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IN VITRO AND *IN VIVO* ANTIOXIDANT EVALUATION AND ESTIMATION OF TOTAL PHENOLIC, FLAVONOID CONTENT OF *SYZYGIUM ALTERNIFOLIUM* LEAVES.

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ABSTRACT

Syzygium alternifolium walp. (Myrtaceae) has been traditionally used for the treatment of various ailments such as stomach ache, ulcers, rheumatism and diabetes. Preliminary phytochemical screening revealed the presence of phenols and flavonoids. Thus the total phenolic, flavonoids contents, *in vivo* and *in vitro* antioxidant potential were evaluated from the ethanolic extract of *Syzygium alternifolium* leaves. The antioxidant activity was determined by *in* vivo methods such as LPO, GSH and catalase levels and in vitro methods such as DPPH scavenging assay, Nitric oxide assay. The extract exhibited potential antioxidant activity in both in vivo and in vitro studies, associated with the total phenolic and flavonoid contents. The percentage inhibition of the extract followed dose-dependency and found significant ($P < 0.001$) as compared to standard (ascorbic acid) for DPPH and NO. The extract showed a significant ($P < 0.001$) increase in GSH levels and serum catalase activity and decrease in LPO levels when compared with the control group. Hence Syzygium alternifolium can be considered as a potent antioxidant in comparision with satandard Ranitidine in a dose dependent manner.

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INTRODUCTION

Oxygen free radicals are detrimental to the integrity of biological tissues and mediate their injury. The mechanism of damage involves lipid peroxidation, which destroys cell membranes with the release of intracellular components such as lysosomal enzymes leading to further damage, the radicals also promote mucosal damage by causing degradation of the cell metabolism and deoxyribonucleic acid damage [1]. The extracts of several plants have been used as therapeutic agents. Spices and herbs are recognised as source of natural antioxidant that can protect from oxidative stress and thus play an important role in the chemo prevention of disease resulting from lipid peroxidation [2]. The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but they may be also influenced by other organic and inorganic compounds such as coumarins, phenolic aids and antioxidant micronutrients, e.g., Cu, Mn and Zn[3]. Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups [4]. The phenolics content may contribute directly to the antioxidative action [5]. It has been suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans [6]. Consequently, the antioxidant activities of plant/herb extracts are often explained by their total phenolics and flavonoid contents with good correlation.

Syzygium alternifolium walp. (Myrtaceae) native to North Arcot, Cuddapah, Kurnool, and the Nagari hills, in eastern Chittoor district, India [7,8]. The juice of the fruits is used to cure stomach ache and ulcers while the external application of the fruit pulp reduces rheumatic pains [9]. The fruit pulp and alcoholic extract of seeds posses anti diabetic properties. The antihyperglycemic activity activity of *Syzygium alternifolium* seed was compared with the treatment of glibenclamide [10]. The juice of fresh leaves and pulp of tender shoots are used to treat bacillary dysentery [11]. The leaves of *Syzygium alternifolium* are reported to contain flavonoids like sitosterol, (+) pintol, sideroxylon, sizalterin and 6.8-di C-methyl flavones [12].

A variety of inherent antioxidants like superoxide dismutase (SOD), reduced glutathione, catalase and peroxidase are present in the organism, which protect them from oxidative stress forming the first line of defence system [13]. Reactive oxygen species have been contributed in the aetiology and pathophysiology of gastrointestinal inflammation and gastric ulcers [14]. Therefore, there is a need for agents to minimize and repair free radical induced damage. The antioxidants play a crucial role in these defence process. Potent antioxidant formulations have been reported to be effective in the healing and cytoprotection of the experimentally induced peptic ulcers [15]. The present study was performed to establish the correlation of antioxidant activity by *in vitro* methods such as DPPH scavenging assay, Nitric oxide assay and *in vivo* methods such as lipid peroxidation (LPO), GSH and catalase levels with the previously reported anti ulcer potential [16] of *Syzygium alternifolium* leaf extract.

