Pharmaceutical and phytochemical evaluation of a novel anti – white spot syndrome virus drug derived from terrestrial plants

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Abstract

White spot syndrome virus (WSSV), the most contagious pathogen of cultured shrimp, causes mass mortality, leading to huge economic loss to the shrimp industry. The lack of effective therapeutic or prophylactic measures has aggravated the situation, necessitating the development of antiviral drugs. With this objective, the antiviral activity of the drug, (TP22C - derived from the terrestrial plant) was evaluated. The phytochemical and pharmaceutical characterization of TP22C was carried out. The in vivo anlysis of TP22C in the host – pathogen interaction model revealed the efficiency of the drug. The survival percentage of the treated (with TP22C) WSSV infected host was 86 %. Neither the VP 28 gene nor the immediate early genes (ie) were expressed in the host at the 42nd and 84th hrs. Significant results were obtained from the toxicity assays of the drug in shrimp model. The phytochemical screening, biochemical constituents assay, elemental analysis, antioxidant property evaluation and phytochemical fingerprint profiling predicts that TP22C can protect L. vannamei from the WSSV infection, at a dose far below the toxicity level.

Keywords: Shrimps, Litopenaeus vannamei, Anti – WSSV, terrestrial plants, TP22C, White Spot Syndrome Virus.

1. Introduction

White spot disease (WSD) has caused severe mortality in farmed shrimp in many countries and is responsible for huge economic losses in the shrimp culture industry. The losses in India alone have been estimated at several million dollars per year [1]. The principal clinical sign of White spot syndrome (WSS) is the presence of white spots in the exoskeleton of the diseased shrimp. Other signs include a rapid reduction in food consumption, lethargy and reddening of appendages. Mortality rates are usually very high and cumulative mortality can reach 100% within 3 to 10 days from the onset of visible gross signs [2]. Antiviral research using plant extracts has gained momentum since 1950. Scores of medicinal herbs have already been tested and used with good results in the control of viral and bacterial diseases in shrimp and fish.

Strategies for the prophylaxis and control of WSSV theoretically include improvement of environmental conditions, stocking of specific pathogen free (SPF) shrimp post larvae and enhancement of disease resistance by using immunostimulants. Several plants from both terrestrial and marine origin have already been tested against viral diseases to judge its immunostimulant efficacy. Aqueous extracts of Cynodon dactylon (terrestrial plant) and Ceriops tagal (mangrove) exhibited protective effects against WSSV in Penaeus monodon [3,4,5]. The aqueous extract of Sargassum weighti (seaweed) showed significant anti-WSSV property against marine shrimp, Penaeus indicus and freshwater crab, Paratelphusa hydrodomomous [6]. The extract of Phyllanthus amarus and Psidium guajava has shown antiviral activity against yellow head baculovirus in P. monodon [7]. The extract of Clinacanthus nutans has been tested against yellow head virus (YHV) in shrimp and the results indicated an effective control of YHV infection in shrimp [8]. Other control measures that have been undertaken against the WSSV in the culture systems are oral administration of peptidoglycan, lipopolysacharides, β-1, 3 glucan [9 & 11] vaccination with inactivated viral preparation and viral envelope protein, VP19 and VP28 [12, 13] feeding with fucoidan extracted from Sargassum polycysticus [15] and antiviral drug supplemented with Spirulina platensis [16]. An aqueous preparation of a
composite mixture of 7 Indian medicinal plants (Aegle marmelos, Allium sativum, Curcuma longa, Cynodon dactylon, Lantana camara, Mimosa pudica, Ocimum sanctum) were developed and patented in 2002 and they concluded that the preparation was effective in controlling WSSV at the rate of 15 ppm [17]. Glycoproteins derived from Celosia cristata similar to the antiviral proteins of Bougainvillea spectabilis [18], exhibited active antiviral property by inhibiting the mechanical transmission of two tobamoviruses, tobacco mosaic virus and sunnhemp rosette virus, and citrus ring spot virus into their hosts [19]. These previous literatures motivated us to work with the commonly available terrestrial plants, for the eradication of WSSV from the shrimp aquaculture industry and at the same time the protocol should be inexpensive for the marginal farmers. The uniqueness of these phytomolecules, that are derived from these plants have prompted us to take up this present investigation, for which we have selected 30 plants. The leaves from each of these plants were studied for their anti-WSSV property in the host, Litopenaeus vannamei. Further the crude drug derived from the terrestrial plant is subjected to an array of phytoconstituents analysis to judge the efficacy of the same as a potent anti – WSSV drug.

