7.0. STANDARDIZATION AND QUALITY EVALUATION OF THE MOST EFFECTIVE HEPATOPROTECTIVE ACTIVITY OF *BRUGUIERA CYLINDRICA* LEAF EXTRACT

7.1. Introduction

Over the past decade, herbal medicines have become a topic of increasing global importance, having repercussion on both world health and international trade. WHO recommends and encourages the use of herbal medicine because of the availability of huge amount of raw materials. The active ingredients of plant material do not depend on single substance, but it influenced by the large number of other components in the herbal medicines. The constituents of plant may differ depending on genetic variations, environmental conditions. Identification of therapeutic indexes is not only authentication of raw material but also specifies the quantitative and qualitative active principles and detecting contaminants. The consistency, purity and potency of an herbal extract should be standardized by chemical or biological marker for its active ingredients. Standardization includes botanical identification of plant drugs by morphological, microscopic, histological characteristics, physicochemical values *viz.,* ash, acid insoluble ash, water/alcohol soluble extractive values, moisture content TLC/HPTLC finger profiles with chemical markers, therapeutic effects and organoleptic

characteristics to ensure the integrity of herb (Murray, 1996). Organoleptic analysis includes sense, smell, taste and touch to identify a plant. Organoleptic analysis is best utilized to separate the desired herb from extemporaneous plant materials. Physical analysis of the crude plant provides initial information on its constituents such as water and fibre content. Chemical analysis determines the actual percentage of active ingredients as well as other characteristics compounds. Therapeutic effect is being evaluated by studying the toxic (Acute and sub acute) effects of the drugs and microbial contamination like bacterial and fungal pathogens, heavy metals (lead, cadmium and aresenic), pesticide residues and aflatoxin. While standardization, the physical and chemical analyses reduce the result of adulteration and provides a measure of quality control for the manufacturing process. In order to ensure the acceptance of phytopharmaceuticals as an integral part of modern drug therapy, the important method of quality assessment is organoleptic and physical standardization of marine herbal products. Hence, the present study has been focused on the organoleptic and physical properties of chosen mangrove plant extracts.

7.2. Materials and methods

7.2.1. Analysis of physical properties (Ameh *et al.*, 2010)

7.2.1.1. Ash values

The ash values are a measure of the inorganic constituents present in the raw drug. High ash content explains its unsuitable nature to be used as a drug. In the present study, the total ash, water soluble ash, acid insoluble ash and ethanol soluble ash have been analyzed.

7.2.1.2. Total ash

One gram of *Bruguiera cylindrica* leaf extract was taken in a previously ignited silica crucible. It was incinerated by gradually increasing the heat not exceeding dull red heat (450˚C) until free from carbon and then cooled and weighed. The procedure was repeated to get the constant weight. The percentage of ash was calculated by using the following formula:

% of total ash = Initial weight/final weightX100

7.2.1.3. Water soluble ash

The total ash was boiled with 25 ml water and filtered through ash free filter paper (Whatmann 4.1). It was followed by washing with hot water. The filter paper was dried and ignited in the silica crucible, cooled

and the water insoluble ash was weighed. The water soluble ash was calculated by following formula:

% of water soluble ash = (Weight of the total ash/
Weight of the water insoluble ash)X100

7.2.1.4. Acid insoluble ash

The total ash obtained was boiled for 5 minutes with 25 ml of 10% (w/v) diluted hydrochloric acid and filtering through ash free filter paper (Whatmann 4.1). The filter paper was ignited in the silica crucible, cooled and the water insoluble ash was weighed. The percentage of acid insoluble ash was calculated by using the following formula:

% acid insoluble ash = (Weight of the total ash/
Weight of the acid insoluble ash)X100

7.2.1.5. Ethanol soluble ash

The total ash obtained was boiled for 5 minutes with 25 ml of 10% (w/v) ethanol and filtering through ash free filter paper (Whatmann 4.1). The filter paper was ignited in the silica crucible, cooled and insoluble ash was weighed. The ethanol soluble ash is calculated by using the following formula:

% of ethanol soluble ash = (Weight of the total ash/
Weight of the ethanol soluble ash) X100

7.2.2. Analysis of organoleptic properties

The organoleptic parameters *viz.,* colour, odour, taste and consistency were also checked as per the standard procedures.

