



SCREENING FOR BIOACTIVE COMPOUNDS OF LEAVES AND ROOT SAMPLES OF *Plantago asiatica*

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ABSTRACT

Phytochemical constituents of medicinal plants are used as traditional medicine to cure various diseases. *Plantago asiatica* from Plantaginaceae family is considered as orthodox medicinal herb throughout the years and used in the treatment of diseases includes, wounds bronchitis, chronic constipation; diarrhea. The aim of the present study is to investigate the phytochemicals, antioxidant activity, antidiabetic, and quantitative estimations of phytochemicals by High Performance Liquid Chromatography (HPLC). Leaves and root samples of plant were dried, powdered and used for phytochemical extraction with successive solvents such as Petroleum Ether, Acetone, Ethyl Acetate, Methanol and Cold extract using Soxhlet apparatus. Alkaloids, Carbohydrates, Flavonoids, Phenols, tannins, terpenoids, saponins were detected both in leaves and root extracts of different solvents. Acetone extract of plant leaves, showed high free radical scavenging activity as evidenced by the low IC₅₀ values in 2,2-diphenyl-1-picrylhydrazil (DPPH) assay (9.95µg/ml), 2,2¹-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay (51.55µg/ml), Reducing power (FRAP)(3.23mg/ml), Superoxide Dismutase(SOD) (2.39mg/ml) and cold extract showed Nitric oxide(NO) (10.5µg/ml), scavenging assays. The HPLC analysis of methanol extract of plant leaves showed phenols (51mg/g), flavonoids (163.4mg/g), ethyl acetate of plant root showed Tannins (35.41mg). The antidiabetic activity of α-amylase was observed in petroleum ether extract of leaves with IC₅₀ of 236.47µg/ml. Our findings provided evidence that the extracts of plant contain medicinally bioactive compounds and it justifies their medicinal uses.

KEY WORDS

Antidiabetic, Antioxidant, HPLC, *Plantago asiatica*, , Superoxide dismutase

INTRODUCTION

Plantago asiatica belongs to the genus *Plantago* (in latin "planta", meaning "sole of the foot") and the family of Plantaginaceae. *Plantago* is considered as the largest genus comprising about 275 annual and perennial species distributed in Eastern Asia¹⁻² and also in Japan, china and many other places³. The bioactive component of plantago seeds were widely reported in recent years. The phytochemical screening of *P. asiatica* as shown to contain polyphenolic compounds such as phenylpropanoid glycosides, coumarins, lignans, triterpenes, flavonoids and phenolic acids in the seeds⁴

⁵, Plants rich with such bioactive compounds shown to reduce the risk of many diseases, including heart disease, cancer, diabetes and age related degenerative disorders and it is also used in the treatment of many diseases including wounds, bronchitis, chronic constipation, diarrhea⁴. *P. asiatica* shown to possess antiviral, antileukemia, anticarcinoma, diuretic, antioxidant⁶ and immune enhancement effects and also used to treat liver disease, Stomach problems and urinary system inflammation⁷.

The report provides studies of bioactive components and medicinal uses of *P. asiatica* seeds since there was

lack of report on *P.asiatica* roots and leaves were known, so our present study is aimed to screen phytochemical compounds, therapeutic activity present in the root and leaves of *P.asiatica* and also quantification of phytochemicals by HPLC method.

MATERIALS AND METHODS

Plant material

P.asiatica leaves and roots were collected from northeast part of India. They were authenticated by D.M.university, G.P.wome's college, Department of environment science, Imphal west-001. The authenticated leaves and roots of *P.asiatica* were collected and washed using distilled water. The plant samples were air dried and were powdered and subjected for phytochemical extraction using soxhlet apparatus.

Preparation of Plant Extraction

Eighty grams of dried and powdered *P.asiatica* leaves and roots were extracted with 1000ml of successive solvents such as Petroleum Ether, Acetone, Ethyl Acetate, Methanol and Cold extract by using Soxhlet apparatus till it reaches a clear solution at a temperature not exceeding the boiling point of the solvents, then extracts were condensed using rotary evaporator. The extraction of plants was then used for Phytochemical Analysis.

