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Phyllanthus amarus enriched *Artemia nauplii* enhanced survival, growth and nutritional quality of early post-larvae of the prawn *Macrobrachium rosenbergii*

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Abstract

The presence of primary and secondary phytochemical compounds in petroleum ether, acetone and ethanol extracts of *Phyllanthus amarus* was detected qualitatively as well as quantitatively. Alkaloids, terpenoids, tannins, polyphenols and quinines were present in the petroleum ether extract of *P. amarus*. Terpenoids, flavonoids, tannins, saponins, cardiac glycosides and quinines were present in the acetone extract, whereas alkaloids and terpenoids were absent in ethanol extracts of *P. amarus*. From the different solvent extracts of *P. amarus*, overall, 20 secondary phytochemicals were identified. Among these, presence of six bioactive compounds {Dodecanoic acid; Propanoic acid, 2-(tricyclo [3.3.1.13, 7] dec-2-ylidene)-; Tetradecanoic acid; Neophytadiene; Hexadecanoic acid, ethyl ester, and 9, 12, 15-Octadecatrienoic acid, (Z, Z, Z)-} have been identified through literature. Among the three solvents, ethanol extract of *P. amarus* enriched Artemia nauplii was found to produce the best survival, growth, nutritional indices (weight gain and specific growth rate) and concentrations of basic biochemical constituents (total protein, amino acid, carbohydrate, and lipid) in *M. rosenbergii* early post larvae. This suggests that the general health of the prawn was improved. This study indicates the fact that the primary phytochemical quality and secondary bioactive compounds of *P. amarus* have the potency to sustainably enhance the survival, growth and nutritional quality of *M. rosenbergii*. Thus, this herb is recommended as a feed additive.

Introduction

Medicinal plants provide an inexhaustible resource of raw materials for the pharmaceutical, cosmetics, food industries and more recently in agriculture for pest control [1]. The herbal extract could be defined as the compounds and /or mixture of compounds obtained from fresh or dried plants, or parts of plants, such as leaves, flowers, seeds, roots and barks. The carry me seed plant, *Phyllanthus amarus* is a common pantropical weed that grows well in moist, shady and sunny places. It is widely spread throughout tropics and subtropics [2]. It has been used in Ayurvedic medicine for over 2,000 years and has a wide number of traditional uses. It has been reported to be used in treating jaundice by blocking DNA polymerase of hepatitis B virus [3-5]. There are also reports that it is used to treat diabetes, otitis, diarrhoea, gonorrhoea, and frequent menstruation, galactogogue, leucorrhoea, menorrhagia, mammary abscess, skin ulcer, hypertension, pain, inflammation, cough, asthma bronchitis, gastrointestinal disturbances, helminth infestation, nociceptive pain, mutation and cancer. It has a urolithic property, dissolving renal calculi [6].

P. amarus contains moderate amount of protein, rich in carbohydrate and low in fat, ash, crude fiber. It also contains Mg, Ca, K, PO and ascorbic acid, Fe; Zn, thiamine, niacin and riboflavin [7]. It has been reported *that P. amarus* contains many valuable compounds, such as lignans, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids [8]. The extracts and the compounds isolated from *P. amarus* reported to have a wide spectrum of pharmacological properties including anti-viral and anti-fungal, anti-bacterial, anti-plasmodial and anti-inflammatory, anti-tumor, anti-cancer, anti-diabetic, anti-oxidative, anti-hyperglycemic, hypolipidemic, immuno-stimulatory, hepatoprotective, nephroprotective and diuretic [7, 9-18].

The research in the use of plant extracts for aquatic animals is increasing with the demand for eco-friendly and sustainable aquaculture [19]. The medicinal plants have phytochemicals, the bioactive substances which are responsible for the biological activities they exhibit. For this reason, herbal extracts were introduced in the pharmacopoeias [20]. The herbal biomedicine active principles in the aquaculture have the characteristics of growth and survival promoting ability, tonic to improve the immune system, anti-microbial capability, anti-stress characteristics and stimulating appetite due to presence of alkaloids, flavonoids, pigments, phenolics, terpenoids, starch, steroids and essential oils. Recent studies showed that the incorporation of medicinal plants (raw material, extracts and phytocompounds) as additives of fish/prawn feeds stimulates the growth and immune responses [21-34].

The freshwater prawn, *Macrobrachium rosenbergii* is the most popular one for the commercial farming in many countries. It gained momentum after the setback in shrimp farming due to disease outbreaks and other factors. Newly-hatched *M. rosenbergii* larvae are around 2 to 2.2 mm in length. They normally start feeding about 1 day after hatching and initially require live zooplankton. Their prey should be of a suitable size for ingestion; the optimal size is estimated at 300 to 500 µm.

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Live organisms serve as living capsules of nutritive elements such as essential proteins, amino acids, lipids, fatty acids, carbohydrates, vitamins and minerals. *Artemia nauplii* are the most widely used live food organism for the seed production of marine as well as freshwater fish and crustaceans [35]. Newly-hatched Artemia nauplii are considered the most successful starter diet during the first rearing week but, after that, are usually fed in combination with prepared diets, since this is an omnivorous [36]. Normally, 2, 00,000 to 3, 00,000 nauplii are hatched from each gram of high-quality brine shrimp cysts [37], Prawn larvae and early post-larvae prefer live organisms than formulated feeds.

Artemia has high nutritive value and high conversion efficiency. All the life stages of *Artemia*, i.e. cysts (after decapsulation), nauplii, juveniles, sub-adults are used as feed. Today, in majority of the commercial aqua hatcheries, Artemia nauplii is virtually used as a sole diet. Frozen adult Artemia, are widely used by aquarists, fish breeders and aqua culturists. Artemia biomass is also used as food additive for domestic livestock or extraction of pharmaceutical products as also in making protein rich food products. It is even used for human consumption in some countries. Owing to its great utility, Artemia trading is a growing business in several parts of the world [38].

Therefore, in the present study, an attempt has been made to check the primary and secondary phytochemical compounds present in petroleum ether, acetone and ethanol extracts of *P. amarus,* the whole plant. These extracts were subjected to enrichment on Artemia nauplii and fed to *M. rosenbergii* early post-larvae (PL) for evaluation of its nutritional indices [survival rate (SR), length gain (LG), weight gain (WG) and specific growth rate (SGR)], and quantification of concentrations of basic biochemical constituents (total protein, amino acid, carbohydrate and lipid) [39].

Materials and Methods

The traditional herb carry me seed plant, *Phyllanthus amarus* was collected at Bharathiar University campus, Coimbatore, Tamil Nadu, India (Figure 1) and authenticated with Botanical Survey of India, Coimbatore. The herb was thoroughly washed with freshwater, blotted and spread out and dried for 2 weeks at room temperature. Shade dried herb was ground to fine powder. The powered sample was stored in sterile containers for further use.

Preparation of extracts

The powdered whole plant sample of *P. amarus* (50 g) was taken and packed in Whatmann No. 1 filter paper and put into soxhlet apparatus. The extracts were successively soaked with 300 ml (1:6 w/v) of petroleum ether (99.98% purity, SRL Pvt. Ltd. India), acetone (99.5% purity, SD Fine Chemicals, India) and ethanol (99.9% purity, Changshu Yangyuan Chemicals, China) individually and sequentially extracted for 6-9 h each (30 to 36 cycle) based on their polarity (non-polar to polar). Repeated extraction was done until a clear colorless solution was obtained. The extracts were filtered by using double layer muslin cloth and concentrated at 40-50 °C using rotary vacuum evaporator (ROTAVAP). The extracts obtained were vacuum-dried under 40 °C and used for further investigation. The extracts obtained were appeared as dark green, gummy solid.

Qualitative analysis of primary phytochemical substances

The extracts were subjected to detection of the presence of primary phytochemical bio-molecules, such as alkaloids, terpenoids, flavonoids, tannins, polyphenols, saponins, and cardiac glycosides using the standard qualitative procedures of Trease & Evans [40].

Figure 1. The carry me seed plant, Phyllanthus amarus Linn. (Schumach & Thonn), Infra Kingdom: Streptophyta; Super Division: Embryophyta; Division: Tracheophyta; Subdivision: Spermatophytina; Class: Magnoliopsida; Superorder: Rosanae; Order: Malpighiales; Family: Phyllanthaceae (Integrated Taxanomic Information System); a). Whole plant; b). Branches of P. amarus with leaves and seeds; c). Root of *P. amarus.*

P. amarus is a small erect annual herb (10-60 cm tall) with numerous small oblong-elliptic or squarish leaves and glabrous (6-12 mm long). The main stem is simple or branched, terrete smooth or scabridulous in younger parts [39]. Flowers are very small, yellow in colour and hang down in beautiful array hidden below the leaves. The flowers produce very small (2 mm) fruits that burst open and the seeds are hurled away. When the plants are picked, the feathery leaves fold in, completely closing themselves. P. amarus is a common pantropical weed that grows well in moist, shady and sunny places. It is widely spread throughout tropics and subtropics [2].

