



Therapeutic Protection from Hepatic Injury and Chemical Constituents of *Buchanania angustifolia* Roxb

Buchanania angustifolia Roxb'un Kimyasal Bileşenleri ve Karaciğer Hasarına Karşı Terapötik Koruyucu Etkisi

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ABSTRACT

Objectives: The use of modern medicines in the treatment of diseases instead of traditional medicine is causing different adverse effects. Therefore, there is need to search new bioactive compounds to treat different diseases. *Buchanania angustifolia* has been used in traditional medicine in the treatment of different diseases. On the basis of folkloric information, *B. angustifolia* aerial parts extracts have been selected for their antioxidant and hepatoprotective potentiality and phytochemical constituents.

Materials and Methods: Qualitative phytochemical screening of *B. angustifolia* extracts revealed the presence of phytochemical constituents such as steroids, terpenoids, flavonoids, alkaloids, glycosides, tannins, carbohydrates, oils and amino acids. BA-1 and BA-2 compounds isolated from hydroalcoholic extract using chromatography and were identified as linolenic acid and mixture of stigmaterol and β -sitosterol.

Results: Antioxidant activity was performed on superoxide, hydroxyl and 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride free radicals. The *B. angustifolia* extracts showed dose-dependent activity on free radicals. The hydroalcoholic extract showed better activity. The extracts showed hepatoprotective activity on thioacetamide-induced liver intoxication in rats, and hydroalcoholic extract exhibited significant restoration of the altered biochemical parameters due thioacetamide-induced liver intoxication.

Conclusion: Among the tested extracts of *B. angustifolia*, hydroalcoholic extract showed higher antioxidant and hepatoprotective activity and stigmaterol and β -sitosterol were isolated. Further research is needed to evaluate other pharmacological activities and to isolate the other bioactive compounds from *B. angustifolia*.

Key words: *Buchanania angustifolia*, chemical constituents, antioxidant activity, thioacetamide, hepatoprotective activity

ÖZ

Amaç: Hastalıkların tedavisinde geleneksel tıp yerine modern ilaçların kullanımı farklı yan etkilere neden olabilmektedir. Bu nedenle, çeşitli hastalıkların tedavisinde kullanılmak üzere yeni biyoaktif bileşiklerin araştırılması gerekmektedir. *Buchanania angustifolia*, geleneksel tıpta farklı hastalıkların tedavisinde kullanılmaktadır. Bu çalışmada, folklorik bilgilere dayanılarak, *B. angustifolia* bitkisinin toprak üstü kısımlarının antioksidan ve hepatoprotektif etkilerinin ve fitokimyasal bileşenlerinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: *B. angustifolia* ekstreleri üzerinde yapılan kalitatif fitokimyasal analizler, ekstrelerde steroid, terpenoit, flavonoid, alkaloid, glikozit, tanen, karbonhidrat, yağ ve aminoasit yapısındaki bileşiklerin varlığını ortaya koymuştur. BA-1 ve BA-2 bileşikler kromatografik teknikler kullanılarak sulu alkollü ekstreden izole edilmiş ve linolenik asit ile stigmaterol ve β -sitosterol karışımı olarak tanımlanmıştır.

Bulgular: Antioksidan aktivite, süperoksit, hidroksil ve 1-(2,6-dimetilfenoksi)-2-(3,4-dimetoksifeniletamino) propan hidroksil serbest radikalleri üzerinde gerçekleştirilmiştir. *B. angustifolia* ekstreleri, serbest radikallere karşı doz-bağımlı aktivite göstermiştir. Sulu alkollü ekstrenin daha iyi aktivite gösterdiği belirlenmiştir. Ekstreler sıçanlarda tiyoasetamid-nedenli karaciğer zehirlenmesinde hepatoprotektif etki göstermiş ve sulu alkollü ekstre, tiyoasetamid-nedenli karaciğer zehirlenmesine bağlı olarak değişen biyokimyasal parametreleri anlamlı derecede iyileştirmiştir.

Sonuç: *B. angustifolia*'nın test edilen ekstreleri arasında sulu alkollü ekstrenin daha yüksek antioksidan ve hepatoprotektif etkinlik gösterdiği belirlenmiştir ve stigmaterol ve β -sitosterol bileşikler izole edilmiştir. *B. angustifolia*'nın diğer farmakolojik etkilerinin değerlendirilmesi ve başka biyoaktif bileşiklerinin izolasyonları için daha fazla araştırmaya ihtiyaç duyulmaktadır.

