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# Estimation of Biomolecules from Selected Medicinal Plants and their Antimicrobial Activity against Flacherie Disease Causing Bacteria of *Bombyx mori* L.

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Abstract

The silkworm, *Bombyx mori* L. is highly susceptible to viral and bacterial diseases. To manage these diseases, chemical based bed disinfectants are being used which are not eco-friendly and have residual toxicity in silkworm rearing bed and environment. The use of *Adhatoda vasica* and *Phyllanthus niruri* leaf extracts in silkworm rearing is one of the strategies to manage viral and bacterial diseases of silkworm which are non-toxic and environmentally safe. In this study, the aqueous and methanolic leaf extracts of above plants were analysed for different biomolecules such as alkaloids, tannins, phenols, carbohydrates, proteins and flavonoids. The alcoholic (Methanol) leaf extract of *A. vasica* had highest content of biomolecules *viz*., alkaloids (1.190 mg/ml), tannins (14.714 mg/ml), phenols (0.358 mg/ml), proteins (2.254 mg/ml), carbohydrates (3.085 mg/ml) and flavonoids (2.759 mg/ml) compared to aqueous extract (0.125, 6.608, 0.150, 1.146, 2.262 and 1.452 mg/ml). The methanolic leaf extract of *Phyllanthus niruri* had 0.403, 16.047, 0.574, 1.532, 2.081 and 2.593 mg/ml and 0.009, 8.937, 0.502, 0.325, 0.714 and 0.694 mg/ml of alkaloids, tannins, phenols, proteins, carbohydrates and flavonoids in methanol and aqueous extracts, respectively. It is confirmed that, the methanolic leaf extracts of both contained significantly more antimicrobial biomolecules because of their more solubility compared to aqueous. Under *in-vitro* conditions, the maximum zone of inhibition was recorded in methanolic extract of *P. niruri* at $10^{-7}$ (7.50 and 8.11 mm) followed by $10^{-5}$ (7.44 and 7.67 mm) and $10^{-7}$ (7.05 and 7.72 mm) and minimum was recorded in aqueous extract of *Adhatoda vasica.* Thus the plant extracts possess antimicrobial property by inhibiting the growth of flacherie causing bacteria.

*Keywords*: *Adhatoda vasica, Phyllanthus niruri,* Biomolecules, Inhibition zone, *Bombyx mori* L.

Silkworms are affected by a number of diseases due to various biological, chemical, physical, nutritional and environmental causes. Among different diseases, flacherie is one of the serious diseases of silkworms causing cocoon crop loss. To manage these diseases, chemical based bed disinfectants are being used. They are not eco-friendly and have residual toxicity in silkworm rearing bed and environment. Phytochemicals are bioactive compounds of plant origin and are said to be secondary metabolites. Knowledge on the chemical constituents of extracts of medicinal plants is helpful in the discovery of new sources of new drugs against different pathogens of silkworms. The biological efficacy of the medicinal plants depends on the presence of the required quantity and nature of the secondary metabolites in their crude extract (Jazani, 2009). These plant extracts are having promising antimicrobial activities which are exploited for disease management and further spread of the disease in silkworm rearing.

Use of *Adhatoda vasica* (Family:Acanthaceae) and *Phyllanthus niruri* (Family: Phyllanthaceae) leaf extracts are one of the organic based management strategies of viral and bacterial diseases of the silkworm. It contains varied range of biomolecules such as alkaloids, phenols, tannins, carbohydrates, proteins and flavonoids having strong antimicrobial property and act against silkworm pathogens. Therefore, the use of plant extracts in silkworm disease management not only reduces the mortality but also improves the larval and cocoon parameters. The major aim of the study was to estimate the different biomolecules having antibacterial and antiviral properties. These properties of biomolecules enhance the trend of proper use of medicinal plants to identify the new indigenous sources of bed disinfectants and

serve as a remedy against silkworm pathogens (Jigna Parekh and Sumitra Chanda, 2007).

