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Original Article

IN VITRO ANTIOXIDANT AND ANTICANCER POTENTIAL OF FLOWERS OF TODDALIA ASIATICA (RUTACEAE)

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ABSTRACT

Objectives: The aim of this research was to evaluate the phytochemical, antioxidant and anti cancer property of the methanol extract of flowers of *Toddalia asiatica.*

Methods: The phytochemical screening of the methanol extract of flowers of *Toddalia asiatica* was performed for carbohydrates, glycosides, cardiac glycosides, terpenoids, triterpenoids, phenols, phytosteroids, phlobatannins, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests. The total antioxidant capacity was evaluated by an array of experiments such as FRAP (Ferric reducing antioxidant power assay), DPPH (2,2-diphenyl-1-picrylhydrazyl), NO (Nitric oxide) and SOD (Superoxide) assays. The annihilation activity of free radicals was calculated in % inhibition and absorbance. The cytotoxicity and cell viability was calculated by using MTT (Microculture Tetrazolium Assay) colorimetric assay.

Results: On quantification, the total phenol and flavonoids content were estimated to be 99.8% and 85.3% respectively. The DPPH study revealed that the methanol extract of flowers of *Toddalia asiatica* has high antioxidant activity about 82.7% when compared with the positive control (Ascorbic acid). Anticancer activities were assayed with standard MTT colorimetric procedure against MCF-7 cell lines. From the analysis, it was found that *Toddalia asiatica* showed 66% of cell death in MCF-7 cell line at 500µg/ml tested dose.

Conclusion: *Toddalia asiatica* flowers extract demonstrated the presence of secondary metabolites with potential antioxidant and cytotoxic activities.

Keywords: *Toddalia asiatica*, Phytochemicals, Antioxidant, MTT assay.

INTRODUCTION

India is endowed with rich wealth of medicinal plants. India recognizes more than 2500 plant species which have medicinal values [1]. Herbal medicines have become more popular in the treatment of many diseases due to popular belief that green medicine is safe, easily available and less side effects [2]. *Toddalia asiatica* (L) Lam. (Rutaceae) is commonly called ''Wild orange tree or Forest Pepper'' which is found in South Africa, Sri Lanka, South India, Western Nilgiri, Palani hills and Tirunelveli district [3]. All parts of the plant are claimed to have medicinal value. The bioactive compounds like alkaloids, flavonoids, tannins and phenolic compounds are responsible for the medicinal value of the plant which produces a definite physiological action on the body [4]. All parts of the plant are used to treat sprains, contusions, cough, malaria, dysentery, gastralgia and furuncle infections. It is also used in the treatment of paralysis, intermittent fever, dyspepsia, bronchitis, nausea and arthritis. The root bark is bitter, astringent, digestive, carminative, constipating, antidiarrhoeal, and diuretic. The fruits are used for cough and throat pain relief. The extracts of the plant have been reported to possess skin whitening property, antifeedent, spasmolytic, wound healing, anti microbial, anti-inflammatory and analgesic activities [5-9].

Based on its medicinal value and presence of rich bioactive secondary metabolites the present study was focused to analyse the Phytochemical screening, Anti-oxidant and Anticancer properties of methanol extract of flowers of *Toddalia asiatica.*

MATERIALS AND METHODS

Chemicals and reagents: Chemicals used in the study were of analytical grade and procured from Merck India Pvt. Ltd

Plant material

The flowers of *Toddalia asiatica* collected from Thandarai, Kanchipuram district, TamilNadu, India and were identified and authenticated by Prof. N. Raaman, Centre for Advanced Studies in Botany, University of Madras.

Preparation of plant extract

The collected plant was subjected to shade drying and the dried plant material was powdered mechanically. The powdered material was extracted with methanol at room temperature for 72 h. The extract was filtered through Whatman No. 1 filter paper and concentrated to dryness. The final residue obtained was then used for further analysis.

Phytochemical screening

Methanolic extract of flowers of *Toddalia asiatica* was subjected to preliminary phytochemical screening for the detection of various phyto constituents.

Qualitative phytochemical analysis

Chemical tests for the screening and identification of bioactive chemical constituents in the medicinal plants under study were carried out in extracts using the standard procedures as reported by Sofowara 1982 [4], Trease and Evans 1989 [5] and Harborne1973 [6].

