CHAPTER 4

STANDARDIZATION AND QUALITY CONTROL OF POLYHERBAL FORMULATION

4.1 INTRODUCTION

A large drift in the field of medicine is found in recent days from synthetic to herbal drugs [288], and it is know that about 80% of the world's population are depending on the herbal medicine. The effectiveness of synthetic drugs is over covered by its risk of side effects which make us look for alternate safe and effective drugs. On looking towards this scenario we get the idea of using naturally available herbs as alternate. There is a high demand for herbal medicines in the developed and developing countries due to their wide range of biological activities, higher safety margins and cost effectiveness [289]. The impediment in acceptance of herbal drugs is due to the lack of their standard quality profile. The profile of the constituents in the final plant product has implications in efficacy and safety. Thus Standardization of herbal formulations is essential in order to assess quality of drugs and this is of paramount important in order to justify their acceptability in modern system of medicine [230]. The World Health Organization (WHO) has recognized the importance of medicinal plants for medical care in developing nations and has laid certain guidelines to support the member states in their efforts to formulate national policies on traditional medicine and to study their potential usefulness including evaluation, safety and efficacy. In order to assess the quality of drugs based on their active compounds standardization is the key factor [231]. WHO provided some guidelines about standardization parameters for herbal formulations [232].

The present work was carried out in accordance to WHO guideline to standardize the polyherbal formulation. The formulation was investigated for their organoleptic characters, physicochemical parameters, phytochemical parameters, Heavy

metal analysis and microbial load assessment along with assessment of bioactive markers using HPLC.

4.2 STANDARDIZATION AND PHYSICOCHEMICAL PARAMETERS

The parameters such as Organoleptic characters, Preliminary phytochemical analysis, Moisture content, Ash values, Sampleive values, Total phenolic content, Total flavonoid content, Heavy metal and microbial load analysis were performed according to standard guidelines. HPLC analysis was conducted to determine the quantity of active compounds.

4.2.1 Evaluation of Organoleptic Properties

Organoleptic evaluation refers to the evaluation of the formulation for its color, odor, taste, texture, etc. This was carried out based on the method as described by Wallis. To find the odor of an innocuous material, a small portion of the sample was placed in the beaker and examined by slow and repeated inhalation of the air over the material. If no odor was perceptible, the sample was crushed between the thumb and index finger or between the palms using gentle pressure. If the material was known to be dangerous it was crushed by other suitable means such as pouring a small quantity of boiling water onto the crushed sample placed in a beaker. The strength of the odor was determined (none, weak, distinct, strong) followed by the odor sensation (aromatic, fruity, musty, mouldy, rancid, etc.) was studied. Taste was distinctively classified as aromatic, pungent, sweet, sour, astringent, mucilaginous or bitter.

4.2.2 Preliminary phytochemical screening of polyherbal formulation

The sample was screened for the various plant constituents following standard procedure. The crude sample was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones using standard procedures.

4.2.3 Test for alkaloids

4.2.3.1 Dragendorff's test: To 1 ml of the sample, add 1 ml of Dragendorff's reagent, an orange red precipitate indicates the presence of alkaloids.

4.2.3.2 Mayer's test: To 1 ml of the sample, add 2 ml of Mayer's reagent, a cream colored precipitate reveal the presence of alkaloids.

4.2.3.3 Wagner's test: To 1 ml of the sample, add 2 ml of Wagner's reagent, the formation of a reddish brown precipitate indicates the presence of alkaloids.

4.2.3.4 Hager's test: To 1 ml of the sample, add 3 ml of Hager's reagent, the formation of yellow precipitate confirms the presence of alkaloids.

4.2.4 Test for carbohydrates

4.2.4.1 Molisch's test: To 2 ml of the sample, add 1 ml of alpha-naphthol solution and concentrated sulphuric acid through the sides of the test tube. Purple or reddish violet ring at the junction of the two liquids reveals the presence of carbohydrates.

