassiana* inoculums. After solidification of the media, in each petriplate 4 holes with a diameter of 6 to 8 mm were made with a sterile Cork borer. Then 100 µL of plant extracts in different solvents such methanol, ethanol and double distilled water were introduced into the wells. The Petri plates were incubated at 25±1^{0}C. Inhibition of growth of *Beauveria bassiana* was indicated by a clear zone around the well filled with plants extracts. The inhibition zone was measured in mm on 2nd and 3rd day of incubation and recorded the data.

III. RESULTS AND DISCUSSION

Plants are great natural sources of nutrition and medicine. Plants consist of many bioactive compounds, such as a**lkaloids, flavonoids, phenols, saponins,** minerals, vitamins, etc., that provide many therapeutic properties. In the present study, antifungal activity of 46 selected medicinal plants in three solvents i.e., **ethanol, methanol and aqueous** was examined under *in vitro* conditions. Results of the study indicate that out of 46 medicinal plants, only nine showed a positive impact on inhibiting the fungal pathogen *Beauveria bassiana* in different solvents i.e., methanol, ethanol and aqueous.

The results in graph-1 indicate that *Phyllanthus emblica* L. (Usiri) showed the highest zone of inhibition (27.26±0.36) in the ethanol solvent, followed by other methanol (16.0±0.48) and aqueous (4.00±0.16) solvents. This is followed by *Azadirachta indica* A.juss. (Neem), which also showed the maximum zone of inhibition (27.1±0.53) with ethanol solvent; it was followed by aqueous (21.03±0.44) and methanol solvent (20.33±0.84) against *Beauveria bassiana. Ocimum tenuiflorum L.*(Thulasi) is also effective in inhibiting the growth of the fungal pathogen (26.23±0.55) with ethanol solvent, followed by methanol plant extract (17.1±0.29), but aqueous plant extract is not effective in inhibiting the growth of the fungal pathogen. The inhibition zone recorded was 22.4±2.28mm and 22.16±0.62 mm with ethanol and methanol solvents, respectively, with *Cymbopogon citrates (DC) Stapf*. (Lemon Grass), but the inhibition zone recorded was 14.93±0.32 mm with aqueous extract. *Psidium guajava* L. (Guava) showed the highest inhibition zone (23.1±0.53 mm) with methanol extract compared to the other two solvents used, i.e., ethanol (**15.93±0.21)** and aqueous extracts (12.96±0.28**)**. *Murraya koenigii L.*(Curry Leaf) also showed maximum inhibition zone (19.76±0.55) in methanol extract, followed by ethanol plant extract (14.06±0.46). At the same time, it was noticed that the aqueous extract is not effective against the *fungal pathogen Beauveria bassiana. Sapindus mukorossi* Gaertn. (Kunkudu) ethanol extract showed a high inhibition zone (14.93±0.21mm) compared to methanol (7.1±0.29) and aqueous (2.93±0.32) solvents. *Manilkara zapota* L. (Sapota) showed the highest inhibition zone (18.03±0.20) with methanol extract, followed by aqueous (16.00±0.40mm) and ethanol (11.00±0.25mm) plant extract. *Lawsonia inermis* L. (Henna) methanol extract was found to be effective in inhibiting *Beauveria bassiana (18.9±0.29)* but not the other two solvents.

Azadirachta indica A.juss. -**Neem**

Azadirachta indica showed the maximum inhibition zone with all the solvents used in the study; this may be due to the presence of photochemicals in the leaves of neem that possess antiviral, antibacterial, and anti-fungal properties. Subapriya and Nagini (2005) stated that the constituents of neem leaf exhibited immune-modulatory, antifungal, antibacterial, and anti-inflammatory properties. Mahmoud *et al.* (2011) observed the effectiveness of *Azadirachta indica* seed extract in methanol solvent against *Aspergillus niger and Fusarium oxysporum.* Saleem *et al*. (2018), Braga *et al.* (2021), and Siddavaram Nagini *et al.* (2023) mentioned that the more abundant phytochemicals in neem are azadirachtin, gedunin, and nimbolide, which have potential antimicrobial properties.