2. Materials and methods

2.1. Screening and isolation of anti – white spot syndrome virus drug

Thirty terrestrial plants were collected from different parts of the West Bengal and Tamil Nadu of India. Four solvents based on their polarity were used to extract phytomolecules from the dry leaves by the soxhlet extraction method. A total of 120 crude isolates thus obtained were coded properly, viz. TP01A (Terrestrial Plant 01 solvent A), TP01B, TP01C, likewise. These coded isolates were administered to Litopenaeus vannamei (white legged shrimp) weighing 5 – 7 gms. post challenge with WSSV to determine the anti – white spot syndrome virus (WSSV) efficacy in the host - pathogen interaction model. Amongst these 120 isolates, 7 showed significant anti – WSSV property. The best anti – WSSV plant isolate, TP22C was derived and purified, and used in further bioassays.

2.2. Toxicological analysis of TP22C in animal model

The lyophilized plant isolate (TP22C) was used to prepare the strength solution for the toxicity studies in L. vannamei (6 – 8 gms.) as the animal model. The stocks having strength of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/ml were prepared in NTE buffer. From each of the preparations, aliquots of 10 μl were administered intramuscularly into the 6th abdominal segment of apparently healthy L. vannamei. The control consisted of animals injected with 10 μl of distilled water alone. For each of the concentrations of the extract, 6 animals were used in triplicates and were monitored for 7 days and subjected for general health assessment following the parameters such as; characteristic colouration, feed intake, moulting, antennal intactness and necrosis. The percentage of survivability obtained with different dilutions of the extract was statistically analyzed by a single factor ANOVA. The differences were considered significant at p ≤ 0.05.

2.3. Determination of the in vivo efficacy of TP22C in host - pathogen model

The plant isolate (TP22C) was dissolved in NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl and 0.02 M EDTA, pH 7.4) and termed as, plant isolate - buffer solution, at the concentration of 10 mg/ml (500mg/kg body weight of shrimp). During the experimental trials, shrimps (TS) (5 shrimps in each tank) were injected intramuscularly with a mixture of viral suspension and the above prepared plant product at the volume of 25 μl per animal (5 μl of viral suspension, 20 μl of plant isolate - buffer solution). The positive control (POS) shrimps were injected with a mixture of 20 μl NTE buffer and 5 μl viral suspension, while the negative control (NEG) shrimps were injected with 25 μl NTE buffer only. All the mixtures were incubated at 29 °C for 3 hrs. before the experimentation. The experimental trial was carried until the absolute mortality of the positive control, post infection with WSSV was observed. During this trial, shrimps [Negative (NEG), Positive (POS) and Test Sample (TS)] were subjected to comprehensive molecular analysis, post infection with WSSV. The genes namely; the VP28 (WSSV gene), ie 1 (immediate early 1 gene – immune related gene of shrimp) and Shrimp β actin gene (internal control gene) were expressed on the 42th hr and 84th hr, after the viral challenge using reverse transcriptase PCR, to find out whether the plant isolate (TP22C) was inhibiting the processes involved in the viral multiplication cycle during host pathogen interaction. The survival percentages in all the three shrimp groups were recorded. The experiments were conducted in triplicates and the results were confirmed and concluded after 100 % mortality was observed in the positive control (POS) group.

