Therapeutic protection from hepatic injury and Chemical constituents of *Buchanania angustifolia* Roxb

Buchanania angustifolia Roxb'un hepatik hasar ve kimyasal bileşenlerinden terapötik koruma

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Short title: Hepatoprotective activity of Buchanania angustifolia

Kısa unvan: Buchanania angustifolia'nın hepatoprotektif etkinliği

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ABSTRACT

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

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

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

Conclusion: Among the tested extracts of *B. angustifolia,* hydro-alcoholic extract showed more antioxidant and hepatoprotective activity and isolated the compounds stigamsterol and β-sitosterol. Further research is needed to evaluate more pharmacological activities and isolation of most active bioactive compounds from *B. angustifolia*.

Key Words: *Buchanania angustifolia,* Chemical constituents, Antioxidant activity, Thioacetamide, Hepatoprotective activity.

ÖZET

Amaç: Geleneksel tıp yerine hastalıkların tedavisinde modern ilaçların kullanımı farklı yan etkilere neden oluyor. Bu nedenle, farklı hastalıkları kontrol etmek için yeni biyoaktif bileşikler aramak gerekir. Buchanania angustifolia, geleneksel tıpta farklı hastalıkların tedavisinde kullanmaktadır. Folklorik bilgilere dayanarak, B. angustifolia anten parçaları, antioksidan ve hepatoprotektif potansiyelleri ve fitokimyasal bileşenler için seçilmiştir.

Gereç ve Yöntem: *B.angustifolia* özütlerinin niteliksel fitokimyasal taramaları, steroidler, terpenoidler, flavonoidler, alkaloidler, glikozitler, taninler, karbonhidratlar, yağlar ve amino asitler gibi fitokimyasal bileşenlerin varlığını ortaya koydu. BA-1 ve BA-2 bileşikleri, hidroklorik asit ve Stigmasterol ve β-sitosterol karışımı olarak tanımlanır ve kromatografi kullanılarak hidroalkolik özünden izole edilir.

Bulgular: Antioksidan aktivitesi Süperoksit, Hidroksil ve, L- (2,6-dimetilfenoksi) -2-(3,4-dimetoksifeniletilamino) propan hidroklorür (DPPH) serbest radikalleri üzerinde gerçekleştirildi. B. angustifolia özleri, serbest radikallere doza bağımlı aktivite gösterdi. Hidroklorik asit ekstraktları arasında daha iyi aktivite görülmüştür. Ekstraktlar sıçanlarda tiyoasetamid kaynaklı karaciğer zehirlenmesinde hepatoprotektif etkinliği gösterdi ve hidroalkol özütü, tiyoasetamidin neden olduğu karaciğer zehirlenmesine bağlı olarak değişen biyokimyasal parametrelerin önemli restorasyonunu sergiledi.

Sonuç: B. angustifolia'nın test edilen ekstraktları arasında hidro-alkollü ekstrakt antioksidan ve hepatoprotektif etkinlik göstermiş ve stigamsterol ve β-sitosterol bileşiklerini izole etmiştir. B. angustifolia'dan en aktif biyoaktif bileşiklerin daha fazla farmakolojik aktivitesinin ve izolasyonunun değerlendirilmesi için daha fazla araştırmaya ihtiyaç vardır.

INTRODUCTION

Plants have been used as medicines since time immemorial around the world. The well known evidences about the use of plants in medicine were ayurveda in India, African traditional medicines, unani medicine in middle east countries, traditional Chinese medicine in China[1-5]. Major problems associated with traditional medicine are the lack of standardization, consistency, safety and quality[6]. The advance in technology provided the perfection in the medicine for treating the different diseases. Simultaneously the use of modern medicine offered a new side effects on their long term usage and the diseases causing microorganisms getting resistance to the drugs[7, 8]. So, the people are turning back to the traditional medicine because of their less side effects and diversity of chemical entities in them for treatment of different diseases. In this point of view the researchers are now functioning on identification, evaluation, standardization of biological activities of traditional medicinal plants[9] using advanced technology in isolations of new bioactive molecules. The present work done on one of the traditional medicinal plant i.e. *Buchanania angustifolia* Roxb (*B. angustifolia*) for the isolation of new bioactive molecules and for providing scientific evidence on its' traditional usage by Indian tribes for a wider range of ailments, including nutritional disorders, skin diseases, gravel, healing of wounds, rheumatic pain, tonic for sexual debility and other urinary problems[10-12]. *B. angustifolia* is mainly grown in dry deciduous forests and distributed around South India and Srilanka regions. However, to the best of our knowledge, the phytochemical constituents, biological activities of *B. angustifolia* have not been reported [13]. Therefore, this study was planned to investigate the phytochemical constituents, antioxidant, hepatoprotective potentials of hexane, ethyl acetate and hydroalcoholic extracts of *B. angustifolia* aerial parts.