Phyllanthus emblica L.**- Usiri**

Wickramaarachchige Harini Sihara *et al.* (2021) observed the presence of cardiac glycosides, flavonoids, and tannins through photochemical screening and stated that *Phyllanthus emblica* possesses anti-fungal properties and is effective against *Candida albicans.* A similar observation was noticed in the present study with *Phyllanthus emblica* in preventing the growth of the fungal pathogen *Beauveria bassiana* on silkworm. Ravindra Shukla *et al.* (2012) reported that *Phyllanthus emblica* is endowed with antioxidant, anti-fungal, and aflatoxin-inhibitory properties with methanol and aqueous extracts. Orabi Mohamed (2023)**observed** the greater antimicrobial activity of the leaves of *Phyllanthus emblica than the fruit. And it was suggested that* polyphenolic compounds, vitamins, and minerals may amalgamate to mitigate oxidative damage and treat microbial infections.

Ocimum tenuiflorum *L.*- **Tulsi**

Several studies revealed that tulsi has anti-fungal, anti-bacterial, and anti-viral properties against many disease-causing agents responsible for infections, and it is dynamic against various animal pathogens (Vasudevan *et al.*, 1999). Singh *et al.* (2010) explained that the major components of *Ocimum sanctum,* such as eugenol and linalool, proved to have anti-fungal properties. Khan *et al.* (2010) reported that the anti-fungal activity and synergistic interaction of Tulsi are due to the presence of azoles. Sethi *et al. (2013)* reported that essential oils of different species of *Ocimum exhibited* different levels of anti-fungal properties against *the fungal* pathogen of the plant *Rhizoctonia solani.* Vidya Shinde and Datta Dhale (2011) observed the anti-fungal activity of *Ocimum tenuiflorum* leaf in chloroform, alcoholic, and aqueous extracts against pathogenic fungi on vegetable crops. The antifungal action of the essential oils of *Ocimum tenuiflorum* L. and *Ocimum basilicum* L. may be owing to disruption of biofilm, suppression of germ tube production, and inhibition of biofilm formation, as elucidated by Alessandra Piras *et al.* (2018). Satish Sharma *et al.* (2019) stated that *Ocimum sanctum* leaf extracts significantly inhibited the growth of fungal pathogens such *as Rhizoctonia solani, Rhizoctonia bataticola, Phoma sorghina, etc.*

Cymbopogon citratus *(DC.)* – **Lemon grass**

Syed Nyamath and Karthikeyan (2018) reported that *Cymbopogon citratus* is a medicinal herb that contains citral, flavonoids, and tannins, which may be responsible for the antimicrobial and anti-fungal properties. Bayala (2018) reported that ethanolic extracts of *Cymbopogon citratus* leaves were effective against *Staphylococcus aureus* in the presence of flavonoids and tannins, and the root extract of *Cymbopogon citratus* had anti-fungal activity in the presence of citral. Oluwole Solomon Oladeji *et al.* (2019) observed antimicrobial, insect-repellant, insecticidal, and cytotoxic properties on some cancer cell lines in humans using lemongrass oil. The authors also stated that the presence of citral in lemongrass induces programmed cell death in several cancer cell lines by activating procaspase-3 in a dose- or time-dependent manner. Mohammed et *al.* (2020) stated that the phytochemical contents and pharmacological activity were highly impressive against a few pathogenic microorganisms isolated from the infective skin with the extracts of lemongrass residues. Gao *et al.* (2020) stated that lemongrass oil and citral were effective in exterminating the *bio-films of Candida albicans and Staphylococcus aureus by hampering the interactions and breaking the composition of the matrix of bio-films.* Sharma *et al.* (2021) mentioned that citral is one of the potent biomolecules of lemon grass with a range of biological activities and therapeutic properties like antioxidant, anti-diabetic, antimicrobial and anti-inflammatory.

The present study also showed that *Cymbopogon citratus* is effective against the fungal pathogen *Beauveria bassiana* in ethanol and methanol extracts. This may be due to the presence of citral in lemongrass.

***Psidium guajava* L.- Guava**

The antibacterial and antifungal activity of *Psidium guajava* leaves was assessed by Rathish Nair and Sumitra Chanda (2007) against gram-positive and gram-negative bacterial strains and fungal strains in various solvents (methanol, acetone, and N, N-dimethylformamide (DMF). They observed that, while all of the extracts were equally effective against gram-negative germs, the acetone extract was particularly effective against gram-positive and fungal strains. On the whole, the acetone extract from *Psidium guajava* was very effective against the 91 microbiological strains that were tested. Bipul Biswas *et al.* (2013) reported that guava leaf extracts in methanol and ethanol solvents inhibited the growth of gram-positive bacteria, but gram-negative bacteria were resistant to all the solvent extracts. They stated that the guava leaves are rich in many bioactive elements, such as cineol, flavonoids, triterpenes, tannins, resin, eugenol, malic acid, etc., that can fight against pathogens.