Anahtar kelimeler: *Buchanania angustifolia*, kimyasal bileşenler, antioksidan aktivite, tiyoasetamid, hepatoprotektif aktivite

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INTRODUCTION

Plants have been used as medicines since time immemorial around the world. The best known uses of plants in medicine were ayurveda in India, African traditional medicines, Unani medicine in Middle East countries, and in traditional Chinese medicine.¹⁻⁵ The major problems associated with traditional medicine are the lack of standardization, consistency, safety, and quality.⁶ Advances in technology provided the perfection in medicine for treating the different diseases. Simultaneously, the use of modern medicine offered a new adverse effects with their long-term use and disease-causing microorganisms gaining resistance to drugs.^{7,8} As such, people are turning back to traditional medicine because of their fewer adverse effects and the diversity of chemical entities in them for the treatment of different diseases. From this point of view, researchers are now focusing on the identification, evaluation, and standardization of the biologic activities of traditional medicinal plants⁹ using advanced technology to isolate new bioactive molecules. The present work was performed on one of the traditional medicinal plants, *Buchanania angustifolia* Roxb (*B. angustifolia*), to isolate new bioactive molecules and to provide scientific evidence of its traditional use by Indian tribes for a wide range of ailments, including nutritional disorders, skin diseases, gravel, healing of wounds, rheumatic pain, tonic for sexual debility, and urinary problems.¹⁰⁻¹² *B. angustifolia* is mainly grown in dry deciduous forests and distributed around South India and Sri Lanka regions. However, to the best of our knowledge, the phytochemical constituents and biologic activities of *B. angustifolia* have not been reported.¹³ Therefore, this study was planned to investigate the phytochemical constituents, antioxidant, and hepatoprotective potentials of hexane, ethyl acetate, and hydroalcoholic extracts of *B. angustifolia* aerial parts.

EXPERIMENTAL

Drug and chemicals

Chemicals used for the study were analytical grade. Silymarin, thioacetamide (TAA) and 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DPPH) were purchased from Sigma-Aldrich, St. Louis, USA, nitroblue tetrazolium was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Riboflavin was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. The kits for assessment of different biochemical parameters such as aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), alkaline phosphatase (ALP), total bilirubin, and total protein were purchased from Span diagnostics Ltd., Gujarat, India. Folin-Ciocalteu reagent, bromocresol green were purchased from Sigma-Aldrich, St. Louis, USA.

Plant material collection and preparation of extracts

The plant material was collected from the Talakona forest region, Tirupathi, Andhra Pradesh, India, during the month of December, 2010 (AUCP/BGR/2010-431). Authentication of the plant was performed by Rtd. Prof. M. Venkai, Department of Botany, Andhra University, Visakhapatnam. The plant material was dried under shade and powdered for separate extraction

processes using maceration with hexane, ethyl acetate, and hydroalcoholic [ethanol (70% v/v)], and then concentrated to dryness under vacuum using rotavapour.

Preliminary phytochemical studies

The extracts of the *B. angustifolia* were subjected to different phytochemical tests for the identification of its phytochemical constituents, using standard procedures.¹³

Quantification of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu reagent. The method is based on blue light absorption measurement due to the chemical reduction of tungsten and molybdenum oxides of Folin-Ciocalteu reagent, when combined with the compounds present in the extracts using colorimetry at 765 nm. The phenolic content in the extract was measured in gallic acid equivalents as mg/g, using a gallic acid calibration curve. The results are shown in mean values.^{14,15}

Quantification of total alkaloid content

The plant extract (1 mg/mL) was dissolved in 2 N hydrochloric acid and the solution was filtered. The phosphate buffer's pH was neutralized to 0.1 N sodium hydroxide. One milliliter of extract solution, 5 mL of phosphate buffer, and 5 mL of bromocresol green solution were placed in separation and then the solution was mixed well. The complex formed in the solution was extract with chloroform. The absorbance of complex color in chloroform was measure at 470 nm. The experiment was performed in triplicate and results were reported in atropine equivalents. The results are presented as mean values.^{14,16}

In vitro antioxidant activity

In vitro antioxidant activity was assessed use prepared extracts of *B. angustifolia* with dimethyl sulfoxide as a vehicle on superoxide, hydroxyl and DPPH free radicals.^{14,17,18} The percentage inhibition and inhibition concentration (IC)₅₀ values were calculated.

Superoxide radical scavenging activity

The superoxide scavenging activities of the selected plant extracts were evaluated as per standard methods, using absorption of light at 560 nm, induction of superoxide free radical generation by riboflavin, and corresponding reduction by nitroblue tetrazolium.