Preliminary qualitative phytochemical analysis

P.asiatica leaves and roots extracts was assessed for the preliminary qualitative phytochemical screening were carried out for Alkaloids (Wagner's reagent), Cardiac glycosides (Keller Kelliani's test), Carbohydrates (Molisch's test), Flavonoids (Alkaline reagent test), Phenols (Ferric chloride test), Phlobatannins (Precipitate test), Amino acids and Proteins (ninhydrin test), Saponins (Foam test), Sterols (Liebermann-Burchard test), Tannins (Braymer's test), Terpenoids (Salkowki's test), Quinones, Oxalate following the protocol mentioned in Solomon Charles Ugochukwu⁸

In vitro antioxidant activity

DPPH radical scavenging assay

The free radical scavenging activity was measured using the DPPH assay according to the method reported by Brand-williams⁹. Stock solution of the radical DPPH was prepared by dissolving 24mg DPPH in 100ml methanol. The working solution of DPPH was prepared by diluting the stock solution with methanol to obtain an

absorbance about 0.99 (± 0.02) at 517nm. Stock solutions (10 mg/mL) of plant extracts were diluted to final concentrations of 10, 20, 30, 40, and 50 μ g/mL, in methanol. DPPH solution (3 ml) was added to different extracts at different concentration. The mixture was shaken vigorously and allowed to stand at room temp for 30 min. Then, absorbance was measured at 517 nm. Reference standard compound being used was Gallic Acid (100 μ g/ml) and experiment was done in triplicate. The percentage DPPH scavenging effect was calculated by using following equation:

DPPH scavenging effect (%) or Percent inhibition = $A_0 - A_1 / A_0 \times 100$. Where A_0 was the Absorbance of control reaction and A_1 was the Absorbance in presence of test or standard sample.

ABTS radical scavenging activity

Antioxidant activity of plant (leaves and root) extracts as per ABTS decolourization assay was measured using the method reported by Re¹⁰. The working solution of ABTS^{•+} radical was made by reacting ABTS (7mM) with potassium persulfate (2.45mM) and raising the volume to 50 mL with Ethanol. The ABTS solution was diluted with ethanol to an absorbance of 0.99 (± 0.02) at 734 nm. Plant samples were prepared in methanol with dilutions 10-50 μ g/ml. Different concentrations of extracts were mixed thoroughly with 3ml mL ABTS radical working solution and incubated for 15min and absorbance was recorded at 734 nm. The percentage antioxidant activity of the sample was determined using the following formula: %Antioxidant activity = $[(Ac - As)/Ac] \times 100$ where Ac and As are the absorbance's of the control and sample, respectively. The control was prepared by adding 10 μ L of methanol in place of the sample. The positive control Gallic acid used as standard solution. Assays were carried out in triplicate.

Nitric oxide scavenging activity

Nitric oxide scavenging activity can be estimated by the use of Griess Illosvoy reaction –Garrat¹¹. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations (10 - 50 μ g/ml) of extract of plant were dissolved in methanol and incubated at 30°C for 2 hours. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% phosphoric acid (H₃PO₄) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added and absorbance was immediately read at 550nm.

Inhibition of nitrite formation by the plant extracts calculated relative to the control Gallic acid and percentage inhibition were linearized against the concentrations of each extract and standard antioxidant.

Superoxide Dismutase (SOD) activity

The procedure adopted was that of Beauchamp and Fridovich¹². The reaction mixture was composed of 2.6 μM riboflavin, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), phosphate buffer saline (PBS- pH 7.4) and various concentrations of test samples. The sample was randomly placed in a light storage box and replaced, randomly, every 5-min for 15-min. The light intensity at the sample level was 5,500 lux. During the light illumination, NBT was reduced to blue formazan formation that was measured by the absorbance at 560 nm. The inhibition of blue formazan formation was taken as a superoxide quenching activity. The positive control Gallic acid used as standard solution. Assays were carried out in triplicate.

Reducing power assay

The reducing power of extracts (leaves and root) of *P. asiatica* was determined by the method of Oyaizu¹³. Various concentrations (10-50 μg/ml) of the plant extracts in corresponding solvents were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). This mixture was kept at 50°C in water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml) and absorbance was measured at 700 nm. Control was prepared in similar manner excluding samples. Gallic Acid at various concentrations (1 mg/ml) was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

Antidiabetic assay

Inhibition of α-amylase activity

The α-amylase inhibitory activity was determined using a modified assay of that described in the Worthington¹⁴ Enzyme Manual. A total of 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) containing 0.5 mg/mL of α-amylase were pre-incubated at 25°C for 10 min. After the pre-incubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) was added to each tube at timed

intervals. The reaction was stopped using 1.0 mL of dinitrosalicylic (DNS) acid colour reagent. The test tubes were incubated in a boiling water bath for 5 min and then cooled to room temperature. The mixture was diluted by adding 5 to 15 mL of distilled water, and the absorbance was measured at 540 nm. The absorbance readings were compared with the controls that contained buffer instead of sample extract. The percentage α-amylase inhibitory activity was calculated.

