Phyllanthus amarus root clone with significant activity against bovine viral diarrhoea virus – a surrogate model of hepatitis C virus

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The present study has been carried out with *Phyllanthus amarus*, a plant identified for its anti-hepatitis B virus property. In a two-step study, the first step involved generation of plant tissue through *in vitro* root culture to get higher biomass within a short period, and the second step dealt with a preliminary investigation on testing the potential antiviral activity of those root tissues against hepatitis C virus (HCV) surrogate, bovine viral diarrhoea virus (BVDV). Root culture by orbital shaking (100 rpm) led the root inocula to a continuous segmentation starting from the primary stage of culture, and as a result of cumulative growth of the resultant small root units in a flask, about 800-fold production of root biomass was achieved after 25 days of culture in 1/4 Murashige and Skoog's medium with IAA 1.0 mg/l. An aqueous crude extract of the harvested roots caused a reproducible, dose-dependent reduction (maximum of 95%) in cytopathic effect (CPE) of BVDV with no cytotoxic effect, whereas the control anti-hepatitis viral compound ribavirin, at its most effective dose, showed comparatively less reduction in viral CPE (75%) and a detectable cytotoxic effect (15% cell killing). This article presents anti-BVDV activity of *P. amarus* crude extract and certainly holds promises for further research on its anti-HCV activity.

PHYLLANTHUS species (family Euphorbiaceae) are generally well known for the biologically active compounds they possess[1]. In particular, the herb *P. amarus* is known for its anti-hepatitis B virus (HBV) activity[2–7]. Though considerable work has been done on exploring the effectivity of the plant against HBV, no such report has yet been published on its utility against hepatitis C virus (HCV). The World Health Organization has estimated 170 million people worldwide are infected with hepatitis C[8]. In India the disease continues to be a major threat[9]. At present, the only specific treatment for chronic hepatitis C is IFN-α therapy, either alone or in combination with the guanosine analogue, ribavirin. But response of patients to the drug is not quite satisfactory. The situation, thus demands alternatives and complements to the current therapies[10].

Investigation on the remedial property of *P. amarus* for HCV is important because, if the plant would possess anti-HCV property in addition to anti-HBV, which together often infect people chronically[10], treatment of both the diseases will be easier. This view is further strengthened by the fact that the defence mechanism of patients against both the diseases is very similar[11].

While working with *P. amarus*, *in vitro*-generated roots have been used for the study. Although roots of the plant were found to have pharmaceutical property[2] like the aerial parts, this plant organ was rarely used for therapeutic evaluation. The low biomass of the root in comparison to the rest of the plant body (approx. 1:8) may be one of the reasons. With the advancement of biotechnology, manyfold production of plant roots within a short time period has become possible, and root culture is now a preferred technique for generating a stable source of secondary metabolites[12]. The stability of root culture is expected to be maintained over a prolonged growth period due to the chromosomal stability promoted by organized meristem in cultured roots[13]. Production of antiviral material *in vitro* from *P. amarus* would be a better alternative to the field harvest, since there are no established methods of organized cultivation of this plant, and depletion of the wild would limit the supply of quality material. Moreover, roots cultivated under controlled condition would obviate any geographical adaptational pressure that might result in degradation or fluctuation in medicinal quality of the extracts produced therefrom.

The present study established a protocol for enhanced production of *P. amarus* roots in liquid culture with a view to investigating any potential anti-HCV activity in extracts prepared from it. The extract was tested against bovine viral diarrhoea virus (BVDV) which is often employed as a surrogate model of HCV replication *in vitro*. To date, research with HCV has been hampered by the inability to propagate the virus *in vitro*. As such, surrogate models of viral replication are often employed. HCV is a member of the hepacivirus genus of the Flavivirus

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family of viruses. BVDV is the type member of the pestivirus genus in the Flavivirus family of viruses. These two viruses share many features including their single-stranded, positive-sense RNA genomes that encode about nine functionally analogous gene products. Studies of the potential anti-HCV antiviral activities of compounds often utilize this surrogate virus which replicates efficiently in tissue culture[10]. Recently, research institutes[14], biopharmaceutical companies[15] and blood product industry[16] have been working with scientists to test compound activity against hepatitis C using BVDV, which reacts to compounds in a manner similar to HCV.