Hydroxyl radical scavenging activity

The scavenging activity of selected plants extracts on hydroxyl radical was measured as per established methods. It was studied by the competition between deoxyribose and the extract's antioxidant molecules for hydroxyl radicals generated from the Fe²⁺/EDTA/H₂O₂ system.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured as per methods. This method is based on measurements of color absorbance of alcoholic DPPH solution (blue color) after adding the antioxidant solution (extract/compound). If antioxidants are present in the test compound, the blue color turns to yellow due to DPPH.

Calculation of percentage inhibition

The percentage inhibition of superoxide production by the extract was calculated using the following formula:

$$\text{Inhibitory ratio} = (A_0 - A_1) \times 100 / A_0$$

A_0 : Absorbance of control, A_1 : Absorbance of plant extract or/and ascorbic acid

IC_{50} calculation from percentage inhibition

The optical density obtained with each concentration of the extract/ascorbic acid was plotted taking concentration on the X-axis and percentage inhibition on the Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ascorbic acid.

Selection of animals

Healthy albino rats of either sex weighing between 180-250 g aged 60-90 days were used for the study. The rats were taken care of with standard light and humidity and with a supply of proper food and water.

Acute toxicity studies

The acute toxicity study was conducted for extracts of *B. angustifolia* extracts as per Organisation for Economic Co-operation and Development (OECD) guidelines 420 (OECD 2001) and regulations of the Institutional Animal Ethics Committee (Regd no: 516/01/A/CPCSEA). The albino mice of single sex, were selected into three groups, each consisting of 6 animals. They were maintained for one week before the experiment under room temperature and allowed free access to food and water. The animals were subjected to an acute toxicity study using each extract at a dose of 2000 mg/kg orally at regular intervals of time, i.e., 1, 2, 4, 8, 12 and 24 h. During this time, the animals were under observation to note different conditions such as skin changes, morbidity, aggressiveness, oral secretions, sensitivity to sound and pain, respiratory movements, and mortality.

Assessment of hepatoprotective activity of *B. angustifolia*

The selected plant extracts were tested for their hepatoprotective nature using TAA-induced liver toxicity in rats. For this experiment, animals were divided into twelve groups of 6 animals. Group I was treated with normal saline (vehicle) for one week through oral administration at 2 mL/kg body weight. Group II and Group III animals were treated with TAA as a 2% w/v solution in water on the first day at 50 mg/kg body weight subcutaneously (s.c.), then Group II was continuously treated with saline and Group III with silymarin at a dose of 25 mg/kg body weight p.o. for three weeks. Groups IV to XII were treated with TAA as a 2% w/v solution in water on the first day at 50 mg/kg body weight s.c., then Groups IV, V, and VI were treated with hydroalcoholic extract, Groups VII, VIII, and IX with ethyl acetate extract, and Groups X, XI, and XII were treated with hexane extract at a doses of 125, 250, 500 mg/kg body weight orally for three weeks. Animals of all groups were anaesthetized using chloroform 48 h after the final dose of extracts. Blood samples were collected from the animal groups from the retro-orbital plexus, then the samples were immediately centrifuged

at 2400 rpm for quarters of an hour. Then, the clearly separated serum after centrifugation was used for measuring the different biochemical parameters using an auto analyzer with reagent kits.¹⁹⁻²¹ All experimental procedures involving animals were conducted according to OECD guidelines and approved by the Institutional Animal Ethics Committee, Andhra University. Results were analyzed using Two-way ANOVA followed by the Bonferroni post-hoc test. All groups were compared with the silymarin group.

Isolation of phytoconstituents (Compounds)

There was very less phytochemical work reported on *B. angustifolia*. So, depends on availability of extracts after pharmacological study, hydroalcoholic extract of *B. angustifolia* was used for separation of compounds using column chromatography.

RESULTS

Qualitative phytochemical screening

Qualitative phytochemical screening of *B. angustifolia* extracts revealed the presence of different phytochemical constituents such as steroids, terpenoids, flavonoids, alkaloids, glycosides, tannins, carbohydrates, oils and amino acids. The extracts gave negative results for the quinines and saponins. All extracts of *B. angustifolia* revealed the presence of phenols, alkaloids, carbohydrates, steroids, terpenoids, and glycosides, and gave negative results to saponins. The hydroalcoholic and ethyl acetate extracts revealed the presence of flavonoids and tannins but the hexane extract gave negative results. The hexane and ethyl acetate extracts revealed the presence of a min amount of oil but hydroalcoholic extracts gave negative results. All extracts gave negative results to amino acids but the hydroalcoholic extracts give a weak positive result for the presence of amino acids (Table 1).