7.2.3. Microbiological studies

One gram of *Bruguiera cylindrica* leaf extract was stored at refrigerator (4°C) and room temperature (27°C) was serially diluted once in a week for up to 45 days with sterile water up to 10^{-10} dilutions. From each dilution, 1ml of sample was taken and used for the enumeration of total heterotrophic bacteria (Nutrient agar, HiMEDIA, Mumbai), fungi (Rose Bengal agar, HiMEDIA, Mumbai), *Escherchia coli* (Mc Conkey agar, HiMEDIA, Mumbai) and *Salmonella* sp. (Bismuth Sulphate agar, HiMEDIA, Mumbai), *Enterobacter* (EMB agar, HiMEDIA, Mumbai) using appropriate culture medium. All the plates were incubated for 24 hrs but the fungal plates were maintained for 72 hrs. After the incubation period, the colony forming unit (CFU) of bacterial and fungal populations were observed and the average values were counted and expressed as number of $CFU.g^{-1}$.

7.2.4 Heavy metal analysis

About 500 mg of crude ethanolic leaf extract of *Bruguiera cylindrica* was taken for heavy metal/trace element analysis. Necessary precautions were taken at every step to avoid metallic contamination in any form. Precleaned silica crucible was maintained at 800°C until the weight of the crucible was constant. Leaf extract was taken in the silica crucible and maintained in a Muffle furnace (TECHNO, HYBRID, India) at 800°C for 4 hrs and further cooled at room temperature by keeping in a desiccator. The ash obtained was ground well with the ratio of 4:1 and further concentrated to 2 ml by heating. The concentrated ash was filtered through Whatman paper No.1. The filtrate was made up to 20 ml using deionised water and stored it in tightly capped plastic tubes. Then the prepared solution was directly used for the determination of various mineral elements *viz.,* iron (Fe), copper (Cu), zinc (Zn), chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), cadmium (Cd) and arsenic (As) using an Atomic Absorption Spectrophotometer (Perkin Elmer Life and Analytical Sciences, Shelton, USA) by following standard procedure (Rajendran *et al.,* 2003).

7.2.5. High performance thin-layer chromatography (HPTLC) fingerprinting

Standardization of herbal extracts depends upon the isolation of active ingredient standards in pure form and in quantities. The qualitative and quantitative analysis of the phytochemical constituents depends upon the HPLC, GCMS and HPTLC chemical fingerprinting analysis. High performance thin layer chromatography helps in the authentication of the plant material (Aggarwal, 2001; Spreeman and Gaedeke, 2000) was performed on a silica gel glass plate (10 × 20 cm, Silica gel 60 F254, Merck). Samples were applied to the plate as 6 mm band using CAMAG linomat applicator. The slit dimension was kept at 6 X 0.45 mm and 20 $mm.s^{-1}$, scanning speed was employed. Leaf extract of *Bruguiera cylindrica* was prepared at the concentration of 1 $mg.ml^{-1}$ in ethanol. 10 µl of extract was used as a constant application rate. The chromatogram was developed by using chloroform: methanol: glacial acetic acid: formic acid (80.0: 10.0: 5.0: 5.0) as the mobile phase.

7.3. Results

The physical and organoleptic property of the most potent hepatoprotective mangrove extract was represented in table 16. The organoleptic properties of the *Bruguiera cylindrica* revealed that, the leaf extract is brown in colour, sour taste, spicy odour and sticky paste

consistency. The physical property of most potent hepatoprotective mangrove extract was represented in table 17. The physical properties of the *Bruguiera cylindrica* revealed that, the leaf extract contains 11.76% of the total ash and were found insoluble in HCl, H_2SO_4 and H_2O. The percentage of extractive value is 6.20%. Whereas, the ethanol soluble ash (23.46%) was found higher than the water soluble ash (12.87%).

Chemical authentication and quality evaluation of the most potent hepatoprotective mangrove extract was carried out during the present study. Chemical standardization such as metal/trace element analysis for the most potent hepatoprotective mangrove extract was listed in table 18. It revealed that, the concentration of trace/heavy metals in crude ethanolic extract of *Bruguiera cylindrica* was found within the permissible limits as per the WHO guidelines (WHO, 2000).

The microbial contaminants such as total heterotrophic bacteria (THB), fungi, *E. coli, Salmonella sp.* and *Enterobacter sp.* were also evaluated in the most potent hepatoprotective mangrove extracts. The microbial counts of *Bruguiera cylindrica* leaf extracts showed that, the THB counts were found maximum (91 $CFU.g^{-1}$) when the extract was stored in 27°C and found minimum at 4°C in the refrigerator. The other microbial counts were not reported in the stored products in both the temperatures.