Gas Chromatography-Mass Spectrum (GC-MS) analysis for secondary phytochemical compounds

Different solvent extracts of *P. amarus* were subjected to GC-MS analysis (Thermo GC-trace ultra ver-5.0; Thermo MS-DSQ-II; ZB 5-MS capillary standard non-polar column (30 mts, 0.25 mm id, 0.25 µm film) for identification of different phytochemical compounds (South India Textile Research Association (SITRA), Coimbatore, Tamil Nadu, India). The working condition of GC-MS was as follows: Carrier Gas, He; Flow, 1.0ml/min; Temperature Programme, oven temperature raised from 70 °C to 260 °C at 6 °C/min; Injection Volume, 1 µl; Detector, mass selective detector MS-DSQ-II with XCALIBUR software; Injector temperature, 250 °C; Ion source temperature, 200 °C; Interface temperature, 250 °C; Total running time, 40 min; Relative percentage constituents was expressed as percentage with peak area normalization.

Peaks resolved with relative abundance of 0-100 were considered as major compounds. To show the minor peaks, the chromatogram was magnified. Identification of various components present in each extract was confirmed based on the peak area, retention time and molecular formula. Identification of various components present in these extracts were assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. National Institute Standard and Technology (NIST4) and WILEY9 [41], on-line library source was also used for matching the identified components.

Proximate composition of *P. amarus*

The proximate composition of *P. amarus* whole plant powder, such as contents of moisture, crude protein, crude fibre, crude fat and ash were estimated following AOAC methodology [42], and total nitrogen free extract (carbohydrate) content was calculated by subtracting all the above contents adopting Castell & Tiews method [43].

Artemia cyst hatching and its enrichment

The brine shrimp, *Artemia franciscana* cysts were purchased from Aqua world, Paris Corner, Chennai, India. The cysts (2 g/ 20 L and 15 g kg-1 body biomass of the prawns for feeding trial) were taken and hydrated in 1 L-1 of purified artificial saltwater (prepared from artificial sea salt powder 35.0 g L-1, pH of 6.5). After 12-15 h, the cysts burst, and the embryo surround by the hatching membrane become visible. After a few hours, the brownish orange colored nauplii came out. The 48-hr old Artemia nauplii were collected on a sieve and enriched with 1% of petroleum ether, acetone and ethanol extracts each of *P. amarus* separately for 1 h at the rate of 1 g in 100 ml.

Procurement and acclimatization of experimental animal

The early post larvae (PL-10) of the freshwater prawn, *M. rosenbergii* were procured from Sri Durgai Hatcheries, Chengalpattu, Tamil Nadu, India. They were transported to the laboratory in polythene bags filled with oxygenated water. The prawns were acclimatized to the ambient laboratory condition with ground water in cement tanks (6 × 3 × 3 feet) for a week (temperature, 26.5±1.3 °C; pH, 7.01±0.12; TDS, 0.80±0.05 g/l; DO, 7.30±0.49 mg/l; BOD, 29.0±1.24 mg/l; COD, 124.0±3.2 mg/l; ammonia, 0.026±0.007 mg/l). During acclimatization the PL were fed with un-enriched Artemia nauplii. About half of the tank water was renewed each day and adequately aerated to maintain a healthy environment. This ensures an environment devoid of accumulated metabolic wastes and sufficient oxygen supply to the prawns. The unfed feeds, faeces, moult and dead prawns were removed by siphoning.

Feeding trails with extracts of *P. amarus* enriched *Artemia nauplii*

M. rosenbergii early PL ranging from 0.8±0.06 cm in length and 0.06 ± 0.02 g in weight were used in a triplicate experimental set up. The prawns were divided into four groups. One group served as control and fed with un-enriched Artemia nauplii and the other three groups were fed with 1% of petroleum ether, acetone, and ethanol extracts of *P. amarus* enriched Artemia nauplii at the rate of three times per day (8.00 a.m., 2.00 pm and 10.00 pm) for 30 days. At the end of the feeding trial the final length and weight were measured for calculating nutritional indices and estimating basic biochemical constituents. During the feeding trial the water medium was renewed daily by siphoning method without severe disturbance to the prawn and was aerated adequately.

Determination of nutritional indices

The survival rate (SR), length gain (LG), weight gain (WG), and specific growth rate (SGR) were individually calculated Tekinay & Davis [44].

i. Survival (%) = Total No. of live animals/Total No. of initial animals × 100

ii. Length gain (cm) = Final length (cm) – Initial length (cm)

iii. Weight gain (g) = Final weight (g) – Initial weight (g)

iv. Specific growth rate, (%) = log W2 – log W1/ t ×100

Where, W1 & W2 = Initial and Final weight respectively (g), and t = Total number of experimental days.

Estimations of basic biochemical constituents

The initial and final concentrations of basic biochemical constituents, such as total protein, amino acid and carbohydrate were estimated in test prawns adopting the methodology of Lowry *et al.* [45], Moore & Stein [46], and Roe [47], respectively, and the total lipid was extracted following the method of Folch *et al.* [48] and estimated gravimetrically following the method of Barnes & Blackstock [49].

Statistical analysis

Data between control versus experiments and between experiments were subjected to statistical analysis through one-way ANOVA and subsequent post hoc multiple comparison with DMRT by adopting SPSS (v20). All the details of statistical analyses were given in respective tables. The P values less than 0.05 were considered statistically (95%) significant.

Results

Primary phytochemicals of *P. amarus*

The petroleum ether extract of *P. amarus* showed presence of 5 primary compounds, such as alkaloids, terpenoids, tannins, polyphenols and quinines. Of which, tannins were luxuriantly present, alkaloids and terpenoids were moderately present. The other compound, such as polyphenols and quinones were poorly present (Table 1). In acetone extract of *P. amarus*, the presence of 6 compounds, such as terpenoids, flavonoids, tannins, saponins, cardiac gylcosides and quinines were detected. Of which, tannins and cardiac gylcosides were luxuriantly present, flavonoids and saponins were moderately present. The other compounds, such as terpenoids and quinones were poorly present (Table 1). Similarly, the ethanol extract of *P. amarus* contains 6 primary compounds, such as, flavonoids, tannins, polyphenols, saponins, cardiac gylcosides and quinines. Of which flavonoids and tannins were luxuriantly present, polyphenols and quinones were moderately present, and other compounds, such as saponins and cardiac gylcosides were poorly present (Table 1). Therefore, overall, *P. amarus* contains 8 primary phytochemical compounds. In this study, ethanol has served as the best solvent for extraction of flavonoids. For the extraction of tannins, all three solvents can be used. Ethanol can be used for the extraction of saponins, while, acetone can be used for the extraction of cardiac glycosides. For extractions of alkaloids and terpenoids, petroleum ether can be used. Ethanol can also be used for extractions of polyphenols and quinines.

Secondary phytochemicals of *P. amarus*

Overall, different solvent extracts of *P. amarus* revealed presence of 20 secondary compounds, of these, 7 are in petroleum ether, 6 are by acetone and 7 are from ethanol extracted samples (Table 2). The details of compounds present in the individual extract, the active principles with their retention time, molecular formula, molecular weight, peak area, similar index and reverse similar index in percentage are presented (Tables 3-5). About 30% of active principal compounds possessed biological properties, which have been identified from known sources.