Antioxidant assay

Determination of DPPH scavenging assay

DPPH radical scavenging activity of methanolic extract was determined according to the method reported by Blois [10]. An aliquot of 0.5 ml of sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the formula.

$$\% \text{ of inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Iron chelating activity (FRAP)

The method of Benzie and Strain (1996) [11] was adopted for the assay. The principle is based on the formation of *O*-Phenanthroline-Fe^{2+} complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations of methanol extract of *Toddalia asiatica* ranging from 10 to 500µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in duplicates. Vitamin C was used as positive control.

Superoxide dismutase (SOD) activity

SOD activity was assayed by determining the inhibition rate of nitro blue tetrazolium reduction with xanthine oxidase as a hydrogen peroxide generating agent [12]. The extract (500 µl of 0.1mg/ml) and allopurinol (100 µg/ml) (in methanol) is mixed with 1.3 ml phosphate buffer (0.05M, pH 7.5) and 0.2 ml of 0.2 units/ml xanthine oxidase solution. After 10 min of incubation at room temperature (25°C), 1.5 ml of 0.15 M xanthine substrate solution is added to this mixture. The mixture is again incubated for 30 min at room temperature (25°C) and then the absorbance is measured at 360 nm using a spectrophotometer against blank (0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase) and the values are expressed as percentage of SOD levels. Vitamin C was used as positive control.

Nitric oxide radical scavenging assay

To 1 ml of various concentrations of methanolic extract taken in different test tubes, 0.3 ml of sodium nitroprusside (5 mM) in phosphate buffer was added. The test tubes were incubated at 25 °C for 5 hr after which, 0.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dichloride and 3% phosphoric acid) was added. The absorbance of the chromophore was read at 546 nm. The experiment was performed in triplicate [13]. Vitamin C was used as positive control.

Cytotoxicity analyses of methanol extract of *toddalia asiatica* on mcf 7 (human breast cancer) cell lines

Cell growth inhibition by MTT assay

The MCF-7 cells were plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 h, the cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37 °C. After starvation, cells were treated with different concentrations of methanolic extract (10-500µg/ml) for 24 h. At the end of the treatment period the medium was aspirated and serum-free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37 °C in a CO_2 incubator. The 50% inhibitory concentration value (IC_{50}) of the crude extract was identified for normal fibroblast cell line.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graphpad prism 5 software.

RESULTS

Phytochemical screening

The Phytochemical screening and quantitative estimation of secondary metabolites of *Toddalia asiatica* showed that the flowers are rich in alkaloids, phenols and flavonoids. The results are summarized in Table-1. Maximum levels of phenols were found in *Toddalia asiatica* (99.8%). Phenols are secondary metabolites. Many of the phenolic have been shown to contain higher levels of antioxidant activities. Antioxidants provide chemical protection for biological systems against harmful effects of reaction or process that cause excessive oxidation, protein and DNA damage and cell death. The levels of flavonoids were found to be 85.3% in *Toddalia asiatica*. Flavonoids are reported to possess many useful properties, including anti-inflammatory, antimicrobial, enzyme inhibition, oestrogenic, anti allergic, antioxidant and anti-tumour activity.

Table 1: Qualitative and quantitative estimation of Phytoconstituents of methanol extract of *Toddalia asiatica*

S. No.	Phytochemicals	Methanol extract
1	Alkaloids	18 µg/ml (0.29)
2	Steroids	-
3	Phenols	99.8%
4	Flavonoids	85.3%

Antioxidant assay

In vitro antioxidant assay was performed with the methanol extract, showed concentration dependent increase in inhibition up to 500 µg/mL using ascorbic acid as the standard. *Toddalia asiatica flowers* have better DPPH activity by photometric assay. DPPH is usually used as a substrate to evaluate anti-oxidative activity of antioxidant. This method is based on the reduction of methanol DPPH solution in the presence of hydrogen donating antioxidant due to the formation of the non radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow colored diphenyl picrylhydrazine. It appears that extracts of *Toddalia asiatica* possess hydrogen donating abilities to act as an antioxidant.