Material and methods

Root culture

Establishment of root culture: Root apical parts of 0.8–1.0 cm (root explant), excised from micropropagated *P. amarus* maintained under tissue-culture condition, were inoculated into 250 ml Erlenmeyer flasks (2 explants/flask), containing 100 ml autoclaved MS (Murashige and Skoog's medium)[17], 1/2 MS, 1/4 MS (1/2 and 1/4 respectively of the original concentration of MS salts) basal liquid medium with 3% sucrose and the growth hormones, indoleacetic acid (IAA), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA) individually, ranging from 0.1 to 3.0 mg l^{-1}, at pH 5.7. Cultures were kept under continuous agitation on a rotary shaker (100 rpm) and incubated under a diffused light/dark (18/6 h) photoperiod at a temperature of 22 ± 2°C. The fresh root-biomass after 25 days of growth in each subculture was weighed (g) and the growth pattern was studied for each type of treatment. Experiments on antiviral activity were conducted with extracts from the root clones showing fastest growth rate as observed in 1/4 MS medium containing IAA 1.0 mg l^{-1}. All operations were performed using aseptic techniques.

Data analysis: Each treatment consisted of ten replicates and the experiment was repeated twice. Standard deviation was calculated for each value and a two-tailed 't'-test was performed between the results obtained from two different treatment groups to find out the significance of the treatment.

Antiviral testing

Preparation of aqueous extract: Fresh root material harvested from liquid medium after 25 days of culture was pressed (2–3 times) against double-lined Whatman filter paper, weighed out to 10 g and homogenized using a mortar and pestle with 30 ml of sterile double-distilled water. The crude extract was centrifuged at 10,000 rpm for 30 min in an IEC B-22M Programmable Centrifuge (International Equipment Company, USA) at room temperature. The pellet was resuspended and centrifuged as above. The supernatant was filtered through a 0.05 µm millipore filter, lyophilized (VIRTIS, Benchtop 3L) and stored at –20°C in 1 ml vials for further use.

Antiviral evaluation using BVDV: Madin–Darby bovine kidney (MDBK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM), 10% horse serum, 1% penicillin/streptomycin, 1% glutamine. On the day preceding the assay, the cells were trypsinized, pelleted, counted and resuspended at 1×10^4/well in 96-well plates in a volume of 100 µl per well. A pretitred aliquot of virus was removed from the freezer (–80°C) just before each experiment. The virus was diluted into tissue-culture medium such that the amount of virus added to each well will give nearly complete cell killing at 6–7 days post-infection (MOI > 1). The lyophilized *P. amarus* root extract was diluted in media to the desired high-test concentration and then further diluted in tissue culture media. The day following plating of cells, the plates were removed from the incubator, and the medium removed. Extract dilutions were added to the appropriate wells of the microtitre plate in a volume of 100 µl per well. Each dilution was set up in triplicate with complete medium (cell control), complete medium containing appropriately diluted virus (virus control), extract toxicity control wells (cells plus extract only), extract colorimetric control wells (extract only), or experimental wells (extract plus cells plus virus).

After 6–7 days of incubation at 37°C in a 5% CO_2 incubator the test plates were analysed by staining with the tetrazolium containing dye mixture Cell Titer 96® (Promega, Madison, WI). The mixture was metabolized by the mitochondrial enzymes of metabolically active cells to a soluble formazan product, allowing the rapid quantitative analysis of cell numbers. The plate was read spectrophotometrically at 490 nm with a Molecular Devices Vmax plate reader. Using an in-house computer program, %CPE reduction, % cell viability, the inhibitory concentration resulting in a 50% inhibition of BVDV-induced cell killing by the extract (IC_{50}), the toxic concentration killing 50% of cells (TC_{50}) and the therapeutic index (TI = TC/IC) were calculated.

A positive control for anti-BVDV activity was set with ribavirin, a standard antiviral compound following similar protocol as described above.

