Research Article

Phytochemical analysis and HPLC estimation of phytoconstituents of *Buchanania lanzan* Spreng

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Received: 14 September 2019

Revised: 11 October 2019

Accepted: 18 October 2019

Abstract

Objective: The present study was designed to investigate cytotoxic active principles from crude extract of *Buchanania lanzan* Spreng based on its ethnobotanical information. The objectives of study are; collection of plant material, extraction and preliminary phytochemical evaluation and preformation of HPLC finger printing of extracts. **Materials and methods:** The bark of *Buchanania lanzan* Spreng was extracted with different solvents by soxhlet method and screened for the presence of its phytochemical composition. The ethanolic and ethyl acetate extracts were selected due to its high percentage yield for HPLC fingerprinting. **Results:** The bark of *Buchanania lanzan* Spreng showed the presence carbohydrates, alkaloids, flavonoids, saponins, proteins, amino acids and phenolic compounds. The comparative study of total flavanoid content and total phenolic content of ethanolic and ethyl acetate extracts shows that ethyl extracts had higher content of galic acid and quercetin as compared to ethanolic extract. **Conclusion:** Phenols are the compound which proves to enhance the body's immune system there by inhibiting the development of new blood vessels (angiogenesis) that is necessary for tumor growth. The present study proved that bark extracts of *Buchanania lanzan* Spreng had higher content of phenols and flavanoids into it and hence it can be a potent anti-cancerous herbal drug in future.

Keywords: *Buchanania lanzan*, anticancer, HPLC, phenols, flavonoids, quercetin, galic acid

Introduction

Cancer is a major public health burden in both developed and developing countries. Several synthetic agents are used to cure the disease but they have their toxicity like bone marrow toxicity, neurotoxicity etc and hence the research is going on to investigate the plant derived chemotherapeutic agents (Hossein and Hassanpour, 2017; Kooti et al., 2017; Kumari and Kakkar, 2008).

In recent times, plants research has increased all over the world and evidences has been collected to show immense potential of medicinal plants used in various traditional system. Many traditional healers and herbalists in the India have been treating cancer patients for many years using various medicinal plant species. Hence, an attempt has been made to screen

Buchanania lanzan Spreng for the prevention and treatment of cancer (Kapoor, 2000; Mehta et al., 2011; Jain and Jain, 2015; Warokar et al., 2010).

Naturally occurring substances of plant origin and dietary components that have been widely studied for their antimutagenic activity, which includes carotenoids, flavonoids, tea-polyphenols, vitamins, cucuminoids, tannins, coumarins, chlorophyllin, porphyrins and alkylresorcinols from cereal grains (Rai, 2010; Mehta, 2011; Puri et al., 2000). Phenols are the compound which proves to enhance the body's immune system there by inhibiting the development of new blood vessels (angiogenesis) that is necessary for tumour growth (Pattnaik et al., 2013; Perveen et al., 2017; Selassie et al., 2013), while flavonoids modulate signal transduction pathways within cancer cells resulting in angiogenesis and metastasis within the cell. It also promotes apoptosis (Chang et al., 2008; Katrin, 2014; Kuntz et al., 1999). Hence, an attempt has been made for accurate scientific

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DOI: <https://doi.org/10.31024/apj.2019.4.5.1>

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documentation of phenolic and flavonoid content of *Buchanania lanzan* Spreng bark extracts by HPLC method to establish its claim for the prevention and treatment of cancer.

Materials and methods

Plant material

The stem bark of *Buchanania lanzan* was collected and the plant specimen was authenticated by Dr. K. Madhava Chetty, assistant professor, Department of Botany, SV University, Tirupati (A.P). The voucher no. for the specimen is 1123. A voucher specimen has been preserved in the laboratory for future reference.

Preparation of crude extract and phytochemical screening

The stem bark of the plant was dried in shade for about 3 weeks and grinded by using a electric mixer to a coarse powder. Powders of stem barks are first extracted with the petroleum ether (60-80°C) for defatting. The successively re-extraction had been performed with various solvents in increasing polarity for 48 hrs in a soxhlet extraction apparatus. The resulting extracts were filtered through whatman no 1 filter paper and then concentrated about one tenth the original volume in a water bath (40°C) for complete dryness. The extract was refrigerated at 2-8°C until use.