MATERIALS AND METHODS

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 tetrozolium 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 like Aspartate Aminotrasferase (AST/SGOT), Alanine Aminotranferase (ALT/SGPT), Alkaline Phosphatase (ALP),

Total bilirubin and Total protein were purchased from Span diagnostics Ltd., Gujarat, India. Folin-Ciocalteau reagent , Bromocresol green were purchased from Sigma-Aldrich, St. Louis, USA.

Plant Material collection and Preparation of extracts

The plant material was collected at Talakona forest region, Tirupathi, Andhra Pradesh, India, during the month December, 2010 (AUCP/BGR/2010-431). The authentication of the plant was done by Rtd. Prof. M. Venkaih, Department of Botany, Andhra University, Visakhapatnam. The plant material was dried under shade and the powdered, it for extraction separately using maceration process with hexane, ethyl acetate, and hydroalcoholic (ethanol (70%v/v) were concentrated to dryness under vacuum using rotavapour.

Preliminary phytochemical studies

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

Quantification of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteau reagent. The method is based on blue light absorption measurement due to the chemical reduction of tungsten and molybdenum oxides of Folin-Coicalteau reagent, when combined with the compounds present in the extracts using colorimetry at 765 nm. The phenolic content in the extract was meassured in the gallic acid equivalents as mg/gm (GAE), using gallic acid calibration curve. The results showed in mean values [14, 15].

Quantification of total alkaloid content

The plant extract (1mg/ml) was dissolved in 2 N HCl and the solution was filtered. The phosphate buffer's pH was neutralized 0.1N NaOH. 1 ml of extract solution, 5 ml of phosphate buffer and 5 ml of bromocresol green (BCG) solution placed in separation and then mixes the solution well. The complex formed in the solution was extract with chloroform. The absorbance of complex color in chloroform was measure at 470 nm. Overall experiment was performed thrice and results were reported in atropine equivalents. The results showed in mean value[14, 16].

In vitro antioxidant activity

In-vitro antioxidant activity was assessed by use of prepared extracts of *B. angustifolia* using Dimethyl sulphoxide (DMSO) as vehicle on superoxide, hydroxyl and DPPH free radicals[14, 17, 18]. The percentage inhibition and IC_{50} values were

calculated.

Superoxide radical scavenging activity

Superoxide scavenging activity of the selected plant extracts were evaluated as per standard methods. It is by 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 method. It was studied by the competition between deoxyribose and the extract's antioxidant molecules for hydroxyl radicals generated from the Fe+2/ EDTA/H_2O_2 system.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured as per methods. This method is based on measure of color absorbance of alcoholic DPPH solution (Blue color) after addition of antioxidant solution (Extract/Compound). If antioxidants present in the test compound blue color turns yellow color due to DPPH.

Calculation of Percentage Inhibition

The percentage inhibition of superoxide production by the extract was calculated using the formula: Inhibitory ratio = $\underline{(Ao\text{-}A_1) \times 100/}\ A_0$

A_0: Absorbance of control; A_1: Absorbance of plant extract or/and Ascorbic acid.

IC_{50} calculation form percentage inhibition

The optical density obtained with each concentration of the extract/ascorbic acid was plotted taking concentration on X-axis and percentage inhibition on 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 at standard light and humidity by supplying proper food and water.

Acute toxicity studies

The acute toxicity study was conducted for extracts of *B. angustifolia* extracts as per 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 in to three groups of consisting of 6 animals. They were maintained for one week before the experiment, under room temperature and allowed free access to water and diet. The animals were subjected for acute toxicity study using each