Table 1. The primary phytochemicals present in *P. amarus* extracted with different solvents

Phytochemicals	Solvents		
	Petroleum ether (non-polar)	Acetone (middle-polar)	Ethanol (Polar)
Alkaloids	++	--	--
Terpenoids	++	+	--
Flavonoids	--	++	+++
Tannins	+++	+++	+++
Polyphenols	+	--	++
Saponins	--	++	+++
Cardiac gylcosides	--	+++	+
Quinones	+	+	++

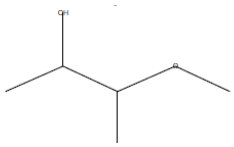
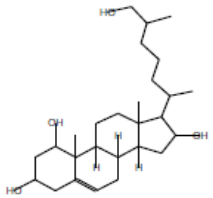
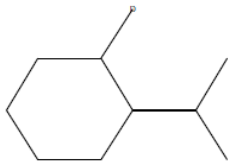
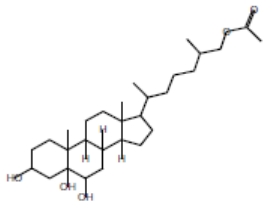
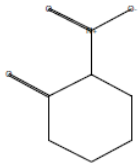
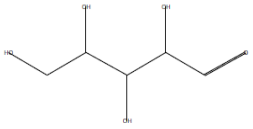
+, Poorly present; ++, Moderately present; +++, Luxuriantly present; --, Absent

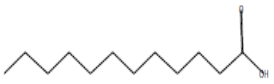
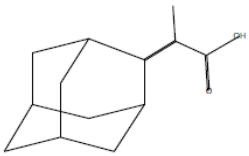
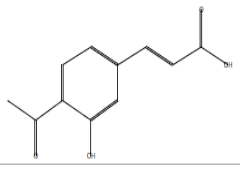
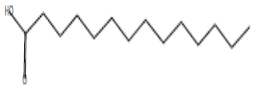
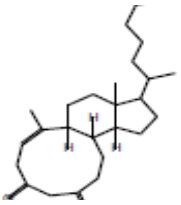
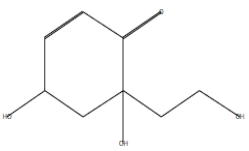
The petroleum ether extract of *P. amarus* revealed the presence of 7 different secondary compounds. They are, (R)-2-(1-Methylethylidene)-cyclohexane-drt; d-Xylose; Dodecanoic acid; Tetradecanoic acid; (E)-5, 10-secocholest-1(10)-en-3, 5-dione; 2, 4-Dihydroxy-2-(2'-hydroxyethyl) cyclohex-5-en-1-one; and 3, 3'-Dibromo-2, 2'-diquinolinyl Disulfide. Of which, 2 compounds, Dodecanoic acid, and Tetradecanoic acid possessed biological properties (Figures 2 and 2a; Table 3).

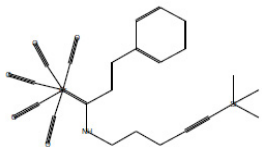
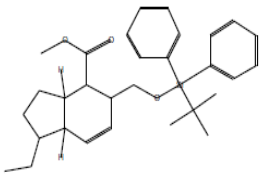
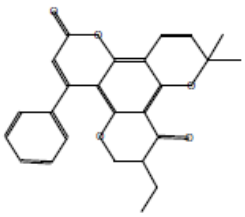
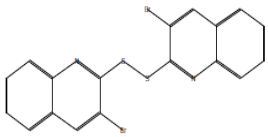
The acetone extract of *P. amarus* showed presence of 6 different secondary compounds. They are, 2-Butanol, 3-methoxy-; 2-Nitrocyclohexanone (CAS); Propanoic acid, 2-(tricyclo[3.3.1.13,7] dec-2-ylidene)-; Neophytadiene; 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-; and Methyl (3R)-3-(tert-butyldimethylsilyloxy)-3-[3-((1S)-1-(tert-butyldimethylsilyloxy)-3-butynyl) phenyl]propanoate. Of which, 3 compounds, Propanoic acid, 2-(tricycle [3.3.1.13,7] dec-2-ylidene)-; Neophytadiene; and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-} possessed biological properties (Figures 3 and 3a; Table 4).

In the ethanol extract of *P. amarus*, 7 different secondary compounds were detected. They are, Cholest-5-ene-1á,3á,16á,26-tetrol; Cholestane-3á,5à,6á,26-tetrol - 26-Acetate; 3,10-Dioxa-2,11-disiladodecane,

Table 2. Overall secondary phytochemical compounds present in different solvent extracts of *P. amarus*

Sl. No.	Peak RT	Solvent	Name of the compound	Molecular formula	Chemical structure
1.	3.98	Acetone	2-Butanol, 3-methoxy-	$C_5H_{12}O_2$	
2	4.71	Ethanol	Cholest-5-ene-1á,3á,16á,26-tetrol	$C_{27}H_{46}O_4$	
3	5.18	Petroleum ether	(R)-2-(1-Methylethylidene)-cyclohexane-drt	$C_9H_{15}D$	
4	8.13	Ethanol	Cholestane-3á,5à,6á,26-tetrol - 26-Acetate	$C_{29}H_{50}O_5$	
5	9.78	Acetone	2-Nitrocyclohexanone (CAS) Neophytadiene	$C_6H_9NO_3$	
6	12.35	Ethanol	3,10-Dioxa-2,11-disiladodecane, 2,2,11,11-tetramethyl-	$C_{12}H_{30}O_2Si_2$	
7.	14.25	Petroleum ether	d-Xylose	$C_5H_{10}O_5$	

8.	18.52	Petroleum ether	Dodecanoic acid* (lauric acid)	$C_{12}H_{24}O_2$	
9.	20.00	Acetone	Propanoic acid, 2-(tricyclo[3.3.1.13,7]dec-2-ylidene)-*	$C_{13}H_{18}O_2$	
10.	20.68	Ethanol	3-(4-Acetyl-3- hydroxyphenyl)-2-propenoicacid	$C_{11}H_{10}O_4$	
11.	22.86	Petroleum ether	Tetradecanoic acid* (myristic acid)	$C_{14}H_{28}O_2$	
12.	24.40	Acetone	Neophytadiene*	$C_{20}H_{38}$	
13.	27.19	Petroleum ether	(E)-5,10-secocholest-1(10)-en-3,5-dione	$C_{27}H_{44}O_2$	
14.	28.16	Ethanol	Hexadecanoic acid, ethyl ester* (palmitic acid)	$C_{18}H_{36}O_2$	
15.	29.99	Acetone	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-* (linolenic acid)	$C_{18}H_{30}O_2$	
16.	30.26	Petroleum ether	2,4-Dihydroxy-2-(2'-hydroxyethyl)cyclohex-5-en-1-one	$C_8H_{12}O_4$	

17.	32.61	Ethanol	(Sax)-(-)-2,2'-Bis(di-2-furylphosphino)-1,1'-binaphthalene	$C_{36}H_{24}O_6P_2$	
18.	35.71	Acetone	Methyl (3R)-3-(tert-butyldimethylsilyloxy)-3-[3-((1S)-1-(tert-but yldimethylsilyloxy)-3-butynyl)phenyl] propanoate	$C_{26}H_{44}O_4Si_2$	
19.	36.28	Ethanol	5-Methoxy-8,8-dimethyl-6-(2-methylbutanoyl)-4-phenyl-2 H,8H-benzo[1,2-b:3,4-b']dipyran-2-one	$C_{26}H_{26}O_5$	
20.	38.42	Petroleum ether	3,3'-Dibromo-2,2'-diquinolinyl Disulfide	$C_{18}H_{10}Br_2N_2S_2$	

RT- Retention Time; *Compounds having bioactive properties

Table 3. GC-MS profiles of secondary phytochemicals of *P. amarus* in petroleum ether extract

RT	Name of the active principle compound	P	MF	MW	Area (%)	SI	RSI	Compound having biological properties (by literature only)
5.18	(R)-2-(1-Methylethylidene)-cyclohexane-drt	32.33	$C_9H_{15}D$	124	18.71	851	902	--
8.11	NV	--	--	--	--	--	--	--
14.25	d-Xylose	11.87	$C_5H_{10}O_5$	150	0.01	421	486	--
18.52	Dodecanoic acid*	69.79	$C_{12}H_{24}O_2$	200	0.04	820	863	Antimicrobial activity **[50]**
22.86	Tetradecanoic acid*	75.87	$C_{14}H_{28}O_2$	228	0.07	858	888	Antioxidant, Anti-cancer, Hyper cholesterolemic, Larvicidal, Repellent activity **[51]**
27.19	(E)-5,10-secocholest-1(10)-en-3,5-dione	59.40	$C_{27}H_{44}O_2$	400	1.25	438	594	---
30.26	2,4-Dihydroxy-2-(2'-hydroxyethyl) cyclohex-5-en-1-one	35.10	$C_8H_{12}O_4$	172	6.76	648	990	--
38.42	3,3'-Dibromo-2,2'-diquinolinyl Disulfide	53.83	$C_{18}H_{10}Br_2N_2S_2$	476	0.33	674	898	--

RT, Retention time; NV, Not validated; P, Probability; MF, Molecular formula; MW, Molecular weight; SI, Similar index; RSI, Reverse similar index; *Compounds having bioactive properties.

2,2,11,11-tetramethyl-; 3-(4-Acetyl-3- hydroxyphenyl)-2-propenoic acid; Hexadecanoic acid, ethyl ester; (Sax)-(-)-2,2'-Bis(di-2-furylphosphino)-1,1'-binaphthalene; and 5-Methoxy-8,8-dimethyl-6-(2-methylbutanoyl)-4-phenyl-2H, 8H-benzo [1, 2-b: 3, 4-b'] dipyran-2-one. Of which, only one compound, Hexadecanoic acid, ethyl ester possessed bioactive properties (Figures 4 and 4a; Table 5).