Instrumentation

The HPLC system, Shimadzu, Japan was used for the study with shimadzu LC-10ATVP, pump, shimadzu spd10A uv-vis detector, phenomenex gemini-NX-5 μm C18(2) 110 Å, LC column 250 x 4.6 mm column and baseline chromatography DS-N2000 software.

Phytochemical Screening

The chemical tests were performed for screening of different chemical groups present in extracts.

Test for carbohydrates

Molisch's test: A test tube containing extract of drug was added with two drop of freshly prepared 20% alcoholic solution of alpha naphthol and mixed con. H_2SO_4 was added from the side wall of the test tube. Colour at the junction of the two liquids was observed.

Benedict's test: A test tube containing extract of drug was added with benedict's solution, mixed well and boiled vigorously for two minutes and then cooled. The colour of the precipitate was observed.

Fehling's test (Reducing sugars): To 2 ml of extract, equal volume of mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes in boiling water bath. Formation of red or brick red coloured precipitate indicated the presence of reducing sugars.

Test for alkaloids

Dragendorff's Test: A test tube containing extract of the drug was added with 5 ml of water mixed with 2M hydrochloric acid. To this 1 ml of dragendorff's reagent (potassium bismuth iodide solution) was added from the side wall of the test tube. Color at the junction of the two liquids was observed.

Wagner's test: A test tube containing extract of drug was added with 1.5 % v/v of hydrochloric acid and few drop of Wagner's reagent. The color of the precipitate was observed.

Mayer's Test: The Mayer's reagent was added to 2 ml of extract solution and the color of the precipitate was observed.

Hager's Test: A test tube containing extract of drug was added with 3 ml of Hager's reagent. Color at the junction of the two liquids was observed.

Test for steroids and sterols

Liebermann's Burchard reaction: A test tube containing extract of drug was mixed with 2 ml of chloroform and added 10 drops of acetic anhydride followed by 2 drops of con. H_2SO_4 . The change in colour of solution was observed.

Salkowsky test: A test tube containing extract of drug was mixed with equal volume of chloroform and con. H_2SO_4 was added. The change in color of acid and chloroform layer was observed.

Test for glycosides

Legal's test: A test tube containing extract of drug was mixed with pyridine then sodium nitroprusside solution was added. The change in colour of solution was observed.

Borntrager's test: A test tube containing extract of drug was added with few ml of dil. H_2SO_4 and boiled with ether or chloroform. To the separated organic layer, ammonia was added and change in colour was observed.

Keller Kiliani test: A test tube containing extract of drug was added with $\text{C}_2\text{H}_4\text{O}_2$. To this 1ml con H_2SO_4 was added from the side wall of the test tube. Colour at the junction of the two liquids was observed.

Test for flavonoids

Shinoda test: A test tube containing extract of drug was added with 10 drops of dil. hydrochloric acid followed by the small piece of magnesium and the color was observed.

Alkaline reagent test: To 2 ml of test solution add 2 ml alkali, gives yellow color, which disappears on addition of

dil HCl it disappears, which indicates presence of flavonoids.

Test of saponins (Foam Test): A test tube containing 1ml extract of drug was added with 20 ml distilled water in graduated cylinder for 15 minutes. Formation of foam layer was observed.

Test for Phenolic and tannins

Ferric chloride test: Extract solutions were treated with 5% ferric chloride solution. Formation of blue colours indicated the presence of hydrolysable tannins and formation of green colour indicated the presence of condensed tannins.

Gelatin test: 3 ml of test solution when treated with gelatin solution (3ml) gave white precipitate.

Lead Acetate test: A test tube containing extract of drug was added with two ml of 40 % sodium hydroxide solution and boiled followed by glacial acetic acid and 1 ml of lead acetate solution. The change in colour of solution was observed after cooling.