The ability of plant extract to reduce ferric ions was determined in FRAP assay. In FRAP assay the reducing power was observed over the concentration range 20 – 500 µg/ml. O-phenanthroline quantitatively forms complexes with Fe+2 which get disrupted in the presence of chelating agents [14]. The methanol extract interfered with the formation of a ferrous-o-phenanthroline complex, thereby suggesting that the extract has metal chelating activity. SOD activity was assayed by determining the inhibition rate of nitro blue tetrazolium reduction with xanthine oxidase as a hydrogen peroxide generating agent. The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic superoxide radical (0_2), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I. N. T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction Fig.4 showed the superoxide inhibition values of *Toddalia asiatica* and standard were 79.7%, 65.1% respectively. Results obtained emphasized the capacity of *Toddalia asiatica* extracts to annihilate the superoxide anions generated through NBT system. The nitric oxide scavenging assay showed the inhibitory concentration of methanolic extract quite equivalent to standard (fig. 4). The result indicated that the extracts might contain compounds able to compete with oxygen to react with NO and thus inhibit the generation of the nitrite and peroxy nitrite anions.

Cell line studies

The MTT assay is a rapid and highly accurate colorimetric approach that widely used to determine cell growth and cell Cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan. The toxicity and cell viability of methanol extract of flowers of *Toddalia asiatica* had been shown in fig. 5.

Table 2: Antioxidant profile of *Toddalia asiatica* methanol extract expressed in % of scavenging

Parameter	Conc µg/ml	Methanol extract	Positive control (Vitamin C)
DPPH scavenging assay	500	82.7	72.1
Iron chelating activity (FRAP)	500	72.1	92.6
Superoxide dismutase	500	79.7	65.1

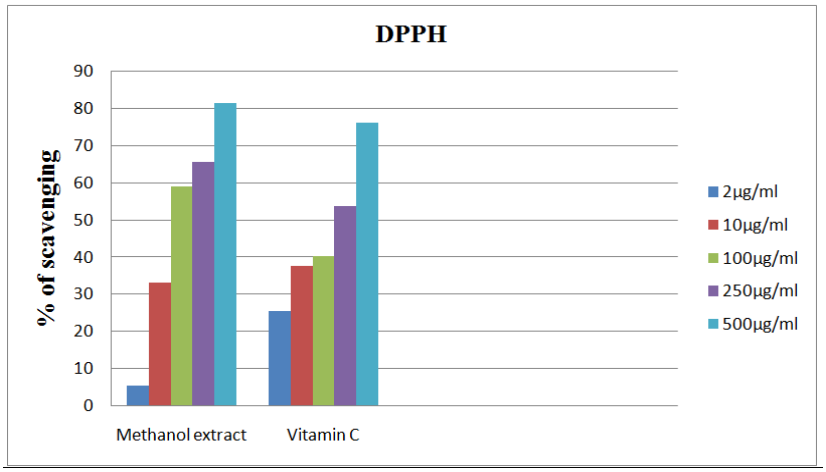

Fig. 1: DPPH scavenging assay of methanol extract of *Toddalia asiatica*

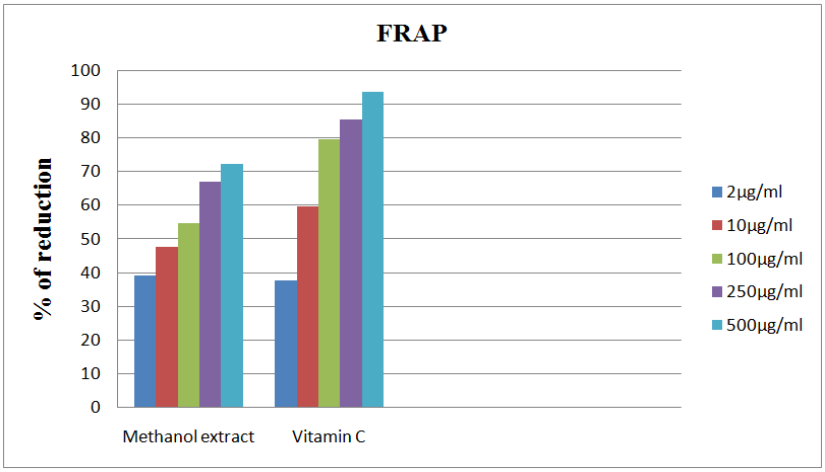

Fig. 2: Iron chelating activity (FRAP) of methanol extract of *Toddalia asiatica*

