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ESTIMATION OF PHENOLIC COMPOUNDS BY RP-HPLC AND ANTIOXIDANT ACTIVITY IN LEAF AND STEM EXTRACTS OF *BARLERIA PRIONITIS* L.

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Key words:

Antioxidant activity, *Barleria prionitis* L., phenolic compounds, RP-HPLC, secondary metabolites.

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ABSTRACT: In the present investigation phenolic contents and antioxidant potential of *Barleria prionitis* L. leaf and stem extracts were analyzed by *in vitro* antioxidant assays and RP-HPLC method. The results indicated that high amount of total phenolics were present in acetone extracts of leaf (20.22±0.57 mg of GAE/g) and stem (21.19±1.25 mg of GAE/g). Highest flavonoid content was observed in ethanolic (44.23±2.82 mg of RE/g) and acetone extracts (44.94±0.85 mg of RE/g) of leaf, while stem extracts were poor in flavonoid contents. RP-HPLC method for determination of specific phenolic compounds was reported for the first time in *B. prionitis* L. Out of the phenolic compounds tested, Gallic acid was most common and more abundantly present in both leaf and stem of *B. prionitis* L. Aqueous extract of leaf showed highest extraction capacity for Gallic acid (211.70 µg/g) and Catechol (112.57 µg/g), while Caffeic acid (37.74 µg/g) and p-Coumaric acid (48.66 µg/g) were highest in methanolic extract. The present study indicated strong influence of extraction solvents and plant parts on antioxidant potential of plant extracts as well as quantity and quality of phytocompounds. The stem of *B. prionitis* L. plants was found to be superior in phenolics and antioxidant potential. RP-HPLC results however noted higher amount and number of phenolic compounds in leaf than stem extracts, indicating presence of other phenolic compounds in stem. Out of the four extraction solvents tested, acetone was best while water was found to be weakest in terms of extraction capacity.

INTRODUCTION: *Barleria prionitis* L. is a perennial medicinal plant belonging to family Acanthaceae. The genus *Barleria* is the third largest genus in the family Acanthaceae comprising of over 300 species that are distributed worldwide. *Barleria prionitis* L. is widely distributed throughout Asia, Africa and Australia and is quite prevalent in the hotter parts of India.

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In Sanskrit it is known as 'Kuranta', 'Kurantaka' owing to the presence of thorns and 'Pita–Saireyaka' meaning yellow flowered. The plant is especially well known for strengthening gums, treating bleeding gums and toothache and hence it is known as 'Vajradanti'. This plant is also used to treat whooping cough, fever, arthritis, gout, leprosy, used as an expectorant, diaphoretic, as a paste it is applied over boils and glandular swellings, and commonly used as wound healer [1, 2]. The plant has antiseptic properties and its decoction is used as a wash in dropsy [3].

Medicinal plants are reservoirs of potentially useful chemical compounds which could serve as clues

for modern drug design [4]. The most important bioactive constituents of plants are alkaloids, tannins, flavonoids, glycosides, steroids and phenolic compounds. The study of phytoconstituents and their bioactivity in plant extracts is desirable in order to exploit it for treating various health ailments and chronic diseases as well [5]. Preliminary phytochemical analysis of extracts of *Barleria prionitis* L. have revealed the presence of glycosides, phenols, flavonoids, saponins, steroids and tannins [3, 6, 7, 8, 9].

The first report on chemical investigation of this plant appeared in 1970 when Moitra *et al.* reported the presence of β-sitosterol. A number of iridoid glycosides like 6-O-trans-p-coumaroyl-8-O-acetylshanzhiside methyl ester and its cis-isomer as well as verbascoside, barlerin were isolated and structural determination of 6-O-trans-p-coumaroyl-8-O-acetylshanzhiside methyl ester and its cis-isomer were undertaken using 2D NMR data [11]. The leaves of *B. prionitis* L. were found to contain scutellarein, melilotic acid, syringic acid, vanillic acid, p-hydroxybenzoic acid, and 6-hydroxyflavones [12]. From the aerial parts of *Barleria prionitis*, phenylethanoid glycoside, barlerinoside along with six iridoid glycosides, shanzhiside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetylshanzhiside methyl ester, barlerin, acetylbarlerin, 7-methoxydiderroside, and lupulinoside were isolated and structures of these compounds were elucidated with the aid of extensive NMR spectral studies and chemical reactions [13].

Antioxidants are universally present in all living systems and play an important role in preventing oxidative damage [14]. Oxidative damage can lead to cancer, cardiovascular diseases and diabetes in humans. Antioxidants are commonly used as dietary supplements to help the human body in reducing oxidative damage by free radicals and reactive oxygen species (ROS). Reactive oxygen species such as hydroxyl radical, nitric oxide, hydrogen peroxide, superoxide etc. are generated from various biological reactions in the body. These ROS can cause cellular injuries, DNA damage and mutations [7, 8, 9, 13, 15, 16, 17]. Medicinal plants are important source of a number of antioxidants, mainly polyphenols. Recently, *in vitro* studies have been undertaken in *B. prionitis* L. for determination of antioxidant properties and free radical scavenging activity [8, 9, 13, 15, 16, 17]. However, the information regarding effects of various factors such as plant parts, solvents used and method of extraction on phytochemical content and antioxidant activity is very limited. Also very little information is available regarding type of phenolic compounds present in different plant parts of *B. prionitis* L. Therefore, in the present study, the different plant parts of *B. prionitis* L. were analyzed for their phytoconstituents and antioxidant potential using various *in vitro* assays while RP-HPLC profiling of phenolic compounds is reported for the first time.

MATERIALS AND METHODS:

Plant Material: The plants of *B. prionitis* L. were collected from Thane in Maharashtra, India and were authenticated at Botanical Survey of India, Pune, India. A voucher specimen (DYP01TH) was deposited at their herbarium. Leaves and stem of collected plants were separated and left to air dry at room temperature in shade for around two weeks. Preparation of crude plant extracts: The dried stem and leaves were pulverized separately in blender in order to obtain fine powder. The ground plant tissues were extracted separately with various solvents like ethanol, methanol, acetone and distilled water, using rotavapour. The plant extracts of dried leaf and stem thus prepared were stored at 0°C until further use [18].