## Material and Methods

### Collection and Sample Preparation

The medicinal plants such as *Adhatoda vasica* (Adusoge) and *Phyllanthus niruri* (Kirunelli) (Fig. 1) were used as the source of phytochemical agents for the study. The leaves were collected from 'Sanjeevini Vatika' (Herbal garden), Department of Horticulture, UAS, GKVK, Bengaluru. The required quantity of fresh leaves of each plant was harvested and surface sterilized with 70 per cent ethyl alcohol then washed with sterile distilled water and shade dried. The shade dried plant samples were then powdered in electric blender at slow speed, sieved and kept stored in desiccators (Krishnaprasad *et al.*, 1979).

Fig. 1: *Adhatoda vasica* and *Phyllanthus niruri* used for the analysis of antimicrobial biomolecules

The extracts were prepared by soxhlet extraction method (Manjunath Ajanal *et al*., 2012) using methanol and distilled water as solvents. 10 g of leaf powder of both the medicinal plants were taken in 250 ml of solvent and prepared the stock. The extract of each was diluted and used for biomolecule estimation.

### Qualitative analysis of antimicrobial biomolecules of *Adhatoda vasica* and *Phyllanthus niruri*

The qualitative analysis of biomolecules present in *Adhatoda vasica* and *Phyllanthus niruri* was done by different tests. Alkaloids by Dragondroff's test, tannins and phenols by Ferric chloride test, proteins by Ninhydrin test, carbohydrates by Fehling's test (Lanjwani *et al.,* 2015) and flavonoids were tested by lead acetate test (Usman *et al*., 2009).

### Quantitative analysis of antimicrobial biomolecules of *Adhatoda vasica* and *Phyllanthus niruri*

#### Total Alkaloids

The total alkaloids present in both the medicinal plants were estimated by using atropine as standard. 10 mg / ml of atropine was measured and prepared reference standard (0.2, 0.4, 0.6, 0.8, 1 and 1.2 ml). For sample estimation, one ml of plant extract was taken in 10 ml of volumetric flask. Then five ml of phosphate buffer (pH 4.7) and Bromocresol green (BCG) solution was added to the mixture and the same was shaken with four ml of chloroform. The absorbance of the complex in chloroform was measured at 470 nm in UV Spectrophotometer against the blank prepared (distilled water). The total alkaloids present in both the extracts were carried out and expressed in mg/ml (Manjunath Ajanal *et al*., 2012).

#### Total Tannins

The total tannin content was present in both the plant extracts was determined by using Folin-Ciocalteu method. About 0.1 ml of the leaf extract was added to 10 ml volumetric flask containing 0.9 ml of distilled water. 0.5 ml of Folin-Ciocalteu phenol reagent and one ml of 35 per cent sodium carbonate solution was added and then diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/ml) was prepared and absorbance for the sample and standard solutions was measured with UV-VIS spectrophotometer against the blank (distilled water) at 700 nm. The total tannin content of the sample was carried out in triplicate. The amount of tannin content present in the sample was expressed in terms of mg/ml (Haile and Kang, 2019).

#### Total Phenols

The total phenol content present in *A. vasica* and *P. niruri* leaf extracts was determined by using Folin-Ciocalteu reagent. The total phenol estimation was arrived by using Gallic acid (20, 40, 60, 80, 100 µg/ml)

as a reference standard for plotting calibration curve. A volume of 0.5 ml of the plant extract of above was taken in 10 ml volumetric flask and two ml of Folin-Ciocalteu reagent was added and mixed. The same was neutralized with four ml of 20 per cent sodium carbonate solution. The reaction mixture of both the medicinal plant extracts was incubated at room temperature for 30 minutes with intermittent shaking for colour development. The absorbance of the resulting blue colour was measured at 765 nm using UV-VIS spectrophotometer. The total phenol content in the leaf extracts of both was determined from the linear equation of a standard curve prepared with gallic acid (Deepika Jain and Sunitha Shrivastava, 2017).