4.2.4.2 Fehling's test: To 1 ml of the sample, add an equal quantity of Fehling's A and B solution and heat. The formation of brick red precipitate indicates the presence of carbohydrates.

4.2.4.3 Benedict's test: To 5 ml of Benedict's reagent add 1 ml of sample solution and boil for 2 minutes and cool. Formation of a red precipitate shows the presence of carbohydrates.

4.2.4.4 Barfoed's test: To 5 ml of Barfoed's solution, add 1 ml of sample solution and heat to boil, formation of a red precipitate of copper oxide was formed and confirms the presence of carbohydrates in the test sample.

4.2.5 Test for steroids and sterols

4.2.5.1 Liebermann & Burchard's test: Dissolve the sample in 2 ml of chloroform in a dry test tube. Add ten drops of acetic anhydride and two drops of concentrated sulphuric acid. The solution becomes red, then blue and finally bluish green, indicating the presence of steroids and Terpenoids

4.2.5.2 Salkowski's test: Dissolve the sample in chloroform and add equal volume of concentrated sulphuric acid. Formation of bluish red to cherry red color in chloroform layer

and the acid layer assumes marked green fluorescence, represents the steroid and sterol components, in the tested sample.

4.2.6 Test for glycosides

4.2.6.1 Legal test: Dissolve the sample in pyridine and add freshly prepared sodium nitroprusside solution to make it alkaline. The formation of pink to red color indicates the presence of glycoside.

4.2.6.2 Baljet's test: To 1ml of the test sample add 1ml sodium picrate solution and the change yellow to orange color reveals the presence of glycoside.

4.2.6.3 Borntrager's test: Add a few ml of diluted sulphuric acid to 1 ml of the sample solution. Boil and filter the sample with chloroform. Separate the chloroform layer and treat with 1 ml ammonia. The formation of red color shows the presence of anthraquinone glycoside.

4.2.6.4 Keller Killiani test: Dissolve the sample in acetic acid containing traces of ferric chloride and transfer it to a test tube containing sulphuric acid. At the junction, formation of a reddish brown color, which gradually becomes blue, confirms the presence of deoxy sugar attached to the aglycon part of glycoside.

4.2.7 Test for Saponins

To about 1 ml of alcoholic sample, add 20 ml of distilled water and shake in a graduated cylinder for 15 minutes. A 1cm layer of foam indicates the presence of saponins.

4.2.8 Test for flavonoids

4.2.8.1 Shinoda's test: To 1 ml of the sample, add magnesium turnings and 1-2 drops of concentrated hydrochloric acid. Formation of pink or red color shows the presence of flavonoids.

To 1 ml of sample, add 1 ml of ferric chloride, the formation of brown color confirms the presence of flavonoids.

4.2.9 Test for tannins and phenolic compounds

To 1 ml of sample, add 5% neutral ferric chloride, a dark blue color product shows the presence of tannins.

To 1 ml of sample, add few ml of gelatin solution, a white precipitate reveals the presence of tannins and phenolic compounds.

To 1 ml of the sample, add lead tetra acetate, a precipitate production shows the presence of tannins and phenolic compounds.

4.2.10 Test for protein and amino acid

4.2.10.1 Biuret's test: To 1 ml of the sample add 1 ml of 40% sodium hydroxide solution and 2 drops of 1% copper sulphate solution. Formation of violet color indicates the presence of protein.

4.2.10.2 Ninhydrin test: Add two drops of freshly prepared 0.2% ninhydrin reagent to the sample solution and heat. Development of a purple color reveals the presence of proteins and amino acids.

4.2.10.3 Xanthoproteic test: To 1 ml of the sample add 1 ml of concentrated nitric acid. The formation of white precipitate confirms the presence of amino acid.

4.2.11 Test for fixed oils

4.2.11.1 Spot test: Press a small quantity of sample between the filter paper. Oil stains on paper indicates the presence of fixed oils.