Chemicals: Methanol, Acetone, Folin-Ciocalteu reagent, sodium carbonate were purchased from SRL Pvt. Ltd. Mumbai, India; Trolox, Rutin, TPTZ (2,4,6- tripyrolyl-5-triazine), and DPPH (2,2-diphenyl-1-picrylhidrazyl) were obtained from Sigma-Aldrich Chemical Co. St. Lois, USA; aluminium chloride, Gallic Acid, acetic acid were acquired from Molychem, Mumbai, India; ferric chloride was from HiMedia Laboratories, Mumbai India; ethanol, HPLC grade water, methanol and acetic acid used for RP-HPLC were procured from Merck, Darmstadt, Germany.

Qualitative phytochemical analysis:

Plant extract samples of *B. prionitis* L. prepared in various solvents, were subjected to preliminary

phytochemical screening for detection of different types of phytochemicals.

Test for tannins [19]:
About 2 ml of filtered extract was taken in a test tube and 2 ml of ferric chloride was added. The presence of blue-black coloured precipitate indicated the presence of tannins.

Test for saponins [19]:
To 0.5 ml of extract, 5ml of distilled water was added in a test tube. The solution was shaken vigorously and observed for stable persistent froth indicating presence of saponins.

Test for terpenoids (Salkowski test) [19]:
To 0.5 ml of the extract, 2 ml of chloroform was added. To this solution, concentrated Sulphuric acid 3 ml was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for cardiac glycosides [19]:
To 2ml of extract 1ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1ml of sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for iridoid glycosides [20]:
0.1 ml of the extract was mixed with 1ml of Trim-Hill reagent (consisting of 10ml acetic acid, 1ml of 0.2% $CuSO_4$,$5H_2O$ in distilled water and 0.5ml of concentrated HCl) and heated for some time. Appearance of cherry red colour in the solution indicates the presence of iridoid glycosides.

Test for flavonoids [19]:
Dilute ammonia about 5 ml was added to the extract. Concentrated sulphuric acid 1ml was added to this reaction mixture. A yellow colouration that disappears on standing indicates the presence of flavonoids.

Test for steroids [19]: To 1ml extract 1 ml of chloroform was added; then 1ml of concentrated sulphuric acid was added carefully to form coloured layer. Upper layer turns red. Sulphuric acid layer forms yellow with green fluorescence, indicates the presence of steroids.

Test for phenolics [19]:
To 2ml of extract, 1ml of ferric chloride was added; a blue or green colour indicated presence of phenolic compounds.

Test for triterpenes [19]:
To 1ml extract 1 ml of chloroform was added, then 1ml of concentrated sulphuric acid was added. Appearance of yellow colour in the lower layer of the solution on standing indicates the presence of triterpenes.

Test for alkaloids [20]:
To 1ml of extract, few drops of Mayer's Reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100 ml of distilled water) were added. The presence of creamy yellow coloured precipitate indicated the presence of alkaloids.

Quantitative Phytochemical Analysis and antioxidant activity study:
The plant extracts were further subjected to various *in vitro* assays for determination of their antioxidant activity and free radical scavenging potential. These phytochemical assays were performed by spectrophotometric methods and absorbance at specific wavelengths was taken using a UV spectrophotometer (Shimadzu – Model UV-1700 Pharma spec). For the total phenolic content, total flavonoid content and FRAP assay, all extracts were diluted to 1% (10 mg of plant material/ml) in their respective solvents whereas various dilutions of extracts (w/v) were used for DPPH and ABTS assays to facilitate IC50 value determination. All readings were taken in triplicates and the activities/concentrations of all three were averaged to give final value.

Total Phenolic Content (TPC):
The TPC assay was carried out by a UV-Spectrophotometric method using 1N Folin-Ciocalteu reagent and 20% sodium carbonate. The absorbance was recorded at 760 nm [17]. Gallic acid was used as phenolic standard, which was analyzed at increasing concentrations to prepare standard

curve of gallic acid. The Phenolic content of each sample was determined from the standard curve of Gallic Acid. The TPC is expressed as milligrams of Gallic Acid Equivalent per gram of sample (mg GAE/g).

Total Flavonoid Content (TFC):

The total flavonoid content assay detects the amount of coloured complex formed between the flavonoids and aluminium ions. The samples were reacted with 2% aluminium chloride and the absorbance was recorded at 368 nm [21]. Increasing concentrations of flavonoid standard Rutin were used to prepare the standard curve and the flavonoid content of each sample was determined from it. The total flavonoid content was thus expressed as milligrams of Rutin Equivalent per gram of sample (mg RE/g).

Ferric Reducing Antioxidant Power (FRAP) assay:

The FRAP assay detects the amount of antioxidants present in the sample quantitatively, based on its ability to reduce Fe^{3+} to Fe^{2+}. The FRAP reagent was prepared by mixing 50 ml of 0.3M acetate buffer, 5ml of 10mM TPTZ solution and 5 ml of 20mM ferric chloride. The three reagents were mixed together and incubated at 37°C for 10 min. The samples were reacted with FRAP reagent and the reaction mixture was incubated at 37°C for 30 min [22]. The antioxidant content based on ferric ion reducing ability, was calculated using a standard curve of ascorbic acid at 593 nm. The FRAP value for each sample was expressed as milligrams of Ascorbic Acid antioxidant capacity per gram of sample (mg AAE/g).

DPPH Radical Scavenging Activity Assay:

This assay measures the antioxidant potential and free radical scavenging ability of phytochemicals present in the sample. Scavenging of stable DPPH radical changes the colour of reagent from purple to colourless, which can be detected at 517 nm. The samples at different concentrations were reacted with DPPH solution prepared in methanol [21]. The absorbance of the DPPH radical was recorded at 517 nm and the radical scavenging activity of the samples were calculated by the given formula:

$$\% \text{ scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}}$$

Thereafter the scavenging activities of each concentration were plotted on the graph and regression analysis was carried out. For every sample, IC_{50} value was determined which represents concentration of the sample at which 50% of the free radicals are scavenged.