2.4. Phytochemistry of TP22C

2.4.1. Phytochemical screening of plant isolate TP22C

The terrestrial plant isolate/extract (TP22C) was subjected to various phytoconstituents analyses to judge the efficacy of the cumulative phytomolecular consortium as a potent anti – WSSV drug. The qualitative phytochemical ingredients present in TP22C such as; alkaloids, flavonoids, glycosides, phyllobatannins, reducing compounds, resins, saponins, steroids, tannins, terpenoids were analyzed using the methods described by the earlier researchers [20, 21].

2.4.2. Quantitative biochemical analysis of the terrestrial plant

Fresh leaves of the terrestrial plant were collected and washed thoroughly with tap water to remove the dust particles followed by sterile distilled water. The washed leaves were also surface sterilized with calcium hypochlorite before they were kept aside for drying at room temperature for 5 - 6 hrs. Based on the requirement for the biochemical analysis, these leaves were used from time to time in different assays as follows; crude protein content was estimated by the Lowry’s method [22], carbohydrate content using anthrone reagent, crude fat content, using chloroform – methanol mixture [23]. Total moisture and ash was estimated by standard protocols [24]. The Calcium (Ca), Copper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn), Potassium (K), Sodium (Na) and Zinc (Zn) content was estimated by Atomic Absorption Spectrometer
The water soluble vitamins [Vit B₃, Vit B₆, Vit B₁₂, Vit C and Folic acid] and the fat soluble vitamins [Vit A and Vit E] were analyzed.  

2.4.3. Estimation of Carbon, Hydrogen and Nitrogen by CHNS/O Analyzer

In CHN analysis, lyophilized samples (dry powdered leaves) were weighed (1.0 – 2.0 mg) in small tin capsules and submitted for combustion at 925 °C for about 2 min in the combustion box of an elemental analyzer (Perkin Elmer CHNS/O Analyser Series II, Model # 2400). The carbonization was performed in the presence of ultrapure O₂, promoting the full oxidation of the organic matter. Ultrapure helium was used as a carrier gas. Carbon, Hydrogen, and Nitrogen present in samples were converted into CO₂, H₂O, and N₂, respectively. The gases were homogenized, depressurized, and separated by analytical columns and quantified through changes in thermal conductivity of the products. The values were registered automatically by the recorder and integrator coupled to the analyzer. Acetanilide was used for calibrating the instrument. Final concentrations of C, H, and N in the samples were stoichiometrically calculated, considering the percentage of the elements in CHN analysis and the total mass of the freeze-dried samples.

2.4.4. Antioxidant properties of the plant isolate TP22C

The antioxidant potentials of the plant isolate (TP22C) were determined employing various in vitro assay methodologies. The total phenolic content was estimated by the standard protocol. The DPPH radical scavenging activity and hydroxyl radical scavenging activity was determined according to the standard protocol.  

2.4.5. Phytochemical fingerprint profiling using thin layer chromatography

Fresh leaves of the terrestrial plant were used for the phytochemical fingerprint profiling. The standard protocol of thin layer chromatography (TLC) was followed. The silica coated aluminum plate was used as stationary phase and a mixture of 100 ml of petroleum ether, 11 ml of isopropanol and 5 drops of distilled water was used as mobile phase. The phytopigments and their respective Rf values were recorded.

3. Results

3.1. Studies on the anti-WSSV efficacy of TP22C

The activity of the crude drug (TP22C) was examined against WSSV in L. vannamei to confirm its efficacy as a potent anti-WSSV drug. On completion of the experiment, after 84 hrs. the shrimps were nested PCR negative, and when the DNA extracted for virus from these shrimps were injected into a fresh batch of shrimps none of them showed any clinical signs of WSSV infection and remained negative to nested PCR (Fig. 1). The survivability was 86% at the end of the 84th hr of the experimentation (Fig. 2).
3.2. Determination of in vivo toxicity of the plant isolate TP22C
L. vannamei (6-8 gms.) (n=6) were injected with the plant isolate at different concentrations ranging from 5-50 mg/ml and monitored for 7 days (Fig. 3). Response of the animals was more or less the same without any significant mortality even up to a concentration 30 mg/ml (p <0.05). However, at 50 mg/ml strength there was significant reduction (43 % average percentage survival) (p <0.05) in survival of shrimps during the experimental period of 7 days.