Table 1. Nature of phytoconstituents present in different extracts of *B. angustifolia* aerial parts

| Phytochemical constituents | Extracts of <i>B. angustifolia</i> aerial parts | | |
|----------------------------|---|---------------|----------------|
| | Hexane | Ethyl acetate | Hydroalcoholic |
| Phytosterols | + | + | ++ |
| Terpenoids | + | + | + |
| Glycosides | + | ++ | ++ |
| Saponins | - | - | - |
| Flavonoids | - | + | + |
| Tannins | - | + | + |
| Carbohydrates | + | + | + |
| Alkaloids | + | + | ++ |
| Amino acids | - | - | + |
| Oils | + | + | + |
| Phenols | + | + | ++ |

+, ++: Present, -: Absent

Quantification of phenolic and alkaloid contents

The quantified phenolic contents of *B. angustifolia* extracts ranged from 13.85±1.22 to 34.10±2.62 mg/g. The hydroalcoholic extract had more phenolic content (34.10±2.62 mg/g) than other extracts. The quantitative alkaloid content ranged from 16.24±2.38 to 31.86±1.88 mg/g (Table 2).

Antioxidant activity

The hydroalcoholic, ethyl acetate and hexane extracts of *B. angustifolia* were found to possess concentration-dependent scavenging activity. The mean IC₅₀ values for the hydroalcoholic, ethyl acetate, and hexane extracts of *B. angustifolia* on superoxide radical were found as 237±0.56 µg, 294±0.22 µg, and 450±0.43 µg, respectively, and for ascorbic acid, it was 54.4±1.1 µg (Figure 1, Table 3). The mean IC₅₀ values for hydroxyl radicals were found as 265±0.82 µg, 231±0.62 µg, and 369±0.52 µg, respectively, and for ascorbic acid it was 68.00±1.3 µg (Figure 2, Table 3). The mean IC₅₀ values of DPPH radicals were found as 206±0.18 µg, 272±0.14 µg, and 295±0.68 µg, respectively. The mean IC₅₀ value of ascorbic acid was found as 22.0±0.5 µg (Figure 3, Table 3). Among all the tested extracts, better free radical scavenging activity was found for the hydroalcoholic extract of *B. angustifolia* aerial parts.

Acute toxicity studies

There were no visible signs of toxicity and mortality, and no behavioral changes such as alertness, motor activity, breathlessness, restlessness, diarrhea, tremor, convulsion, and coma were observed at the administered doses. The animals were physically active and no deaths were recorded, even at doses of up to 2000 mg/kg body weight. Hence, all the tested extracts were considered as safe and nontoxic.

Table 2. Total phenolic and alkaloid contents (mg/gm) of *B. angustifolia* aerial parts' extracts

| S. No | Name of the extract | Total phenolic content (mg/gm) | Total alkaloid content (mg/gm) |
|-------|------------------------------|--------------------------------|--------------------------------|
| 1 | Hexane | 13.85±1.22 | 16.24±2.38 |
| 2 | Ethyl acetate | 26.28±0.66 | 22.40±1.36 |
| 3 | Hydroalcoholic (ethanol 70%) | 34.10±2.62 | 31.86±1.88 |

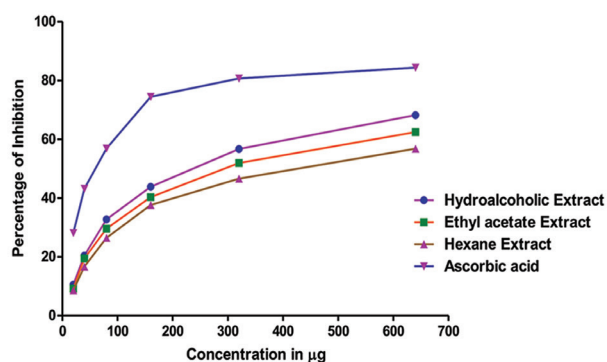


Figure 1. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on superoxide radical

Hepatoprotective activity

We examined the *B. angustifolia* aerial parts at three dose levels, 125 mg/kg, 250 mg/kg, and 500 mg/kg, which was assessed by measuring liver-related biochemical parameters (AST, ALT, ALP, total serum bilirubin, and total protein) levels for their hepatoprotective nature using TAA-induced hepatotoxicity in rats.