ABTS Radical Scavenging Activity Assay:

The antioxidants in a sample were quantified by decolorizing reaction with ABTS radicals generated during the reagent preparation. For carrying out ABTS assay, equal quantities of 7mM solution of ABTS and 4.95mM potassium persulphate solution were mixed. This mixture was incubated for 12 hours in dark at 4°C. This mixture was then used to freshly prepare ABTS reagent in methanol giving stable absorbance of 0.706±0.001 at 734 nm. Absorbance of the ABTS reagent was recorded spectrophotometrically as the control at 734 nm and the ABTS radical scavenging activity of the samples at various concentrations was calculated by the given formula:

$$\% \text{ scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}}$$

Thereafter the scavenging activities of each concentration were plotted on the graph and regression analysis was carried out to determine IC_{50}.

Detection and quantification of Phenolic compounds by RP-HPLC:

Detection and quantification of various phenolic compounds present in the extracts was done using reverse-phase HPLC technique. In this experiment, Waters HPLC (Model 2487) instrument with a UV spectrophotometer detector was used. The column was a 15 cm hypersil C18 reverse phase column with 5µ particle packing. The mobile phase (Composition – 20% methanol, 1% acetic acid and 79% water) was passed through the column at the rate of 1ml/min. Five phenolic standards namely Gallic acid, Catechol, p-Coumaric acid, Ferulic acid and Caffeic acid were used for their detection and quantification in the plant extracts. Each phenolic standard stock was prepared (1mg/ml) in HPLC grade methanol and stored at 0°C till further use. All five phenolic standards were mixed freshly for analysis, to make 100ppm strength for each standard. This phenolic mix was injected as a

single injection to obtain standard chromatogram. Run time was 50 minutes. For quantification of phenolic compounds in individual samples, plant extracts were first diluted to 100 mg/ml. All the extracts were then filtered through 0.45µm pore size cellulose syringe filters (Sigma-Aldrich). Injection volume for each sample as well as standard was 20 µl. A linear gradient elution scheme was used in this method and detection was done at 280 nm. The phenolic compounds were detected on the basis of retention time as identified by a standard chromatogram of a mixture of the pure phenolic compounds obtained beforehand. The concentration of individual phenolic compound was estimated from the peak area measurements in comparison to standards and the output was given in the units of ppm using Empower software. The results were then converted from ppm to µg/g. All the solvents and chemicals used were of HPLC grade.

Data analysis:

The mean and standard deviation of the triplicate readings of each sample for each assay were determined and the mean values were analyzed using Microsoft excel. Regression analysis was also carried out using same software. Results were expressed as mean ± standard deviation of three replications. The statistical analysis was carried out by one way analysis of variance (ANOVA) combined with Fisher's LSD test to determine the differences of means among the samples using GraphPad Prism 5.0 software. A significant difference was considered at the level of $p<0.05$.

RESULTS AND DISCUSSION:

The environmental factors such as climatic conditions, soil type, nutrition, biotic and abiotic stress are known to influence presence as well as amount of important phytochemicals *in vivo* in medicinal plants. In case of *in vitro* phytochemical and antioxidant potential studies in plants, factors like type of solvents, method of extraction, type of plant parts and geographical location of plant material can influence the results significantly [18, 23]. Different solvents dissolve different compounds due to differences in their polarity. The factors such as rate of extraction, diversity of different compounds to be extracted, ease of subsequent handling of the extracts, toxicity of the solvent and potential health hazard of the extractant are considered when choosing type of solvent for extraction process [18, 23]. Also it has been reported that different plant parts differ in the quantity as well as quality of phytoconstituents [23]. Therefore, their comparative evaluation becomes necessary in determining the superiority and desirability of a particular plant part and solvent type in terms of medicinal properties.

TABLE 1: LABELS OF VARIOUS EXTRACTS OF LEAF AND STEM PLANT PARTS IN *B. PRIONITIS* L. COLLECTED FROM THANE

Plant part	Extracts in various solvents			
	Ethanol	Methanol	Acetone	Distilled water
Dry Leaf	BPTDLE	BPTDLM	BPTDLA	BPTDLDW
Dry Stem	BPTDSE	BPTDSM	BPTDSA	BPTDSDW

In the present study, four types of extracts using solvents such as ethanol, methanol, acetone and distilled water were prepared from dried leaf and stem of *B. prionitis* L. (**Table 1**). Qualitative tests, quantitative as well as RP-HPLC analysis were performed on these extracts to determine their phytoconstituents and antioxidant potential.

Qualitative phytochemical analysis:

Qualitative phytochemical testing is a rapid method for screening of plant extracts for presence or absence of secondary metabolites. In the present study, leaf and stem extracts of *B. prionitis* L. were subjected to qualitative phytochemical screening which revealed presence of secondary metabolites viz. saponins, flavonoids, phenols, alkaloids, terpenoids, triterpenes and cardiac glycosides (**Table 2**). Out of the various phytochemicals tested, saponins, phenols and flavonoids were present in most of the extracts. Similar results were reported in extracts of leaves [3, 6, 7, 9] whereas contrasting results were reported by Sharma *et al.* showing absence of saponins in both methanolic and aqueous extracts of stem of *B. prionitis* L. The current study revealed that steroids, tannins and iridoid glycosides were absent in all extracts of both leaf and stem while alkaloids were uniquely present only in acetone extract of leaf. Similar results were reported by Sharma *et al.* indicating absence of steroids, tannins and alkaloids in aqueous extracts of leaf and stem whereas, contrasting results were recorded by Aishwarya and Ravikumar in leaf of *B. prionitis* L.

TABLE 2: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L.