MATERIALS AND METHODS

Drugs and chemicals

2,2´-diphenyl-1-picrylhydrazyl, sodium nitroprusside, Griess reagent, sulfosalicylic acid were obtained from Sigma-Aldhrich chemical, Bangalore, India. Thiobarbituric acid, Folin-ciocalteu's reagent, ascorbic acid and trichloroacetic acid, were purchased from Hi-Media Pvt. Ltd., Bombay, India. All other chemicals and reagents used in this study were of analytical grade.

Plant Material

The leaves of *Syzygium alternifolium* were collected from the local area of Tirupati during the month of July. The plant was identified and authenticated by Dr. Madhava Setty,Sri Venkateswara University,Tirupati, where the voucher specimen was deposited for future reference.

Preparation of extract

Fresh leaves were collected, shade dried and powdered mechanically. About 150gm of the leaf powder was extracted with 1000ml of 95% ethanol by reflux heating over water bath. The extract was concentrated in Vacuo and then air dried at room temperature, weighed and percentage yield was calculated and stored in air tight container in a desscicator until used.

Phytochemical screening

Preliminary phyto chemical screening of the powdered leaves was performed for the presence of flavanoids, tannins and poly phenols.

Experimental animals

Albino rats of wistar strain of either sex weighing between 150-200 gms were used. They were housed in polypropylene cases at room temperature (25 ± 2^{0} c) and provided food and water *ad libitum.* The ethical approval was permitted from Sri Sivani College of Pharmacy, Srikakulam, Andhra Pradesh and the approval no is 1427/PO/a/11/CPCSEA .

Acute oral toxicity studies and Gross behavioural changes

Healthy adult male albino rats were fasted overnight with free access to drinking water. They were divided into five groups each consisting of six animals. Group-1 animals were treated with distilled water (2ml/kg/p.o) and Group-2 to Group-5 animals received 500 mg, 1 gm, 2 gm, 4 gm/kg/p.o of ethanolic extract of leaves of *Syzygium alternifolium* respectively. The animals were observed continuously for 2 hours, then intermittently and at the end of 24 hours. The numbers of the deaths were noted to calculate LD_{50} [17]. The animals were observed for behavioural, neurological and autonomic profiles during acute toxicity studies [18].

Estimation of total phenolic content and total flavonoid content

The total phenolic content of extracts was measured using the Folin-Ciocalteu method [19]. To the 0.3 mL sample extract Folin-ciocalteu's reagent (1.5 mL, 10% v/v) and 1.2 ml 7.5% w/v Na_2CO_3 were added. The reaction mixture was thoroughly mixed and was incubated in the dark for 30 min. The absorbance of the reaction mixture was then measured at 765 nm.

The total flavonoid content of ethanolic extract was measured using the aluminium chloride [20]. Ten milligram of quercetin was dissolved in 80% ethanol and then diluted to 25, 50, and 100 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm by a UV-visible spectrophotometer. The amount of 10% aluminum chloride was substituted for the same amount of distilled water in blank.

IN VIVO ANTIOXIDANT STUDIES

Estimation of lipid peroxidant from PMS

0.5ml of TCA was added to 0.5ml PMS and incubated at 37°C for 2 hrs. To this added 1ml of ice cold TCA and centrifuged at 1000 rpm for 10 minutes. From the above, 1ml of supernatant was taken and 1ml of TBA was added, tubes were kept in boiling water bath for 10 minutes. The tubes were removed and brought up to room temperature and 1ml of distilled water was added. Absorbance was measured at 532nm [21].

Estimation of reduced glutathione from PMS

1.0ml of PMS was precipitated with 1.0ml of sulphosalicylic acid. The samples were kept at 4°C for at least 1 hour and then subjected to centrifugation at 1200 rpms for 15 minutes at 4°C. From the above,0.1ml supernatant was taken, to this added phosphate buffer and made total volume of 3.0ml. The yellow colour developed was read immediately at 412nm on a spectrophotometer [22].