Test for protein and amino acid

Biuret test: A test tube containing extract of drug was added with 1 ml of 40 % NaOH solutions and two drops of 1 % Cu_2SO_4 solution. The change in colour of solution was observed.

Ninhydrin's test: 2 drops of freshly prepared 0.2 % ninhydrin reagent was added to extract of drug and heated to boiling for 2 min. The change in colour of solution was observed after cooling.

Xanthoprotein test: A test tube containing extract of drug was added with conc. nitric acid. The change in colour of precipitate was observed.

Millon's test: Six drops of Millon's reagent was added to the extract of drug and the colour was observed.

Test of resins: A test tube containing extract of drug was added with acetone and distilled water. The turbidity of the solution was observed.

Test of fats or fixed oils (Saponification): Four ml of 2 % sodium carbonate solution was mixed with extract of the drug and boiled. To soapy solution few drops of conc. HCl was added and observed for fatty precipitate formation.

Determination of percentage yield

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

Percentage yield = {Weight of Extract/ Weight of powder material taken} x 100

Estimation of Gallic acid by Rp-HPLC

Mobile phase preparation: Mobile phase was prepared by mixing Methanol: Water (60:40). pH of the solution was adjusted to 3.5 with dilute phosphoric acid. This solution was filtered using a 0.45 micron Millipore filter paper and was sonicated for

10mins. The total volume of the mobile phase prepared was 500ml.

Standard preparation: Accurately 10mg of Gallic acid was taken in 10ml volumetric flask and make up the volume to 10 ml with methanol (the concentration of this solution is 1000 $\mu\text{g}/\text{ml}$). From this above solution working solution 20, 40, 60, 80 and 100 μg was pipetted into 10ml volumetric flask and volume was made up to the mark with Methanol. This is a working solution. This was sonicated for 8 minutes then the solution was filtered using 0.45 micron Millipore filters.

Sample preparation: To 1mg of the given sample was taken in 1ml of respective solvents. The solution was vortex for 5mins. The sample was filtered using 0.45 micron Millipore filters. 20 μl of this sample was injected in the HPLC system.

The concentration of unknown sample was calculated as follows = Sample area/ STD area x STD wt. / Sample wt. x Sample dilution/STD dilution x purity/100 x 100

Estimation of Quercetin by Rp-HPLC

Mobile phase preparation: Mobile phase was prepared by mixing Methanol: 0.005mM phosphate buffer (70:30). pH of the solution was adjusted to 3 with dilute phosphoric acid. This solution was filtered using a 0.45 micron Millipore filter paper and was sonicated for 10mins. The total volume of the mobile phase prepared was 500ml.

Standard preparation: 10mg of Quercetin was taken in 10ml volumetric flask and make up the volume to 10 ml with methanol (the concentration of this solution is 1000 $\mu\text{g}/\text{ml}$). From this above solution working solution 10, 20, 30, 40 and 50 μg was pipetted into 10ml volumetric flask and volume was made up to the mark with Methanol. This is a working solution. This was sonicated for 8 minutes then the solution was filtered using 0.45 micron Millipore filters.

Sample preparation: To 1mg of the given sample was taken in 1ml of respective solvents. The solution was vortex for 5mins. The sample was filtered using 0.45 micron Millipore filters. 20 μl of this sample was injected in the HPLC system.

The concentration of unknown sample was calculated as follows= Sple area/ STD area x STD wt. / Sple wt. x Sple dilution/STD dilution x purity/100 x 100.

Results

Percentage yield

As a result of successive solvent extraction the total yield of

various extracts of *Buchanania lanzan* bark obtained is tabulated in the table 1. The ethanolic extract of *Buchanania lanzan* bark showed a highest yield of about 5.55g/200g (2.7%), whereas the chloroform extract has showed the lowest yield of 1.8g/ 200g (0.8%).