Group I, which was treated with vehicle, showed no significant changes in liver enzyme biomarker levels (AST, ALT, ALP, total bilirubin, and total protein). Group II was treated TAA, there were significant changes in levels of biomarker enzymes. The animals of Group III were administered TAA and then silymarin (Table 4). There were significant changes in biomarker enzymes levels compared with Group II rats' enzymes levels and the percentage protection offered by the silymarin against the changes in AST, ALT, ALP, total bilirubin, and total protein levels were 96.24%, 95.25%, 93.90%, 97.83%, and 96.14%, respectively.

The percentage protection produced by the hydroalcoholic extract (Groups IV, V and VI) on the enhancement of AST, ALT, ALP, total bilirubin, and total protein levels were 45.45%, 45.01%, 38.35%, 35.38%, and 32.87%; 57.04%, 58.40%, 51.79%, 48.21%, and 46.05%; 76.39%, 78.77%, 69.45%, 62.74%, and 65.81%, respectively.

The percentage protection produced by the ethyl acetate extracts (Groups VII, VIII, and IX) the enhancement of AST,

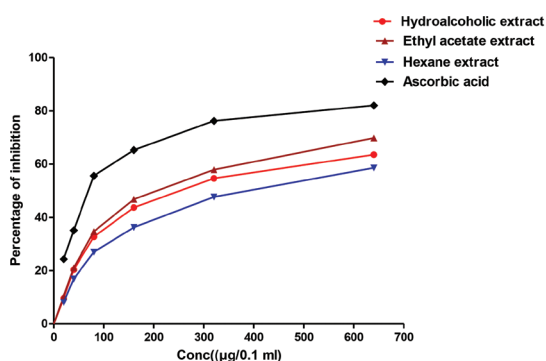


Figure 2. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on hydroxyl radical

Table 3. Fifty percent IC₅₀ of different extracts of *B. angustifolia* against superoxide, hydroxyl and DPPH radicals

| Name of the extract | 50% IC ₅₀ (µg/mL) | | |
|---------------------|------------------------------|------------------|--------------|
| | Superoxide radical | Hydroxyl radical | DPPH radical |
| Hydroalcoholic | 237±0.56 | 265±0.82 | 206±0.18 |
| Ethyl acetate | 294±0.22 | 231±0.62 | 272±0.14 |
| Hexane | 450±0.43 | 369±0.52 | 295±0.68 |
| Ascorbic acid | 54.4±1.1 | 68.00±1.3 | 22.0±0.5 |

DPPH: 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride, IC₅₀: Inhibition concentration

ALT, ALP, total bilirubin, and total protein levels were 43.03%, 41.03%, 36.08%, 29.40%, and 26.94%; 54.20%, 55.41%, 49.57%, 45.64%, and 47.36%; 66.36%, 67.38%, 60.04%, 59.32%, and 61.20%, respectively.

The percentage protection produced by the hexane extracts (Groups X, XI, and XII) on the enhancement of AST, ALT, ALP, total bilirubin, and total protein levels were 37.20%, 37.18%, 30.55%, 25.98%, and 24.97%; 48.29%, 47.72%, 42.43%, 40.51%, and 40.78%; 58.75%, 59.83%, 53.60%, 51.62%, and 52.64%, respectively.

The decrease in the AST, ALT, ALP and total bilirubin levels, and increase in levels of protein to normal, and percentage protection produced by the higher doses of the extracts were comparable to those of silymarin. Among all the extracts, the hydroalcoholic extracts of selected plants showed better activity (Table 5).

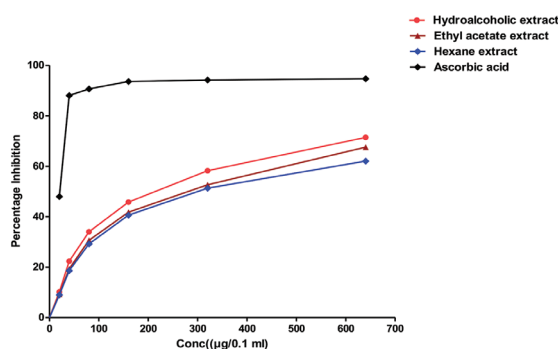


Figure 3. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride radical

Isolation of compounds

The selected plant extracts (hexane, ethyl acetate, and hydroalcoholic) on thin-layer chromatography showed different spots with different retention factor values, but on the basis of biologic activities (antioxidant and hepatoprotective), the hydroalcoholic extract of *B. angustifolia* was used for the separation of compounds using column chromatography.