Proximate composition of *P. amarus*

The proximate composition of *P. amarus* was found as follows, crude protein (6.13%), crude fat (6.3%), crude fibre (24.66%), total ash (6.78%) and total nitrogen free extract (43.38%). It has 2567 k.cal/kg of gross energy.

Table 4. GC-MS profiles of secondary phytochemicals of *P. amarus* in acetone extract

RT	Name of the active principle compound	P	MF	MW	Area (%)	SI	RSI	Compound having biological properties (by literature only)
3.98	2-Butanol, 3-methoxy-	4.18	$C_5H_{12}O_2$	104	84.04	993	999	--
9.78	2-Nitrocyclohexanone (CAS)	6.82	$C_6H_9NO_3$	143	0.00	369	868	--
16.07	NV	--	--	--	--	--	--	--
20.00	Propanoic acid, 2-(tricyclo[3.3.1.13,7]dec-2-ylidene)-*	29.76	$C_{13}H_{18}O_2$	206	0.02	641	678	Antioxidant **[52]**
24.40	Neophytadiene*	24.32	$C_{20}H_{38}$	278	0.01	702	809	Antipyretic, Analgesic, Anti-inflammatory, Antimicrobial, Antioxidant **[53]**
29.99	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-*	9.57	$C_{18}H_{30}O_2$	278	0.71	740	783	Antimicrobial agent **[53]**
35.71	Methyl (3R)-3-(tert-butyldimethylsilyloxy)-3-[3-((1S)-1-(tert-but yldimethylsilyloxy)-3-butynyl) phenyl] propanoate	51.59	$C_{26}H_{44}O_4Si_2$	476	7.74	955	996	--

RT, Retention time; NV, Not validated; P, Probability; MF, Molecular formula; MW, Molecular weight; SI, Similar index; RSI, Reverse similar index; *Compounds having bioactive properties.

Table 5. GC-MS profiles of secondary phytochemicals of *P. amarus* in ethanol extract

RT	Name of the active principle compound	P	MF	MW	Area (%)	SI	RSI	Compound having biological properties (by literature only)
4.71	Cholest-5-ene-1á,3á,16á,26-tetrol	10.63	$C_{27}H_{46}O_4$	434	2.43	338	524	--
8.13	Cholestane-3á,5à,6á,26-tetrol - 26-Acetate	88.65	$C_{29}H_{50}O_5$	478	0.12	521	541	--
12.35	3,10-Dioxa-2,11-disiladodecane, 2,2,11,11-tetramethyl-	13.28	$C_{12}H_{30}O_2Si_2$	262	0.06	439	554	--
15.75	NV	--	--	--	--	--	--	--
20.68	3-(4-Acetyl-3- hydroxyphenyl)-2-propenoic acid	37.27	$C_{11}H_{10}O_4$	206	0.37	646	663	--
28.16	Hexadecanoic acid, ethyl ester*	83.88	$C_{18}H_{36}O_2$	284	0.46	824	857	Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Lubricant, Anti-androgenic, Flavour, Haemolytic, 5-Alpha reductase inhibitor **[54]**
32.61	(Sax)-(-)-2,2'-Bis(di-2-furylphosphino)-1,1'-binaphthalene	45.31	$C_{36}H_{24}O_6P_2$	614	71.70	897	990	--
36.28	5-Methoxy-8,8-dimethyl-6-(2-methylbutanoyl)-4-phenyl-2 H,8H-benzo[1,2-b:3,4-b'] dipyran-2-one	57.04	$C_{26}H_{26}O_5$	418	12.64	670	750	--

Nutritional indices and basic biochemical constituents in *M. rosenbergii* PL fed with *P. amarus* extracts enriched *Artemia nauplii*

The morphometric data, length and weight, and nutritional indices, such as SR, WG, and SGR were found to be significantly increased ($P<0.05$) in petroleum ether, acetone and ethanol extracts of *P. amarus* enriched Artemia nauplii fed PL groups when compared with control (Table 6). Similarly, the contents of biochemical constituents, such as total protein, total amino acid, total carbohydrate and total lipid were found to be significantly higher ($P<0.05$) in petroleum ether, acetone and ethanol extracts enriched *Artemia nauplii* fed PL when compared with control (Table 6). Among these, ethanol extracts enriched Artemia nauplii produced the best results followed by acetone and petroleum ether extracts.

Discussion

Primary phytochemicals of *P. amarus*

Similar to that of the present study, the presence of alkaloids, tannins, terpenoids, saponins, phenolics, flavonoids and steroids have been reported in the hexane extract of *P. amarus* [55]. Alkaloids in ethyl acetate extracts of leaf and root, saponins in aqueous and methanol extracts of the stem, and flavonoids in petroleum ether extract of all parts of *P. amarus* have been reported [56]. Phenols and flavonoids from the root, stem and leaf of *P. amarus* have also been reported using different solvents, ethyl acetate, dimethylformamide, chloroform, dichloromethane and n-Hexane [57]. Dimethylformamide was reported to be the best solvent for extraction of phenols and flavonoids from the root, stem and leaf of *P. amarus* [58]. Qualitative phytochemical studies have also been reported in different species of Phyllanthus [59, 60].

It has been reported that alkaloids, such as morphine, atropine and quinine are biologically and therapeutically active [61]. In the present study, alkaloids are moderately present in petroleum ether extract of *P. amarus*.

Terpenoids are known to possess antimicrobial, antifungal, anti-parasitic, anti-viral, antiallergenic, anti-spasmodic, anti-hyperglycaemic, anti-inflammatory, immunomodulatory and anticancer properties, and they have inhibition of cholesterol synthesizing property as well [62, 63]. In the present study, terpenoids are moderately present in petroleum ether extract of *P. amarus*.

The flavonoids and other polyphenols are potentially beneficial to human health. They are known to contain a broad spectrum of chemical and biological activities including antioxidant, free radical scavenging, anti-ageing, anti-allergic, anti-inflammatory, anti-microbial, anti-

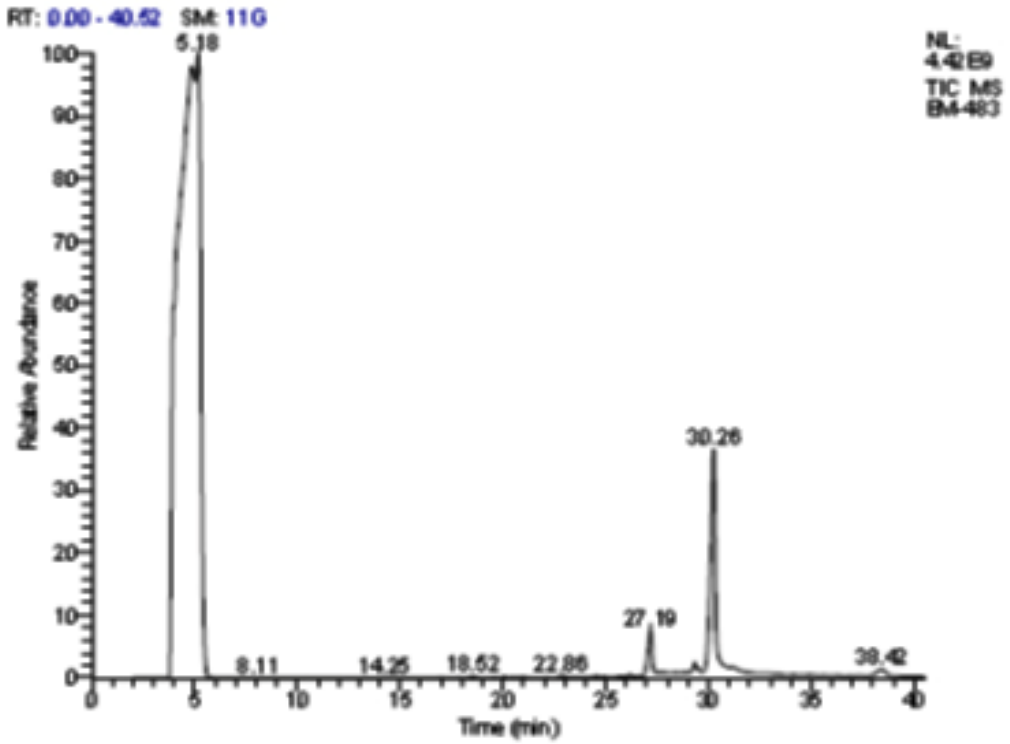


Figure 2. GC- MS chromatogram of petroleum ether extracted *P. amarus* (Relative abundance, 0-100)