Estimation of catalase from PMS

3ml of H_2O_2 Phosphate buffer was mixed with 0.05ml of PMS for Test sample and Blank was prepared by mixing 0.05ml of PMS with Phosphate buffer. Absorbance was measured for both blank and test at 240nm for 2 minutes with 60 seconds interval. Catalase activity was calculated in terms of k/minutes [23].

IN VITRO ANTIOXIDANT STUDIES

Assay for Nitric Oxide scavenging activity

The reaction mixture (3ml) containing sodium nitroprusside (10µM) in Phosphate buffer and various concentrations of drug i.e., 10,20,40,80,100 µg/ml of ascorbic acid were incubated at 25°C for 120 minutes. Control without test compound kept in identical manner. After incubation 0.5ml of the incubation solution was removed and diluted with 0.5ml of Griess reagent. The absorbance of the chromophore was read at 546nm [24]. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

$$\text{NO scavenged (\%)} = A_{control} - A_{test} / A_{control} * 100$$

Diphenyl-1-Picrylhydrazyl(DPPH) scavenging effect

0.1mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 3ml of extract solution in water at different concentrations i.e. 10,20,40,80,100 µg/ml. After 30mins absorbance was measured at 517nm. Lower absorbance of the reaction mixture indicates free radical scavenging activity [25]. The capability of scavenging the DPPH radical was calculated using the formula

$$\text{DPPH scavenged (\%)} = A_{control} - A_{test} / A_{control} * 100$$

STATISTICAL ANALYSIS

The results are expressed as mean ±SEM. Comparison between the treatment groups and control were performed by Two way analysis of variance (ANOVA) followed by Turkey multiple comparison. Statistical significance was set accordingly.

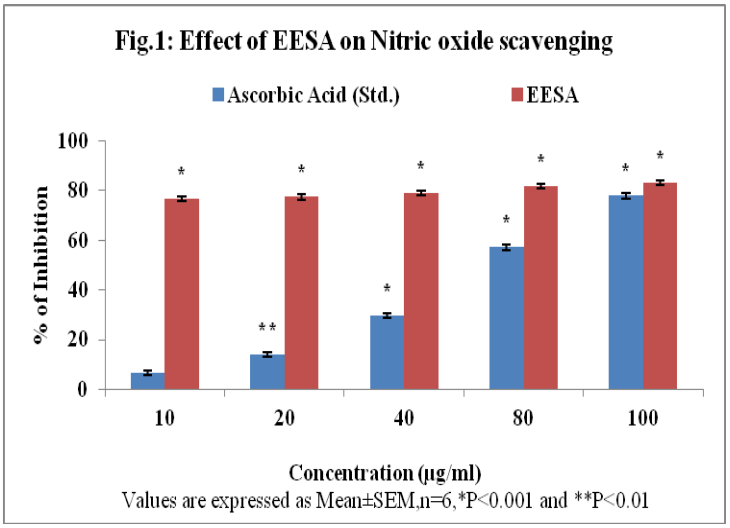
RESULTS

The total phenolic content in the ethanolic extract of *Syzygium alternifolium* was 27.44 ± 0.55 mg/g while the flavonoid content was 18.72 ± 0.41 mg/g. These results demonstrate that flavonoids represent the main group of phenolic compounds in *Syzygium alternifolium* leaves.