***Murraya koenigii* L. -** Curry Leaf

Murraya koenigii extracts were evaluated by Fernandes *et al.,* (2012) against various fungal pathogens on *Manilkara zapota* in different solvents, namely ethanol, chloroform, and aqueous. They revealed that the aqueous extract was active against *Penicillium* and *Aspergillus niger*. while the ethanol and chloroform extracts were active against *Aspergillus flavus* in maintaining the levels of sugar and *Aspergillus niger* in maintaining the levels of protein. And conclude that *Murraya koeinigii* extracts have proved to be antifungal against *Aspergillus flavus* and *Fusarium.* Rao *et al.* (2007) stated that *Murraya koenigii* has antioxidant activity with immense radical-scavenging properties. Jayaprakash Arul and Ebenezer (2012), observed that *Murraya koenigii* ethanolic extract is effective against *Trichophyton mentagrophytes* and *Microsporum gypseum*. Gabriel and Izionworu (2014), **studied the anti-fungal activity of** *Murraya koeingii* in aqueous and organic solvents, and he noticed a high inhibition zone against *Candida albicans, Penicillium funiculosum, Penicillium camemberti,* and *Aspergillus niger.* Phytochemicals such as saponins, steroids, tannin, alkaloids, flavonoids etc., in *Murraya koeinigii* has antifungal, antimicrobial, and anti-inflammatory properties. Dhamane *et al.* (2019) stated that gallic acid and other phenolics in *Murraya koenigii* possess anti- bacterial activity.

Sapindus mukorossi Gaertn -Indian soap berry

Sapindus mukorossi is a plant with extremely high medicinal properties, distributed in tropical and sub-tropical regions of Asia. Bibi George and Shanmugam (2014) reported that ethanol extract was effective in inhibiting *Aspergillus fumigates* and *Aspergillus niger* at 100% plant extract and effective in inhibiting both bacteria and fungi compared with the aqueous extract of *Sapindus mukorossis.* The researchers stressed that the ethanol extract showed a greater number of compounds, such as tannins, flavonoids, alkaloids, phenolic compounds, phytosterols, and saponins, than the aqueous extract of *Sapindus mukorossis;* this may be the reason for efficient control of both bacteria and fungi. The present study also indicates that the ethanol extract of *Sapindus mukorossi* is more effective compared to methanol and aqueous extracts. The presence of saponins, in *Sapindus mukorossi* may be the reason for inhibiting fungal growth.

***Manilkara zapota* L.- Sapota**

Osman *et al.* (2011) examined the antimicrobial properties of ***Manilkara*** *zapota* using stem bark and leaves of ethyl acetate extracts against some pathogenic microorganisms like bacteria and fungi and found that the extract of stem bark showed antimicrobial activity against all the pathogenic bacteria tested (*Bacillus megaterium, Bacillus subtilis, Escherichia coli, Salmonella typhi*, and *Sarcina lutea*) with a zone of inhibition in the range of 08–16 mm, whereas leaf extracts had mild activity against these bacterial strains with inhibition zones in the range of 06–09 mm. While examining the anti-fungal activity of ***Manilkara*** *zapota*, the same researchers also observed that the ethyl acetate extract of stem bark showed positive effects against some fungal strains like *Aspergillus flavus, Fusarium* sp., and *Vasinfectum* sp., with a zone of inhibition between 8 - 13 mm. The ethyl acetate extract of leaves had no anti-fungal activity. These studies indicate that the antibacterial constituents in the bark and leaves of *Manilkara zapota* are present in very low concentrations, and therefore they show antibacterial activities at high doses. In contrast to the above study, *Manilkara zapota* methanol extracts showed a higher inhibition zone (18.03±0.20), followed by aqueous (**16.00±0.40) and ethanol (11.00±0.25) extracts in the present study.**