Results

Establishment of root culture of P. amarus

Explants (4–5 mg/flask) commenced growth in an orbital shake culture after 5–6 days of inoculation, when small bead-like root lateral initials appeared throughout their

length. Explants thereafter went on producing laterals and increasing in total biomass and weight until the twenty-first day, the peak of the growth phase, followed by a stationary growth phase (data not presented). The general feature of the root culture as observed during each subculture under a circular shaking speed of 100 rpm, was that, after attaining a certain volume, small root segments got continuously torn-off from the principal body. These segmented roots started to grow individually as separate entities, giving a puffy ball-like look (Figure 1), increased in volume, and finally each of the bodies rounded-up forming circular to oval semi-compact objects with all the emerging laterals in it (Figure 2 *a*, *b*). The effect of 1/4 MS was most beneficial for *P. amarus* roots and IAA was best-suited of all the growth hormones tested, as an enormous number of root laterals differentiated in its presence. NAA-induced growth was always different from that of IAA or IBA due to the fact that in the former, roots were associated with callusing at an early stage of lateral branch development (Figure 2 *c*). As a result, increase in weight of culture in this growth hormone was mostly presented by the root callus mass. The maximum yield (3.679 g/flask) was obtained in the presence of 1.0 mg l^{-1} IAA with 1/4 MS. More than a 800-fold increase in the weight of *P. amarus* roots was obtained in this medium and this increase was significant to that observed in supplementation of other growth hormones, IBA or NAA. The interaction of full-strength MS salts with growth hormones had least impact on root growth (Figure 3 *a*–*c*).

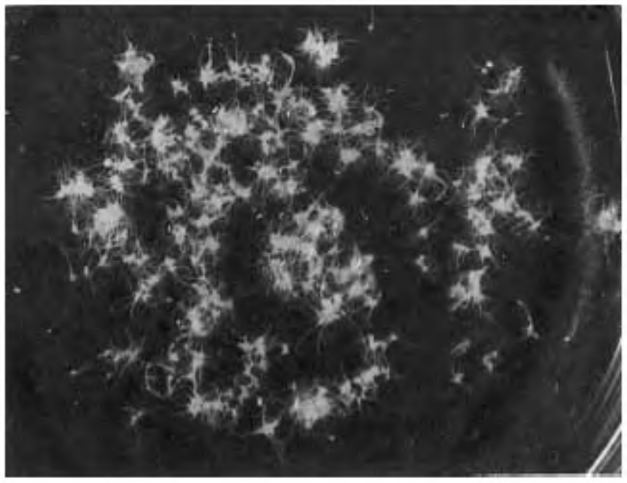

Figure 1. Segmented roots with emerging laterals giving puffy appearance.

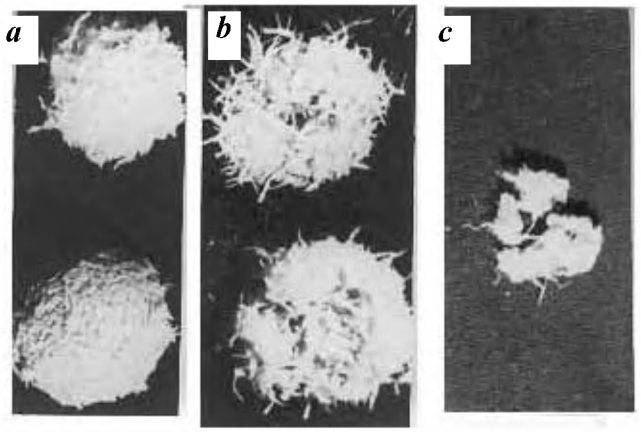

Figure 2 ***a***. Surface view of a fully-grown semi-compact root mass; ***b***, Cross-sectional view of the root mass; ***c***, Root callus in presence of NAA.

Effect of root extract on BVDV

Aqueous extract of *P. amarus* roots from culture stock was used in antiviral assay using the HCV surrogate model, BVDV. The extract caused a reproducible dose-dependent reduction in BVDV-induced cytopathic effect (CPE) (95% reduction by 200 µg ml^{-1} extract) with an IC_{50} = 33 µg ml^{-1} and the cytotoxic effect of the treatment was as little (almost nil) as TC_{50} value was never reached (Figure 4 *a*). The TI value was calculated to be > 6 (> 200 µg ml^{-1}/33 µg ml^{-1}). The result of the experiment with ribavirin, a standard anti-hepatitis viral compound showed 75% CPE reduction at its most effective concentration, resulting in IC_{50} = < 0.78 µg ml^{-1} and this concentration had a detectable cytotoxic effect on BVDV as TC_{50} reached 8.33 µg ml^{-1}, causing 15% cell killing (Figure 4 *b*). TI value for ribavirin was > 10.67 (Figure 4 *b*).