Phytochemical screening of the plants

The secondary metabolites and other phytochemical constituents of medicinal plants account for their medicinal value. Hence phytochemical screening plays a major role in all kind of scientific studies. Dried extracts of the plants were subjected to the phytochemical test using methods reported (Khandelwal, 2005; Kokate, 1994; Tiwari et al., 2011) to test for presence of

alkaloids, glycosides, tannins, phenols, saponins, flavonoids and terpenoids. Small amount of each extract

Table 1. Total yield of *Buchanania lanzan* bark extract in various solvents

Solvents	<i>Buchanania lanzan</i> bark	Percentage (%)
Petroleum ether	1.8	0.9
Chloroform	1.7	0.8
Ethyl acetate	3.1	1.5
Ethanol	5.5	2.7
Water	2.8	1.4

Table 2. Phytochemical screening of *Buchanania lanzan* bark extract

Chemical Tests	Pet. ether	Chloroform	Ethyl acetate	Ethanol	Aqueous
Alkaloids					
Mayer's reagent	-	-	-	-	-
Hager's reagent	+	-	-	-	-
Wagner's reagent					
Dragendorff's reagent	-	-	+	-	-
Glycosides					
Legal's test	-	-	-	-	-
Keller-Kiliani	-	+	+	+	-
Brontager's Test	-	-	+	+	-
Phenols/Tannins					
Ferric chloride	-	-	+	+	+
Gelatin Solution	-	-	-	+	-
Lead acetate test	-	-	+	+	+
Flavonoids					
Alkaline reagent test	-	-	+	+	+
Shinoda test	-	-	-	+	+
Saponins					
Foam test	-	-	+	+	+
Carbohydrates					
Molisch Test	-	-	+	+	+
Benidicts Test	-	-	+	+	+
Fehlings Test	-	-	+	+	+
Fixed oil/Fats					
Saponification Test	+	-	-	-	-
Phytosterols					
Liebermann Burchards Test	+	+	+	-	+
Salkowaski Test	+	+	+	-	-
Protein & Amino acids					
Millon's Test	+	-	-	+	-
Biuret Test	+	-	-	+	-
Ninhydrine Test	+	-	-	+	-
Xanthoproteins Test	-	-	-	+	-
Resins					
Acetone-Water Test	+	+	-	-	-

(+) Indicates 'Presence'; (-) Indicates 'Absence'

was suitably suspended into the methanol to make the concentration of 1 mg per ml. The outcomes of the results are shown in table 2.

Phytochemical screening of different solvent extracts of *Buchanania lanzan* bark uncovered the existence of various phytochemicals as presented in Table 2. In particular the ethanolic and ethyl acetate extracts of *Buchanania lanzan* bark showed the presence of carbohydrates, alkaloids, flavonoids, saponins, proteins, aminoacids and phenolic compounds. The

presence of phytochemical constituents are maximum in ethanolic and ethyl acetate extracts as compared to other extracts, hence further investigations has only been concentrated on the ethanolic and ethyl acetate extract of *Buchanania lanzan* bark.

Quantification of bioactive compounds

Total Phenols content

Numerous phytochemicals which contain phenolic component proved to be possessing significant cytotoxic activity. Phenols are the compound which proves to enhance the body's immune system there by inhibiting the development of new blood vessels (angiogenesis) that is necessary for tumour growth. Hence the quantity of total phenol was estimated by HPLC method using gallic acid as standard drug (Table 3, Figure 1, 2, 3).

The presence of phenolic compounds (TPC) is expressed as mcg of gallic acid equivalent of dry concentrate test utilizing the mathematical expression obtained from the calibration curve: $Y = 10,656.7913x + 130,157.5190$, $R^2 = 0.999$, where X is the absorbance and y is the Gallic acid equivalent (GAE).

