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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF *Toddalia asiatica* (Linn.) FRUIT EXTRACTS: AN *In vitro* STUDY

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Abstract

The objective of the present study was to examine the preliminary phytochemicals and antioxidant activity of *Toddalia asiatica* (Linn) fruit extracts using *In vitro* study. The *Toddalia asiatica* fruits were used for the different extract and used for the analysis. Antioxidant capabilities were measured by supperoxide scavenging activity, DPPH Assay and Nitric oxide scavenging activity. In addition, phytochemical constituents of different extracts were determined. The results demonstrated that all three extracts concentration-dependently scavenged free radicals, reactive oxygen species and shows significant total polyphenol contents all the extracts. In particular, methanolic extract showed significantly greater antioxidant activity and as compared others, which could be due to the presence more amount of polyphenol contents. In conclusion the extracts can be used against the oxidative stress. However, further studies are warrant to analysis the active components present in the extracts.

Key words: *Toddalia asiatica,* Supperoxide scavenging activity, DPPH assay, Nitric oxide scavenging activity and Polyphenol contents.

1. Introduction

Plants, animals and the humans have intimate biological relationships and have evolved along parallel lines cooperating and depending upon each other for existence. In the present scenario, greater emphasis is being laid on the traditional knowledge of ethnic people imparting that the use of knowledge in biological resources as a new source of drugs, medicine, food, and pharmaceutical raw materials and thus maintains maintenance of good health (Dixit *et al*., 2010). It estimated that about 80 % of the world's population still relies on plant-based medicines for their primary health care (Martins, 2013). This is a clear indication of the role of medicinal plants in the maintenance of health and treatment of diseases, further as therapeutic alternatives throughout the world (Abayomi *et al.*, 2013).

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The effects of plants are preliminary due to the presence of secondary metabolites produced by plant species. The secondary metabolites include phenol, alkaloids, terpins, flavinoids etc., A medicinal herb is different from botanic term "herb". It refers to any plants used for medicinal purposes. Public, academic and government interest in traditional medicines is growing exponentially due to the increased incidence of the adverse drug reactions and economic burden of the modern system of medicine (Dubey *et al.*, 2004).

Reactive oxygen species (ROS), the highly reactive oxygen-containing molecules, including free radicals are produced in very low levels during normal cell metabolism. Excess of ROS produced and not scavenged; these species may lead damage of macro molecules such as lipid, proteins and DNA damage, which results in the implication of the pathogenesis of numerous disorders, e.g. cardiovascular, atherosclerosis,

reperfusion injury, cataractogenesis, rheumatoid arthritis, inflammatory disorders and cancer.ROS may also be very damaging, they can attack lipids in cell membranes that cause membrane damage such as membrane lipid peroxidation and a decrease in membrane fluidity and also cause DNA mutation leading to cancer. (Antonio *et al*., 2013).

Exposure of cellular macromolecules to ROS and reactive nitrogen species (RNS) is strongly controlled by enzymic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non enzymic antioxidants such as vitamin C, Vitamin E, glutathione reduced (GSH), carotenoids and other low molecular weight compounds such as Lipoic acid that function interactively and synergistically to neutralize free radicals. Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions (Farombi *et al*., 2002). Antioxidant compounds are widely used compounds to counter the free radicals mediated oxidative stress in the cells. These antioxidant compounds can be derived from natural and chemical sources. Natural sources are much safer to use due to less toxicity and side effects, so the production of antioxidant compound from the natural sources such as plants and algae is in great demand.

Toddalia asiatica (TA) (L.) Lam. (Rutaceae), also known as Wild Orange tree, is a green leafy climber growing in the evergreen forests. It is commonly known as Lopez root, forest pepper, wild orange tree (English); Kanchanah, Tiksnaksah, Dahana (Sanskrit); Jangli Kali Mirch, Kanch, Dahan (Hindi) Konda Kasinda, Vana Kasinda, Mulla Kasinda, Erra Kasinda, Mirapakanda, Mulla Morinda (Telugu); Milagaranai, Kattumilagu (Tamil). It is vastly distributed in the tropical regions of Africa, Asia and Madagascar. *Toddalia asiatica*, has been in folklore use in India and China from 18th century. *Toddalia asiatica* (Linn), belongs to family Rutaceae. *Toddalia asiatica* is one such resource being threatened. *Toddalia asiatica* commonly grows in forests, near anthills in dry areas like the tropics, near rivers or streams and grows fairly well in clay soils (Meyer, 2005). *Toddalia asiatica* has been in folklore use in India and China from 18th century. *Toddalia asiatica* is commonly known as Lopez root, forest pepper and wild orange tree. T*oddalia asiatica* is an important plant used for the treatment of a range of diseases like cough, malaria, ingestion, influenza, lung diseases, rheumatism, fever, stomach ailments, cholera and diarrhea. Coumarine derivatives with anti plasmodial and antimicrobial activity have been isolated from its leaves (Malmoori *et al*., 2008).