Discussion

The significance of the present study depended mainly on two major factors: growth rate of *P. amarus* roots (under *in vitro* condition) and the activity of the root extracts against hepatitis viruses. The *P. amarus* root culture grew rapidly in 1/4 strength MS medium in presence of 1.0 mg l^{-1} IAA. It is interesting to note that initiation of new root laterals in presence of IAA or IBA always took place in prime root explants as well as in multiple units of small root-masses that originated by being torn-off from the root masses during the course of culture. As a result, total root yield increased rapidly due to multiplication effect. This phenomenon was different from that typically observed in cultured roots where the explant itself grew by emerging laterals, which in turn elongated, thickened and then formed a root-woven, mat-like structure as described by Sudha and Seeni[18] in *Decalepis arayalpathra*. Decreased strength of MS served better for *P. amarus* isolated root culture.

The major advantage of culturing medicinal plant roots is that this method provides an alternative source of bioactive medicinal compounds of the plant. Root culture has been considered to be an important area of biotech-

nological research since 1942 (ref. 19). Later on, this technique was exploited for producing commercially important pharmaceutical compounds at higher levels than plant roots[20–24].

It is important to note that the *P. amarus* root extract not only caused reproducible dose-dependent reduction in

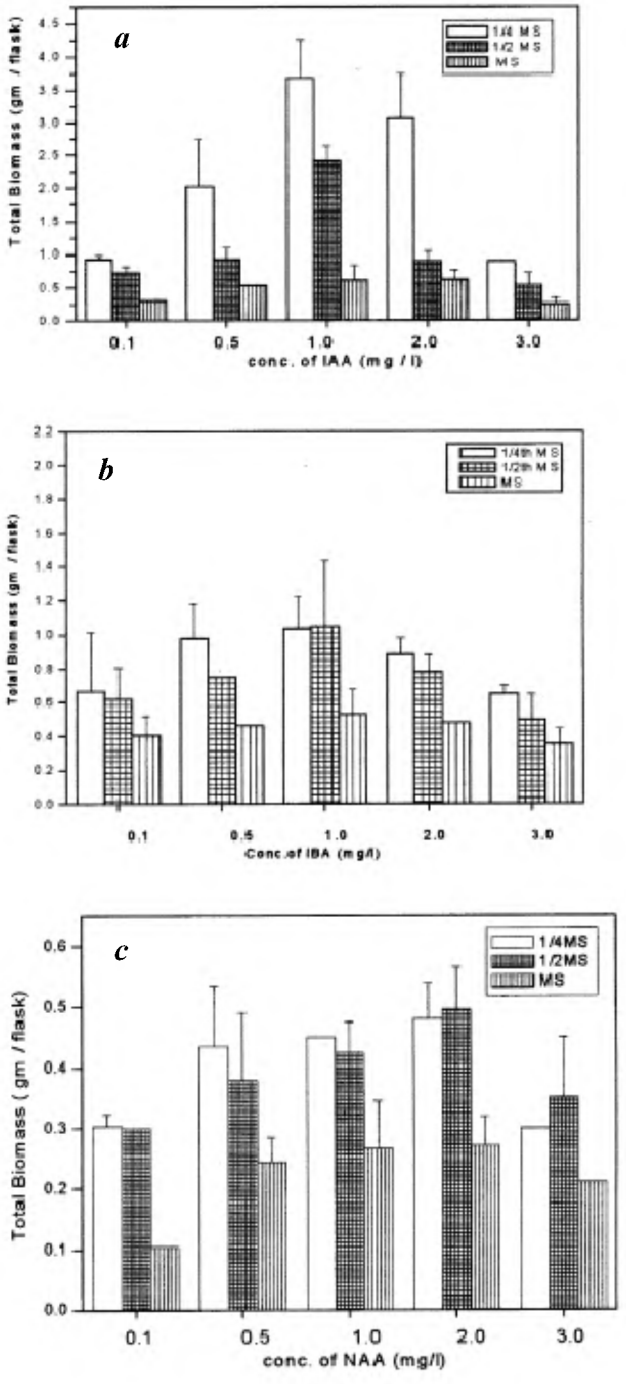

Figure 3. Effect of different auxins on biomass production of *P. amarus* roots in MS liquid medium containing macro and micro salts at 1/4, 1/2 and full strength. Effect of (***a***) IAA, (***b***) IBA, and (***c***) NAA on root growth.