Column chromatography was performed using a standard procedure. Silica gel (Qualigens), 60-120 mesh was used as an absorbent for column chromatography. The column was eluted using hexane, hexane-ethyl acetate, ethyl acetate, and ethyl acetate-methanol mixtures by gradient. BA-1 and BA-2 compounds were isolated in the study.

Structure elucidation and characterization of compound BA-1

Compound BA-1 was isolated in the combination of hexane and ethyl acetate (95:05) in the form of colorless oil. The proton nuclear magnetic resonance (¹H-NMR) spectrum (Figure 4) of compound BA-1 in acetone exhibited signals due to olefinic

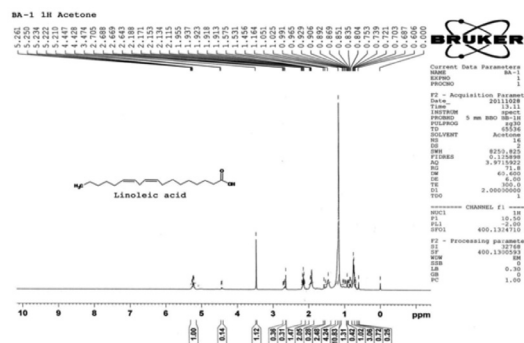


Figure 4. The proton nuclear magnetic resonance spectrum of compound BA-1

Table 4. Enzymes levels due to the effect of *B. angustifolia* extracts at different doses on TAA-induced liver toxicity in rats

| Name of extract/compound | Quantity of extract (mg/kg b.w.) | Enzymes levels of TAA-induced hepatotoxicity | | | | |
|--------------------------|----------------------------------|--|-------------|--------------|-------------------------|----------------------|
| | | AST (U/L) | ALT (U/L) | ALP (U/L) | Total bilirubin (mg/dL) | Total protein (g/dL) |
| Normal (drug vehicle) | 2 mL | 96.17±6.97 | 56±3.58 | 217.5±2.59 | 0.17±0.01 | 7.40±0.46 |
| TAA | 50 mg | 330.5±6.53 | 173±5.22 | 548.67±21.60 | 2.123±0.27 | 4.87±0.78 |
| Silymarin | 25 mg | 87±2.83 | 50.17±4.96 | 196.00±5.10 | 0.13±0.05 | 7.50±0.37 |
| | 125 mg | 224±3.03 | 120.3±4.32 | 421.67±6.65 | 1.43±0.29 | 5.7±0.37 |
| Hydroalcoholic | 250 mg | 196.8±2.04 | 104.67±3.01 | 377.17±3.13 | 1.18±0.27 | 6.03±0.29 |
| | 500 mg | 151.5±2.66 | 80.8±2.32 | 318.67±2.58 | 0.9±0.18 | 6.53±0.32 |
| | 125 mg | 229.67±3.72 | 125±4.00 | 429.17±2.32 | 1.55±0.23 | 5.55±0.22 |
| Ethylacetate | 250 mg | 203.5±4.28 | 108.17±2.23 | 384.5±4.97 | 1.23±0.24 | 6.07±0.18 |
| | 500 mg | 175±2.83 | 94.17±3.71 | 349.8±2.14 | 0.97±0.18 | 6.42±0.35 |
| | 125 mg | 243.3±4.46 | 129.5±2.43 | 447.5±3.21 | 1.62±0.27 | 5.5±0.37 |
| Hexane | 250 mg | 217.3±3.50 | 117.17±2.23 | 408.17±2.79 | 1.33±0.20 | 5.9±0.41 |
| | 500 mg | 192.8±2.64 | 103±2.19 | 371.17±2.40 | 1.12±0.17 | 6.2±0.34 |

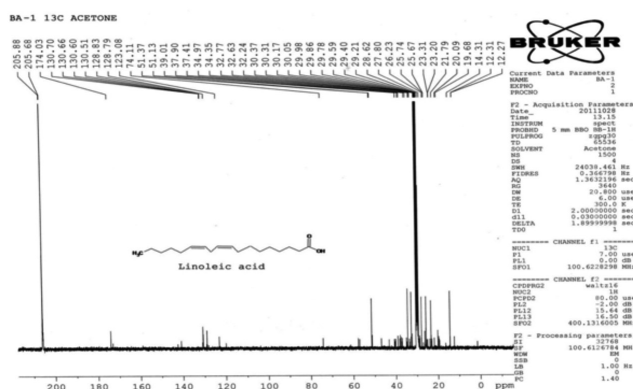
TAA: Thioacetamide, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase

