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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND ESTIMATION OF COMPOUND BY HPLC IN ETHANOLIC EXTRACT OF *PIPER BETLE* L.

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Abstract
Present investigation deals with Present investigation deals with *Piper betle* L. obtained from ethanolic extract for estimation of phytoconstituents and qualitative estimation of phytoconstituents by HPLC. The study revealed that total phenolics and total flavonoid content obtained were found to be more in ethanolic extract. The results of this study suggest that the plant material contain phenols and flavonoids and can be used as therapeutic agents for various pharmacological activities.

Keywords: *Piper betle* L, Phytoconstituents, HPLC

INTRODUCTION

Piper betle L. (Piperaceae) leaves is widely used as a mouth freshener after meal.[1,2]*Piper betle* Linn. (Local name 'Paan') Piperaceae, a dioecious, annual creeper, climbing by many small adventitious rootless, grows to a height of about one metre, generally grown in hotter and damper parts of the country.[3,4] It is extensively found in damp forests and is propagated in India and other countries in South-East Asia, such as Vietnam and China. In India it is found in Uttar Pradesh, Bihar, Bengal, Orissa, Tamilnadu, Andhra Pradesh and Karnataka. In Tamilnadu, three varieties of *Piper betle* leaves, Sirugamani, Karpoori and Vellaikodi are accessible mostly.[5] It is used in variety of decoction, in curing wounds, burns, impectigo, furuneloris, eczema, lymphangits and juice is beneficial stomatic. Kammaru (a variety of *Piper betle*) leaf has a good level of juice that heals pharyngitis, abdominal pain and swelling. Generally,betle leaf cures urticaria and as per ayurvedic medicine, it recovers the loss of equilibrium between the three 'humours,' namely, Vatha, Pitha and Kapha. The roots and fruits are well-known for treatment of malaria, asthma. [6,7]

Material and Method

Extraction Procedure

Following procedure was adopted for the preparation of ethanolic extracts from the shade dried and powdered herbs.

Defatting of Plant Material

Powdered plant material of *Piper betel* Linn. was shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether (60-80°C) in a soxhlet apparatus. The extraction was continued till the defatting of the material had taken place.

Extraction by hot continuous percolation process

100 g. of *Piper betel* Linn dried plant material were exhaustively extracted with ethanol. The extracts were evaporated above their boiling points. Finally, the percentage yields were calculated of the dried extract.[8]

Determination of Percentage yield

Calculation of percentage yield

The percentage yield of yield of each extract was calculated by using formula:

$$\text{Percentage yield} = \frac{\text{Weight of extract x 100}}{\text{Weight of powdered drug taken}}$$

Qualitative Phytochemical Tests

The extracts obtained by solvent extraction were subjected to various qualitative tests to detect the presence of plant constituents.

A. Carbohydrates

B. Alkaloids

C. Steroids

D. Glycosides

E. Saponins

F. Tannin

G. Triterpenoids

H. Proteins and amino acids

I. Resins

J. Fixed oils, fats and waxes

The extracts were subjected to various qualitative tests to detect the presence of plant constituents. The results have been shown in table.

Preparation of Test Solution

The test solution was prepared by taking 1 g of the extract in 25 ml of methanol.[9,10]

Test for Carbohydrates

Following tests were carried out for carbohydrates.

a) **Molisch's test:** In a test tube containing extract of drug, added two drop of freshly prepared 20% alcoholic solution of α- napthol and mixed concentrated sulphuric acid along the sides of the test tube. If carbohydrate present purple color or reddish violet color produce at the junction between two liquids.

b) **Benedict's test:** In a test tube containing extract of drug add benedict's solution, mix well, boiled the mixture vigorously for two minutes and then cooled. Formation of red precipitate due to presence of carbohydrates.

c) **Barfoed's test:** The barfoed's solution added to 0.5 ml of solution under examination, heated to boil. Formation of red precipitate of copper oxide was indicated the presence of carbohydrates.

d) **Anthrone test:** To the two ml of anthrone test solution, add the extract of drug. A green or blue colour indicated the presence of carbohydrate.

Test for Alkaloids

a) **Dragendorff's Test**: Few mg of extract of the drug dissolved in 5 ml of water added 2 M hydrochloric acid until an acid reaction occurred; 1 ml of dragendorff's reagent (potassium bismuth iodide solution) was added an orange red precipitate indicated the presence of alkaloids.

b) **Wagner's test**: Acidify the extract of drug with 1.5 % v/v of hydrochloric acid and added a few drop of Wagner's reagent (iodine potassium iodide solution). Formations of reddish brown precipitate indicated the presence of alkaloids.

c) **Mayer's Test**: Two ml of extract solution was treated with 2 - 3 drops of Mayer's reagent was added (potassium mercuric iodide solution) formation of dull white precipitate indicated the presence of alkaloid.

d) **Hager's Test**: Extract of the drug solution was treated with 3 ml of Hager's reagent (saturated solution of picric acid) formation of yellow precipitate confirmed the presence of alkaloids.