3.3. Reverse transcription PCR analysis of various genes expressed during the host - pathogen interaction
The expressions of the genes on the 42nd hr and 84th hr after the challenge with the virus were examined to find out whether the plant isolate (TP22C) was inhibiting the processes involved in the viral multiplication cycle during host - pathogen interaction. The gene expression study was conducted in three groups (POS, NEG and TS) of animals. Viral genes were not amplified in the test group (TS) of animals and appeared exactly like the negative controls (NEG). In the case of positive control (POS), the viral genes such as immediate early gene (ie 1) and VP28 were found to be expressed at both 42nd hr and 84th hr after challenge with WSSV. It was observed that, as the time passed by, there was an increase in the intensity of bands of these genes suggesting more multiplication of the virus in the positive control shrimps (Fig. 4).

In the case of positive control animals which received the virus intramuscularly, total mortality was observed at the 84th hr itself. Hence, animals were not available to assay beyond that timeline.

3.4. Phytochemistry of TP22C
3.4.1. Phytochemical screening of plant isolate TP22C
The results of the phytochemical screening of plant isolate (TP22C) are tabulated below (Fig. 5.). The proximate micronutrients composition of the leaves are Calcium (20513.77 ppm), Copper (31.96 ppm), Iron (98.12 ppm), Magnesium (255.23 ppm), Potassium (412.98 ppm), Sodium (2201 ppm), Zinc (120.45 ppm), Vit A (0.031 ppm), Vit B3 (0.08 ppm), Vit B6 (98.38), Vit B12 (361 ppm), Vit C (66025 ppm), Vit E (805 ppm) and Folic acid (20632 ppm) (Fig. 7).

Results are presented as mean ± SEM and the difference between the data sets are significant (p < 0.05).

<table>
<thead>
<tr>
<th>Phytochemical Constituents</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Glycosides</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Phylobatannins</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Reducing compounds</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Resins</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
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<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Tannins</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Reverse transcription PCR analysis of VP28, immediate early (ie 1) and β actin genes in host. (M = Marker lane, Ts = Test sample, Neg = Negative, Pos = Positive).

Fig. 6. Proximate composition of leaves of plant.
3.4.3. Estimation of Carbon, Hydrogen and Nitrogen by CHNS/O Analyzer

Acetanilide (standard) used to calibrate the system resulted in a Carbon, Hydrogen and Nitrogen composition of 71.08 %, 7.03 % and 10.54 % respectively. The Carbon, Hydrogen and Nitrogen content of the freshly dried leaf is 41.39 %, 6.04 % and 3.95 % respectively. It indicates that the majority of the chemical compounds present in the samples are having long carbon chains, and the presence of the nitro group is very less in comparison to the former. The details of the output (Fig. 8) from the CHNS/O Analyzer system are elaborated below.

3.4.4. Antioxidant properties of the plant isolate TP22C

The total phenolics content is 474 ± 0.71. Decrease in absorbance of the DPPH radical caused by the antioxidant was attributable to radical scavenging by hydrogen donation. The reaction was visible as a color change from purple to yellow. The half maximal inhibitory concentration (IC$_{50}$) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of plant isolate (inhibitor) is needed to inhibit a given biological process (or component of a process, by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC$_{50}$). It is commonly used as a measure of antagonist drug potency in pharmacological research. According to the FDA, IC$_{50}$ represents the concentration of a drug that is required for 50% inhibition in vitro. The plant isolate TP22C reveals considerate radical scavenging assay in both DPPH and OH group. The particulars of the in vitro assay systems after appropriate statistical analysis are represented in Table 1.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Name of the assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total phenolic content</td>
<td>474 ± 0.71 (GAE/mg/100gm sample)</td>
</tr>
<tr>
<td>2</td>
<td>DPPH radical scavenging activity</td>
<td>9.72 ± 0.25 mg/ml (IC$_{50}$)</td>
</tr>
<tr>
<td>3</td>
<td>Hydroxyl radical scavenging activity</td>
<td>167 ± 0.96 mg/ml (IC$_{50}$)</td>
</tr>
</tbody>
</table>