**Total Protein**

The total protein content in the methanolic and aqueous extracts of both the medicinal plants was estimated by Lowry's method. The standard protein solution was prepared by dissolving five mg standard protein Bovine Serum Albumin (BSA) in five ml of 2N NaoH and serial dilutions were prepared (20, 40, 60, 80, 100 µg/ml). The blank and sample tubes contained 0.5 ml of distilled water and plant extracts (methanol and aqueous). Add five ml of Lowry's reagent (Reagent, 10% sodium carbonate in 0.1 N NaoH + 1 % copper sulphate and 1 % sodium potassium tartarate) to each tube and kept undisturbed for 15 min. Then add 0.5 ml of folin reagent with equal amount of water in each test tube with vigorous shaking and kept undisturbed for 30 minutes. The intensity of blue colour was observed at 660 nm on the spectrophotometer. The standard graphs were plotted with known values of BSA and determined the actual amount of total protein content of both the samples of *A. vasica* and *P. niruri* (Lowry *et al*., 1951).

**Total Carbohydarte**

The total carbohydrate content present in *A. vasica* and *P. niruri* was estimated by Anthrone method in both aqueous and methanolic extracts. The standard carbohydrate solution was prepared by dissolving five mg of glucose in five ml of distilled water to arrive working standard (0, 0.2, 0.4, 0.6, 0.8 and 1 ml). Make up the volume to one ml in all the tubes including the extract sample tubes (aqueous and methanolic) by adding distilled water. In each test tube four ml of anthrone reagent (Dissolve 200 mg of anthrone reagent in 100 ml of concentrated $H_2SO_4$) was added. The test tubes were heated for eight minutes in boiling water bath. Cool rapidly and read the green to dark green colour at 630 nm. Draw a standard graph by plotting concentration of the standard on the *X*-axis versus absorbance on the *Y*-axis and calculated the amount of carbohydrate present in the methanol and aqueous extracts of both the plants (Yemm and Willis, 1954).

**Total Flavonoids**

The total flavonoid content of plant extracts was determined by using aluminium chloride assay. An aliquot (0.5 ml) of plant extracts was taken in different test tubes and added two ml of distilled water followed by the addition of 0.15 ml of sodium nitrite (5 %) and allowed to stand for six minutes. Later 0.15 ml of aluminium trichloride (10 %) was added and incubated for six minutes followed by the addition of two ml of sodium hydroxide (4 %) and the mixture volume was made up to five ml with distilled water. After 15 minutes of incubation, the mixture of standard and plant extracts in the tubes turns to pink and the absorbance was measured at 510 nm against blank by using spectrophotometer. The total flavonoid content present was expressed in mg / ml of *A. vasica* and *P. niruri* leaf extracts (Deepika Jain and Sunitha Shrivastava, 2017).

**Antimicrobial Activity of Plant Extracts**

A survey was undertaken during the months of August and September 2020 in Chintamani and Department of Sericulture, UAS, GKVK, Bengaluru for collecting silkworms infected by NPV and bacterial flacherie.

The haemolymph from diseased silkworms were collected by pricking the abdominal legs of silkworm in small vials containing thiourea. A loopful of the haemolymph was taken with inoculation needle and streaked on the Nutrient Agar medium and incubate at room temperature (Govindan *et al*., 1998). The bacteria were purified by re-culture on nutrient agar

medium. From the grown bacteria, the dilutions $10^{-3}$, $10^{-5}$ and $10^{-7}$ were prepared and used for *in-vitro* studies.

Sterilized whatman No.1 filter paper discs of five mm diameter were dipped in botanical extracts for one minute and drained by the edges of petriplate, then placed at the centre of the petriplate. Three replications were maintained for each treatment, further control (Distilled water, Methanol and Absolute control) was used for comparison. The bacteria inoculated plates were incubated for 48 hours at room temperature. The diameter of inhibition zone (mm) of botanical extracts against bacteria was measured at 24 and 48 hours of incubation.