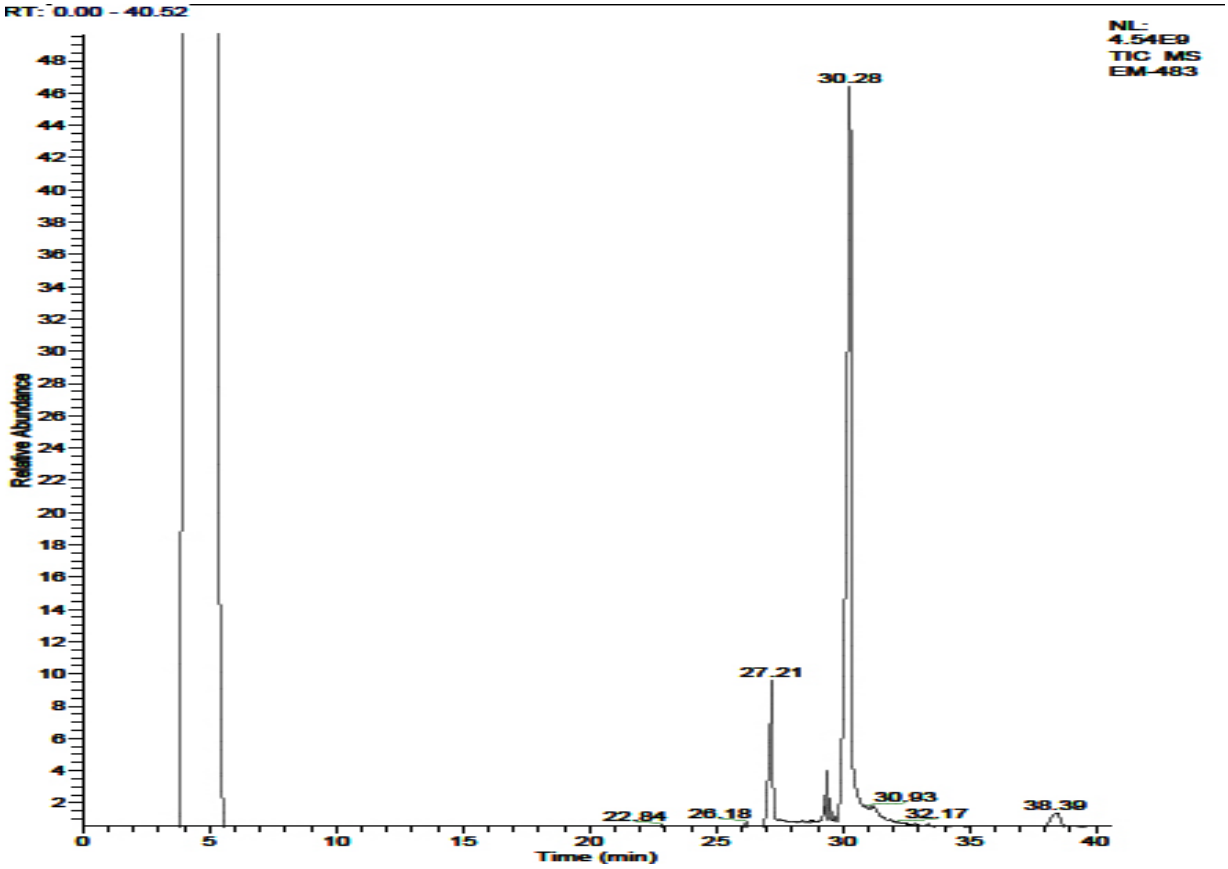


Figure 2a. GC-MS chromatogram of petroleum ether extracted *P. amarus* (with relative abundance, 0-40.52)

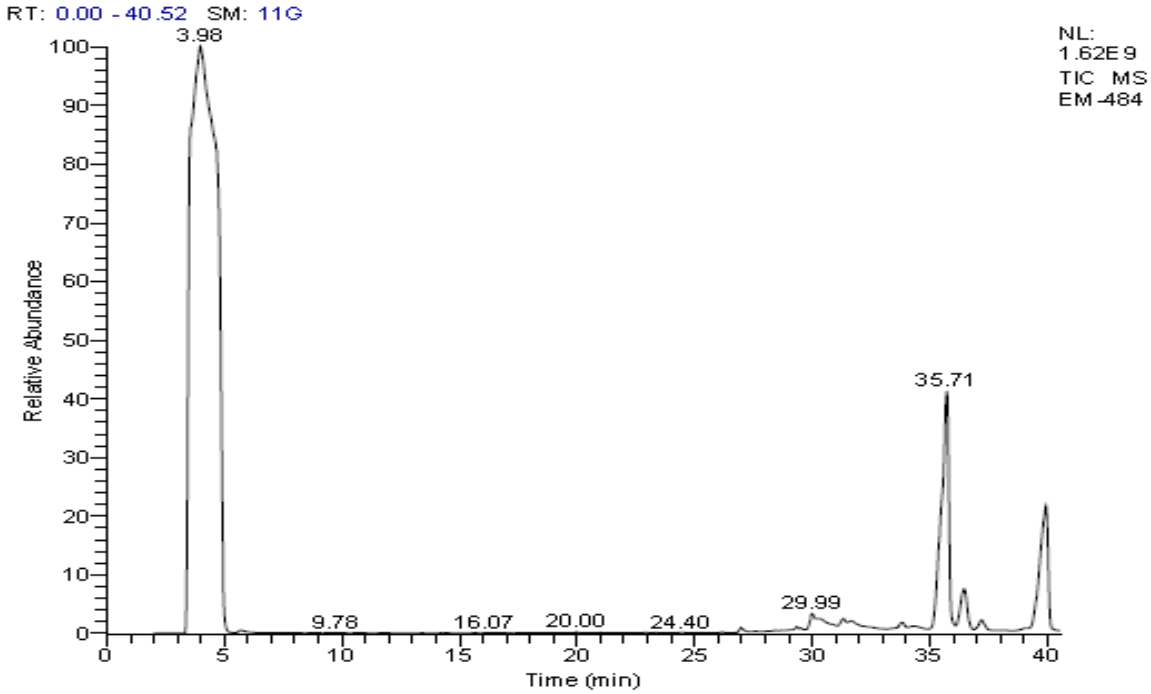


Figure 3. GC- MS chromatogram of acetone extracted *P. amarus* (Relative abundance, 0-100)