4.2.12 Determination of moisture content

Moisture content of the sample was determined by loss on drying (LOD) at 105°C by Karl Fischer method [293]. 1 gm of weighed quantity of sample was taken in a pre-weighed crucible and kept in an oven at 105^{0}C. The crucible was cooled in desiccator and weight was taken. The procedure was repeated till a constant weight was obtained. The loss of weight was calculated as the amount of moisture content in mg per gm of air dried material. Weighed quantity of sample was also subjected to Karl Fischer titration to determine the moisture content present in the prepared polyherbal formulation.

4.2.13 Determination of Ash values

4.2.13.1 Total ash

Two gm of grounded air-dried material was accurately weighed in a previously ignited and starred silica crucible. This was gradually ignited by raising the temperature to 450°C until it was white. The sample was cooled in a desiccator and weighed. The percentage of total ash was calculated with reference to air-dried sample.

4.2.13.2 Acid insoluble ash

The ash obtained was boiled with 25 ml of hydrochloric acid (2M) for 5 minutes, the insoluble matter was collected on an ash less filter paper and washed with hot water. This was ignited, cooled in a desiccator and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried material.

4.2.13.3 Water soluble ash

The ash was boiled with 25 ml of water for 5 minutes, the insoluble matter on ash less filter paper was collected, washed with hot water, ignited, cooled in a desiccator, and weighed. The weight of the insoluble matter from the weight of the total ash was subtracted; the difference represents the water soluble ash. The percentage of water insoluble ash was calculated with reference to the air-dried material.

4.2.14 Determination of extractive values

4.2.14.1 Water soluble extractives

Five grams of coarsely powdered air-dried material was macerated with 100 ml of water in closed conical flask for 24 hours, the flask was shaken frequently for the first 6 hours and allowed to stand for 18 hours. This was then filtered through Whatman filter paper grade no.100. 25 ml of the filtrate was evaporated to dryness in petri dish, dried at 105°C and weighed. Percentage of water soluble extractive with reference to air-dried material was calculated.

4.2.14.2 Alcohol soluble extractives

Five grams of air-dried and coarsely powdered plant material was macerated with 100 ml of 70% ethanol in a closed conical flask for 24 hours the flask was shaken frequently during the first 6 hours and allowed to stand for 18 hours. This was then filtered rapidly taking precaution against loss of ethanol. 25 ml of the filtrate was evaporated to dryness in a petri dish, dried at 105°C and weighed. Percentage of alcohol soluble extractive value was calculated with reference to air-dried drug.

4.2.15 Total phenolic content

Total phenolic content was determined by Folin–Ciocalteu reagent assay [294]. 1 ml of methanolic sample was mixed with 1ml Folin-Ciocalteu reagent (1N). After 3 min, 1 ml of sodium carbonate solution (20%) was added. The volume was then made up to 10 ml with distilled water. The mixed solution was allowed to stand for 90 min before the absorbance was read at 765 nm. Gallic acid was used as standard for calibration curve. The total phenolic content was expressed as mg Gallic Acid Equivalents (GAE) per 1 mg of sample.

4.2.16 Total flavonoid content

Total flavonoid content was measured by the method described by the Moreno et al. [295]. 1 ml of methanolic sample solution was added to 10 ml volumetric flask containing 0.2 ml of aluminium nitrate (10%), 0.2 ml of potassium acetate solution (1M) and made up to 10 ml with methanol. The mixture was placed in room temperature for 40 mins and the absorbance was measured at 415 nm. Quercetin was used as standard for calibration curve. The total flavonoid content was expressed as µg quercetin equivalent (GAE) per 1mg of sample.

4.2.17 Heavy metal analysis

Heavy metals are released from many different anthropogenic sources in the environment. These heavy metals and trace elements differ with geographical sources and may lead to sever toxicity. Lead and cadmium are the most abundant heavy metals and are

highly toxic. Excessive accumulation of these metals in food is associated with number of diseases, especially of the cardiovascular, renal, nervous and skeletal systems [296-298]. They are also associated with carcinogenesis, mutagenesis and teratogenesis.