Tests	Dried leaf extracts				Dried stem extracts			
	BPTDLE	BPTDLM	BPTDLA	BPTDLDW	BPTDSE	BPTDSM	BPTDSA	BPTDSDW
Steroids	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-
Saponins	+	+	+	+	+	+	-	+
Terpenoids	+	+	-	-	-	+	-	-
Triterpenes	+	-	-	-	+	-	-	+
Cardiac Glycosides	-	+	+	-	-	-	-	-
Iridoid Glycosides	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+
Phenolics	+	+	+	+	+	+	+	+
Alkaloids	+	-	-	-	-	-	-	-

+ = present; - = absent

The present investigation revealed presence of terpenoids in methanolic leaf and stem extracts as well as ethanolic extract of stem of *B. prionitis* L. Previous reports however indicated their absence in ethanolic leaf extract of *B. prionitis* L.[7] Out of the two plant parts compared in the present study, leaf was found to be richer in secondary metabolites that stem, which was supported by observations of Sharma *et al.* in *B. prionitis* L.

Total Phenolic Content (TPC): Polyphenols are the most commonly found secondary metabolites in plants of high medicinal value. Phenolic compounds are known to be responsible for antioxidant potential of plants by neutralization of free radicals that are generated during stress, metabolism, diseases and other numerous factors [22]. High phenolic content in plant extracts is thought to be responsible for high antioxidant potential in plants [18].

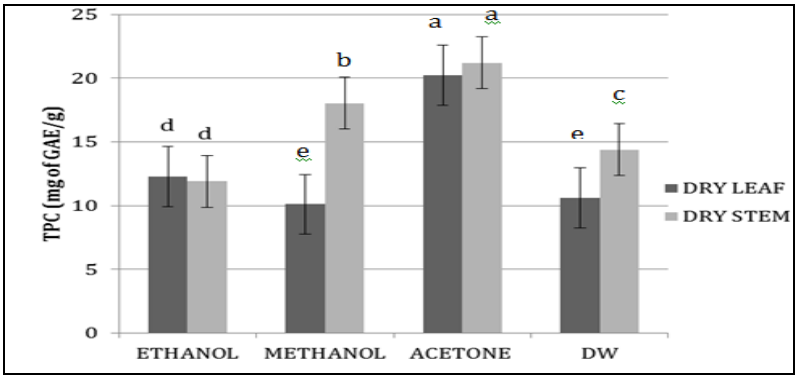

FIG. 1: TOTAL PHENOLIC CONTENTS IN LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PREPARED IN DIFFERENT SOLVENTS

In the present study, significant difference in total phenolic contents was recorded among various solvents used for extract preparation in both leaf and stem of *B. prionitis* L. (**Fig. 1**). Highest phenolic contents were observed in acetone extracts for both leaf (20.22±0.57 mg of GAE/g) and stem (21.19±1.25 mg of GAE/g) whereas lowest were found in methanolic and aqueous leaf extracts indicating strong non-polar nature of phenolic compounds present. Similar to our observations, Moteriya *et al.* as well as Olabindri *et al.* have also reported acetone to be the best solvent for extraction of total phenolics in *Maytenus emarginata* and *Jatropha gossypifolia* respectively. Our study revealed that in acetone extract, total phenolic content was two times higher than that observed in rest of the extracts of leaf. The acetone extract of stem showed TPC two times higher than that of ethanolic and 1.5 times higher than methanolic and aqueous extracts of stem. In contrast to this, Sharma *et al.* reported methanol as the best solvent for extraction of total phenolics from leaf and stem of *B. prionitis* L. In the present study, it was observed that, TPC of leaf and stem were significantly different in methanol and water solvents. Similar results were reported by Sharma

et al. indicating different extraction capacity of methanol and water. When two plant parts were compared, stem of *B. prionitis* L. was found to be richer in phenolic contents as compared to leaf. In contrast to this, Sharma *et al.* reported leaf of *B. prionitis* L. as a better source of phenolic compounds than stem.

Total Flavonoid Content (TFC):

Flavonoids are most commonly found in plant kingdom. They belong to universally occurring group of plant polyphenolic compounds. Flavonoids are also known to be responsible for free radical scavenging activity, *in vivo* as well as *in vitro* [21]. In the current investigation, highest flavonoid content was observed in ethanolic (44.23±2.82 mg of RE/g) and acetone extracts (44.94±0.85 mg of RE/g) of *B. prionitis* L. leaf, among all extracts tested (**Fig. 2**). Similar to our observations, Moteriya *et al.* as well as Olabindri *et al.* have also reported acetone to be the best solvent for extraction of total flavonoids in *Maytenus emarginata* and *Jatropha gossypifolia* respectively.

In the present study it was observed that, methanolic extracts of leaf and stem as well as ethanolic extract of stem showed no significant difference in the total flavonoid contents. The TFC of acetone and ethanolic leaf extract was found to be 3.5 times higher than aqueous extracts and 2 times higher than methanolic extracts of both leaf and stem. Aqueous extracts of leaf and stem showed low TFC values indicating poor extraction capacity of water as compared to other solvents. However, contrasting results were recorded by Narmadha and Devki for *Barleria cristata* leaf ethanolic and aqueous extracts that showed similar TFC values.

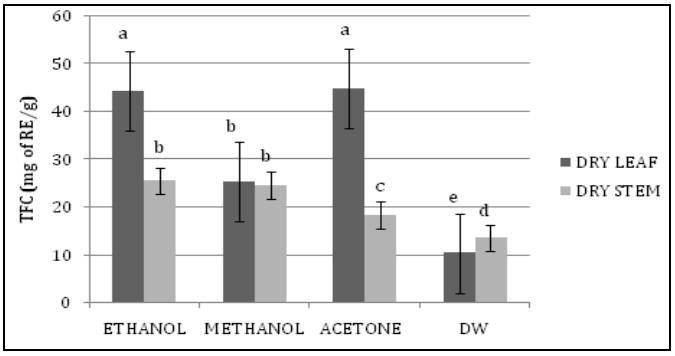

FIG. 2: TOTAL FLAVONOID CONTENTS IN LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PREPARED IN DIFFERENT SOLVENTS

The current study revealed that leaves of *B. prionitis* L. are richer in flavonoids than stem, which was confirmed by several reports in *Gloriosa superba* L. [27], *Maytenus emarginata* [24], *Cassia siamea* and *Cassia tora* [28].