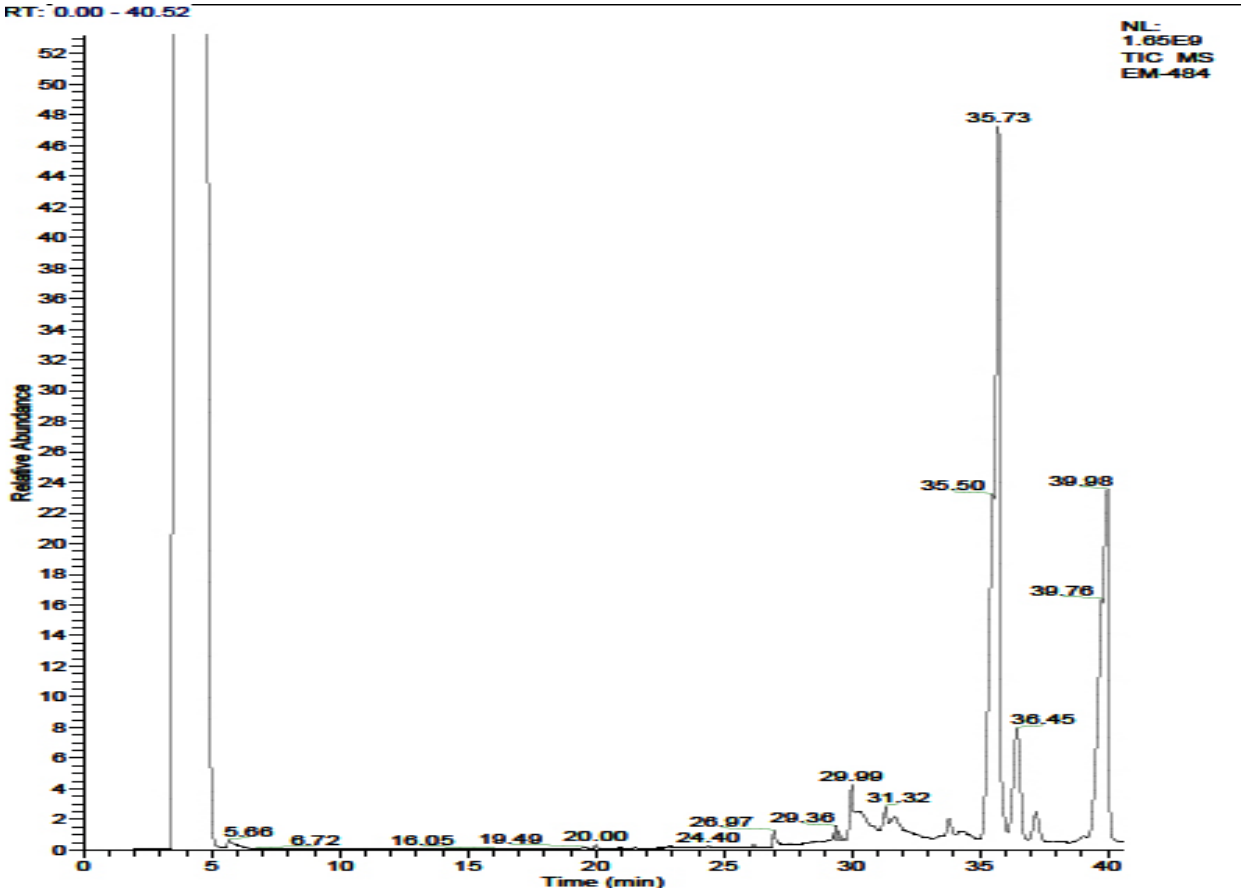


Figure 3a. GC- MS chromatogram of acetone extracted *P. amarus* (Relative abundance, 0-40.52)

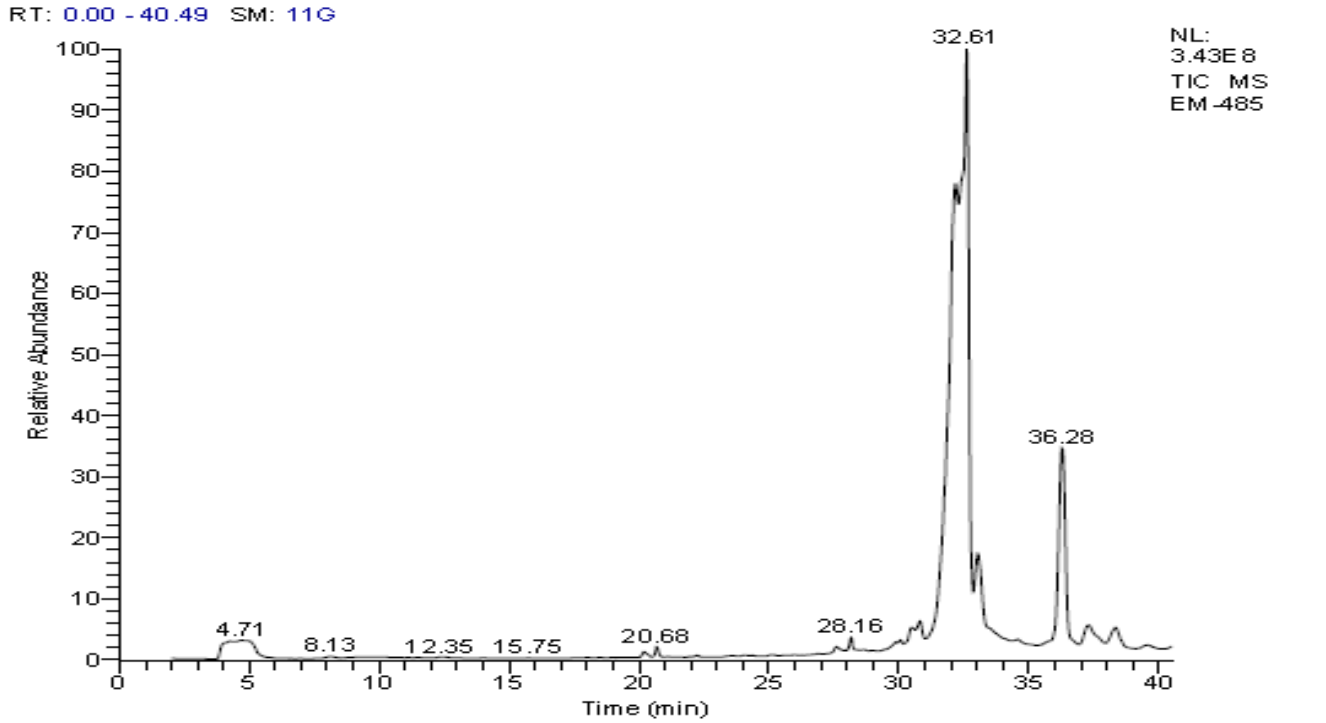


Figure 4. GC- MS chromatogram of ethanol extracted *P. amarus* (Relative abundance, 0-100)

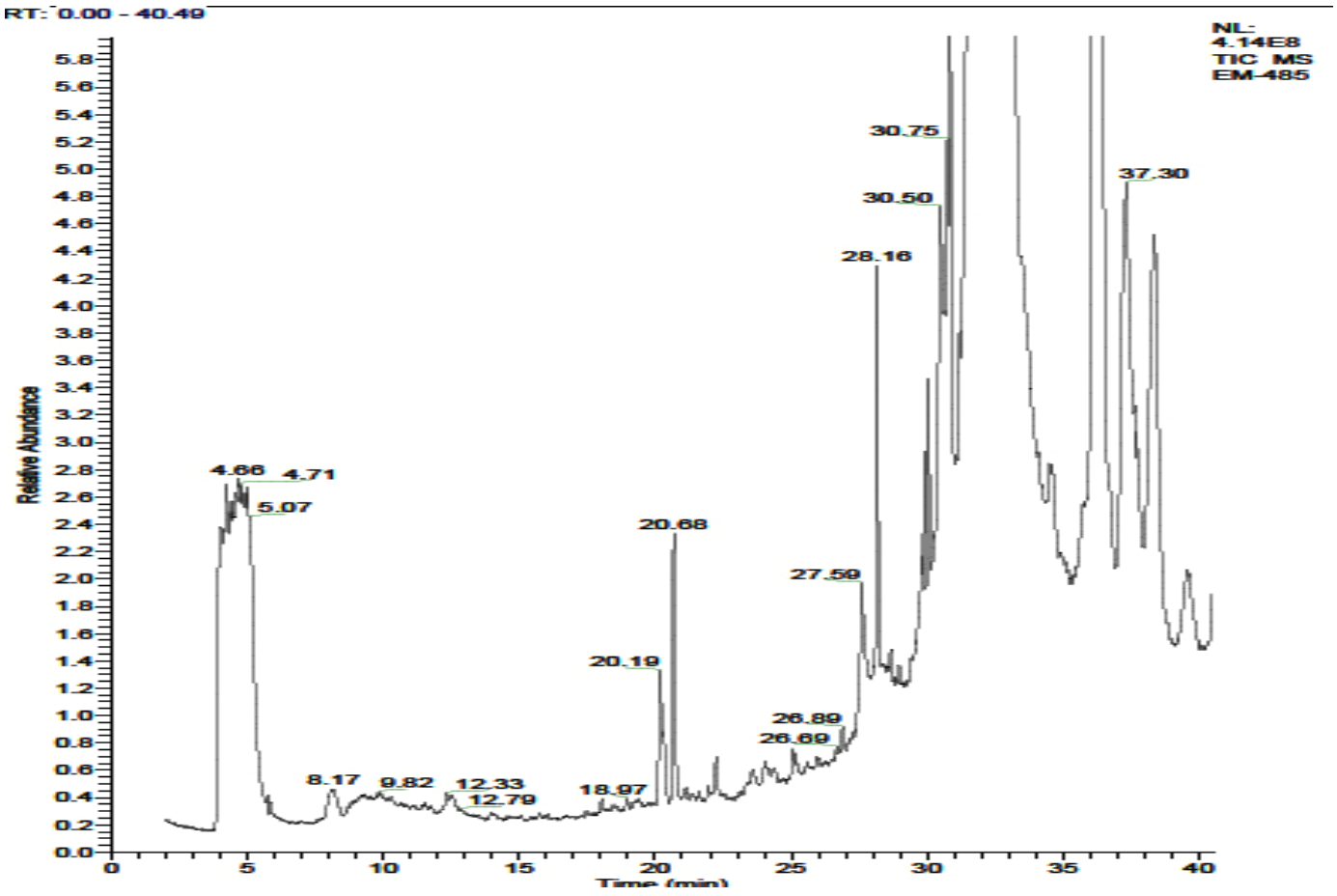


Figure 4a. GC- MS chromatogram of ethanol extracted *P. amarus* (Relative abundance, 0-40.49)