4.2.17.1 Determination of Heavy metals by AAS instrumentation

Two gm of the sample was weighed accurately and digested with 25 ml of 0.5 Nitric Acid. This was mixed properly and heated on a water bath for 15 mins. Filter the sample and make up with 25 ml, 0.5M Nitric acid. Aspirate blank, standards and sample solutions separately by using above parameters.

4.2.18 Determination of Microbial load

The polyherbal formulation was tested for the presence of microorganisms- *Escherichia coli, Salmonella sp., Shigella sp., Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterobacter* in the plant samples being used for the preparation of herbal formulations following WHO guidelines.

4.3 Results and Discussion

In the present research work the polyherbal formulation was subjected to physiochemical, phytochemical and analytical analysis.

The Organoleptic analysis of the formulation shows powder nature with dark green color, bitter taste and characteristic property.

The phytochemical analysis of the polyherbal formulation revealed the presence of various phytochemical constituents which represents the various traditional medicinal claim. The preliminary phytochemical analysis of the formulation is given in the Table 4.1.

Table 4.1 Preliminary phytochemical studies of the polyherbal formulation

S. No	Tests	Presence
1	**Test for carbohydrates**	
	a.Molisch's test	+
	b.Fehings's test	+
	c.Benedict's test	+
	d.Barfoed's test	+
2	**Test for Proteins and Amino acids**	
	a.Ninhydrin test	+
	b.Biuret test	+
	c.Zanthoproteic test	+
3	**Test for Fixed oils and Fats**	
	a.Spot test	+
4	**Test for Alkaloids**	
	a.Mayer's test	+
	b.Dragendorff's test	+
	c.Wagner's test	+
	d.Hager's test	+
5	**Test for Saponins**	
	a.Frothing test	+
6	**Test for Glycosides**	
	a.Legal's test	+
	b.Baljet's test	+
	c.Borntrager's test	+
	d.Keller-kiliani test	+
7	**Test for Phytosterols**	
	a.Libermann's test	+
	b.Libermann Burchard test	+
	c.Salkowski's test	+
	Test for Flavonoids	
	a. Shinoda's test	+

8	**Test for Tannins and Phenolic compounds**	
	a.Ferric chloride (5%) test	+
	b.Reaction with Lead acetate	+
	c.Gelatin test	+

'+' is denoted as present, '-' was denoted as absent

4.3.1 Ash Value and Moisture Content

Ash values are useful in determining the authenticity and purity of any drug and also these values are important for quantitative standards. The total ash value of polyherbal formulation was 21% w/w, water soluble ash was 1.14% w/w and acid soluble ash was 0.15% w/w. All these values were considered to be within the limits as per WHO guidelines. Moisture content is also one of the quality determinants of a drug. The lesser the moisture content lower are the chances to get contaminated by bacterial, fungal or yeast growth. Moisture content of the formulation was found to be 0.6% w/w.

4.3.2 Extractive values

The extractive values of individual plants are given in Table 4.2. The alcohol soluble extractive values revealed the presence of polar chemical component present in the plant ingredients, which includes glycosides of steroids, flavonoids, diterpenoids, quinones and lignans. Water soluble content of plant ingredients was high, which is due to the presence of inorganic matters, sugars, carboxylic acids, saponins and tannins. *B. monnieri* was found to have the highest water soluble extractive value (22.9%w/w).

Table 4.2 Extractive values of plants present in the polyherbal formulation

S.No	Herbal drug	Water soluble extract (%w/w)	Alcohol soluble extract (%w/w)
1	*Bacopa monnieri*	32.9	6.2
2	*Dioscorea bulbifera*	19.5	7.6
3	*Hipphophae rhamnoides*	14.38	7.5

4.3.3 Total phenolic content and Total flavonoid content of the polyherbal formulation

Total phenolic and flavonoid content of the sample represent the antioxidant efficacy of the samples. Phenolic content of the formulation was found to be 0.163 mg eq wt of Gallic acid respectively. Flavonoid content of the formulation was found to be 0.044 mg eq wt of Quercetin respectively.