IN VITRO ANTIOXIDANT PARAMETERS

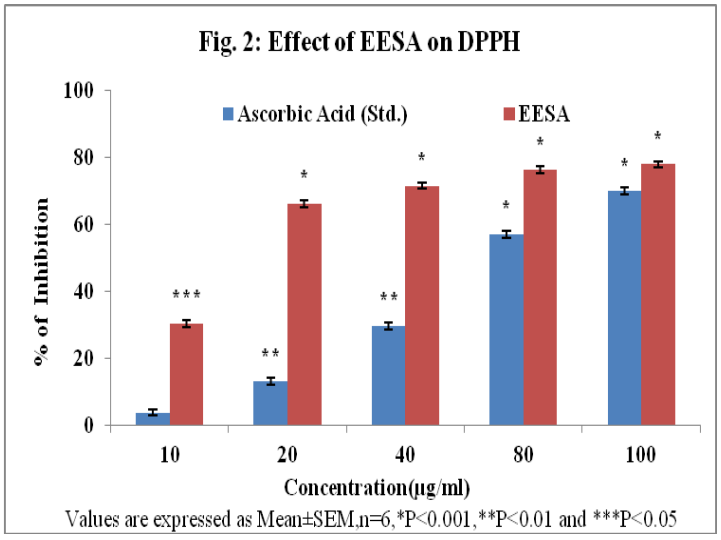
Nitric oxide scavenging

The ethanolic extract of *Syzygium alternifolium* showed promising free radical scavenging action against NO induced release of free radicals at the concentration 100 µg/ml showing 83.0±0.74% of NO inhibition. The reference standard ascorbic acid also demonstrated significant ($p<0.001$) radical scavenging potential in the concentration of 100 µg/ml (77.8±0.19). The free radical scavenging effect of NO was observed in a concentration dependent manner (Fig.1).



DPPH scavenging

The ethanolic extract of *Syzygium alternifolium* exhibited significant free radical scavenging effect of DPPH. The reference ascorbic acid also showed significant free radical scavenging effect. The DPPH radical inhibition was (69.9±1) and (77.85±0.8) for ascorbic acid and *Syzygium alternifolium* respectively at concentration of 100 µg/ml (Fig.2). These results suggest potential scavenging activity for *Syzygium alternifolium* extract.



***IN VIVO* ANTIOXIDANT PARAMETERS**

Estimation of MAD by lipid peroxidation

The MDA levels were significantly increased in control group-I upon pylorus ligation. This indicates role of LPO in ulcer formulation. Ulcer induced rats, when treated with standard drug (Ranitidine 150 mg/kg,p.o) had significant decrease in MDA levels ($p<0.001$), when compared to the control group. The III and IV groups treated with different doses of ethanolic extract of *Syzygium alternifolium* also exhibited a significant decrease in MDA levels ($p<0.001$) when compared to control group-I(Table.1).

Table 1: Effect of ethanolic extract of *Syzygium alternifolium* on *in vivo* antioxidant Parameters.

GROUP	DRUG	DOSE	GSH	CATALASE	LPO
I	Saline	2ml	1.98±0.05	0.091±0.002	0.15±0.007
II	Ranitidine	150mg/kg	5.50±0.11*	0.117±0.0016*	0.049±0.002*
III	Ethanolic extract of *S.alternifolium*	1gm/kg	3.93±0.05*	0.107±0.0009*	0.082±0.002*
IV	Ethanolic extract of *S.alternifolium*	2mg/kg	5.52±0.02*	0.116±0.001*	0.053±0.002*

*P<0.001, when compared to control group (I)

Estimation of catalase

A significant decrease in the levels of catalase was observed in the control group-I. The group (II), that received standard drug (Ranitidine mg/kg) had significant ($p<0.001$) increase in the catalase levels when compared to the control group. Similarly, III and IV groups treated with different doses of ethanolic extract of *Syzygium alternifolium* also exhibited a significant increase in the catalase levels ($p<0.001$) when compared to the control group-I(Table.1).

Estimation of GSH

A significant decrease in the levels of GSH was observed in the control group-I. The group (II), received standard drug showed significant ($p<0.001$) increase in the GSH levels when compared to the control group-I. Similarly, III and IV groups treated with different doses of ethanolic extract of *Syzygium alternifolium* also exhibited a significant increase in the GSH levels ($p<0.001$) when compared to the control group.