Table 6. Nutritional indices and concentrations of biochemical constituents in *M. rosenbergii* PL fed with different solvent extracts of *P. amarus* enriched *Artemia* nauplii

Parameter		*Artemia* nauplii	*Artemia* nauplii enriched with extracts of *P. amarus* (1.0%)		
			Petroleum ether	Acetone	Ethanol
Nutritional Indices	SR (%)	**82.22±5.09^{c}**	93.33±3.33^{ab}	94.44±3.09^{ab}	**96.66±3.33^{a}**
	Length (cm)	**1.62±0.11^{d}**	1.95±0.14^{c}	2.17±0.12^{b}	**2.34±0.15^{a}**
	Weight (g)	**0.68±0.03^{cd}**	0.86±0.04^{c}	1.02±0.04^{b}	**1.07±0.05^{a}**
	LG (cm)	**1.55±0.03^{d}**	1.88±0.04^{c}	2.01±0.03^{b}	**2.27±0.05^{a}**
	WG (g)	**0.62±0.02^{cd}**	0.80±0.02^{c}	0.96±0.01^{b}	**1.01±0.02^{a}**
	SGR (%)	**3.90±0.02^{cd}**	4.01±0.03^{c}	4.08±0.03^{ab}	**4.10±0.04^{a}**
Biochemical constituents (mg/g wet wt.)	Total protein	**62.63±1.60^{d}**	97.96±3.21^{c}	106.52±3.34^{b}	**112.41±2.78^{a}**
	Total amino acid	**22.03±1.41^{d}**	41.94±3.55^{c}	48.54±2.15^{b}	**55.61±2.15^{a}**
	Total carbohydrate	**11.66±1.13^{d}**	25.56±1.13^{b}	27.04±1.13^{b}	**31.76±1.54^{a}**
	Total lipid	**7.18±0.75^{d}**	13.36±0.21^{c}	16.14±0.78^{b}	**18.03±0.57^{a}**

Each value is mean ± standard deviation of three individual observations.
Initial length and weight were 0.80±0.06 cm and 0.06±0.02 g respectively
Mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $P < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT).
SR, survival rate; LG, length gain; WG, weight gain, SGR, specific growth rate

leukemic, vasodilator, anticancer and antibacterial properties and are reported to be useful for improving blood circulation in brain of Alzheimeric patients [64 - 68]. The flavonoids, anthrax quinines and terpenes stimulate glucose uptake in cells [69]. Certain flavonoids exhibited hypoglycemic activity [70] and also beta cell regeneration in pancreas [69]. The leaf was reported to have the highest activity of phenols and flavonoids compared to root and stem. The antioxidant activity of flavonoids is efficient in trapping superoxide anion (O2), hydroxyl (OH), peroxyl (ROO-) and alcohoxyl (RO) radicals [71]. In the present study, ethanol extract of P. amarus contains luxuriant presence of flavonoids.

Tannins are used as the astringent substance in treatment of burns. They precipitate the proteins of exposed tissues to form a protective covering. They are used as mild antiseptics in treatment of diarrhoea, and to check small haemorrhages [72]. They are also used as healing agents in inflammation, leucorrhoea, gonorrhoea and piles. Tannins have been found to have antiviral, antibacterial, anti-parasitic, anti-inflammatory, anti-ulcer and antioxidant properties [73]. In the present study, all the three solvent extracts of *P. amarus* contain luxuriant presence of tannins.

Phenols are structural and allelopathic components which are associated with diverse functions, like nutrient uptake, protein synthesis, enzyme activity and photosynthesis [74]. Phenolic compounds have biological and pharmacological properties, such as anti-microbial, anti-viral, anti-inflammatory, cytotoxic, anti-mutagenic and anti-carcinogenic activities [67, 75]. In the present study, moderate presence of polyphenols was detected in the ethanol extract of *P. amarus.*

Saponins have hypo-cholesterolemic, anti-carcinogenic, anti-inflammatory, antimicrobial, antioxidant activities, anti-haemolytic and antibacterial activities [76,77]. In the present study, ethanol extract of *P. amarus* contains luxuriant presence of saponins.

The cardiac glycosides are one of the most naturally occurring plant phytoconstituents and are basically steroid drug with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection. Cardiac glycosides and catecholamines are agent of choice in treatment of congestive cardiac failure [78]. In the present study, luxuriant presence of cardiac glycosides was detected in the acetone extract of *P. amarus.*

Quinonines have anti-inflammatory, anti-bacterial and immunomodulatory potentials, and are very much used in the treatment of malaria and more recently of tumors [79,80]. In the present study, moderate presence of quinones was detected in the ethanol extract of *P. amarus.* The primary phytochemicals serve as health tonic in aquaculture nutrition [31,81,82].

Secondary phytochemicals of *P. amarus*

In the present study, dodecanoic acid ($C_{12}H_{24}O_2$), propanoic acid, 2-(tricyclo[3.3.1.13,7]dec-2-ylidene)-($C_{13}H_{18}O_2$), tetradecanoic acid ($C_{14}H_{28}O_2$), neophytadiene ($C_{20}H_{38}$), hexadecanoic acid, ethyl ester ($C_{18}H_{36}O_2$) and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- were the active principle compounds of secondary phytochemicals/ bioactive substances identified from *P. amarus*. The dodecanoic acid (lauric acid: N-dodecanoic acid) is a common saturated fatty acid with a 12-carbon atom chain. Lauric acid is found in coconut and palm kernel oils. It is also found in human breast milk, cow's milk and goat's milk [83, 84]. It is used for treating viral infections including influenza (the flu), swine flu, avian flu, the common cold, fever blisters, cold sores, genital herpes caused by herpes simplex virus (HSV), genital warts caused by human papilloma virus (HPV) and HIV/AIDS. It is also used for preventing the transmission of HIV from mothers to children. Further, it is also used in treatment of bronchitis, gonorrhoea, yeast infections, chlamydia, intestinal infections caused by a parasite called Giardia lamblia, and ringworm. Some research suggests lauric acid might be a safer fat than trans-fats in food preparations. It is safe for pregnant and breast-feeding women in food amounts (Lauric acid Wikipedia).

The propanoic acid is a naturally occurring carboxylic, short chain fatty acid. Its anion $CH_3CH_2CO_2$ as well as the salts and esters are known as propionates/ propanoates. Propionic acid inhibits the growth of mould and some bacteria, as a result, used as a food preservative. It is also used to make pesticides and pharmaceuticals. The esters of propionic acid are used as solvents or artificial flavorings [85]. In biogas plants, propionic acid is a common intermediate product, which is formed by fermentation with propionic acid bacteria. Its degradation in anaerobic environments requires the activity of complex microbial communities [86]. In propionic acidemia, a rare inherited genetic disorder, propionate acts as a metabolic toxin in liver cells by accumulating in mitochondria as propionyl-CoA (the initial metabolic product of propanoic acid) and its derivative, methylcitrate, two tricarboxylic acid cycle inhibitors [87,88]. In a study, Al-Lahham et al. [89] demonstrated that propionic acid lowers fatty acids content in liver and plasma, reduces food intake, exerts immunosuppressive actions and probably improves tissue insulin sensitivity. Thus, increased

propionic acid in the body might be considered beneficial in the context of prevention of obesity and diabetes.

The tetradecanoic acid (myristic acid: 1-tetradecanoic acid) is also a common saturated fatty acid. Its salts and esters are commonly referred to as myristates. It was first isolated from nutmeg (*Myristica fragrans*) by Playfair Lyon [90]. Both lauric acid and myristic acid have positive effects on HDL (good cholesterol) level [91-93]. They have high hydrophobicity and act as lipid anchors in bio membranes of eukaryotic cells.

Terpenes and terpenoids are the primary constituents of the essential oils of many types of medicinal plants and flowers. The neophytadiene is a naturally occurring diterpenoid branched hydrocarbon belongs to the group of compounds known as phytanes. It is a member of the class of compounds known as sesquiterpenoids, which are terpenes with three consecutive isoprene unit makes neophytadiene a potential biomarker for the consumption of plant food product. Its presence can be estimated in the subcutaneous fat of animals [94]. These hydrocarbons are derived mainly from plant origin, being important components of vegetable wax [95]. They often have a strong odour and may protect the plants that produce them by deterring herbivores and by attracting predators and parasites of herbivores [96, 97]. Terpenes have desirable properties for use in food, cosmetics and pharmaceutical products [98, 99]. They are usually active ingredients of natural agricultural pesticides [100].