Ferric Reducing Antioxidant Power (FRAP) assay:

In FRAP assay, antioxidant capacity is based on the ability of a compound to reduce ferric ions. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and produce a colored complex of ferrous tripyridyltriazine (Fe^{2+}–TPTZ). The FRAP assay is widely used for the evaluation of the antioxidant potential in the dietary polyphenols [22].

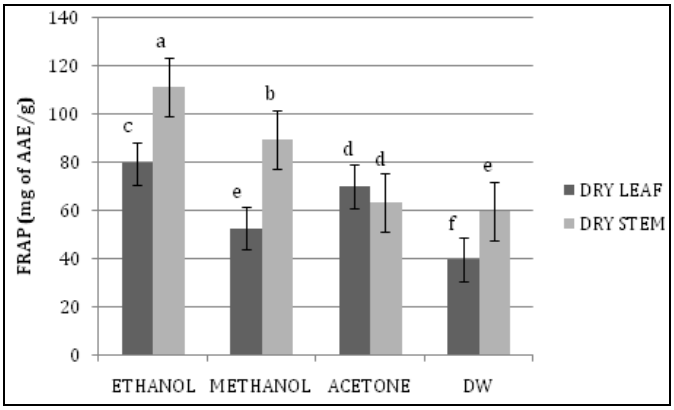

FIG. 3: FERRIC REDUCING ANTIOXIDANT POTENTIAL (FRAP) IN LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PREPARED IN DIFFERENT SOLVENTS

In the present investigation, significant variation in FRAP values was observed among different extracts of leaf and stem of *B. prionitis* L. (**Fig. 3**). Highest FRAP value was observed in ethanolic extract of stem (111.58±1.80 mg of AAE/g), which was around 2.5 times higher than methanolic and aqueous leaf extracts and 1.5 times higher than acetone and aqueous stem extracts.

No significant difference was observed in ferric reducing antioxidant potential between acetone extracts of both leaf and stem. In the present study, stem of *B. prionitis* L. was found to have more antioxidant potential indicated by higher FRAP values than leaf. Similar to our observations, Adedapo *et al.* reported higher ferric reducing antioxidant potential in stem of *Celtis africana* than leaf.

DPPH Radical Scavenging Activity Assay:

In this assay, the antioxidant potential of extracts was estimated from IC_{50} values obtained by regression analysis. The IC_{50} values denote the concentration of a sample required to scavenge 50% of the DPPH free radicals; hence lower the IC_{50} values, higher the antioxidant potential of samples[18].

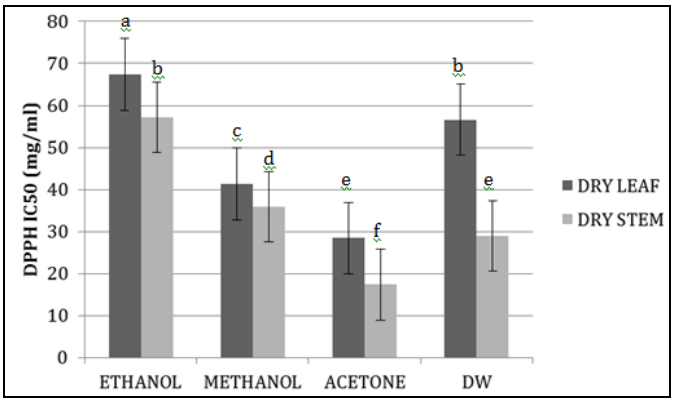

FIG. 4: DPPH FREE RADICAL SCAVENGING ACTIVITY IN LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PREPARED IN DIFFERENT SOLVENTS

In all extracts of *B. prionitis* L. DPPH radical scavenging activity was found to increase with increase in concentration of the sample. Significant difference was recorded in DPPH radical scavenging potential among various extracts of leaf and stem of *B. prionitis* L. In the current study (**Fig. 4),** lowest IC50 value (17.34±0.06 mg/ml) was observed in acetone stem extract, implicating highest antioxidant and free radical scavenging activity among all of the extracts tested. Similar to our findings, Chanda *et al.* have reported acetone extracts having maximum potential for DPPH radical scavenging activity in three species of *Cassia* namely *C. auriculata*, *C. fistula* and *C. siamea.* In the present investigation, free radical scavenging potential of acetone stem extract was recorded about 3 times more than ethanolic leaf and stem extracts as well as aqueous leaf extract and around 2 times more than methanolic leaf and stem extracts. Out of the four extraction solvents tested, acetone was best while ethanol was weakest in antioxidant capacity. Our results clearly indicated higher antioxidant potential of stem than leaf of *B. prionitis* L. Chanda *et al.* reported similar results in three *Cassia* species. Contrasting observations were reported in *B. prionitis* L.[9] and also in *Piper betel* [30] where antioxidant potential was higher in leaf than stem.

ABTS Radical Scavenging Activity Assay:

Medicinal plants contain several secondary metabolites that have free radical scavenging and antioxidant activity. Different antioxidant and free radical scavenging assays differ in principle and experimental conditions. Therefore assessment of antioxidant potential of such phytocompounds, single assay methods may not be sufficient. ABTS free radical scavenging assay is one of the commonly used methods for assessment of free radical scavenging potential of phytoconstituents present in medicinal plants [31].