4.3.4 Heavy metal analysis

The analysis of the levels of heavy metal present in the formulation is as follows the amount of arsenic, lead, mercury and cadmium in the plant samples and formulations are Arsenic- 0.0046 (ppm), Lead- 0.1652 (ppm), Mercury- Not Detectable, Cadmium-0.0198 (ppm). The heavy metals analyzed in the formulation are less than the permissible limits.

4.3.5 Microbial load Analysis

All the primary tests were performed to determine the presence of microorganisms. To find the presence of microorganisms- *E.coli, Salmonella sp., Shigella sp., Enterobacter, Pseudomonas aeruginosa, Staphylococcus aureus,* in the polyherbal formulation. Secondary tests like Coagulase test and DNase test were performed for *Bacopa monnieri* samples which have showed positive results for the primary tests.

Table 4.3 Microbial load analysis of polyherbal formulations

S.No	Bacterial Name	WHO Limit	Cells in Sample/g	Inference
1.	*Escherichia. Coli*	10^2	$1*10^2$	Within Limit
2.	*Salmonella sp.*	Absence	Nil	Within Limit
3.	*Shigella sp.*	Absence	Nil	Within Limit
4.	*Enterobacter sp.*	10^4	Nil	Within Limit
5.	*Staphylococc aureus*	Absence	Nil	Within Limit
6.	*Pseudomonas aeruginosa*	Absence	Nil	Within Limit

4.4 Instrumentation

The analyses were performed on a Shimadzu LC-20AD HPLC system equipped with Rheodyne 7725 injection valve furnished with 20µL loop, an SPD-M20A photodiode array detector and Labsolutions software. Separation was carried out using a Phenomenex C_{18} column (250mm × 4.6mm i.d., 5µm pore size). The column was optimized and maintained at 28ºC throughout analysis.

4.4.1 Quantification of biomarkers from the formulation

4.4.2 Chromatographic conditions for estimation of Bacoside, Quercetin and Rutin in the polyherbal Formulation

Quercetin and Rutin

Mobile phase- Methanol:Water:Acetonitrile (45:15:40), Column Phenomenex C18, Temperature 30ºC, Program Isocratic, Wavelength- 254 nm, Detector Photo diode array, Flow rate 0.8mL/min.

Bacoside

Mobile phase- Water: Acetonitrile (35:65), Column Phenomenex C18, Temperature 30ºC, Program Isocratic, Wavelength- 205 nm, Detector Photo diode array, Flow rate 1.8mL/min,

4.4.3 Sample preparations

For the estimation of Bacoside, Quercetin and Rutin from the formulation, 20 capsules of the formulation was weighed along with the powder and empty gelatin capsule to calculate the average weight. Equivalent to 100 mg of and *B. monnieri* and *H. rhamnoides* for the formulation were weighted respectively and extracted three times with 100 mL methanol. Mixed standard stock solution was prepared by accurately (1.0 mg/mL) weighing three markers Bacoside in acetonitrile: water (1:1) and Quercetin and Rutin dissolved in methanol: acetonitrile: water (45:15:40). The working standard solutions were prepared by diluting the mixed standard solution with the same to a series of proper concentrations to construct calibration curve. The standard stock and working solutions were all stored at 4ºC until use.

4.4.4 Simultaneous estimation of Bacoside, Quercetin and Rutin in formulation

The HPLC conditions for the two flavonoids and saponin were optimized after running it in different mobile phases. The optimal condition allowed the two flavones (Quercetin and Rutin) and saponin to be suitably separated within 10 min. As shown in Figures 4.1 & 4.2 the retention times were 4.5 min for Rutin and 9.6 min for Quercetin and 4.9 min for bacoside respectively. Selecting 254 nm for Quercetin and Rutin as the detection wavelength and 205 nm for bacoside resulted in an acceptable response and enabled the detection of the marker compounds used. The column was maintained at 30ºC throughout analysis with flow rate of 0.8 mL/min and 1.8 ml/min with acetonitrile.