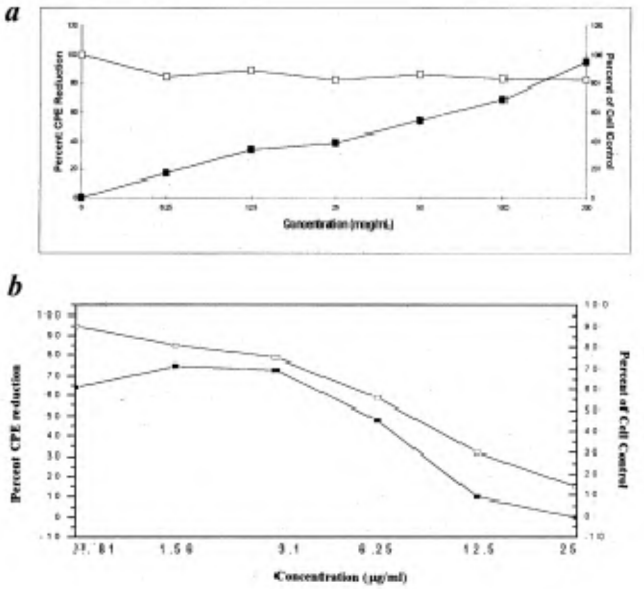

Figure 4. ***a***, Anti-BVDV antiviral activity of *P. amarus* root extract. Various concentrations of root extract were applied to MDBK cell cultures infected with BVDV. Per cent inhibition of BVDV-induced cytopathic effects by the extract is plotted as black boxes. The cytotoxicity of the extract alone related to the cell control is plotted as white boxes. Concentration is in $\mu g\ ml^{-1}$. ***b***, Anti-BVDV antiviral activity of ribavirin, a standard antiviral compound. Different concentrations of ribavirin ($\mu g\ ml^{-1}$) were applied to MDBK cell culture infected with BVDV. Per cent inhibition of BVDV-induced cytopathic effects by ribavirin is plotted as filled boxes and cytotoxicity of the compound related to the cell control is plotted as empty boxes.

BVDV-induced CPE with an $IC_{50} = 33\ \mu g\ ml^{-1}$, but the extract caused a little, if any cytotoxic effect and a TC_{50} value was not reached. Interestingly, ribavirin, a drug used for hepatitis C treatment, and used as positive control, had a toxic effect on the viability of the treated cells, giving TC_{50} to be $8.33\ \mu g\ ml^{-1}$. It is also worth mentioning that TI for root extract is >6, which is close to the value of TI for a coventional drug, ribavirin (>10.67). This is encouraging for an antiviral drug. If the active compound of the extract could be further purified, it might be found to have a much lower IC_{50} and a higher TI. Currently, HCV infection is treated using a combination of interferon-α and ribavirin. This therapy is successful in about 40–50% of patients[25]. Unfortunately, there are considerable side effects associated with this therapy. Ribavirin can cause anaemia[25], while interferon treatment can cause a wide range of side effects, including flu-like symptoms, fatigue and depression[26]. In addition, this combination therapy is expensive. A twelve-month course of treatment may cost US $26,000 (ref. 27). Due to these deficiencies in our ability to treat HCV-infected patients, new antiviral agents are actively being sought. The cultured root extracts of *P. amarus*, described here, provide an economical material source, showing good antiviral activity against the HCV surrogate virus BVDV, with no toxicity. It is relevant to mention in

this context that the anti-hepatitis B surface antigen (HBsAg) activity of the same material observed by us (communicated for publication) will further enhance the value of the present material for conducting therapeutic research with the plant. It is also worth mentioning that hairy root culture of *P. amarus* has been established and experiments have been undertaken to evaluate antiviral activity of the transformed roots.

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