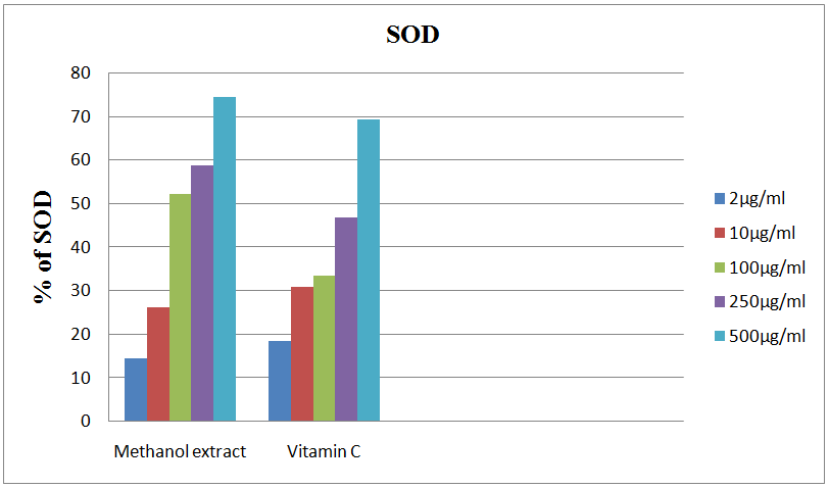

Fig. 3: Superoxide dismutase activity (SOD) of methanol extract of *Toddalia asiatica*

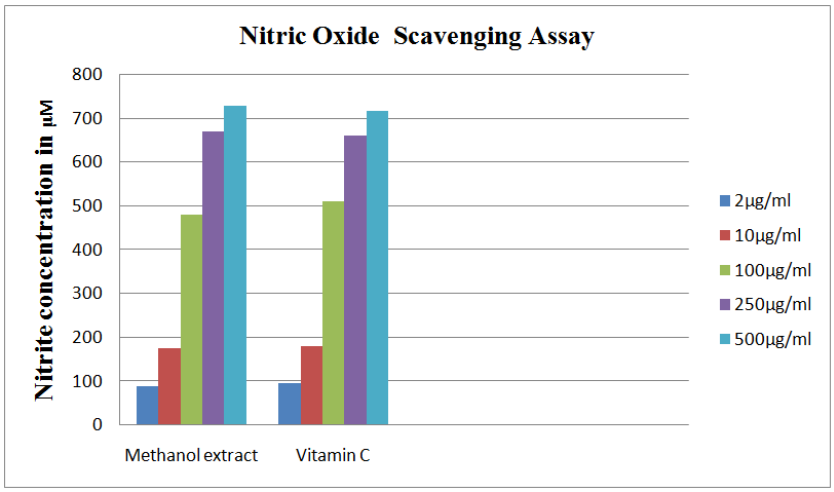

Fig. 4: Nitric oxide scavenging assay of methanol extract of *Toddalia asiatica*

Table 3: Cytotoxicity of methanol extract of flowers of *Toddalia asiatica* on MCF7 cell line

S. No.	Concentration, µg/mL	Optical density	% of cell death
1	10	0.263	18.63
2	50	0.273	19.36
3	100	0.461	32.67
4	250	0.686	48.63
5	500	0.933	66.12