Quantitative analysis Phenols, Flavonoids and Tannins by HPLC

The plant extracts samples were subjected to HPLC analysis to determine the quantitative estimations of phenols, Flavonoids and Tannins using Shimadzu HPLC system with C18 column, UV-Vis detector. The phenols and flavonoids analysis was estimated using mobile Phase HPLC grade Methanol: HPLC grade water (70:30, v/v) and for Tannins using mobile Phase HPLC grade Acetonitrile: HPLC grade water (70:30, v/v) with flow rate 1 ml/min and detection at 254 nm and 264 nm respectively. 10 mg/ml of each plant extract samples and standards Gallic acid, Rutin and Tannic acid were dissolved in respective solvents and filtered through 0.45 μm syringe filter. Filtered samples and standards were then injected into HPLC and chromatogram was obtained. The samples chromatogram was then compared with standard chromatogram.

RESULTS AND DISCUSSION

Preliminary qualitative phytochemical analysis

Qualitative screening of phytochemicals in leaves and roots extracts of *P. asiatica* was summarised. Of the fourteen-phytochemical screened for, ten were found in various solvent extracts. The ethyl acetate, cold extract and acetone extracts of leaves were showed the presence of Alkaloids, carbohydrates, cardiac glycosides, Flavonoids, Phenols, Proteins, Saponins, Tannins, Terpenoids and oxalates, whereas in plant root acetone, methanol, cold extract were showed the presence of Alkaloids, carbohydrates, cardiac glycosides, Flavonoids, Phenols, Proteins, Saponins, Tannins.

Invitro Antioxidant Activity

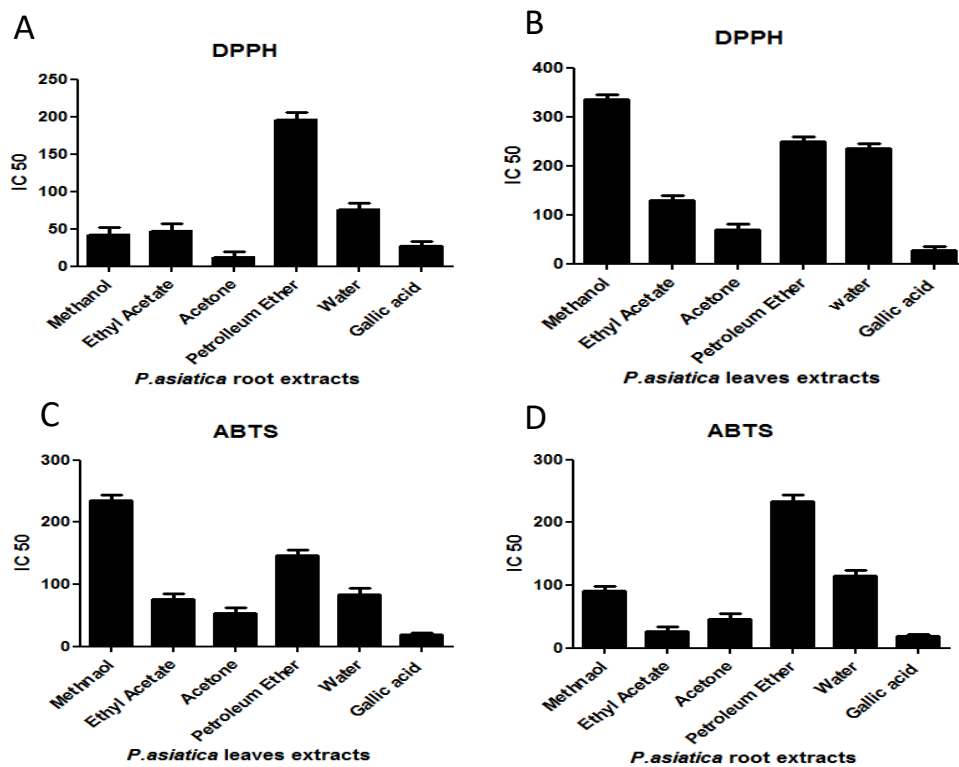
DPPH and ABTS scavenging Activity

The DPPH method is simple and sensitive and measures the compounds that are radical scavengers. Acetone extract of root and leaves of plant showed maximum