extract at a dose of 2000 mg/kg orally in 3 groups 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 like skin changes, morbidity, aggressiveness, oral secretions, sensitivity to sound and pain, respiratory movements and finally their 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 grouped into twelve (N=6). The group I were treated with normal saline (Vehicle) for one week through oral administration at 2ml/ kg body weight. Group II and Group III animals were treated with TAA as a 2% w/v solution in water on first day at 50mg/kg body weight by s.c. then group II continuously treated with saline and group III with silymarin at a dose of 25mg/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 first day at 50mg/kg body weight by s.c. then groups IV, V, VI were treated with hydroalcoholic extract, groups VII, VIII, IX with ethyl acetate extract and groups X, XI, XII were treated with hexane extract at a doses of 125, 250, 500 mg/kg body weight by orally for three weeks. Animals of all groups were anaesthetized using chloroform after 48h of final dose administration of extracts. The blood samples were collected from animal groups by retro-orbital plexus, then samples were without delay centrifuged at 2400rpm for quarters of an hour. Then clearly separated serum after centrifugation was used for measuring the different biochemical parameters using auto analyzer with the help of reagent kits[19-21]. All the experimental procedures involving animals were conducted according to OECD guidelines and approved by the institutional animal ethics committee, Andhra University. Results were analysed by using Two-way ANOVA followed by Bonferroni post-hoc test. All gropus were copmared with Silymarin group.

Isolation of Phytoconstituents (Compounds)

There was very less phytochemical work reported and availability of the extract quantity 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 like steroids, terpenoids, flavonoids, alkaloids, glycosides, tannins, carbohydrates, oils and amino acids. The extracts gave negative

results for the quinines and saponins. The all extracts of *B. angustifolia* revealed the presence of phenols, alkaloids, carbohydrates, steroids, terpenoids and glycosides and gave negative result 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 reveals the presence of minute amount of oils but hydroalcoholic extracts gave negative results. All the extracts gave negative result to amino acids but the hydroalcoholic extracts give minute result for the presence of amino acids (Table 1).

Quantification of phenolic and alkaloid contents

The Quantified phenolic contents of *B. angustifolia* extracts were ranging from 13.85±1.22 to 34.10±2.62 (mg/gm). The hydroalcoholic extract have more phenolic content i.e. 34.10±2.62 (mg/gm) than other extracts. The quantitative alkaloid content was ranging from 16.24±2.38 to 31.86±1.88 (mg/gm) (Table 2).

Antioxidant activity

The hydroalcoholic, ethyl acetate and hexane extracts of *B. angustifolia* were found to possess concentration dependent scavenging activity. The mean IC_{50} values for hydroalcoholic, ethyl acetate and hexane extracts of *B. angustifolia* on superoxide radical were found to be 237±0.56µg, 294±0.22µg and 450±0.43µg respectively and for ascorbic acid was found to be 54.4±1.1µg (Figure 1 and Table 3). The mean IC_{50} values for hydroxyl radical were found to be 265±0.82µg, 231±0.62µg and 369±0.52µg respectively and for ascorbic acid was found to be 68.00±1.3 µg (Figure 2 and Table 3). The mean IC_{50} values on DPPH radical were found to be 206±0.18µg, 272±0.14µg and 295±0.68µg respectively. The mean IC_{50} value of ascorbic acid was found to be 22.0±0.5µg (Figure 3 and Table 3). Among all tested extracts, better free radical scavenging activity was found for hydroalcoholic extract of *B. angustifolia* aerial parts.

Acute toxicity studies

There were no visible sign of toxicity, 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 death was recorded even at the dose of up to 2000 mg/kg body weight. Hence, all the tested extracts were considered as safe and non toxic.

Hepatoprotective activity

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

Group I was treated with vehicle showed no significant changes in the biomarkers of liver enzymes (AST (SGOT), ALT (SGPT), ALP, total bilirubin and total protein) levels. Group II was treated TAA, there is significant changes in levels of biomarker enzymes. The animals of group III were administered with TAA and then silymarin (Table 4). There is significant changes in biomarker enzymes levels compared to group II rats enzymes levels and the percentage protection offered by the silymarin against the changes in AST (SGOT), ALT (SGPT), 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 (SGOT), ALT (SGPT), 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 (SGOT), ALT (SGPT), 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 (SGOT), ALT (SGPT), 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 SGPT, SGOT, ALP and Total bilirubin levels, increase in levels of protein to normal and percentage protection produced by the higher dose of the extracts was comparably similar to silymarin. Among all extracts hydroalcoholic extract of selected plants showed better activity (Table 5).

Isolation of compounds

The selected plant extracts (Hexane, Ethyl acetate and hydroalcoholic) on TLC over showed different spots with different retention factor (R_f) values, but on the basis of

biological activities (Antioxidant and Hepatoprotective) hydroalcoholic extract of *Buchanania angustifolia* was used for separation of compounds using column chromatography.