## RESULTS AND DISCUSSION

The qualitative and quantitative analysis of biomolecules of methanolic and aqueous plant extracts of *Adhatoda vasica* and *Phyllanthus niruri* were determined. The screening of biomolecules from both the plants showed the presence of various types of

TABLE 1
Qualitative analysis of antimicrobial biomolecules of *Adhatoda vasica* and *Phyllanthus niruri*

| Biomolecules | *Adhatoda vasica* | | *Phyllanthus niruri* | |
|---|---|---|---|---|
| | Methanolic extract | Aqueous extract | Methanolic extract | Aqueous extract |
| Alkaloids | + | + | + | + |
| Tannins | + | + | + | + |
| Phenols | + | + | + | + |
| Proteins | + | + | + | + |
| Carbohydrates | + | + | + | + |
| Flavonoids | + | + | + | + |

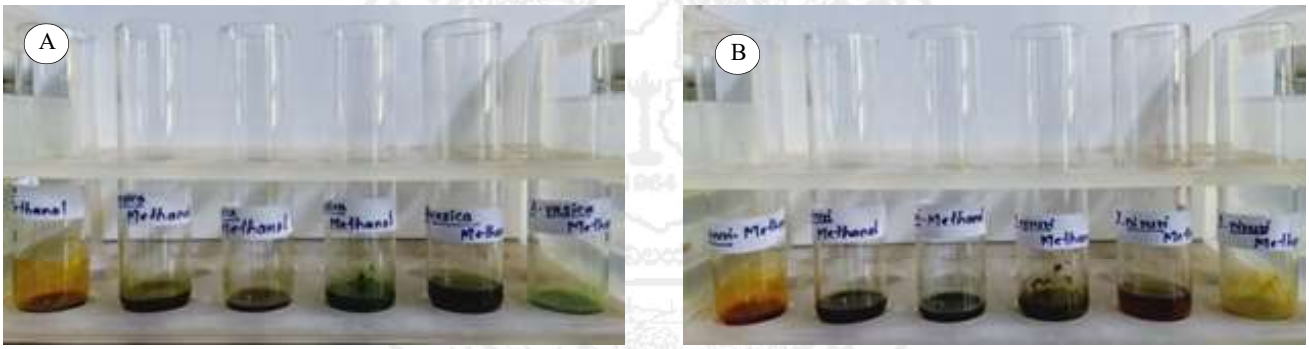


Fig. 2 : Qualitative analysis of antimicrobial biomolecules of A) *Adhatoda vasica* and B) *Phyllanthus niruri*

TABLE 2
Quantitative analysis of antimicrobial biomolecules of *Adhatoda vasica* and *Phyllanthus niruri*

| Antimicrobial biomolecules | Alkaloids (mg/ml) | Tannins (mg/ml) | Phenols (mg/ml) | Proteins (mg/ml) | Carbohydrates (mg/ml) | Flavonoids (mg/ml) |
|---|---|---|---|---|---|---|
| *Adhatoda vasica* | | | | | | |
| Methanolic extract | 1.190 | 14.714 | 0.358 | 2.254 | 3.085 | 2.759 |
| Aqueous extract | 0.125 | 6.608 | 0.150 | 1.146 | 2.262 | 1.452 |
| *Phyllanthus niruri* | | | | | | |
| Methanolic extract | 0.403 | 16.047 | 0.574 | 1.532 | 2.081 | 2.593 |
| Aqueous extract | 0.009 | 8.937 | 0.502 | 0.325 | 0.714 | 0.694 |
| F test | * | * | * | * | * | * |
| S.Em± | 0.006 | 0.187 | 0.008 | 0.030 | 0.079 | 0.030 |
| CD 5 % | 0.018 | 0.568 | 0.027 | 0.091 | 0.240 | 0.091 |

* Significant

biomolecules such as alkaloids, tannins, phenols, proteins, carbohydrates and flavonoids.

The qualitative tests were done by different reagents which exhibit specific reactions with the biomolecules (Fig. 1). The above biomolecules were prominently present in both methanolic and aqueous extracts of both the plants (Table 1).

Nithyatharani and Kavitha, 2018, studied the qualitative phytochemical screening of *Adhatoda vasica* leaves in different solvents like methanol, ethanol, chloroform, petroleum ether and aqueous. The results confirmed the presence of phytocomponents such as, alkaloids, flavanoids, phenols, tannins, carbohydrates and proteins as it was observed in the present study.