The hexadecanoic acid (palmitic acid) is the most common saturated fatty acid found in animals, plants and microorganisms [101]. Palmitates are the salts and esters of palmitic acid. The palmitate anion is the observed form of palmitic acid at physiologic pH (7.4). The excess carbohydrates in the body of human and animals are converted to palmitic acid. It is the first fatty acid produced during fatty acid synthesis and is the precursor to longer fatty acids. Palmitate negatively feeds back on acetyl-CoA carboxylase, which is responsible for converting acetyl-CoA to malonyl-CoA, which in turn is used to add to the growing acyl chain, thus preventing further palmitate generation. According to the WHO excess consumption of palmitic acid increases the risk of developing cardiovascular disease due to increase in LDL levels in the blood [102]. Among all fatty acids, palmitic acid has the strongest effect in boosting the metastatic potential of CD36+ metastasis-initiating cells in mouse models [103].

9,12,15-Octadecatrienoic acid, (Z,Z,Z)- ($C_{18}H_{30}O_2$), Linolenic acid; α-Linolenic acid (ALA); (Z,Z,Z)-9,12,15-Octadecatrienoic acid, methyl ester ($C_{19}H_{32}O_2$); Methyl linolenate) is an n-3 (ω-3) polyunsaturated fatty acid with 18-carbon chain and three cis double bonds found mostly in plant foods such as hempseed, chiaseed, walnuts, tofu, and vegetable oils, including flaxseed (linseed oil), rapeseed (canola oil), soybean oils and Perilla seed oil (mint family, Lamiaceae), pumpkin seed oil. It is precursor for the other long-chain n-3 fatty acids, 20:5, all-cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and 22:6, all-cis-4,7,10,13,16,19 docosahexaenoic acid (DHA) [104]. It is associated with cardiovascular (by helping to maintain normal heart rhythm, heart pumping and reduces blood clots) and neuropathologic diseases, and type 2 diabetes [105]. It is a potential nutraceutical to protect the brain from stroke, characterized by its pleiotropic effects in neuroprotection, vasodilation of brain arteries, and neuroplasticity [106]. It is also used to reduce high cholesterol, high blood pressure, asthma (decrease inflammation and improve lung function). ALA is used to treat rheumatoid arthritis, multiple sclerosis, lupus, diabetes, renal disease, ulcerative colitis, and Crohn's disease. It is also used to prevent pneumonia, chronic obstructive pulmonary disease, migraine headache, skin cancer, depression, and allergic and inflammatory conditions such as psoriasis and eczema. Ironically, ALA may raise some men's risk of getting prostate cancer. Its isomer is γ-linolenic acid (GLA, 18:3, n-6).

According to Arun et al. [107], acetone and ethanol extracts of *P. amarus* leaf contain the following secondary phytochemicals: The predominant compounds present in acetone extract of *P. amarus* were 3, 4-Dimethoxy-di-phenylanine; Phenethylamine, 2-methoxy-alpha-methyl-4, 5- (Methlenedioxy); and 5, 8, 11, 14- Eicosa tetraynoic acid. The compounds at least level presence were Benzhydrazide,4-methoxy-N2-(2-bromo-5-(2-prophnyloxy) benzylideno; 2(3H)-Cyclopent(e)-1,3-oxaxin-2-one, hexahydro, 2H-Pyan-2,6(3H)-dione, dihydro-4,4-dimethyl, Hex-5-encylamine, Cycloheptylamine; and Cyclooctanamine. In the case of ethanol extract, the major compounds reported were, benzene, 1, 2–dimethoxy–4-[[(4-methylphenyl) sulfonyl] methyl]; Phenethylamine, 2-methoxy-alpha-methyl-4,5-(methylenedioxy); and phenanthylamine, 2-methoxy. The minor compounds were, cyclopentane, phentyl; 3-(3-(1-Axirdinyl) propoxy)-2, 5-dimethylpyrazine; 3-(Cycloprophylamino) propioitrile; and3, 5-di-t-butyl phenol. According to Mamza *et al.* [108], the ethanol extract of P. amarus contains, methyl 14–methyl pentadecanoate; palmitic acid (hexadecanoic acids; 10–octadecanoate; 9–hexadecenal; glycerol 1, 3-dipalmitate; 2, 13- octadecadiene-1-ol; Dioctyl ester; and Heptanoic acid (9-dece-1-yl ester). The reported hexadecanoic acid was matched with the detected bioactive compound in the present study. Therefore, detection of phytochemical compounds are based on various factors like the quantity of their presence, soil type, soil nutrients and climatic conditions under which the plant was grown, and age of the plant.

Survival, growth and biochemical constituents of *M. rosenbergii* PL fed with *P. amarus* extracts enriched Artemia nauplii

In the present study, ethanol extract of *P. amarus* enriched Artemia nauplii produced the best SR, WG and SGR (96.6%, 1.01g, and 4.1%, respectively against the control 82%, 0.6g and 3.9%, respectively) in *M. rosenbergii* PL than that of other two solvent extracts (Table 6). This is because of the bioactive principle compounds like dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid), hexadecanoic acid (palmitic acid) and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-(linolenic acid) present in *P. amarus* might have been taken up by Artemia nauplii and transferred to *M. rosenbergii* PL. In addition to polyunsaturated fatty acid (PUFA), the saturated fatty acids (SFA) also required for better survival and growth of prawns. This in turn reflected on significant ($P<0.05$) elevations of total protein, amino acid carbohydrate and lipid levels (112, 55, 31 and 18 mg/g wet tissue, respectively against the control 62, 22, 11 and 7 mg/g wet tissue, respectively) in *M. rosenbergii* PL fed with ethanol extract of *P. amarus* enriched Artemia nauplii than that of other two solvent extracts (Table 6). The antimicrobial, immunomodulatory and protective effects of other bioactive compounds, like propionic acid and neophytadiene present in *P. amarus* might have helped in maintenance of general health of *M. rosenbergii* PL. *Artemia nauplii* itself a very good live feed, it serves digestive enzymes and PUFA for larval growth of fishes and prawns. Herbal enriched Artemia has additional advantage that it carries bioactive substances.

The herbal products are eco-friendly, and have the characteristics ability of growth promoting, immune boosting, and appetizing effects in aquaculture animals. They increase consumption, induce maturation, and have antimicrobial as well as anti-stress capacities in aquaculture of shrimps and fin fishes [109]. It has been reported that in P. monodon PL fed with *A. franciscana* nauplii enriched with diets prepared by using

Hygrophila spinosa, *Withania somnifera*, *Zingiber officinalis, Solanum trilobatum, Andrographis paniculata, Psoralea corylifolia* and cod-liver oil has significantly been improved the survival, growth and resistance to environmental stresses due to salinity [110]. In fish, *Heros severus* larvae, canola oil enriched *A. nauplii* produced better growth and stress resistance to temperature and oxygen deficiency and has converted n-3 fatty acids to EPA and DHA [111]. In rainbow trout, Oncorhynchus mykiss, the *Artemia urmiana* enriched with fish and plant oils (sunflower and canola oils) have produced significantly higher survival rate, specific growth rate and lower food conversion ratio. Further, they have improved the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in Artemia nauplii, and the fish fed with canola oil enriched Artemia showed significantly higher stress resistance against thermal, salinity and hypoxia [112]. In *Penaeus monodon*, the methanol extract of Mucuna pruriens enriched *Artemia* had improved larval survival, whereas methanol extract of *Withania somnifera* showed improved total protein and lipid levels [113]. The survival, growth, and disease tolerance have been reported to increase in shrimp, *P. indicus* fed with individual herbal extract (*Ricinus communis, Phyllanthus niruri, Leucus aspera, and Manihot esculenta*) and seaweed (*Ulva lactuca, and Sargassum wightii*) enriched *Artemia* [114]. The enriched *Artemia nauplii* with sunflower oil, Spirulina and cod liver oil have been produced better survival, growth and concentrations of biochemical constituents in *M. rosenbergii* PL also [25, 115, 116]. No report available so far for *P. amarus* in Macrobrachium.

Conclusion

In the present study, petroleum ether extract, acetone extract and particularly ethanol extract of *P. amarus* enriched *Artemia nauplii* produced significant elevations in survival, growth, and concentrations of total protein, amino acid, carbohydrate and lipid levels in *M. rosenbergii* PL when compared with un-enriched *Artemia nauplii* fed PL. This clearly indicates the fact that the primary phytochemical constituents and bioactive substances present in *P. amarus* should have been taken to prawn through *Artemia*. Thus this herb sustainably exerted growth promotion in *M. rosenbergii*. Hence, *P. amarus* can be incorporated or taken as a feed additive in low cost on-farm feed formulations. This study can be extended further for isolation, purification and characterization of different bioactive substances of *P. amarus* for various biological applications.

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