The effect of ethanolic extract of *Syzygium alternifolium* on all *in vivo* anti oxidant parameters was dose dependent(Table.1).

DISCUSSION

In this study high gastric mucosal MDA levels in animals with peptic ulcer were observed levels of MDA are thought to reflect free radical mediated cell membrane damage. It is known that radical scavengers such as α-tocopherol, carotenoids and glutathione redox system play a significant role in protecting membranes from oxidative damage.

The free radical lipid peroxidation products were formed and accumulated in the membranes, supporting their structures and function. The lipid peroxidation content is regulated by the ratio of the peroxidative and anti-oxidative process. The intensity of the peroxidative process is enhanced by unsaturated fatty acids, active oxygen and the activity of the oxidative enzymes [26]. Thus on one hand lipid peroxides are constantly produced but, on the other hand they are constantly destroyed as a result of the other processes. In ulcerogenic condition there in increase in the gastro-duodenal membrane which leads to membranes deformation and there by become more permeable for hydrogen ions. The increased concentrations of hydrogen ions evoke enzyme inhibition, catabolic processes in the cell and cell death. In the present study, on treatment with ethanolic extract of *Syzygium alternifolium* showed significant decrease in MDA levels when compared with controlled animals.

In ulcer pathogenesis an important role belongs also to hydroxyl radicals which emerge under the conditions of oxidative stress in which glutathione content and peroxidase activity decrease [27]. GSH is a well known antioxidant, which is usually present as the most abundant low molecular mass thiol in most organisms. It has various functions in the defence against oxidative stress and xenobiotic toxicity. It can act as an electron donor for glutathione peroxidase in animal cells, and also directly reacts with ROS. GSH is readily oxidised to glutathione disulfide (GSSG) by glutathione peroxidise, as well as by the reaction with ROS which may subsequently cause the reduction in GSH levels.

In the present study, on treatment with ethanolic extract of *Syzygium alternifolium* showed significant increase in GSH levels when compared with control animals. Catalase in an endogenous oxidant enzyme present in the peroxisomes, reduces hydrogen peroxide produced by dissimulation reaction and prevents the generation of hydroxyl radicals there by, protecting the cellular constituents from oxidative damage [28]. The lowered activity of catalase in ulcer conditions indicates intense peroxidation and suppression of the antioxidant process.

The NO generated from sodium nitroprusside in aqueous solution at physiological P^H interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. Scavengers of NO complete with oxygen leading to a reduced production of NO [24]. The results of the present study postulated that ethanolic extract of *Syzygium alternifolium* has ability to scavenge NO by the above mentioned mechanism.

Among the principle properties that may account for the potential health benefits of flavonoids is their antioxidant activity. Several *in vitro* studies have demonstrated that flavonoids can scavenge superoxide, hydroxyl and peroxyl radicals, affecting various steps in the archidonate cascade via cycloxygenase-II or lipoxygenase [29]. Three distinct mechanisms of production by flavonoids have been identified. The alteration of GSH mechanism, Quenching of reactive oxygen species and the inhibition of Ca^{2+} influx that signals the last step in the cell death cascade induced by glutamate [30].

CONCLUSION

On the basis of results obtained from the above *in vivo* and *in vitro* studies, it was concluded that the ethanolic leaf extract of *Syzygium alternifolium* possess significant antioxidant activity that may associated with the total phenolic and flavonoid content supporting the role anti oxidant in the pathophysiology of peptic ulcer. Further studies are required for the isolation, characterisation of antioxidant compounds and that are responsible for the anti-ulcer activity.

Abbreviations:

EESA : Ethanolic Extract of *Syzygium alternifolium*,
LPO : Lipid peroxidation,
GSH : reduced glutathione,
DPPH : 2,2-diphenyl-1-picrylhydrazyl,
PMS : Post Mitochondrial supernant,
MAD : Malondialdehyde,
TCA : Trichloroacetic acid,
TBA : Thiobarbutaric acid

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Conflicts of interest. None

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