It was further confirmed by Lanjwani *et al.,* 2015 that, the qualitative examination of phytochemicals from ethanol, methanol, chloroform and aqueous extract in indiginous medicinal plants *viz., Solanum surattense, Rhazya stricta*, *Moringa oleifera* and *Cichorium intybus*. The tests revealed the presence of phytochemicals such as alkaloids, phenols, flavonoids, tannins, carbohydrates and proteins. Basavaraju and Nanjappa (2011) reported the presence of alkaloids and proteins in different medicinal plants *viz*., makoi, coleus, arrow root and kalmegh.

The total alkaloid, tannin, phenol, protein, carbohydrate and flavonoids were estimated by different methods. The methanolic leaf extract of *Adhatoda vasica* contained 1.190 mg/ml of alkaloids, 14.714 mg/ml of tannins, 0.358 mg/ml of phenols, 2.254 mg/ml of proteins, 3.085 mg/ml of carbohydrates and 2.759 mg/ml of flavonoids, whereas the aqueous extract contained 0.125, 6.608, 0.150, 1.146, 2.262 and 1.452 mg/ml of of alkaloids, tannins, phenols, proteins, carbohydrates and flavonoids, respectively (Table 2).

The biomolecule content of *Phyllanthus niruri* revealed 0.403, 16.047, 0.574, 1.532, 2.081 and 2.593 mg/ml and 0.009, 8.937, 0.502, 0.325, 0.714 and 0.694 mg/ml of alkaloids, tannins, phenols, proteins, carbohydrates and flavonoids in methanol and aqueous extract, respectively. The methanolic leaf extracts had significantly higher biomolecules compared to aqueous extract in both the plant (Table 2). The methanolic leaf extract of *Adhatoda vasica* found comparatively higher contents of biomolecules (alkaloids, proteins,

TABLE 3

Antibacterial activity of extracts of selected medicinal plants against *Staphylococcus* sp.

| Treatments | Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-3}$ | | $10^{-5}$ | | $10^{-7}$ | |
| | 24 hours | 48 hours | 24 hours | 48 hours | 24 hours | 48 hours |
| $T_1$: *A. vasica* aqueous extract | 5.14(2.38) | 5.28(2.40) | 5.28(2.40) | 5.78(2.51) | 5.72(2.49) | 6.04(2.56) |
| $T_2$: *A. vasica* methanolic extract | 5.28(2.40) | 5.41(2.43) | 5.65(2.48) | 5.94(2.54) | 6.00(2.55) | 6.16(2.58) |
| $T_3$: *P. niruri* aqueous extract | 5.72(2.49) | 5.83(2.52) | 5.22(2.39) | 5.61(2.47) | 6.05(2.56) | 6.77(2.70) |
| $T_4$: *P. niruri* methanolic extract | 7.05(2.75) | 7.72(2.87) | 7.44(2.82) | 7.67(2.86) | 7.50(2.83) | 8.11(2.93) |
| $T_5$: Aqueous control | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) |
| $T_6$: Methanol control | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) |
| $T_7$: Absolute control | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) | 0.00(0.71) |
| F test | * | * | * | * | * | * |
| S. Em ± | 0.26(0.87) | 0.25(0.87) | 0.08(0.76) | 0.21(0.84) | 0.18(0.82) | 0.23(0.85) |
| CD 5% | 0.81(1.14) | 0.77(1.13) | 0.24(0.86) | 0.64(1.07) | 0.54(1.02) | 0.70(1.10) |

* Significant



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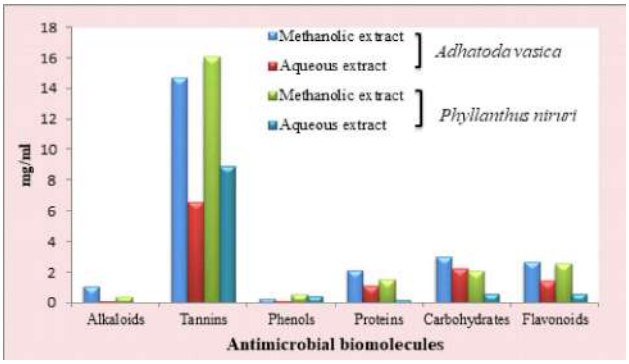


Fig. 3 : Antimicrobial biomolecules of methanolic and aqueous leaf extracts of *Adhatoda vasica* and *Phyllanthus niruri*

carbohydrates and flavonoids) than *P. niruri* except tannin and phenol content (Fig.3).