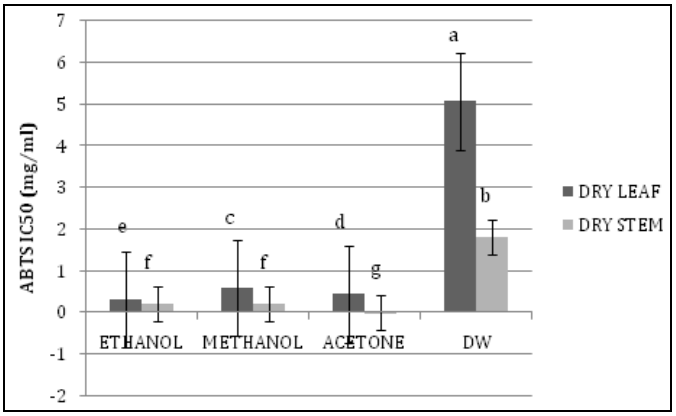

FIG. 5: ABTS FREE RADICAL SCAVENGING ACTIVITY IN LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PREPARED IN DIFFERENT SOLVENTS

In all extracts of *B. prionitis* L., ABTS radical scavenging activity was found to increase with increase in concentration of the sample and significant differences in IC_{50} values were observed in various leaf and stem extracts (**Fig. 5**). In the present study, highest ABTS radical scavenging activity was observed in acetone extract of stem (IC50=0.0039±0.01 mg/ml) while aqueous extracts of both leaf and stem showed low antioxidant potential. Similar results were reported by Rakholiya *et al.* in four species of *Terminalia* i.e. *T. arjuna*, *T. bellerica*, *T. chebula* and *T. catappa.* In the current study, IC50 values of methanolic and ethanolic stem extracts were found to be uniquely similar showing no significant difference. In the present investigation, it was observed that stem of *B. prionitis* L. has more free radical scavenging potential than leaf as depicted by lower IC50 values in all solvent extracts. Similar results were reported in leaf and stem extracts of *Cyamopsis tetragonoloba* [33] however contrasting results were

reported by Alimpic *et al.* in *Salvia juriscii* Kosanin.

There are several methods that can be employed to evaluate free radical scavenging potential in extracts of medicinal plants. Two free radical scavenging activity assays that are most commonly used for this purpose are DPPH and ABTS. In the present study, in both DPPH and ABTS assay, free radical scavenging activity of stem was higher than leaf while acetone being the best extraction solvent. However, Shalaby and Shanab have reported that different free radical scavenging activity assays give varying results in the same set of extraction solvents and samples.

Detection and quantification of Phenolic compounds by RP-HPLC:

Typical phenolics are mainly polyphenols, phenolic acids and flavonoid compounds. Phenolic compounds contain aromatic benzene ring having one or more hydroxyl groups. It has been reported that plants in response to environmental stress and physical injury produce a number of phenolic compounds [18].

RP-HPLC method for determination of polar phenolic compounds was reported for the first time in *B. prionitis* L. This method facilitated simultaneous detection and quantification of five phenolic compounds in samples. In the present study, individual phenolic compounds like Gallic acid, Catechol, Caffeic acid, p-Coumaric acid and Ferulic acid were analyzed and quantified using RP-HPLC for all the plant extracts of *B. prionitis* L. (**Fig. 6-12**). Gallic acid was present most abundantly in extracts of leaf and stem (Table 3) among the five compounds tested (1.02-211.70 µg/g) followed by Catechol (11.63-112.57 µg/g). Highest amount of Gallic acid was found in aqueous extract of leaf whereas minimum in aqueous extract of stem (**Table 3**). Ethanolic extracts of leaf and stem did not show the presence of any of the five phenolic compounds. Ferulic acid was totally absent in all extracts of both leaf and stem. P-coumaric acid was uniquely present only in methanolic extract of dry leaf while Catechol and Caffeic acid were detected only in methanolic and aqueous extracts of both leaf and stem (**Table 3).**

Contrasting to our results, Luis *et al.* reported abundance of Ferulic acid, Ellagic acid and Quercetin in methanolic extracts of *Echinospartum ibericum, Pterospartum tridentatum, Juniperus communis, Ruscus aculeatus, Rubus ulmifolius, Hakea sericea, Cytisus multiflorus, Crataegus monogyna, Erica arborea and Ipomoea acuminate*.

In the present investigation, water showed highest extraction capacity for Gallic acid and Catechol in both leaf and stem plant parts of *B. prionitis* L. Similar results were reported in aqueous extracts of *Helicteres isora* [18]. The Caffeic acid (37.74 µg/g) and p-Coumaric acid (48.66 µg/g) were more in methanolic extract of *B. prionitis* L. leaf as compared to other extracts as per our observations. The leaf of *B. prionitis* L. was found richer than stem in all the phenolic compounds tested.

TABLE 3: QUANTIFICATIONS OF FIVE PHENOLIC COMPOUNDS IN DRIED LEAF AND STEM EXTRACTS OF *B. PRIONITIS* L. PLANTS COLLECTED FROM THANE BY RP-HPLC ANALYSIS

Extracts	Gallic acid (µg/g)	Catechol (µg/g)	Caffeic Acid (µg/g)	p-Coumaric Acid (µg/g)	Ferulic Acid (µg/g)
BPTDLE	-	-	-	-	-
BPTDLM	107.92	11.63	37.74	48.66	-
BPTDLDW	211.70	112.57	12.10	-	-
BPTDSE	-	-	-	-	-
BPTDSM	13.98	-	-	-	-
BPTDSDW	1.02	-	-	-	-