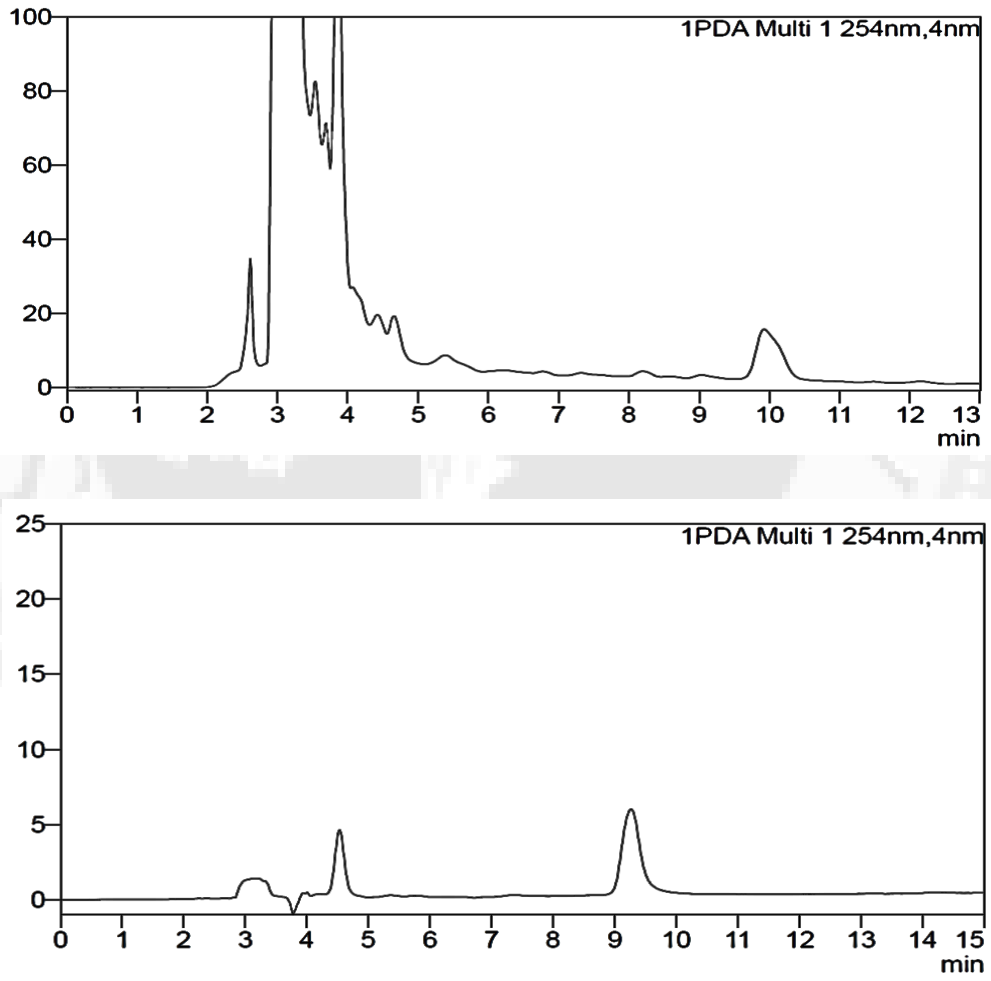

Figure 4.1 HPLC Chromatogram of Quercetin and Rutin Mixture- The content of Quercetin and Rutin in the formulation was estimated and found to be 0.09665%w/w and 0.0541%w/w by HPLC method respectively.

