Research Article

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ASSESSMENT OF ANTIOXIDANT EFFECTS OF THE FORMULATION KUTAJA TRIPHALA: AN IN-VITRO STUDY

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ABSTRACT

Ayurveda is one of the indigenous traditional systems of medicine for which scientific validation is useful for its more acceptances. Kutaja triphala (KT) mentioned in Panduroga Chikitsa of Ashtanga Hridaya. This formulation mainly used for the treatment of Pandu, kushta, jwara, pleeha, tamakaswasa, arsas, bhagandara, hritroga, gara, udara and prameha. The present study was aimed to evaluate the antioxidant activity of Kutaja triphala. Total Phenolic and flavonoid content of Kutaja triphala was determined with Folin – Ciocalteu and aluminum chloride method, respectively. The activity of Kutaja triphala on scavenging DPPH and hydroxyl radicals were analyzed. The reducing activity of Kutaja triphala was evaluated using FRAP (Ferric reducing antioxidant power) and PM (Phosphomolybdenum) assays. Kutaja triphala effectively scavenges DPPH and hydroxyl radicals with IC₅₀ value at 8.5 ± 2.17 and 44 ± 0.6 µg/mL respectively. The FRAP and PM assays had shown the dose dependent reducing activity of this drug. Kutaja triphala possess high Phenolic content (125.45 µg/mg GAE) and flavonoid content (72.25 µg/mg quercetin equivalent). In conclusion, the high Phenolic content and antioxidant property of this drug justify its uses in the treatment of diseases where oxidative stress plays a major role.

Keywords: antioxidants; Ayurveda, Kutaja triphala, free radicals, oxidative stress

INTRODUCTION

Oxidative stress is an important risk factor involved in the pathogenesis of numerous chronic diseases including asthma, inflammatory arthropathies, diabetes, Parkinson's and Alzheimer's diseases, cancers and atherosclerosis¹. It is initiated by highly reactive free radicals which paired with bio molecules like proteins, lipids and nucleic acids for attaining stability². Our body have an innate antioxidant enzyme defence system against free radicals. Antioxidants are helping to stop, or limit damage caused by free radical either by their reducing capacity or free radical scavenging activity. Natural antioxidants have a significant role in preventing oxidative damage in diseases were oxidative stress played a major role³. There is an increasing demand for antioxidant supplements to fight against oxidative damage in sometimes here protective mechanisms are disrupted by various pathological processes⁴.

Ayurveda is one of the indigenous traditional systems of medicine for which scientific validation is useful for its more acceptances. Most of the individual ingredients of poly herbal formulations used in Ayurveda have inbuilt antioxidant activity which may be contribute their therapeutic efficacy⁵. Kutaja triphala (KT) is mentioned in Panduroga Chikitsa of Ashtanga Hridaya. In Charaka Samhita it is termed as Shilajatu vataka because major ingredient of Kutaja triphala is shilajatu.

The ingredients of Kutaja triphala includes kutaja (*Holarrhena antidysenterica*), amalaki (*Emblia officinalis*), haritaki (*Terminalia chebula*), bibhitaki (*Terminalia bellirica*), nimba

(*Azadirachta indica*) patola (*Trichosanthes dioica*), musta (*Cyperus rotundus*), nagara (*Zingiber officinale*), vamsalochana (*Bambusa arundinacea*), pippali (*Piper longum*), karkatakasringi (*Pistacia integerrima*), kandakari (*Solanum surattense*), tvak (*Cinnamomum zeylanica*), ela (*Elettaria cardamomum*) and patra (*Cinnamomum tamala*). Shilajatu is taken in adequate quantity and impregnated for thirty days with the decoction of the fruit of Kutaja, haritaki, bibhitaki, amalaki, nimba, patola, musta and nagara; to this Shilajatu, sugar and powdered tvak-ksheri (vamsalochana), pippali, dhatri, karkatakasringi and kandakari. Additionally, adequate quantity of powders of tvak, ela and patra and honey were added prepared Kutaja triphala in lehya (semisolid) form.⁶ This formulation is mainly used for the treatment of pandu, kushta, jwara, pleeha, tamakaswasa, arsas, bhagandara, hritroga, gara, udara, arsas and prameha. Various studies suggest the pharmacological effects of individual ingredients in Kutaja triphala. Yet there has been no study done in Kutaja triphala as a single formulation. Considering this fact, the present study is planned to analyze the antioxidant, effects of Kutaja triphala using various *in-vitro* antioxidant assays.

MATERIAL AND METHODS

Description of Kutaja triphala (KT)

Kutaja triphala was purchased from Vaidyamadam Vaidyasala, Mezhatthur and Kerala, India for the study. The required amount of this lehya was dissolved in water for further analysis.

Chemicals

The chemicals Folin - Ciocalteu reagent, 2, 2, Diphenyl-1-picryl hydrazyl (DPPH), riboflavin, TPTZ (2, 4, 6-tripyridyl-S-triazine) were purchased from Sigma Aldrich USA. All the reagents and chemicals used in the experiments were of analytical grade.