High performance thin layer chromatography fingerprinting was also performed to detect the active hepatoprotective and antioxidant substances in the *Bruguiera cylindrica* leaf extract and represented in table 20 and figure 35. The HPTLC fingerprinting of ethanolic extract gave ten spots with the following Rf values: 0.10 (15.34%), 0.23 (2.02%), 0.25 (2.85%), 0.36 (10.17%), 0.51 (33.11%), 0.57 (3.00%), 0.65 (5.16%), 0.71 (20.90%), 0.88 (1.60%), 0.91 (5.85%) and most pronounced peak with maximum area was identified with fifth peak Rf (0.51) which is identified as phenol derivatives based on the base line data available (Apaydin and Bilgener, 2000) and also other biochemical constituents are identified as glycosides (Rf=0.10). Moreover, the HPTLC fingerprinting shows the profile of the several quenching zones (Fig. 36) under UV light (254 and 364 nm) after spraying with 90:10 methanol/sulphuric acid reagent, this may due to the presence of several other biochemical constituents. Further detailed studies are in progress to find out the other unknown biochemical constituents.

Table 16. Organoleptic properties of the leaf extract of *B. cylindrica*

Parameters	Characteristics
Colour	Brown
Taste	Sour
Odour	Spicy
Consistency	Pasty, sticky

Table 17. Physical properties of the leaf extract of *B. cylindrical*

Parameters	Values in percentage
Total ash (%)	11.76±1.65
Acid insoluble ash (%)	-
Sulphated ash (%)	-
Extractive value (%)	6.20±0.86
Ethanol soluble ash (%)	23.46±5.92
Water soluble ash (%)	12.87±1.56

± Standard deviation and values are average of three replicates

Table 18. Content of heavy metal/trace metal in the leaf *B. cylindrica*

Heavy metal/ Trace metal	Observed level	Permissible limits (ppm) as per the WHO guidelines per gram sample
Fe (ppm.g^{-1})	0.86±0.007	30
Cu (ppb.g^{-1})	366.40±25.98	150
Zn (ppb.g^{-1})	380.40±43.98	20
Cr (ppb.g^{-1})	5.15±0.78	2
Mn (ppb.g^{-1})	354.00±24.87	30
Ni (ppb.g^{-1})	108.00±9.76	30
As (ng.g^{-1})	2.55±0.08	5
Pb (ppb.g^{-1})	198.80±17.65	10
Cd (ppb.g^{-1})	176.00±21.88	0.3

± Standard deviation and values are average of three replicates

Table 19. Microbial counts in the leaf extract of *B. cylindrical*

Microorganisms	Number of counts (CFU.g^{-1})		Permissible limits as per WHO (CFU.g^{-1})
	27°C	4°C	
THB	91	74	10^5
Fungi	Nil	Nil	10^3
E. coli	Nil	Nil	10^2
Salmonella sp.	Nil	Nil	Absent
Enterobacter sp.	Nil	Nil	10^3

Fig. 35. Picture showing the HPTLC fingerprinting of *Bruguiera cylindrica* leaf extract