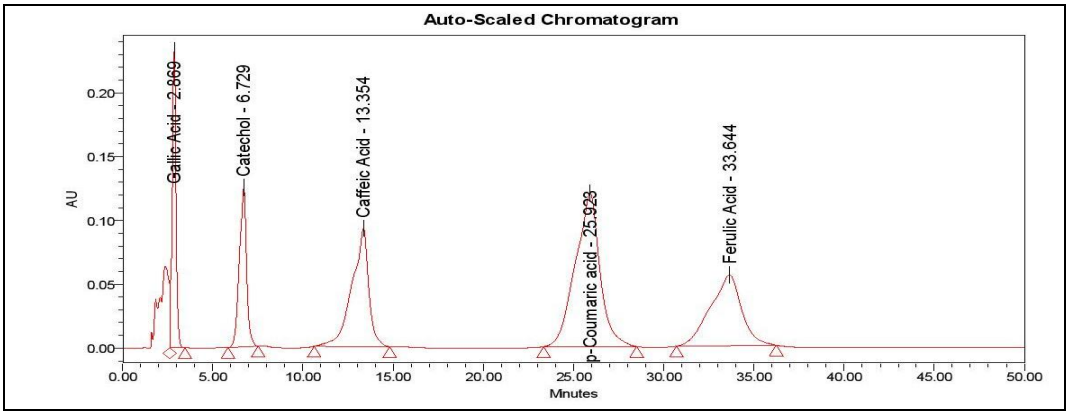

FIG. 6: CHROMATOGRAM OF FIVE PHENOLIC STANDARDS OBTAINED BY RP-HPLC ANALYSIS

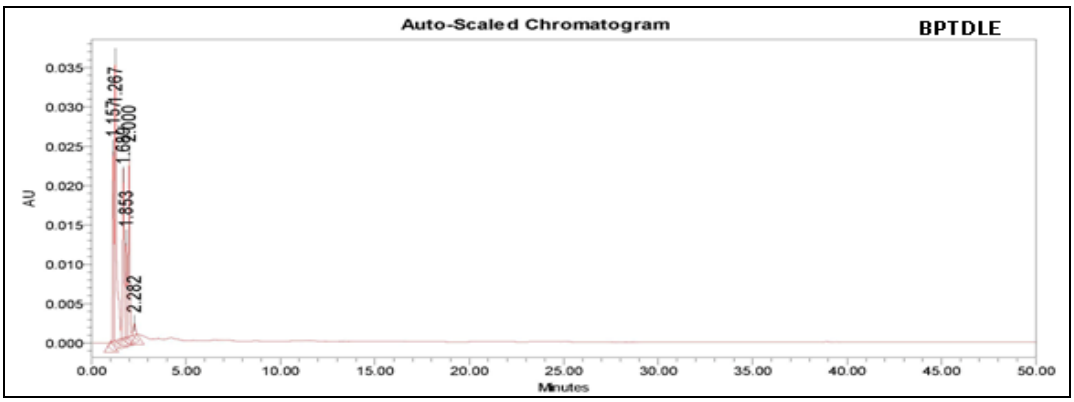

FIG. 7: CHROMATOGRAM OF ETHANOLIC EXTRACT OF LEAVES OF *B. PRIONITIS*

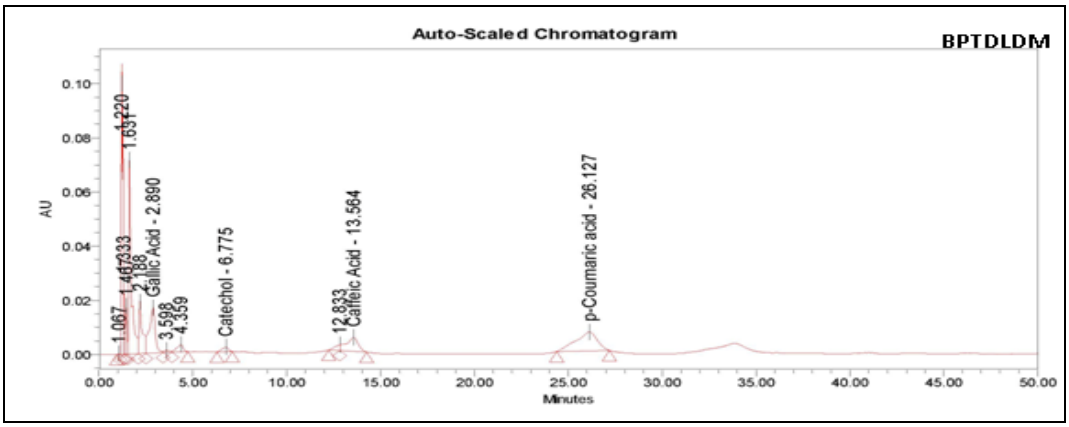

FIG. 8: CHROMATOGRAM OF METHANOLIC EXTRACT OF LEAVES OF *B. PRIONITIS*

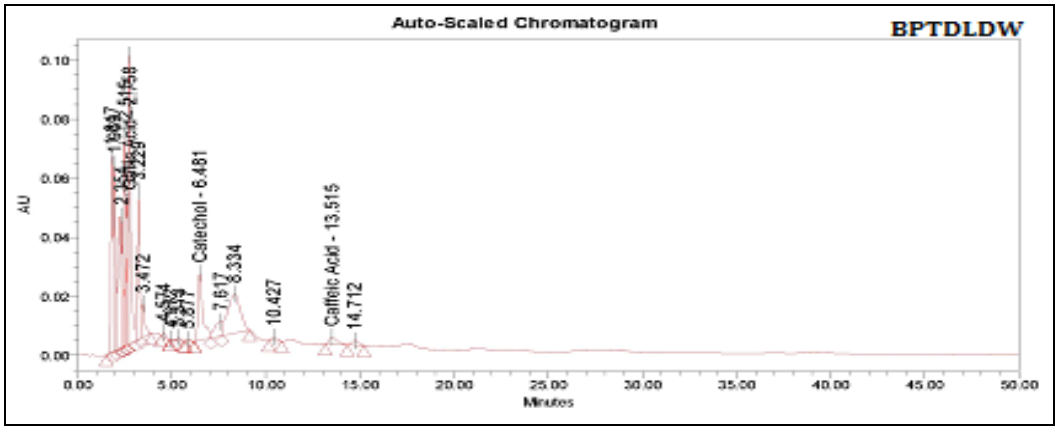

FIG. 9: CHROMATOGRAM OF AQUEOUS EXTRACT OF LEAVES OF *B. PRIONITIS*

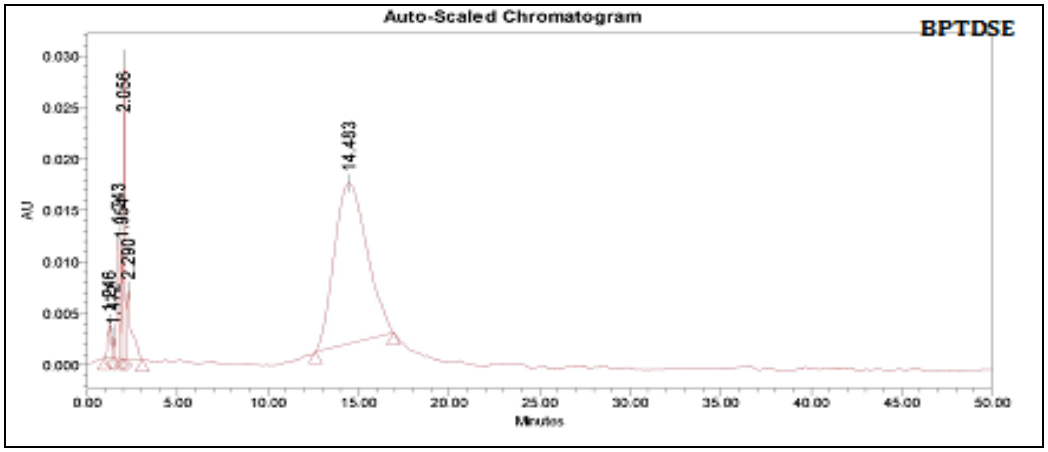

FIG. 10: CHROMATOGRAM OF ETHANOLIC EXTRACT OF STEM OF *B. PRIONITIS*

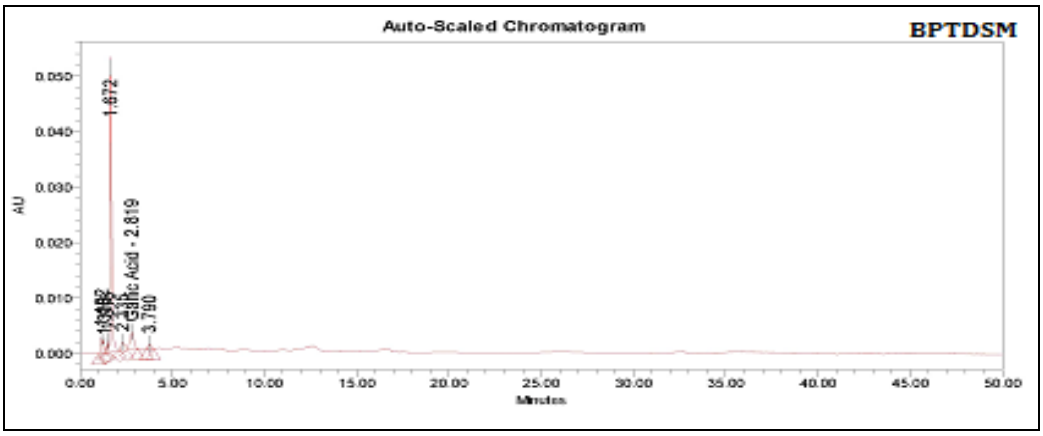

FIG. 11: CHROMATOGRAM OF METHANOLIC EXTRACT OF STEM OF *B. PRIONITIS*

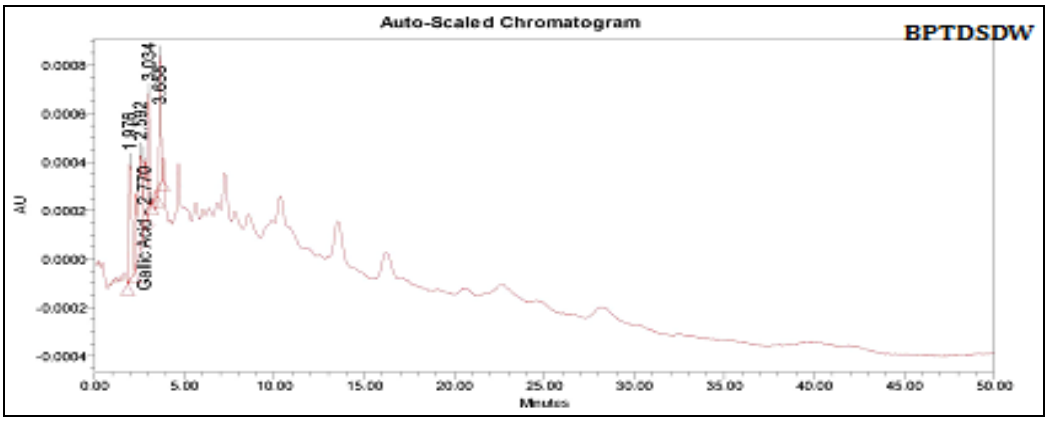

FIG. 12: CHROMATOGRAM OF AQUEOUS EXTRACT OF STEM OF *B. PRIONITIS*

CONCLUSION: The influence of extraction solvents on extractability and availability of secondary metabolites as well as antioxidant capacity of plant extracts was strongly demonstrated in our study. Out of the four extraction solvents tested, acetone was the best solvent for phenols and flavonoids whereas water was found weakest in both leaf and stem of *B. prionitis* L. Acetone extracts of both plant parts also proved to be higher in antioxidant potential than aqeous extracts according to our observations in FRAP, DPPH and ABTS assays. Comparison of DPPH and ABTS assays revealed similar results and trends and therefore either of them can be used to assess free radical scavenging potential of *B. prionitis* L. Different plant parts of *B. prionitis* L. varied significantly in their phytochemical compositions. The secondary metabolites like phenols, flavonoids, alkaloids, saponins, cardiac glycosides, terpenoids etc. were more in leaf than stem. Total phenolics were higher in stem while leaves were richer in flavonoids. Out of the two

plant parts tested, stem of *B. prionitis* L. was superior in antioxidant and free radical scavenging potential than leaf. RP-HPLC analysis of five phenolic standards however revealed higher amount and number of phenolic compounds in leaf than stem extracts, indicating presence of other phenolic compounds in stem.

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CONFLICT OF INTEREST: Authors declare that they have no conflict of interest.

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