Test for Steroids and Sterols

a) **Liebermann's Burchard reaction**: The test extract solution was dissolved in 2 ml of chloroform in a dry test tube. Now 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The solution became red, then blue and finally bluish green in color.

b) **Salkowsky test**: The extract of test solution dissolved in chloroform and equal volume of conc. sulphuric acid was added. Bluish red cherry, red and purple color was noted in chloroform layer, whereas acid assumes marked green fluorescence.

Test for Glycosides

a) **Legal's test**: Extract solution dissolved in pyridine then sodium nitroprusside solution was added to it and made alkaline. Pink red colour indicated the presence of glycosides.

b) **Baljet's test**: To the drug extract, sodium picrate solution was added, yellow to orange colour was indicated the presence of glycosides.

c) **Borntrager's test**: Few ml of dilute sulphuric acid solution, the test solution of extract was added. It was filtered and the filtrate was boiled with ether or chloroform. Then organic layer was separated to which ammonia was added, pink, red or violet colour was produced in orange layer confirmed the presence of glycosides.

d) **Keller Kiliani test**: Extract was dissolved in glacial acetic acid containing trace of ferric chloride one ml concentrated sulphuric acid was added carefully by the side of the test tube. A blue colour in the acetic acid layer and red colour at the junction of the two liquid indicated the presence of glycosides.

Test of Saponins

a) 1 ml of alcoholic extract was diluted with 20 ml distilled water and shaken in graduated cylinder for 15 minutes. One cm layer of foam indicated the presence of saponins.

Test for Flavanoids

a)**Shinoda test**: In the test tube containing alcoholic extract of the drug added 5 - 10 drops of dil. hydrochloric acid followed by the small piece of magnesium. In presence of flavonoids a pink, reddish pink or brown color was produced.

Test for Tannins

a) To the sample of the extract, ferric chloride solution was added appearance of dark blue or greenish black colour indicated the presence of tannins.

b) To the sample of extract, potassium cyanide was added, deep red colour was confirmed the presence of tannins.

c) To the sample of extract, potassium dichromate solution was added, yellow precipitate was produced.

Test for Triterpenoids

a) In the test tube, 2 or 3 granules of tin was added, and dissolved in 2 ml of thionyl chloride solution and test solution was added. Pink colour was produced which indicates the presence of triterpenoids.

b) Two ml of acetic anhydride solution was added to 1 ml of extract of drug in chloroform followed by one ml of conc. sulphuric acid, a violet colored ring was formed indicating presence of triterpinoid.

Test for Protein and Amino acid

a) Biuret's test: To 2 - 3 ml of the extract of drug added in 1 ml of 40 % sodium hydroxide solutions and 2 drops of 1 % copper sulphate solution mix thoroughly, a purplish - violet or pinkish - violet colour produced that indicates the presence of proteins.

b) Ninhydrin's test: Two drops of freshly prepared 0.2 % ninhydrin reagent was added to the extract and heated to boiling for 1 - 2 min. and allow cooling. A blue colour developed that indicating the presence of proteins, peptides or amino acids.

c) Xanthoprotein test: To the extract in a test tube, add conc. nitric acid. A white precipitate was obtained and upon heating turns to yellow and cool the solution carefully. Added 20 % of sodium hydroxide solution in excess orange colour indicated presence of aromatic amino acid.

d) Millon's test: The small quantity of extract of the drug dissolved in distilled water added 5 - 6 drop of millon's reagent. A white precipitate was formed which turned red on heating, indicated the presence of proteins.

e) Lead Acetate test: The extract was taken and two ml of 40 % sodium hydroxide solution was added and boiled, glacial acetic acid was added and cooled than added 1 ml of lead acetate solution, gray black precipitate was formed which indicated presence of sulphur containing amino acid.

Test of Resins

Dissolved the extract in the acetone and pore the solution in the distilled water. Turbidity indicated the presence of resin.

Test of Fats or Fixed oils

a) Using sodium hydroxide: The extract was mixed in one ml 1 % of copper sulphate solution then added 10 % sodium hydroxide solution a clear blue solution was obtain which showed glycerin present in sample.

b) Using sodium hydrogen sulphate: The extract was taken in test tube added a pinch of sodium hydrogen sulphate pungent odour was formed which showed glycerin present in sample.

c) Saponification: Four ml of 2 % sodium carbonate solution was taken and the extract was added. Shaked vigorously and boiled. A clean soapy solution was formed cooled and added few drops of conc. HCl and observed that fatty separate out and float up.