antioxidant activity compared to different extracts with IC₅₀ of 9.95µg/ml and 70.95µg/ml respectively, using Gallic acid as standard with IC₅₀ of 28.21µg/ml. Hence acetone extracts of plant at high concentration captured more free radicals formed by DPPH resulting in decrease in absorbance and increase in IC₅₀ value. When similar work showed, there was a minimum scavenging activity was seen in *P.coronopus* extract with the percentage of inhibition 21.56 and maximum scavenging activity was seen in *P.lanceolata* with the percentage of inhibition 94.17 (Opric¹⁵) compared to *P.asitica* extracts. The ABTS method is commonly used to access scavenging activity of sample on the inhibition of ABTS radical, which is a synthetic compound and produced by

oxidation of ABTS. Acetone extract of leaves and ethyl acetate of root showed maximum antioxidant activity compared to different extracts with IC₅₀ of 51.55µg/ml and 27.74µg/ml respectively, using Gallic acid as standard with IC₅₀ of 17.41µg/ml. The presence of specific chemical compounds in extract may inhibit potassium persulfate activity and hence reduced ABTS. Similar work showed *P.coronopus* root ethyl acetate and methanol extract showed maximum ABTS scavenging activity With IC₅₀ of 0.57mg/ml and 0.56mg/ml (Pereira⁵). Our results with compare to other data evidencing that extracts of plantago species possess significant antioxidant activity. The results of DPPH and ABTS are resulted in graph1.

Graph 1: DPPH and ABTS scavenging activity



A and B represents DPPH scavenging assay, C and D represents ABTS scavenging assay of different extracts of *P.asiatica*

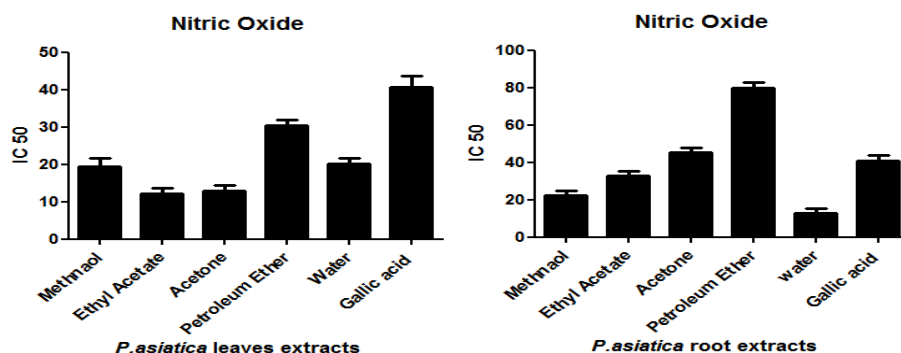
Nitric Oxide scavenging Activity

Nitric oxide involved in many functions, modulating inflammatory response and in regulating physiological process. Leaves and root extracts of plant were found to be very much efficient antioxidant activity through competing with oxygen to scavenge nitrite radical. Cold extract of root sample displayed the highest activity. The IC₅₀ of cold extract of root samples was 8.98µg/ml

and IC₅₀ of ethyl acetate and acetone extracts of leave sample was 10.19µg/ml and 10.5µg/ml compared to other extracts. The results of nitric oxide scavenging activity are resulted in figure 2. Gallic acid was used as reference compound with IC₅₀ of 54.26µg/ml. Similar work showed *P.coronopus* water extract exhibit most potent scavenging activity with IC₅₀ of 3.83mg/ml (Pereira⁵). Thus, plantago species possess potent nitric

oxide scavenging activity. The results of nitric oxide scavenging activity are resulted in graph 2