3.4.5. Phytochemical fingerprint profiling using thin layer chromatography

The Thin Layer Chromatography (TLC) fingerprint profile of the leaf exhibited a chromatogram of 7 key phytopigments viz. carotenes, pheophytin, chlorophyll A, chlorophyll B, lutein, violaxanthin and neoxanthin. The detailed chromatograms and Rf values of the phytopigments are elaborated in Fig. 9, 10.
4. Discussion
Antiviral activities of aqueous extracts from plants are well established [31 – 35] that also includes reports on the anti-WSSV activity of plant extracts [3 – 5, 36 – 39]. A combination of herbal extracts and probiotics as medicated diet could decrease the prevalence of WSSV in Litopenaeus vannamei [40]. Even though reports are available on the protective effect of plant extracts against WSSV, information on their mode of action are scanty. In this present study, an attempt has been made to look into the possibilities of using terrestrial plants as sources of anti-WSSV drugs. With this objective, 30 terrestrial plants abundantly found in different places of West Bengal and Tamil Nadu, were subjected to soxhlet extraction to procure a combination of phytomolecules, potent enough to be an anti-WSSV drug and at the same time applied along with diet as a prophylactic measure. In this study, 7 plant isolates were found to be effective against WSSV. Finally, the plant isolate TP22C proved to be the potent anti-WSSV drug in our research. As TP22C alone could give protection to all animals tested against WSSV, under the experimental conditions, this terrestrial plant species was identified for further studies. The viral DNA was not detected in the tissue which suggested that the virus was either had not invaded the host tissue and multiplied or it was getting eliminated subsequent to the infection. The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose [41]. In animal model the highest non-toxic concentration went up to 30 mg/ml, from which 10 μl extract was injected to shrimps (6-8 gms.). We found that the crude drug TP22C was less toxic to the shrimps at the concentrations required for the antiviral activity. Similarly, the highest non-toxic level of Ceylon cal in P. monodon is 50 mg/ml [5]. The average percentage of survivability of shrimps injected with the TP22C was 86 %, at a concentration of 10 mg/ml. Marginal mortality was due to cannibalism subsequent to moulting may be considered. The result indicated that the minimum concentration of the extract required for extending the virucidal activity was less than its in vivo toxic level with high selectivity index, which is the ratio of toxic concentration to the effective concentration, and shows higher antiviral activity at a concentration below the toxic value. The results generated unambiguously suggest that the virucidal property of TP22C is concentration dependent. Different concentrations of Cidofovir (an antiviral drug) were injected and observed that it was nontoxic to shrimps up to a concentration of 200 mg/kg of body weight and they could successfully use the same for further assays [16]. In a similar pattern on screening 20 Indian medicinal plants, anti-WSSV activity was exhibited by the aqueous extract of Cynodon dactylon on administering 100 mg/kg of body weight when injected intramuscularly. Dosage dependent antiviral effects against WSSV have been reported in the case of antimicrobial peptide mytilin when injected after incubating with WSSV. It was proposed that the antiviral activity of mytilin was mediated by its binding onto the viral envelope [32, 43].