Estimation of total Phenolic and flavanoid Content

Total Phenolic content estimation

Principle: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method.[11]

Preparation of Standard: 50 mg Gallic acid was dissolved in 50 ml methanol, various aliquots of 5- 25µg/ml was prepared in methanol.

Preparation of Extract: 1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of phenols.

Procedure: 1 ml of extract or standard was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Total flavonoid content estimation

Principle: Determination of total flavonoids content was based on aluminium chloride method.

Preparation of standard: 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.

Preparation of extract: 1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; absorbance was measured at 420 nm.

Identification of Marker Compound (Quercetin) by HPLC

Reagents and chemicals

Quercetin was kindly provided by Scan Research Laboratories, Bhopal (India). Methanol and acetonitrile were of HPLC grade and purchased from Merck Ltd, New Delhi, India. Water used was of HPLC grade water from Merck Ltd, New Delhi, India.

Instrumentation

Athermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of λmax. The HPLC system (Waters) consisted of a pump, a U.V. Visible detector, a Thermo C18 (250 X 4.6 mm, 5µm) column, a Data Ace software.

Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 mL min-1. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 256 nm.

Preparation of standard stock solution

10mg of quercetin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm.

Preparation of working standard solution

From stock solutions of quercetin 1 ml was taken and diluted up to 10 ml. from this solution 1.0, ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with mobile phase, gives standard drug solution of 10µg/ ml concentration.

Analysis of extracts

Sample Preparation 10 mg ethanolic extract was taken in 10 ml volumetric flask and dilute upto the mark with Methanol; resultant solution was filtered through Whatmann filter paper and finally volume made up to mark with same solvent to obtain concentration of 1000 µg/ml. The resulting solution was again filtered using Whatmann filter paper no.41 and then sonicated for 10 min.

RESULT AND DISCUSSION:

Percentage Yield of Ethanolic Extract of *Piper Betle* L.

The crude extracts so obtained after the soxhlet extraction process, extract was further concentrated on water bath evaporation the solvents completely to obtain the actual yield of extraction. To obtain the percentage yield of

extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from different samples using Ethanol solvent is depicted in the table. 5.1.

Table No. 1 Result of Percentage Yield of Different Extract

S. No.	Solvents	Percentage Yield (%)
1.	Ethanol	2.1%

Phytochemical Screening of Ethanolic extract

A small portion of the dried extracts were subjected to the phytochemical test using standard methods to test for alkaloids, glycosides, tannins, saponins, flavonoids and steroids separately for extracts of all samples. Small amount of each extract is suitably resuspended into the sterile distilled water to make the concentration of 1 mg per ml. The outcomes of the results are discussed separately in the table 2.

Table No. 2 Result of Phytochemical Screening of Ethanolic extract

S.No.	Constituents	Ethanolic extract
1.	Alkaloids	+
2.	Glycosides	-
3.	Flavonoids	+
4.	Diterpenes	+
5.	Phenolics	+
6.	Amino Acids	+
7.	Carbohydrate	+
8.	Proteins	+
9.	Saponins	+

From the results obtained it is clear that the *Piper betel* L. plant shows the presence of alkaloids, glycosides, saponins, tannins, flavonoids, amino acid terpenoids, were found present in leaf parts when extracted with different solvents using soxhlet extraction procedure. The phytochemical analysis of *Piper betel* L. plant indicates the

presence of phenols and flavonoids present in sufficiently enough quantity according to preliminary phytochemical analysis. Phenolic and Flavonoids are the phytochemicals that are present in ethanol.

Total Phenolic content estimation (TPC)

The content of total phenolic compounds (TPC) and total tannin content was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: **Y = 0.008x + 0.009, R^2= 0.999,** where × is the absorbance and y is the Gallic acid equivalent (GAE).

Total flavonoids content estimation (TFC)

Total flavonoids content was calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: **Y=0.008 X+0.007, R^2=0.999**, where X is the absorbance and Y is the quercetin equivalent (QE).

The total phonolic content in ethanolic extract was 8.874mg of gallic acid equivalent/g of dry extract and total flavonoid content was 8.449 mg of quercetin equivalent/g of dry extract. The results are given in Table 3.