Graph 2: Nitric oxide scavenging activity



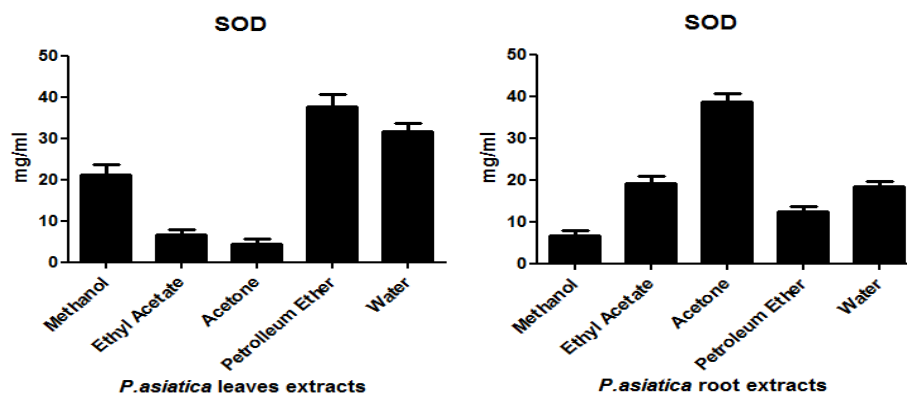
Nitric oxide scavenging activity of different extracts of *P. asiatica*

Superoxide Dismutase (SOD) activity

The superoxide radicals generated can be measured by ability to reduce NBT. SOD is the first of line of defence against oxidative stress in plants. The decrease in absorbance at 560nm with root and leaves extract of plant and the reference compound Gallic acid indicates their ability to quench superoxide radicals in a reaction

mixture. Acetone leaves extract of plant showed concentration of 2.39mg/ml and Methanol root extract of plant showed concentration of 6.84mg/ml compared to other extracts. Similar work showed *P. coronarius* leaves highest SOD activity with concentration of 190 (Nadgórska-Socha¹⁶). The results of superoxide dismutase activity are resulted in graph 3.

Graph 3: Superoxide Dismutase (SOD) activity



Represents Superoxide Dismutase activity of different extracts of *P. asiatica*

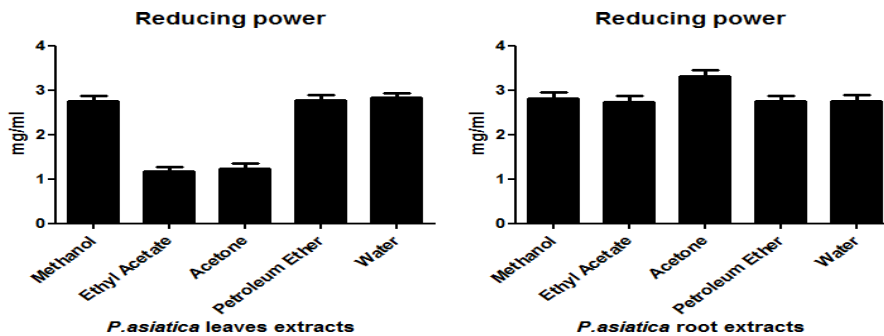
Reducing power assay

In this assay, yellow colour of the test solution changes to various shades of green and blue depending on reducing power each compound. Presence of reducers causes the conversion of the Fe³⁺/ferricyanide complex used in this method to the ferrous form. The reducing power of all extracts increased with increase in concentration. The reducing capacity of each extract depends on electron donating power and terminating free radical chain formation by forming most stable

compounds. The results revealed that methanol, petroleum ether and cold extract of leaves showed increased reducing power with increase in concentration 2.71mg/ml, 2.73mg/ml and 2.83mg/ml respectively and acetone extract of root with concentration of 3.23mg/ml compare to other extracts, against control Gallic acid with concentration of 1.76mg/ml. The results of reducing power assay are resulted in figure 4. Similar work showed methanolic extract of *P. major* showed maximum absorbance at

concentration of 800 μ g/ml (Selamoglu¹⁷). The results of reducing power are resulted in graph 4.

Graph 4: Reducing power assay



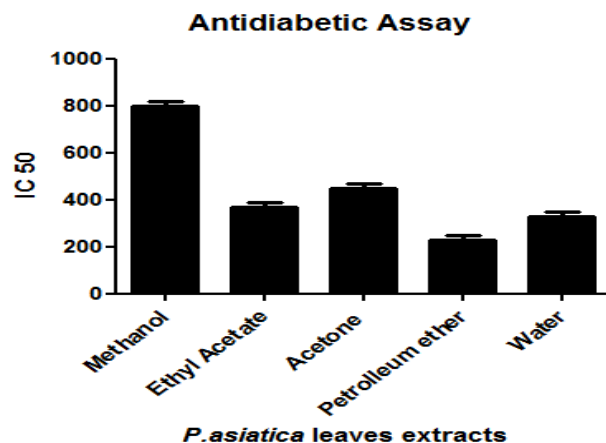
Represents reducing power assay (FRAP) of different extracts of *P.asiatica*