Animals

Male Wistar rats (150-180 g) were purchased from Small Animal breeding station, College of Veterinary and Animal Sciences, Mannuthy, Thrissur. They were maintained under standardized environmental conditions (22 – 28°C, 60 – 79% relative humidity, 12 hr dark/ light cycle) and fed with standard rat feed (Sai Durga feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were conducted after obtaining prior permission from Institutional Animal Ethics Committee (Approval no ACRC/IAEC/2018[1]P4) were following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Estimation of total Phenolic content

The total Phenolic content of Kutaja triphala was determined with Folin - Ciocalteu method⁷. The reaction mixture containing 0.1 mL of Kutaja triphala and 10% Folin - Ciocalteu reagent (2 mL) were incubated at room temperature for 6 min in dark. After adding 7.5% sodium carbonate (2 mL) solution, this mixture was further incubated at room temperature for 30 min. Absorbance of the mixture was read at 760 nm and total phenolics present in the sample was calculated as equivalent of per mg gallic acid (GAE) per gram of sample.

Estimation of total flavonoids

A volume of 0.1 ml of Kutaja triphala made up to 2 mL with methanol. To this add 10% ammonium chloride (100 µL) and 1 M potassium acetate (100 µL) final volume was made up to 3 mL by adding distilled water. This reaction mixture was incubated for 10 min at room temperature and absorbance was measured at 415 nm. Total flavonoids present in Kutaja triphala were expressed as mg equivalent of Quercetin per gram of sample.

Antioxidant (*in-vitro*) activity

a. Hydroxyl Radical Scavenging Activity

The effect of Kutaja triphala on scavenging hydroxyl radicals was measured by its competition between deoxy-2-ribose⁸. The hydroxyl radicals are generated from Fe^{2+} /EDTA/ascorbate/ H_2O_2 system. The reaction mixture contained 100 µM ferric chloride, 100 µM ascorbic acid, 2800 µM deoxy-2-ribose, 100 µM hydrogen peroxide, 100 µM EDTA, 100 µM phosphate buffer (pH 7.4) and various concentrations of the Kutaja triphala (10-50 µg/mL) in a final volume of 1 mL and incubated at 37° C for 1 hr. At the end of incubation, 20% acetic acid (1.5 mL, pH 3.5), 8.1% SDS (0.2 mL) and 0.8% TBA (1.5 mL) were added to 0.4 mL of reaction mixture. After proper mixing, incubated this reaction mixture at 100°C for 1 hour and formation of TBARS was measured by spectrophotometrically at 532 nm. All the experiments were carried out in triplicate and percentage of inhibition was calculated using following formula; Percentage inhibition = $[\text{AC}-\text{AS}]/\text{AC} \times 100$, where AC and AS is the absorbance of control and sample respectively.

b. DPPH (2,2'-Diphenyl -1-Picrylhydrazyl) Radical Scavenging Activity

DPPH radical scavenging activity was analyzed as per the method of Aquino *et al.*, 2001⁹. DPPH, a stable free radical produces an intense purple colour and have maximum absorption peak at 517 nm. Due to the electron donating nature of antioxidants, DPPH radical reduced into DPPH-H and results in decrease of purple colour to yellow. Different concentrations of kutaja triphala (5-25 µg/mL) and 187 µL of DPPH were incubated at room temperature in dark for 20 min. Read the absorbance at 517 nm against methanol as reference. Percentage of inhibition was calculated using following formula; Percentage inhibition = $[\text{AC}-\text{AS}]/\text{AC} \times 100$, where AC and AS is the absorbance of control and sample respectively

c. Ferric Reducing Antioxidant Power Assay (FRAP Assay)

FRAP measures the capacity of test compounds to reduce ferrous ions at acidic pH¹⁰. The FRAP reagent was prepared freshly by mixing 0.3 M acetate buffer (25 mL), 10 mM TPTZ (2.5 mL) and 20 mM FeCl_3 (2.5 mL). About 0.9 mL of FRAP reagent with varying drug concentration of Kutaja triphala (10-50 µg/mL) were incubated at 37°C for 15 minutes. Then, absorbance of blue coloured reaction mixture was measured at 595 nm. The absorbance of drug was directly proportional to its reducing power and reducing activity of Kutaja triphala was calculated from standard graph was plotted using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

d. Phosphomolybdenum Assay

The phosphomolybdenum assay measures the total antioxidant capacity of sample as per the method of Prieto *et al.*, 1999¹¹, which is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by the sample. The reaction mixture was prepared by mixing 4 mM ammonium molybdate, 28 mM sodium phosphate, 0.6 M H_2SO_4 and distilled water (50 mL); a volume of 1 mL of reagent solution and different concentration of Kutaja triphala (10 – 50 µg/mL) which was kept in boiling water bath at 95° C for 90 minutes. Then, the absorbance was read at 695 nm and total antioxidant capacity was expressed in terms of mg/g of trolox equivalent.