Table no. 3: Total Phenolic and flavonoid content

S. No.	Extracts	Total Phenol (mg/gm)	Total Flavanoid (mg/gm)
1.	Ethanolic	8.874	8.449

HPLC Estimation of Marker Compound

Results of High Performance Liquid Chromatography

Identification of Marker Compound (Quercetin) by HPLC

A reverse phase C-18 column equilibrated with mobile phase methanol: acetonitrile (50:50, v/v) was used. Mobile phase was filtered through Whatmann filter paper and degassed. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 256 nm. The sample was injected using a 20 µl fixed loop, and the total run time was 10 min. The sample solution was chromatographed and a concentration of quercetin in Extract sample was found out using regression equation.

Chromatogram of standard quercetin

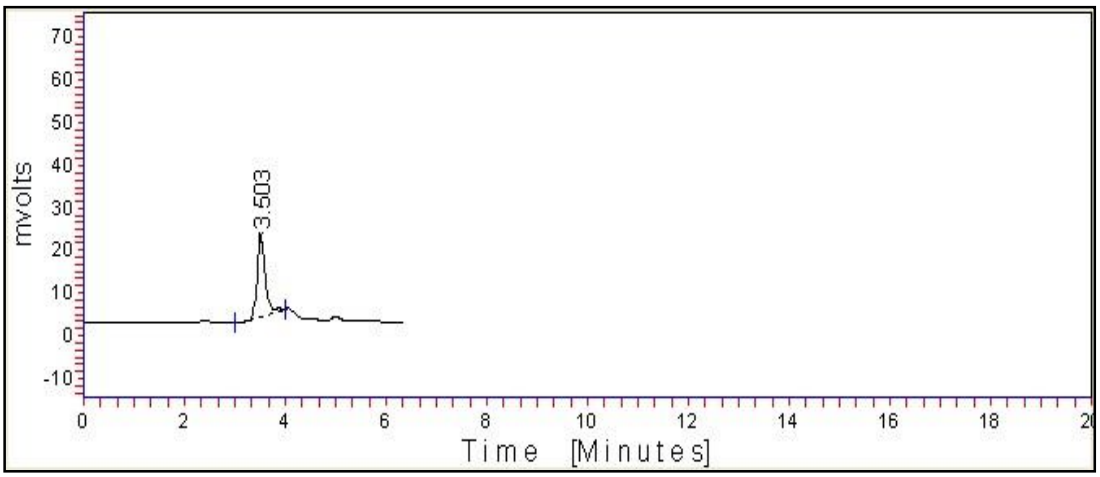

Figure 1: Chromatogram of standard quercetin

Chromatogram of ethanolic extract

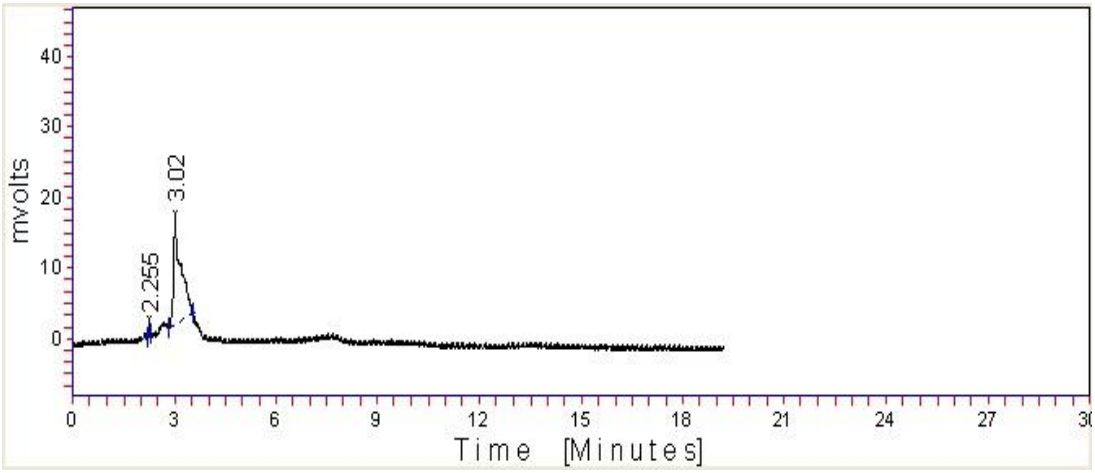

Figure 2: Chromatogram of ethanolic extract

Conclusion

Piper betle obtained from ethanolic extract for estimation of phytoconstituents and qualitative estimation of phytoconstituents by HPLC. The study revealed that total phenolics and total flavonoid content obtained were found to be more in ethanolic extract. The results of this study suggest that the plant material contain phenols and flavonoids and can be used as therapeutic agents for various pharmacological activities.

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