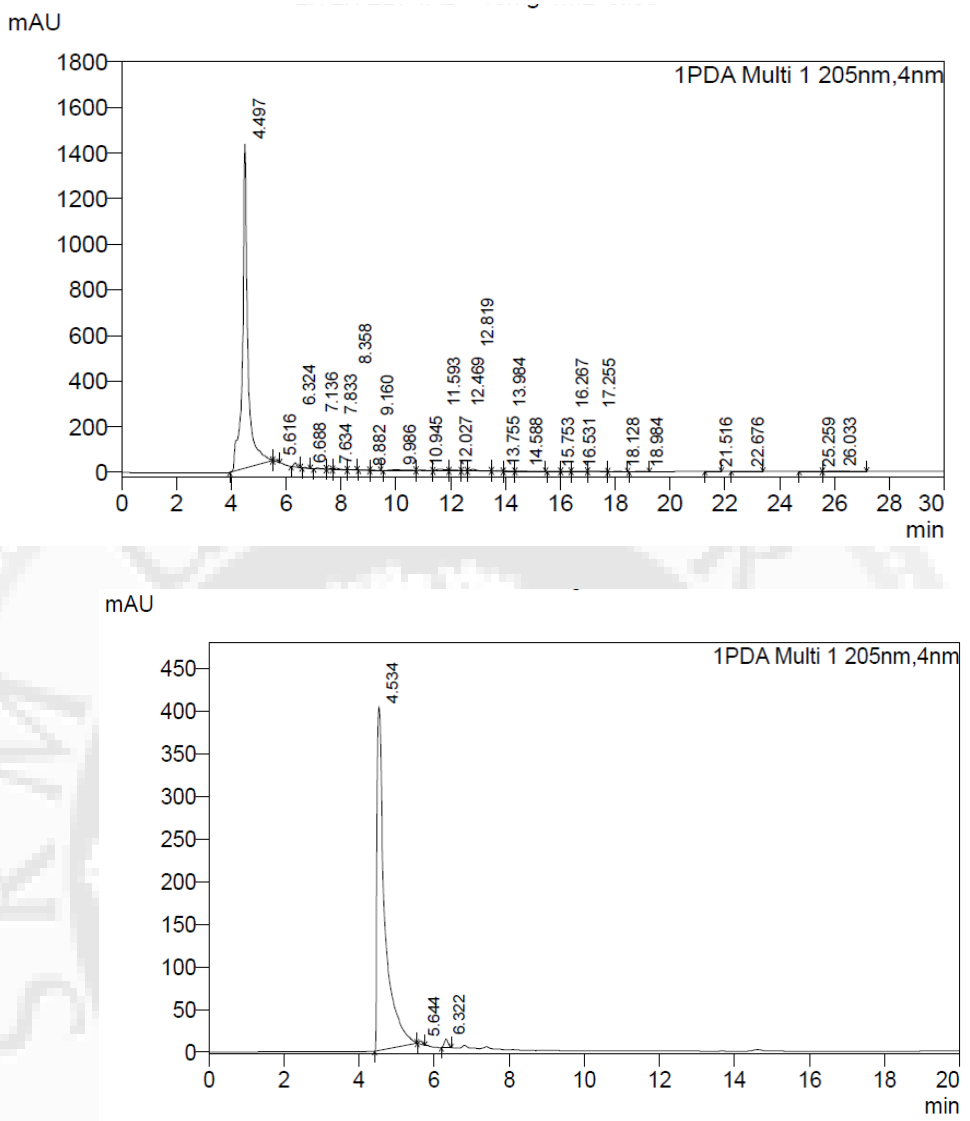

Figure 4.2 HPLC Chromatogram of Bacoside- The content of bacoside in the formulation was estimated and found to be 3.2%w/w by HPLC method respectively

4.5 CONCLUSION

The polyherbal formulation was subjected to physiochemical and phytochemical analysis, which was helpful in establishing the standard. Heavy metal analysis results concluded that levels of heavy metals were within the permissible range and can be preferred to consume by mankind for various medicinal purpose.