***Lawsonia inermis* L. (Henna)**

Babu and Subhasree, (2009) found that *Lawsonia inermis* leaves had strong fungicidal property. Arun *et al*. (2010) stated that *Lawsonia inermis* had an ability to treat skin infections such as tinea, by the presence of naphthoquinones that have anti-inflammatory, anti-fungal and anti-viral properties. In the present investigation *Lawsonia inermis* effectively inhibited the growth of *Beauveria bassiana* in methanol solvent, it may due to the presence of naphthoquinones that have anti-fungal, anti-inflammatory and antibacterial properties. Ali Elnaeim Musa *et al* (2011) stated that the aqueous extract of *Lawsonia inermis* displayed anti-fungal activity against the fungal strains viz., *Aspergillus niger, Aspergillus flavus* and *Penicillium notatum.*

IV. CONCLUSION

From the findings of the study, it was found that *Azadirachta indica* (Neem), *Phyllanthus emblica* (Usiri), and *Ocimum tenuiflorum* (Thulasi) showed the maximum inhibition zone with an ethanol solvent. The methanol solvents of *Psidium guajava* (Guava), *Cymbopogon citratus* (Lemon Grass), and *Azadirachta indica* showed a higher inhibition zone against *Beauveria bassiana.* Aqueous extract of *Azadirachta indica* (Neem) is effective in inhibiting the growth of *Beauveria bassiana*, followed by *Manilkara zapota* (Sapota), *Cymbopogon citratus* (Lemon Grass), and *Psidium guajava* (Guava). It is very clear that *Azadirachta indica* (Neem) showed the highest inhibition zone against *Beauveria bassiana,* the fungal pathogen, on silkworm *Bombyx mori* with all the solvents. The anti-fungal compounds present in the selected plants may be used in formulating fungicides to control the infection with *Beauveria bassiana* and to harvest quality cocoons and increase productivity.

In the present investigation, among 46 plants screened, nine plant extracts were found effective against the fungal pathogen *Beauveria bassiana.* These extracts may be used to reduce mortality and improve economic parameters in muscardine-infected larvae under *in vivo* conditions by lowering the incidence of disease, as well as to improve the general health of the silkworm. Further, *in vivo* studies need to be carried out to determine the accurate concentration and dosage required and understand the potentiality of these plant extracts in controlling the white muscardine disease caused by the fungal pathogen *Beauveria bassiana* in the silkworm *Bombyx mori.*

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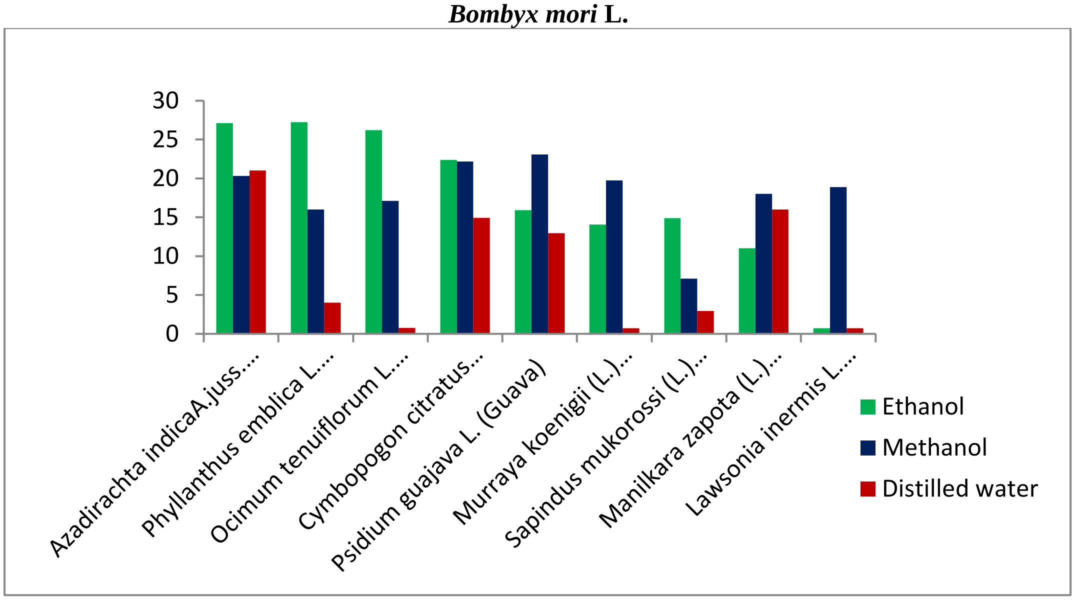
Graph-1: Screening of medicinal plant extracts in three solvents against fungal pathogen *Beauveria bassiana* (Bals) Vuillon. silkworm *Bombyx mori* L.



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