Deepika Jain and Sunitha Shrivastava, 2017, studied the estimation of total phenolic and flavonoid contents in ethyl acetate and methanol extracts of *Butea monosperma* bark. The total phenolic content was determined by folin-ciocatechu reagent and total flavonoid content by aluminium chloride assay as it is revealed in the present study. Further, they also confirmed that, the methanolic extract of bark showed highest amount of total phenolic content (295.83 ± 0.57 mg/g equivalent of Gallic acid) and total flavonoid content (372.55 ± 0.50 mg/g equivalent of rutin). Sunitha Maurya and Dhananjay Singh (2010) analysed the total phenols in *Adhatoda vasica* leaves with Folin-Ciocalteu reagent. The phenol content in the aqueous extract was 92.4 ± 0.14 mg/g compared to petroleum ether. Proteins (19.86 to 25.42 g/100 g) and carbohydrates (41.18 to 53.28g/100 g) were present in shade dried leaves of *Moringa oleifera* (Deepa and Revanna, 2019) which supported the present study in which the shade dried medicinal plants (*Adhatoda vasica* and *Phyllanthus niruri*) found more proteins and carbohydrates.

The aqueous and methanolic leaf extracts of *Adhatoda vasica* and *Phyllanthus niruri* revealed significant results for zone of inhibition against *Staphylococcus* sp. at spore dilutions of $10^{-3}$, $10^{-5}$ and $10^{-7}$. The maximum zone of inhibition was recorded in methanolic extract of *P. niruri* at $10^{-7}$ (7.50 and 8.11 mm) followed by $10^{-5}$ (7.44 and 7.67 mm) and $10^{-3}$ (7.05 and 7.72 mm) due to the presence of phenols and tannins and the minimum zone of inhibition was observed in aqueous extract of *A. vasica* $10^{-3}$ (5.14 and 5.28 mm) followed by $10^{-5}$ (5.28 and 5.78 mm) and $10^{-7}$ (5.72 and 6.04 mm) at 24 and 48 hours of incubation (Table 3).

The results were confirmed by the study as, the aqueous and alcoholic extracts of *Acalypha indica, Leucas aspera* and *Ocimum sanctum* showed antimicrobial activity at different concentrations (50,100 and 150 µl) against *Staphylococcus* sp. infecting mulberry silkworm and reported highest zone of inhibition at 150 µl in both the extracts of *L. aspera* (37.1 and 39.1 mm) compared to *Ocimum sanctum* (29.3 and 32.2 mm) and *Acalypha indica* (28.2 and 29.5 mm) (Karthikairaj *et al.,* 2014). Similarly, the methanolic plant extract exhibited 33.66 mm and hexane exhibited 30.66 mm of inhibition zone at 100 per cent concentration followed by 50 (29 and 23.33 mm) and 25 (22.66 and 18.33 mm) per cent against *Staphylococcus aureus* using agar well diffusion method (Sharath *et al.,* 2016).

*Adhatoda vasica* and *Phyllanthus niruri* are medicinally important plants due to the presence of various biomolecules. The methanolic and aqueous leaf extracts of above plants are considered to be rich indigenous sources of biomolecules such as alkaloids, tannins, phenols, proteins, carbohydrates and flavonoids. Further, the methanolic leaf extract found higher contents of biomolecules compared to aqueous extract of both the plants due to the solubility of compounds in methanol compared to aqueous. The antimicrobial activity was highest in methanolic extract of *P. niruri* in which the zone of inhibition found maximum compared to other extracts. The organic biomolecules present in medicinal plant extracts possess antimicrobial property, non-toxic, biodegradable, non-pollutant and safe for environment.

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