Toddalia is a monotypic genus of flowering plants in the citrus family containing the single species T*oddalia asiatica*, which is known by the English common name orange climber. It is native to many countries in Africa and Asia. It grows in forested riparian habitat with high rainfall. The destruction of forest habitat in Africa threatens the species' survival. This is a liana with woody, corky, thorny stems that climb on trees, reaching up to 10 meters in length. It has shiny green citrus-scented leaves, yellow-green flowers, and orange fruits about half a centimeter wide that taste like orange peel. The seeds are dispersed by birds and monkeys that eat the fruits. In particular, the Scaly-breasted Munia prefers to nest in these trees. Extracts of the plant have demonstrated antiviral activity against H1N1 influenza in the laboratory. The harvest of this slow-growing plant from the wild for medicinal use may cause its populations to decline. Protocols for domestication or propagation of the tree are being researched. As per literature survey, there is no earlier report revealed that the antioxidant activity of various extracts of fruit of *Toddalia asiatica*. Hence, the attempt made on evaluation of *in vitro* antioxidant potential of *Toddalia asiatica*.

2. Materials and Methods

The 2,2‟-azinobis-(3-ethyl benzo thiazoline-6-sulfonic acid) (ABTS+), 2,2-diphenyl-1-picryl hydrazyl (DPPH) were purchased from Sigma Aldrich, USA. Gallic acid, ascorbic acid. The 2-deoxyribose and naphthyl ethylene diamine dihydrochloride (NEDD) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai. Thio barbituric acid (TBA) and trichloroacetic acid

(TCA), reduced nicotinamide adenine dinucleotide (NADH), nitrobluetetrazolium (NBT) and phenazine methosulphate (PMS) were purchased from S.D. Fine-Chem Pvt. Ltd., Mumbai. All other chemicals and solvents were purchased from Ranbaxy Laboratories Pvt. Ltd., New Delhi.

Standard antioxidants

The standard antioxidants used in the study namely Gallic acid, BHC and ascorbic acid were dissolved in double distilled water. BHT was dissolved in methanol and the *Toddalia asiatica* in DMSO.

Radical – scavenging activity

Superoxide anion scavenging activity

Superoxide anion scavenging activity of *Toddalia asiatica* was determined by the method of Nishimiki *et al*. (1972) with modifications. The assay was based on the oxidation of NADH by phenazine methosulphate (PMS) to liberate PMS red. PMS red converted oxidized nitro bluetetrazolium (NB Toxi) to the reduced form NBT red, which formed a violet colour complex. The colour formation indicated the generation of superoxide anion, which was measured spectrophotometrically at 560 nm. A decrease in the formation of colour after addition of the antioxidant was a measure of its superoxide radical scavenging activity**.**

Nitric oxide radical inhibition assay

The nitric oxide radical inhibition activity of *Toddalia asiatica* was measured by the method of Garrat (1964). Sodium nitro prusside in aqueous solution at physiological pH spontaneously produces nitric oxide which interacts with oxygen to produce nitrite ions. This can be estimated using Griess reagent.

DPPH radical scavenging assay

The radical scavenging activity of *Toddalia asiatica* against DPPH was determined by spectrophotometrically by the method of Brand Williams *et al*. (1995). DPPH is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in color (from deep violet to light yellow) was measured. The intensity of the yellow colour depends on the amount and nature of radical scavenger present.

Total polyphenolic content

Total phenol present in the *Toddalia asiatica* extract was determined using Folin-Ciocalteau reagent by the method of Singleton and (Singleton and Rossi, 1965) using Gallic acid as the standard.

Statistical analysis

All the values were expressed as means ± SD of 6 determinations. The data were statistically evaluated by ANOVA. Significance was set at $p < 0.05$.