Table 5. Percentage (%) protection of enzymes levels due to the effect of *B. angustifolia* extracts at different doses on TAA-induced liver toxicity in rats

| Name of extract | Quantity of extract | Percentage protection on enzymes levels of TAA-induced hepatotoxicity | | | | |
|-----------------|---------------------|---|-----------|-----------|-------------------------|----------------------|
| | | AST (U/L) | ALT (U/L) | ALP (U/L) | Total bilirubin (mg/dL) | Total protein (g/dL) |
| Silymarin | 50 mg/kg b.w. | 96.24 | 95.25 | 93.90 | 97.83 | 96.14 |
| | 125 mg/kg b.w. | 45.45** | 45.01** | 38.35** | 35.38** | 32.87** |
| Hydroalcoholic | 250 mg/kg b.w. | 57.04** | 58.40** | 51.79** | 48.21* | 46.05* |
| | 500 mg/kg b.w. | 76.39** | 78.77*** | 69.45** | 62.74** | 65.81*** |
| Ethylacetate | 125 mg/kg b.w. | 43.03* | 41.03** | 36.08* | 29.40* | 26.94* |
| | 500 mg/kg b.w. | 66.36** | 67.38*** | 60.04** | 59.32** | 61.20** |
| Hexane | 125 mg/kg b.w. | 37.20* | 37.18* | 30.55* | 25.98* | 24.97* |
| | 250 mg/kg b.w. | 48.29* | 47.72** | 42.43** | 40.51* | 40.78** |
| | 500 mg/kg b.w. | 58.75** | 59.83*** | 53.60** | 51.62** | 52.64*** |

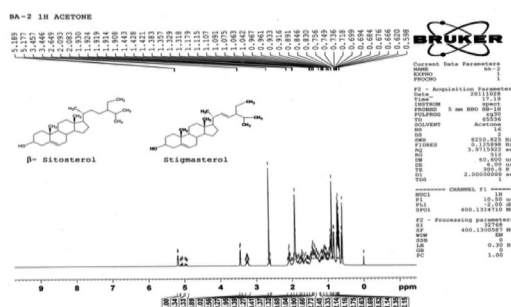
TAA: Thioacetamide, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase

***p<0.001, **p<0.01, *p<0.05

**Figure 5.** ^{13}C -nuclear magnetic resonance spectrum of compound BA-1

protons at δ 5.3–5.4, triplet methylene protons at δ 2.8, 11 methylene protons at δ 2.3, 2.0, 1.6 and 1.3, and methyl protons at δ 0.8. These signals were well matched to corresponding signals of linoleic acid, suggesting that this compound was linoleic acid or an unsaturated fatty acid. In the electrospray ionisation-mass measurement, its molecular weight was determined as 280 through a quasi-molecular ion peak at m/z 279 $[\text{M}-\text{H}]^-$ in the negative mode. This molecular weight was consistent with linoleic acid. Therefore, compound BA-1 was identified as linoleic acid. The introduction of a second double-bond to give linoleic acid or methyl linoleate (methyl 9(*Z*),12(*Z*)-octadecadienoate) gives rise to a peak at 2.8 ppm caused by the *bis*-allylic protons located at C11. The theoretical integration value of the olefinic protons increases to four, whereas that of the large methylene peak decreases further to 14.

Generally, many effects observed in ^1H -NMR are also found in ^{13}C -NMR (Figure 5). For example, the methyl and methylene

**Figure 6.** The proton nuclear magnetic resonance spectrum of compound BA-2

signals are up field in the spectrum, and signals of olefinic carbons are farther downfield. The number and nature of double-bonds affects the chemical shifts, as do the proximity of multiple double-bonds to each other and the presence of functional groups.

Structural elucidation of compound BA-2

Compound BA-2 was isolated as a white solid with colorless needles, a mixture containing two sterolic compounds from the hexane soluble in combination of hexane and ethyl acetate (90:10). The mixture gave a positive color reaction with sulphuric acid indicating the sterolic nature. The molecular formula $\text{C}_{29}\text{H}_{48}\text{O}$ (stigmasterol) and $\text{C}_{29}\text{H}_{50}\text{O}$ (β -sitosterol) were established showing molecular ion peak $[\text{M}]^+$ at m/z 412.3920, $[\text{M}]^+$ at 414, respectively, from the previous data as explained earlier. The ^1H and ^{13}C -NMR data are provided in Figures 6 and 7.