To evaluate the efficacy of TP22C for protecting L. vannamei from WSSV infection, expression of immediate early gene (ie 1) and VP28 and B actin genes were investigated. This study indicated that the viral transcripts involved in viral replication were not expressed in the animals (TS) that were administered with the crude drug. This was alike for both the 42th hr and 84th hr, post challenge with WSSV. The striking observation was that immediate early gene (ie 1) failed to be expressed in this group of animals. The expression of viral ie gene occurs independently of any viral de novo protein synthesis as the primary response to the viral invasion [56]. Once expressed, the ie gene products may then function as regulatory transacting factors and serve to initiate viral replication events during infection. Recently, it was found that WSSV used a shrimp STAT as a transcription factor to enhance viral gene expression in the host cells. STAT directly transactivates WSSV ie 1 gene expression and contributes to its strong promoter activity [44]. In the cascade of viral regulatory events, successive stages of viral replication are dependent on the proper expression of the genes in the preceding stage. In the present study none of these genes, [immediate early gene (ie 1) and VP28] was found to be expressed, that might be due to inactivation of the virus by the virucidal activity of TP22C. The results of different types of assays, viral and immune gene expression indicates that shrimps were protected from disease, either by getting protection from infection, or by getting the same from early dissemination of the infection in the presence of the crude drug.

The extraction of phytochemical constituents from the plant matrix is complex and is influenced by their chemical nature, extraction method, sample particle size, solvent used, as well as the presence of interfering substances. Phytochemicals can also make complexes with different carbohydrate, proteins and other plant by-products. The molecular weights of the vital phytochemicals are often confused with the complexes of the same, and the solubility is also a function of the solvent polarity [45]. The extraction process is an important factor for accessing the biological activity of the plant isolate [46], as it influences the yield of the isolate, the extractive capacity of the extractant and quality of the phytochemical composition [47].

The medicinal values of the leaves may be directly related to their constituent phytochemicals. In the present study the phytochemical screening of the plant isolate (TP22C) revealed the presence of alkaloids, flavonoids, glycosides, phyllobatannins, reducing compounds, resins, saponins, tannins and terpenoids. The secondary metabolites (phytochemicals) and other chemical constituents of medicinal plants account for their medicinal value [48]. For example, saponins are glycosides of both triterpene and steroids having hypotensive and cardio depressant properties [49], while anthraquinones possess astrigent, purgative, anti-inflammatory, moderate antitumor, and bactericidal effects [50]. Cardiac glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia [51].

The biochemical composition of leaves is the most common parameter used for the characterization of plants.
However, the biochemical as well as the micronutrient compositions of the concerned plant parts is often influenced by different origins, environmental, and seasonal factors. Seasonal changes in the chemical composition of essential oils in more than seventy L. camara from different parts of the world has been reported previously. The carbohydrates and proteins present in the plant may be a conglomerate of bioactive sugars, glycoproteins or proteins, which gives the plant its medicinal potency against certain diseases. Some plants are known to contain certain sugars which are biologically active against some diseases. Also, some plant proteins such as trichosanthin (isolated from tubers of Trichosanthes kirilowii), β-trichosanthin (isolated from tubers of Trichosanthes cucumeroides), luffaculin (isolated from seeds of Luffa acutangula) and luffin-a and luffin-b (isolated from seeds of Luffa cylindrica) have been reported to exhibit abortifacient, antitumor, ribosome inactivating and immunomodulatory properties. The total ash content of the plant materials are low, indicating the presence of less amount of mineral elements in the plant materials. However, these values are comparable to values reported for some Nigerian leafy vegetables. The elements such as calcium, magnesium, potassium, zinc, iron, manganese and sodium found in reasonable amount in the leaf are nutritionally and biochemically important for metabolic activities in the host body. Calcium is known to play a significant role in muscle contraction and connective tissue clotting mechanism, etc. Some of these minerals such as magnesium and zinc are required as cofactor in enzyme catalysis in the body. Sodium and potassium, that are present in the intracellular and extracellular fluid helps to maintain electrolyte balance and membrane fluidity. Iron is known to be a component of some metalloenzymes, myoglobin and hemoglobin, which is needed in the transport of oxygen and carbon dioxide during respiration or cellular metabolism. It is known that inorganic mineral elements such as potassium, calcium and zinc play important roles in the maintenance of normal glucose-tolerance in the body. The crude fat may add to the caloric value extractable from the plant for metabolic activities. Vitamins A, B₁, B₂, B₆, B₁₂, C, E and Folic acid were found to be present in the plant. Some of these vitamins, such as Vit B₁₂ found majorly in animal sources function as part of coenzymes methylcobalamin and deoxyadenosylcobalamin used in new cell synthesis. In addition to the antioxidant property of vitamin C and E, vitamin C also strengthens immunity of the body against infections, helps in collagen and thyroxin synthesis and enhance iron absorption while vitamin E play a role in immune function, cell growth, reproduction and DNA repair. Vitamin A, an antioxidant is a component of the visual pigments in the retina; regulates gene expression and cell differentiation. Folic acid is a hematopoetic vitamin and its deficit in the body, leads to anaemia. Vitamins are a diverse group of organic molecules required in very small quantities in the diet for health, growth, and survival. The absence of a vitamin from the diet or an inadequate intake results in characteristic deficiency symptoms and, ultimately leads to death.