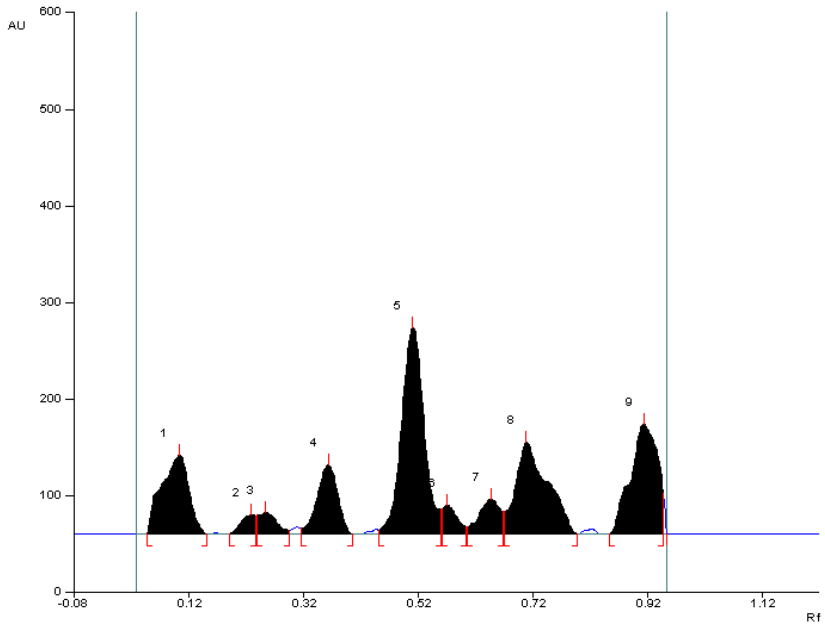
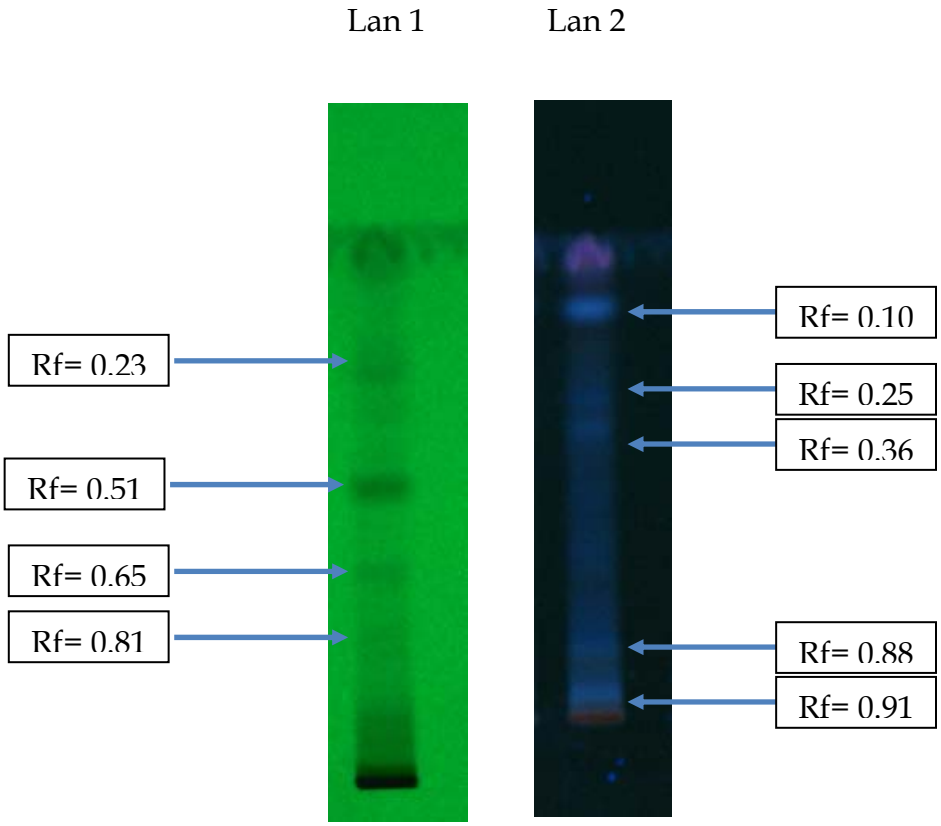


Table 20. HPTLC Rf values of *Bruguiera cylindrica* leaf extract

Peak	Start Rf	Start height	Max Rf	Max height	Height %	End Rf	End height	Area	Area %
1.	0.05	1.8	0.10	81.8	12.55	0.15	0.0	2769.0	15.34
2.	0.19	0.3	0.23	20.0	3.07	0.24	19.0	364.6	2.02
3.	0.24	19.2	0.25	22.1	3.39	0.29	3.6	515.3	2.85
4.	0.31	5.9	0.36	71.2	10.92	0.40	0.0	1835.0	10.17
5.	0.45	4.1	0.51	213.7	32.78	0.56	25.6	5976.4	33.11
6.	0.56	26.0	0.57	29.5	4.53	0.60	7.0	541.4	3.0
7.	0.60	7.1	0.65	36.0	5.53	0.67	22.9	931.7	5.16
8.	0.67	23.0	0.71	95.4	14.64	0.80	0.2	3773.2	20.90
9.	0.86	0.9	0.88	24.2	3.72	0.89	18.6	289.5	1.60
10.	0.89	19.0	0.91	57.9	8.88	0.93	19.1	1055.5	5.85

Fig. 36. Picture showing the HPTLC fingerprinting of different zones of the *Bruguiera cylindrica* leaf extract under UV light



Lan 1 = 254 nm
Lan 2 = 366 nm