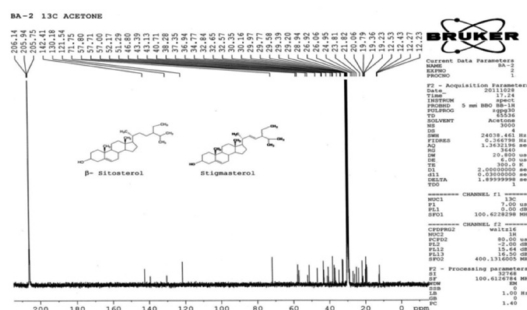


Figure 7. ¹³C-nuclear magnetic resonance spectrum of compound BA-2

DISCUSSION

Modern medicine significantly decreased mortality from different diseases compared with before the mid-nineteenth century. However, these developed medicines that cure diseases cause different adverse effects with long-term use, and their injudicious use has led to the development of drug-resistant diseases.^{22,23} Medicinal plants have been used for the prevention and treatment of diseases for a long time.^{24,25} There is a necessity to identify new bioactive molecules or drugs through science and technology from natural sources including medicinal plants, which are safer with no adverse effects and low cost.²⁴ In recent years, many researchers have reported on new drugs and validated traditional medicinal plants.²⁵ The results of the present study provide scientific evidence for the traditional use of *B. angustifolia*.¹⁰⁻¹² The qualitative phytochemical analysis and quantification of total phenolic and alkaloid contents and biologic activities were studied for *B. angustifolia*. Qualitative phytochemical screening of different extracts revealed the presence of different phytochemical constituents such as steroids, terpenoids, phenols, flavonoids, alkaloids, glycosides, phenols, tannins, carbohydrates, oils, and amino acids. The extracts on qualitative analysis on phenolic and alkaloid contents of hydroalcoholic extract showed greater content in it compared with other extracts.

Many investigations have reported that medicinal plants contain a wide variety of natural bioactive compounds that possess biologic activities.²⁶⁻³⁰ Accordingly, the extracts were tested for their antioxidant capacity and hepatoprotective activity on TAA-induced liver toxicity. Many investigations explained the positive correlation between oxidative stress and liver toxicity.³¹ The extracts of *B. angustifolia* showed a dose-dependent percentage inhibition on tested free radicals, but it was lower activity compared with ascorbic acid; as the concentration increases, the percentage inhibition may increase. The extracts reduced the formation of superoxide free radicals in the tested method; superoxide radical is the main free radical for oxidative stress by being involved in the formation of other free radicals.³² At the same time, the extracts also showed hepatoprotective activity on TAA-induced liver toxicity in rats.¹⁹⁻²¹ TAA-induced liver toxicity was established as the method to evaluate natural products for hepatoprotective activity and its mechanism involved for liver toxicity by damaging the mitochondria and dysfunction of

intracellular organelles of hepatic cells. This ultimately leads to an increase in the amount of bile acid, greater amounts of which generally promote liver damage by oxidative stress³¹ and often elevate liver biomarker enzymes in the body i.e. AST, ALT, ALP, total bilirubin, and total protein. Thus, biomarker enzymes were analyzed in the present study. The results of the hepatoprotective activity in the present study indicate that *B. angustifolia* extracts possesses moderate hepatoprotective activity compared with the standard drug silymarin. The extracts were significantly ($p < 0.05$) normalized the elevated biomarker enzymes levels compared with the toxic group (Group II). As mentioned earlier, there may be a relationship between the antioxidant activity and hepatoprotective activity of *B. angustifolia* extracts. Among all the extracts, the hydroalcoholic extract showed more activities and higher phenolic and alkaloid contents. From this point of view, we tried to isolate new bioactive molecules/compounds using hydroalcoholic extract using column chromatography, but unfortunately, known compounds i.e. mixtures of stigmasterol and β -sitosterol and linolenic acid were isolated. However, these compounds are reported from this plant species for the first time. The isolated compounds, stigmasterol and β -sitosterol were isolated from *B. angustifolia* are may be responsible for the activities of selected plants because there were some earlier reports of different biologic activities of these compounds³³⁻³⁹ or some other unknown compounds may be acting individually or may be synergistically.

CONCLUSIONS

The results of the present study provide evidence about traditional medicinal use of *B. angustifolia*, which is a traditional medicinal plant in India, and isolated known compounds stigmasterol and β -sitosterol from hydroalcoholic extract, were first reported from this species and maybe from this genus. Further research is needed to evaluate more pharmacologic activities of *B. angustifolia* and to isolate the more bioactive compounds through chromatographic techniques. Standardization of these isolates can be carried out by obtaining a chemical fingerprint/profile.

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