The CHNS(O) Analyzer finds utility in determining the percentages of Carbon, Hydrogen, Nitrogen, Sulphur and Oxygen of organic compounds, based on the principle of “Dumas method” which involves the complete and the instantaneous oxidation of the sample by “flash combustion”. The combustion products are separated by a chromatographic column and detected by the thermal conductivity detector (T.C.D.), which gives an output signal proportional to the concentration of the individual components of the mixture. The Carbon, Hydrogen and Nitrogen contents were also estimated, which was far less than the standard.

The antioxidant activity of the plant extracts may be attributed to the presence of the identified phytochemicals. Flavonoids and tannins are phenolic compounds, and the plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Similarly, terpenoids, as vitamins, act as regulators of metabolism and play a protective role as antioxidants. The antioxidant property of the extracts may be a strong contributing factor, for the applications of the plants in the management and treatment of various diseases. Antioxidants prevent oxidative stress caused by free radicals that damage cells and vital biomolecules. They terminate chain reactions triggered by free radicals by removing the free radical intermediates and inhibit other oxidation reactions. The presence of the phytochemicals makes the leaves pharmacologically active and at the same time their antioxidant activity may be responsible for their usefulness in the management and treatment of various diseases. The antioxidant potential of leaves of terrestrial plant projected significant bioactivity and the phytochemicals and antioxidant compounds in TP22C might be useful in controlling the WSSV in the host. Thin layer chromatography (TLC) fingerprinting was used as a quantitative method to characterize and document the phytochemical profiles and pigment constituents of the leaves as a fingerprint. The phytochemical constituents of plants depend on several factors including seasonal changes, biotic (genetic) and abiotic (climatic stress, infection and soil fertility) factors. TLC analyses help in monitoring the phytochemical composition of the leaf extracts and fractions to ensure that no component(s) are lost during processing. In our study, the TLC chromatogram of the leaves depicted the chief phytopigments present in them that acts as a good source of antioxidants.

5. Conclusion

The antiviral activity of the TP22C could be confirmed, and the dosage for conferring protection was standardized. We found that the extract was non-toxic to the shrimps at the concentrations that were effective for the antiviral activity. The survival percentage of the WSSV infected host treated with TP22C was 86 %. Neither the VP 28 gene nor the immediate early genes (ie) were expressed in the host at the end of 42nd and 84th hrs. The pharmaceutical and phytochemical evaluation predicts that TP22C can protect L. vannamei from the WSSV infection. To comprehend the mechanism of the host – drug – pathogen interaction and to further characterize the plants isolate (TP22C), an Initial
Drug Substance Characterization (IDSC) analysis is an urgent need of the hour.

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