Figure 1. Calibration curve of Gallic acid

Figure 2. Chromatogram of Galic acid content of Ethanolic Extract of *Buchanania lanzan* bark

Table 3. Gallic acid content of extracts of *Buchanania lanzan* bark

Extracts of <i>Buchanania lanzan</i> bark	Ethanolic extract (BE)	Ethyle acetate extract (BEA)
Purity	98	98
Sample area	110509.797	870498.25
Standard area	1198023.625	1198023.63
Sample weight in mcg	1000	1000
Standard weight in mcg	100	100
Sample dilution ml	1	1
Standard dilution ml	1	1
Content of Gallic acid mcg	0.903985521	7.12079685

Figure 3. Chromatogram of Gallic acid content of ethyl acetate extract of *Buchanania lanzan* bark

In the present study, the ethyl acetate bark concentrate of *Buchanania lanzan* displays 7.12 mcg of gallic acid equivalent phenols where as the ethanolic concentrate shows 0.9 mcg of gallic acid equivalent phenols (Table 4, Figure 4 & 5).

Total flavonoids content

Flavonoids are polyphenolic compounds that occur ubiquitously

in foods of plant origin. Flavonoids of the flavone, flavanol, flavanone, and isoflavone classes possess antiproliferative effects in different cancer cell lines and anticancer effects of these polyphenols depend on factors like chemical structure and concentration. Hence quantification of flavonoid content has been done in ethanolic and ethyl acetate extracts of *Buchanania lanzan bark* by HPLC method using quercetin as standard compound (Table 5, Figure 4).

Absolute concentration of flavonoids was computed as quercetin proportionate (mcg) utilizing the mathematical statement in light of the calibration curve: $Y = 31,884.2250x + 23,504.2589$, $R^2 = 0.999$, where X is the absorbance and Y is the quercetin comparable (QE).

The result reveals that the ethyl acetate bark concentrate of *Buchanania lanzan* exhibits 0.34 mcg of quercetin equivalent of flavonoids where as the ethanolic concentrate exhibits 0.25 mcg of quercetin equivalent of flavonoids (Figure 5 & 6).

The measurement of aggregates of phenols and flavonoid was resolved and it was found that the ethyl acetate

Figure 4. Calibration curve of Quercetin

Table 4. Quercetine content of extracts of *Buchanania lanzan* bark

Extracts of <i>Buchanania lanzan</i> bark	Ethanolic extract (BE)	Ethyle acetate extract (BEA)
purity	98	98
sample area	850073.375	1141022
standard area	1612042.75	1612043
sample weight in mcg	1000	1000
standard weight in mcg	5	5
sample dilution ml	1	1
standard dilution ml	1	1
Content of Quercetin mcg	0.258390141	0.346828

Figure 5. Chromatogram of Quercetine content of Ethanolic extract of *Buchanania lanzan* bark

Figure 6. Chromatogram of Quercetin content of ethyl acetate extract of *Buchanania lanzan* bark

Figure 7. Comparative representation of total flavonoid and phenolic content in ethanolic and ethyl acetate Extract of *Buchanania lanzan* bark

concentrate of *Buchanania lanzan* bark has higher contents of phenols and flavonoids in comparison to ethanolic extract. Hence more emphasis can be given to the ethyl extract for in-vitro and in-vivo to show plants cytotoxic potential in future (Figure7).

Conclusion

Herbal drugs can play important beneficial roles in human nutrition and health status as it influence the many immunological events associated with the development and progression of cancer. Diet and health relationships are very complex, as food items usually act through multiple pathways and each ingredient can have different molecular targets. Flavonoids and Phenols have the potential of modulating many biological events in cancer such as apoptosis, vascularization, cell differentiation, cell proliferation, etc hence both additive as well as several

dietary flavonoids and phenols have been reported contributing to the health. The higher content of phenols and flavonoids in *Buchanania lanzan* bark extracts keep it as a promising anticancerous herbal drug lead in future. However, further efforts are necessary to understand its potential for the cancer prevention in future.

Acknowledgment

The authors are very grateful to Rajiv Gandhi University of Health and Sciences (RGUHS), Bangalore for providing financial assistance. The authors are also like to thanks Dr. Paadma M Paarakh, Principal of the oxford college of pharmacy, Hongasandra, Bangalore for providing laboratory facilities to carry out work and Dr. Rajesh shukla for his constant scientific support.

Conflict of interest

None

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