3. Results and Discussion

The results obtained in the present study show that TA fruit extracts can effectively scavenge ROS and free radicals including superoxide radical, nitric oxide radical and DPPH *In vitro*. The superoxide anion scavenging ability of various fraction of TA and standard antioxidants such as gallic acid are presented in Figure - 1. The concentration of the antioxidants was from 100 to 500 µg/ml. The inhibition was observed to be concentration-dependent. Gallic acid inhibited superoxide production by about 60 % when added to the reaction mixture even at a very low concentration. The superoxide anion scavenging ability of TA (80.60 %) at 500 µg/ml was comparable with that of gallic acid (84.69 %). Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that could be generated; it also has the ability to change to other harmful reactive oxygen species and free radicals within the living cells (Phaniendra *et al.*, 2015). Superoxide dismutase (SOD) is the cellular antioxidant enzyme, which removes this ubiquitous superoxide metabolic product by converting it into hydrogen peroxide and oxygen in biological systems.

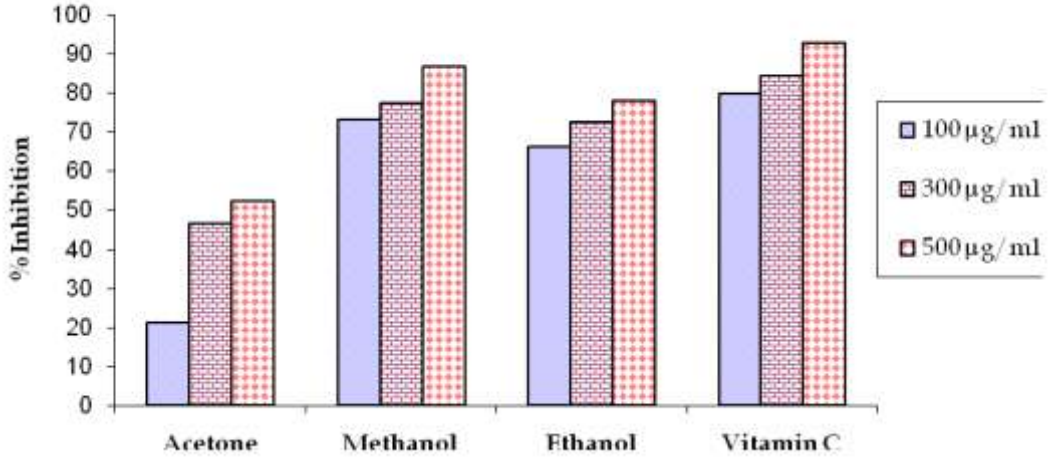

Figure - 1: Supperoxide scavenging activity of *Toddalia asiatica*

Values are mean ± SD of six determinants

The Phenolic compound present in the various fractions of TA and standard antioxidants such as gallic acid and ascorbic acid effectively scavenged superoxide in a concentration-dependent manner. TA showed greater inhibition of superoxide radical production in a dose response manner when compared standard antioxidants used in this study.

The DPPH radical has been used widely as a model system to investigate the radical scavenging activities of several natural compounds occurring in plant extract including phenolic compounds, flavonoids. The effect of antioxidants on DPPH was thought to be due to their hydrogen donating ability (Prathapan *et al*., 2010). The DPPH radical scavenging abilities of the TA extracts are observed in all the extracts under study in a concentration dependent manner. They are significantly comparable to that of Gallic acid (100 %) showing that the extracts have proton-donating ability and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants. It is clear that the antioxidant activity of TA extracts in DPPH assay increased proportionally to the polyphenol content.

In this study, TA and other antioxidants were tested for scavenging activity against $DPPH^{\bullet}$ radicals. The percentage of inhibition was higher for that of TA than known antioxidants. The percentage of inhibition of TA was 79.40 %, gallic acid 85.77 %. The result of DPPH scavenging activity assay in this study indicates that the extract was potently active. The ability of extract to scavenge DPPH could also reflect its ability to inhibit the formation of $ABTS^{+}$. The scavenging activity of $ABTS^{+}$ radical by the extract was found to be significant. This implies that the plant extract may be useful for treating radical-related pathological damage.

The results of total phenolic content of different fruit extracts of TA were given in Table - 1. The total phenol content in the fruits extracts (Acetic acid, Methanol and Ethanol) expressed as gallic acid equivalent (GAE) was in the range of 1.54 to 3.84 mg GAE/g dw. Methanolic extract had the highest content as 3.84 mg GAE/g dw, whereas acetic acid contained a much smaller amount as 1.54 mg GAE/g dw. Plant polyphenol are very important constituents because of their multiple biological effects and direct contribution to antioxidative activity (Lee *et al*., 2002). The results of our study reveal that there is a strong coincidence between antioxidant activity and phenolic content. Several studies on total phenolic content had been published over the years demonstrating its importance in the medicinal field (Abdalbasit *et al*., 2009).