Acute Toxicity

The acute toxicity of kutaja triphala was done as per the guidelines of OECD¹². The male Wistar rat weighing 150-180 g were divided into two groups (n = 6). Group I was kept as normal and group II was treated with single oral administration of Kutaja triphala at a dose of 3 g/ kg b. wt. The feed and water were removed 4 hours before drug administration and replacing them 1 hour after the drug administration. All animals were carefully observed for 14 days for any toxicity symptoms. If mortality is not there, 1/5th and 1/10th of acute dosage was selected as high dose and low dose respectively for further analysis.

RESULTS

Estimation of Total Phenolic and Flavonoid Content

In this study, the Phenolic content of kutaja triphala estimated was 125.45 µg/mg gallic acid equivalents. The results reveals kutaja triphala bears high Phenolic content and that may the reason behind its antioxidant activity. The total flavonoid content of Kutaja triphala was 72.2 µg/mg quercetin equivalents.

Antioxidant Activity of Kutaja triphala

Figure 1: The hydroxyl radicals were generated from Fe3+/ascorbate/EDTA/H2O2 system was markedly decreased by the kutaja triphala in dose dependent manner with IC50 value at 44 ± 0.6 µg/mL

Figure 2: Kutaja triphala was very effective in reducing the stable radical DPPH into DPPH-H by donating hydrogen and concentration of Kutaja triphala required to scavenge 50% DPPH radical (IC₅₀) was 8.5 ± 2.2 µg/mL

Figure 3: In FRAP assay, Kutaja triphala was found to be effectively reduces ferrous ion in to ferric in dose dependent manner and ferric reducing activity of 10 µg/mL of Kutaja triphala was 0.12 ± 0.02 µmol/mL

Figure 4: Similar trend was observed in PM assay, here total antioxidant capacity of Kutaja triphala was found to be increases with increasing concentrations

Acute Toxicity Study of Kutaja triphala

Animals administrated with Kutaja triphala at a dose of 3 g/kg b. wt didn't show any toxic symptoms. The behavioral patterns of animals were observed for 14 hours after Kutaja triphala administration. The animals were found to be normal and not displayed significant changes in behavior, breathing, impairment in food intake and water consumption, postural abnormalities and hair loss. There were no significant changes in body weight. All animals exhibited a normal increase in body weight without drastic difference between both normal and treated groups.

DISCUSSION

Antioxidants are tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. The antioxidant potential of kutaja triphala was investigated in the search for new bioactive compounds from natural resources. The global trends towards the traditional medicine were increased due to its ecofriendly and less toxic nature. Kutaja triphala, an Ayurvedic formulation contains various plant parts shows versatile pharmacological properties.

Phytochemicals contained in the drug are mainly responsible for the antioxidant activity of a drug such as poly phenols and flavonoids. Antioxidant activities of the drug are mainly due to the combined action of these phytochemicals through a variety of mechanisms. Several studies reported the correlation of

antioxidant capacity and Phenolic content¹³ that also provides protection against gastro-duodenal pathogenesis, premature aging, inflammation, metabolic dysfunction and cancer, neuro-degenerative and cardiovascular diseases. The antioxidant effects the combination is extensively determined by poly phenols analysis. Phenolic compounds are powerful donors of hydrogen, which makes them good antioxidants¹⁴. In the present study, the Phenolic content of the drug kutaja triphala estimated was 125.45 µg/mg gallic acid equivalents. The results reveal kutaja triphala bears high Phenolic content and that may the reason behind its antioxidant activity.

The effect on the DPPH and hydroxyl radical scavenging activity reflects the hydrogen donating ability of kutaja triphala. Assay based upon the use of DPPH radicals is among the most popular spectrophotometric methods for determination of the antioxidant capacity of plant extracts, foods, beverages and vegetable extracts because the radical compounds can directly react with antioxidants. Additionally, DPPH scavenging method has been used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive, and reproducible procedures¹⁵. As the concentration of kutaja triphala increases it effectively scavenges free radical.

Kutaja triphala act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups. It was suggested that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with

antioxidant activity. Antioxidants can be reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other¹⁶. The presence of reductants, such as antioxidant substances in the samples, causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. The reducing power of the extracts increased with increasing concentration, which suggests that the electron donating ability of the extracts is concentration dependent. A strong reducing power was noted for aqueous extract of kutaja triphala the values are comparable with the standard antioxidant ascorbic acid.

The total antioxidant capacity (TAC) was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid pH. It evaluates both water-soluble and fat-soluble antioxidants (total antioxidant capacity). The results indicate higher TAC (expressed as ascorbic acid equivalent) of kutaja triphala.

The formulation kutaja triphala has more reducing power and free radical scavenging activity than individual drugs. The present study indicated a strong antioxidant activity of kutaja triphala, which may be responsible for many of the medicinal properties of the drug.

CONCLUSION

The aqueous fraction of kutaja triphala had the best antioxidant effects against the DPPH and hydroxyl radicals and a strong ferric reducing power. This suggests that kutaja triphala can be used to prevent and control the oxidative stress induced by free radicals.

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