Antidiabetic assay

The enzyme activity of α -amylase and α -glucosidase is responsible for postprandial hyperglycemia by breaking down of dietary carbohydrates into glucose. The present study involves the inhibitory effect of α amylase activity by plant extracts may lead in to reduction in postprandial hyperglycemia in diabetic condition. Only leaves extract of plant inhibited α -amylase activity and

there was no significance results observed in root extract of plant. Petroleum ether of leaves extract showed IC₅₀ of 230.62 μ g/ml compared to other extracts and can be concluded that leaves extract of plantago will be beneficial to reduce the rate of digestion and absorption of carbohydrates by decreasing postprandial hyperglycemia. The results of antidiabetic assay are resulted in graph 5

Graph 5: Antidiabetic assay



Represents alpha amylase inhibition activity of different extracts of *P.asiatica*

Quantitative phytochemical analysis

Determination of Total Phenolic, Flavonoid and Tannin content by HPLC

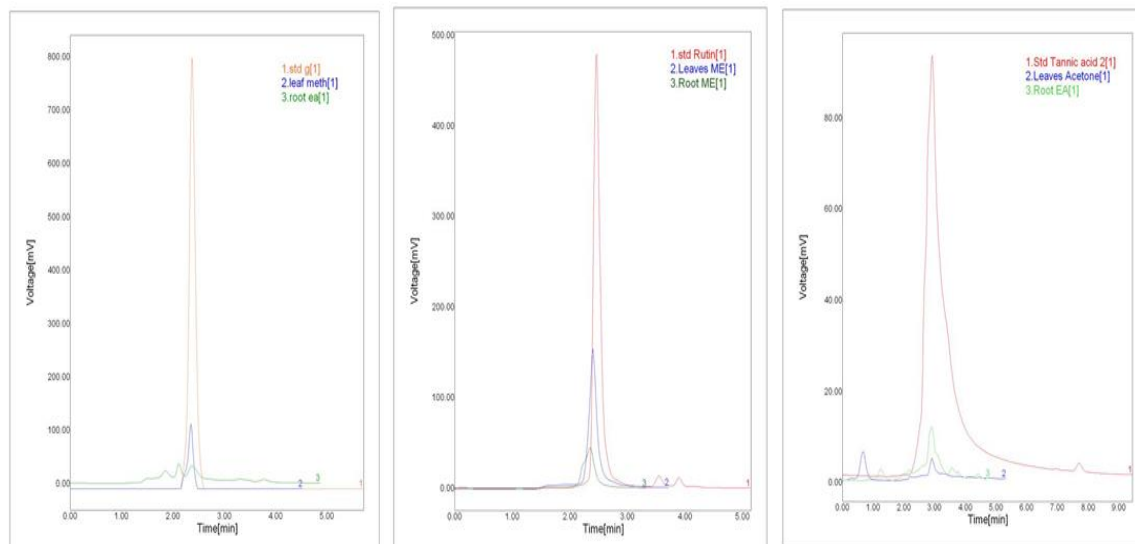
The quantitative estimations of phenols, Flavonoids and Tannic acid was analysed for different extracts of root and leaves. Methanol extracts of leaves showed highest phenolic and flavonoid content with concentration of 51mg/g and 1.63mg/g respectively. Ethyl acetate of root

extract showed maximum quantitative of phenols and flavonoids with concentration of 35mg/g and 52.60mg/g compared to other extracts, using standards Gallic acid for phenols and Rutin for flavonoids. Also quantitative estimation of Tannic acid was observed in acetone and ethyl acetate extracts of leaves and root with concentration of 14.27mg/g respectively. Similar work showed *P.coronopus* ethyl acetate extract with

phenolic content of 0.10mg/g in methanolic extract, flavonoids content of 0.57mg/g in methanolic extract was observed in the work studied by Pereira⁵.

The results of quantitative estimations of phenols, Flavonoids and Tannic acid are resulted in graph 6.

Graph 6: Phytochemicals analysis by HPLC



Determination of Total Phenolic, Flavonoid and Tannin content by HPLC

CONCLUSION

From the results of present study it can be concluded that stem and root extract of *P.asiatica* showed potential antioxidant activity, reducing capacity in acetone, ethyl acetate and cold extract and also α -amylase inhibition activity in petroleum ether extract. Therefore, it explains about medicinal uses of plant. The petroleum ether extract of leaves also revealed a good alpha amylase inhibition percentage; However, extracts of both revealed that it could be used for potential drugs and due its medicinal uses.

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