Column chromatography was done by standard procedure. Silica gel (Qualigens), 60-120 mesh was used as absorbent for column chromatography. The column was eluted using hexane, hexane-ethyl acetate, ethyl acetate, 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 color less oil. The 1H NMR spectrum (Figure 4) of compound BA-1 in Acetone exhibited signals due to olefinic 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 is linoleic acid or an unsaturated fatty acid. In the ESI-mass measurement, its molecular weight was determined to be 280 by a quasi-molecular ion peak at m/z 279 $[M-H]^-$ in the negative mode. This molecular weight was consistent with linoleic acid. Therefore, compound BA-1 was identified as linoleic acid. 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 while that of the large CH_2 peak decreases further to 14.

Generally, many effects observed in 1H-NMR are also found in 13C-NMR (Figure 5). For example, the methyl and methylene signals are up field in the spectrum, while 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 positive colour reaction with sulphuric acid indicating the sterolic nature. The molecular formula $C_{29}H_{48}O$ (Stigmasterol) and $C_{29}H_{50}O$ (β-Sitosterol) were established showing molecular ion peak [M]+ at *m/z* 412.3920 ,$[M]^+$ at 414 respectively from the previous data as explained in the earlier pages. The 1H and 13C NMR data were provided in Figure 6 and Figure7.

DISCUSSION

The modern medicine significantly decreased the mortality of people from different diseases compared to the before mid nineteenth century. However, the developed medicines curing diseases, on long term use causing different side effects and on their injudicious usage leading to the development of drug resistance diseases [22, 23]. Since, medicinal plants have been using for the prevention and treatment of diseases for a long time [24, 25]. There is necessitate to identify new bioactive molecules or Drugs through science and technology from natural sources including medicinal plants which are safer without any side effects and low cost [24]. In recent years, many researchers reporting the new drugs and validating the traditional medicinal plants [25]. The present study results, provides scientific evidence for traditional usage of *B. angustifolia*[10-12]. The qualitative phytochemical analysis and quantification of total phenolic, alkaloid contents and biological activities were studied for the *B. angustifolia*. Qualitative phytochemical screening of different extracts revealed the presence of different phytochemical constituents like steroids, terpenoids, phenols, flavonoids, alkaloids, glycosides, phenols, tannins, carbohydrates, oils and amino acids. The extracts on qualitative analysis on phenolic and alkaloid contents, hydro-alcoholic extract showed more content in it compared to other extracts.

Many investigations were reported that medicinal plants contain a wide variety of natural bioactive compounds, which possess biological activities[26-30]. So, the extracts were tested for their antioxidant capacity and hepatoprotective activity on TAA-induced liver toxicity. Many investigations explained the positive correlation between the oxidative stress and liver toxicity [31]. The extracts of *B. angustifolia* showed dose dependent percentage inhibition on tested free radicals, but it was less moderate activity compared to ascorbic acid and as the concentration increase may be the percentage inhibition may increase. The extracts reduced the formation of superoxide free radicals in the tested method, superoxide radical is main free radical for oxidative stress by involving in 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 method to evaluate the natural products hepatoprotective activity and its mechanism involved for liver toxicity by damaging the mitochondria and dysfunction of intracellular organelles of hepatic cells finally leads to the increase in the bile acid amount in it, which more amount generally

promotes liver damage by oxidative stress [31] and often elevates the liver biomarker enzymes in body i.e. SGOT, SGPT, 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 indicates that, *B. angustifolia* extracts posses moderate hepatoprotective activity compared to standard drug silymarin. The extracts were significantly ($p<0.05$) normalized the elevated biomarker enzymes levels comparing with toxic group (Group II). As earlier said, may be there was a relationship between antioxidant activity and hepatoprotective activity of *B. angustifolia* extracts. Among all extracts hydroalcoholic extract showed more activities and high phenolic and alkaloid contents. In this point of view, we tried to isolate new bioactive molecules/compounds using hydro-alcoholic extract using column chromatography, but unfortunately, known compounds i.e. mixture of Stigmasterol and β-sitosterol and linolenic acid were isolated. However, these compounds were first reports from this plant species. The isolated compounds, Stigmasterol, and β-sitosterol was isolated from *B. angustifolia* are may be responsible for the activities of selected plant because there were some earlier reports on different biological activities of these compounds[33-39] or some other unknown compounds may acting individually or may be synergistically.

CONCLUSION

The present study results in conclusion, provides the evidence about traditional medicinal usage of *B. angustifolia,* is an traditional medicinal plant in India and isolated known compounds stigmasterol and β-sitosterol from hydro-alcoholic extract, were first reported from this species and may be from this genus. Further research is need to evaluate more pharmacological activities of *B. angustifolia* and in isolation of the more bioactive compounds through chromatographic techniques and standardization of them can be carried out by obtaining a chemical fingerprint/profile.

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