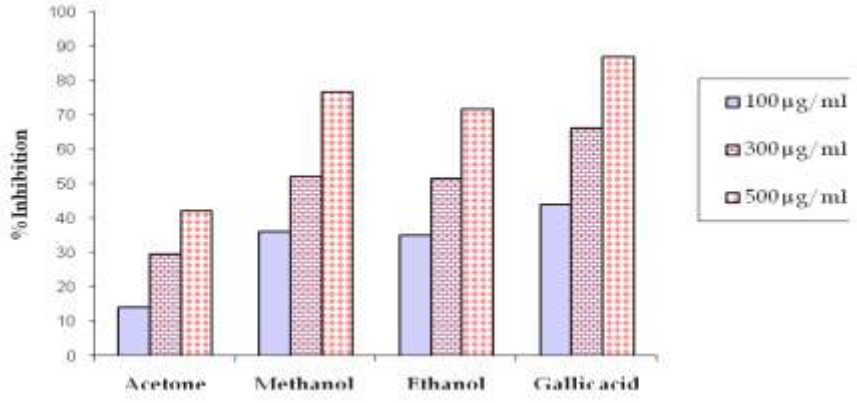

Figure – 2: DPPH Assay of *Toddalia asiatica*

Values are mean ± SD of six determinants

Table - 1: Total pholyphenol content of *Toddalia asiatica*

Extract	Pholyphenol[a]
Acetone	1.75 ± 0.93
Methanol	3.75 ± 0.23
Ethanol	2.45 ± 0.13

a: Gallic acid equivalents mg/g dw plant material respectively; Results represented in means ± standard deviation (n =6).

Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3 and NO_2 are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. All the extracts of TA showed significant nitric oxide radical scavenging activity. The 500 µg/ml of methanolic extract showed more NO scavenging activity (85.54 %), while standards ascorbic acid 91.80 %. This indicates higher scavenging potential for TA. Sayyed *et al.* (2011) demonstrated that plant derived polyphenols are excellent nitric oxide scavengers. Our results on the nitric oxide scavenging ability of TA might be due to the presence of polyphenol present in the extract. The various extract of TA show significant reducing power activity with compared to standard antioxidant. The reducing power of Methanolic extract of TA was found to be 79.2 % followed by standard antioxidant BHA (88.55 %).

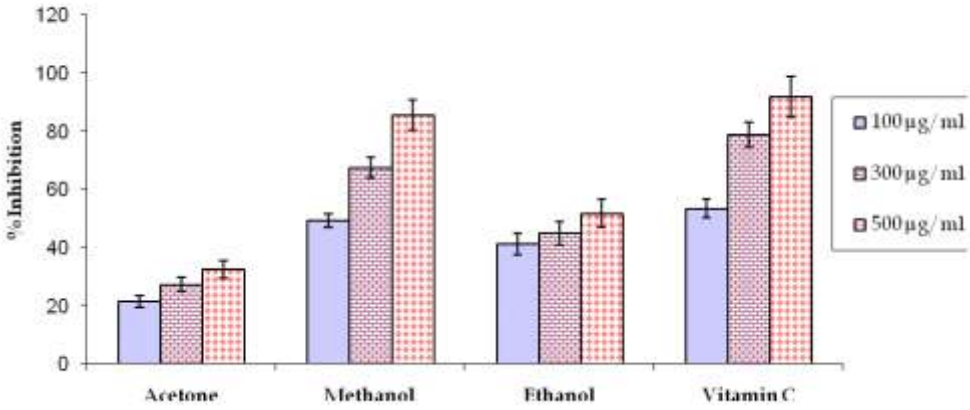

Figure - 3: NO scavenging activity of TA

Value mean ± SD of six determinants

Plants with antioxidant activities have been reported to possess free radical scavenging activity (Das and Pereira, 1990). Free radicals are known as a major contributor to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a

result of deficient natural antioxidant defense mechanism (Parr and Bolwell, 2000). Therefore, the reductive capabilities in terms of $Fe3^{+}$ - $Fe2^{+}$ transformation were measured in the presence of TA with standard antioxidant. Like the antioxidant activity, the reducing power of antioxidants increased with increasing concentration of samples. The methanolic extract shows more potent reducing power than that other extract. But they also have certain reducing power. The reducing power of TA might be due to the presence polyphenolic content present in the extract.

4. Conclusion

In conclusion, the TA fruit extracts showed significant antioxidant activity. But, it was found that methanolic (500 µg/ml) extract shows more potent antioxidant as similar to standard. Hence, it is expected TA could be useful in treating disease related to oxidative stress and gives an idea for the further studies.

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