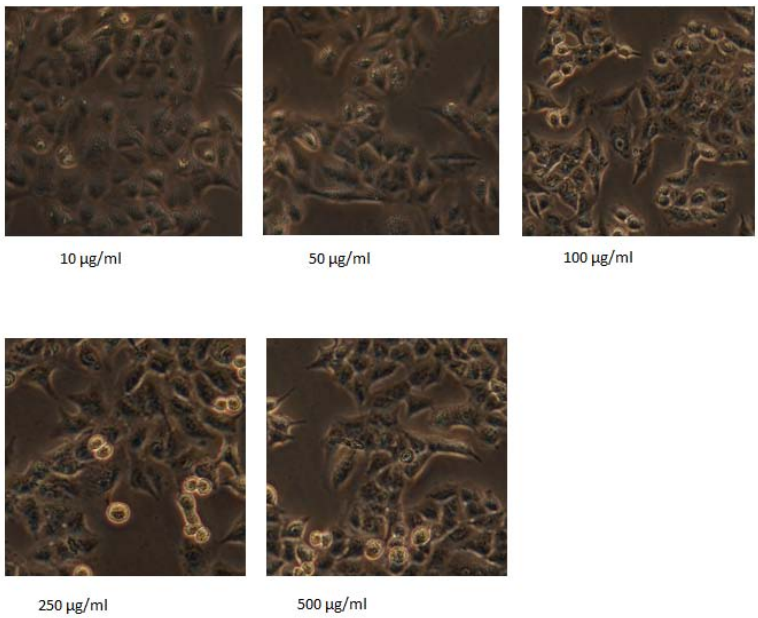

Fig. 5: Morphological representation of Cytotoxicity of methanol extract of leaves of *Toddalia asiatica* on MCF7 cell line

DISCUSSION

The preliminary phytochemical tests result indicates the presence of alkaloids, flavonoids and phenols (table 1). The presence these phytochemical constituents indicate that the *Toddalia asiatica* can be used in a multitude of ways which will be beneficiary to the population. Alkaloids play some metabolic role and control development in the living system which is involved in protective function in animals and the steroidal alkaloids are used as medicine [15]. Flavonoids (specifically flavonoids such as the catechins) are "the most common group of poly phenolic compounds in the human diet and are found ubiquitously in plants.

Flavonoids have been shown to have a wide range of biological and pharmacological activities in invitro studies [16]. In the present investigation, the different methods of in vitro assays were performed to evaluate the antioxidant activity of methanol extract of flowers of *Toddalia asiatica.* DPPH radical scavenging activity of different concentration of methanolic extract of *Toddalia asiatica* is presented in fig.1. The reducing ability of the antioxidants present in the methanolic extract of *Toddalia asiatica* is determined by the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction [17]. The extract has significant scavenging

effect on DPPH and it increased with the increasing concentrations from 2 – 500 µg/ml and at 500 µg/ml concentration the extract was higher than that of standard. This study shows that the extraction of *Toddalia asiatica* has Phytochemicals which have the very high antioxidant activity which might be helpful in preventing diseases induced by oxidative stress. From the table 2 it is revealed that the methanolic extract has high antioxidant activity of about 82.7%. Fig. 2 represents the iron chelating activity of different concentrations of the methanolic extract of *Toddalia Asiatica* which showed an increase in activity with increasing concentration of methanolic extract. Fe^{2+} ion chelating ability was found to be 72.1% at 500 µg/ml concentrations (table 2).

Percentage scavenging of superoxide anion examined at different concentrations of methanolic extract of *Toddalia Asiatica* (2 – 500 µg/ml) is given in Fig. 3. The scavenging activity was found to increase with increase in concentration. The maximum scavenging activity of methanolic extract at 500 µg/ml concentration was found to be 79.7% (table 2). Superoxide scavenging ability of methanolic extract might primarily be due to the presence of flavanoids [18]. The nitric oxides radical inhibition study showed that *Toddalia asiatica* methanol extract was a potent scavenger of nitric oxide (Fig. 4). *Toddalia asiatica* methanolic extract inhibited nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis.

The cytotoxic effect of different concentrations of methanolic extract of *Toddalia asiatica* was evaluated by MTT assay and presented in Fig. 5. MTT assay is a well established in vitro method for assessing Cytotoxicity against cancer cell lines. From the table 3 it is clear that the methanolic extract caused various morphological changes in MCF-7 cells, depending on the concentration administered. Particularly in 500 µg/mL concentrations, the percentage of cell death was observed as 66%. The overall result indicates that the methanol extract of flowers of *Toddalia asiatica* showed promising baseline information for the potential use as an anti cancer agent.

CONCLUSION

This study suggested that the methanol extract of *Toddalia asiatica* possesses high anti-oxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related disorders. Moreover it has potent anti-cancer properties against MCF-7 cell lines. Further studies can be done to identify the active principles to establish the in vivo activities. In an overview of the bioactivity data obtained from the current investigation, it can be highlighted that the tested extracts have many phytoconstituents. Bioactive substances from this plant can therefore be employed in ethnomedicine. Determination of respective antimicrobial potential and toxicological evaluation of these extracts with the view to formulate novel chemotherapeutic agents to be used in future is worth mentioning. As a therapeutic source, the standardised study is warranted in order to exhibit *Toddalia asiatica* as an effective medicinal plant in the near future.

CONFLICT OF INTERESTS

Declared None

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