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Research Article

In Vitro Mutagenicity Study (Bacterial Reverse Mutation Test) of Extracts Prepared from Leaves of *Eucalyptus globulus*

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ABSTRACT

Eucalyptus plant belongs to the Myrtaceae (Myrtle) family. Eucalyptus is a tall evergreen tree native to Australia, nowadays found around the world. Eucalyptus Plants have been used for the treatment of diseases for a very long time. The leaves and oil of the eucalyptus plant are used for medicinal purposes. The plant and its extracts have been evaluated for a number of activities, namely, antimicrobial, antispasmodic, antifungal, antiseptic, bronchitis, deodorant, diabetes, skin diseases, anti-inflammatory, analgesic, anti-arthritic and antipyretic. During the present study, the plant was subjected to genotoxicity studies viz. bacterial reverse mutation test (Ames Test) in order to ascertain an aspect of the safety of the drug and eco-system. *Eucalyptus globulus*leaf extracts showed no mutagenicity when tested with difference revertants of *Salmonella typhimurium*viz. TA98, TA100, TA102, TA1535 and TA1537in presence and absence of metabolic activation system (S9). In addition, the extract showed significant protective effect against mutagenicity induced by mutagen in *S. typhimurium* TA98 and TA100 strains with or

without metabolic activation.The results of the study indicate that *Eucalyptus globulus* is non-mutagenic in Ames test, and is protective against the mutagenicity induced by 4-nitroquinolene-1-oxide, sodium azide and 2-aminoflourene in TA98 and TA100 strains.

KEY WORDS: *Eucalyptus globules*, Mutagenicity, *Salmonella typhimurium.*

INTRODUCTION

*Eucalyptus*is a long lived plant which belongs to the Myrtaceae (Myrtle) family and most often associated with its native Australian environment and fun-loving koalas feasting on its branches. There are many species of eucalyptus trees, including popular varieties like Gum tree and Silver-Dollar tree, which can be grown in the home landscape. The eucalyptus tree that grows in Southern Anatolia is known as 'Adana eucalyptus'(Young-Cheol et al., 2004). *Eucalyptus globulus*leaf extractshave been shown to have many medicinal properties including the treatment of inflammatory diseases in Ayurvedicsystem of medicines. The extract is gummy in nature and is used for a variety of medical conditions including arthritis, diarrhea, dysentery, pulmonary diseases and fungal infections such as ring worm (Boland et al., 1991; Egwaikhide et al., 2008).

*Eucalyptus*plant contains volatile oils which are classified into three major types viz. medicinal, perfumery and industrial (Young-Cheol et al., 2004). *Eucalyptus* oil consists of the volatile oil distilled from the fresh leaves and branch tops of the eucalyptus plant. Topical ointments containing eucalyptus oil have been used in traditional Aboriginal medicines to heal wounds and fungal infections. Teas containing eucalyptus leaves were also used to reduce fevers. The therapeutic uses of eucalyptus soon spread to other traditional medicine systems, including Chinese, Indian (Ayurvedic), and Greco-European (Trivediand Hotchandani, 2004).Some studies have demonstrated that leaf extract and essential oil of *Eucalyptus*spp. have antifungal, repellent, antibacterial, analgesic and anti-inflammatory activities. In India, the leaf essential oil is traditionally used externally as a mosquito repellant and as an insecticide (Boland et al., 1991).

Since *Eucalyptus* tree is long-lived, therefore, it can accumulate mutations throughout their lifetimes that may influence biotic and abiotic interactions hence show marked variation in their defense mechanisms (Padovan, 2013).Leaf extract of medicinal plants have a complex mixture of certain phytochemicals that may contain both mutagenic and antimutagenic properties (Sazada, 2014). It is therefore, recommended to evaluate the mutagenicity study of the plant extract before being applied for any medicinal purpose, otherwise it could be fatal for human being. It has been shown by Yasin and Ahmet (2014) that during the study with *Salmonella typhimurium* (strain TA100) S9(-), *L. globuliferum* stem and leaf extracts showed mutagenic effect. This study is initially derived by Ames (1975) by carrying the mutagenicity effect in certain bacterial cells i.e. *Salmonella typhimurium*, thus called as Bacterial Reverse Mutation Test. Since then, this study had been carried out in almost all kind of substances before being released in to the market for commercial or human uses. The absence of mutagenicity is not characteristic of all natural products in use, since other medicinal plants assayed with the Ames test, with or without the S9, have

yielded positive results for mutagenicity (Magesh et al., 2008). Ames test is commonly used with plant extracts for possible gene mutation determination and carried out using aqueous extracts of root, stem and leaves.

In the present study, authors have attempted to investigate the mutagenic activity of the leaf extract of *Eucalyptus globulus*, a widely used medicinal plant in India, by Ames test as per the guidelines of Organization for Economic Co-operation and Development (OECD) (OECD, 1997).Thequantification and enhanced understanding of the ecological manifestations resulting from exposure to genotoxic sediments will require researchers to broaden their scope beyond simple hazard assessment. It will also embark upon the ecotoxicological significance of genotoxic effects in aquatic systems (Guosheng and White, 2004).

MATERIALS AND METHODS

Preparation of plant extract

E. globulus leaves were collected from few *E. globulus* trees nearby the Faculty of Science, C.C.S. University Campus, Meerut, India. The leaves were air dried and milled into powder. 200 g of the powder was percolated in 500 ml of distilled water for two weeks. The percolated mixture was filtered and evaporated on a water bath. A homogenous aqueous suspension of the extract was made before being used for experiment. *E. globulus* aqueous extract has been reported to be rich in steroids, flavonoids and tannins (Egwaikhide et al., 2008).

Bacterial Test Organisms:

Following five mutants of *Salmonella typhimurium* were used during the evaluation of mutagenic activity of extracts (OECD, 1997).

a) *Salmonella typhimurium* TA-98

b) *Salmonella typhimurium* TA-100

c) *Salmonella typhimurium* TA-102

d) *Salmonella typhimurium* TA-1535

e) *Salmonella typhimurium* TA-1537

Bacterial culture medium is inoculated with the appropriate *Salmonella* strain, sub culture was prepared in nutrient broth and grown overnight at 37°C in an incubator. The overnight culture was evaluated for concentration of cells grown and has been estimated approximately $1x10^9$ cells per ml, this culture is used as the standard bacterial suspension for performing the bacterial reverse mutation test.

Bacterial Reverse Mutation Test:

The Ames test was carried out by using the plate incorporation method (Ames et al., 1975; Maron and Ames; 1983). The potential to induce reverse mutation of *E. globulus* was studied using mutants of *Salmonella typhimurium viz.* TA98, TA100, TA102, TA1535, TA1537. The test was carried out at the highest recommended dose of 5000 µg/plate

and 4 subsequent doses of 4000, 3000, 2000 and 1000 µg/plate as the dose ranges in the presence (+S9) and absence (-S9) of Metabolic activation system (S9 Mix)(OECD, 1997).

The negative control used in this study was solvent i.e. DMSO. Specific positive controls were used in order to confirm the reversion properties and the specificity of each tested strain of *Salmonella typhimurium* and of course, the efficacy of the metabolic activation system. Positive controls used during the study were procured from Sigma Aldrich Chemical Co. Ltd., India and are summarized in Table 1.

Table 1: Mutagens (Positive Control) with and without metabolic activation

***S. typhimurium*Strains**	**Metabolic Activation System**	
	-S9	**+S9**
	Positive control(s) and their concentrations used during the study	
TA-98	2-Nitrofluorene (10 µg/plate)	2- Aminoanthracene (2 µg/plate)
TA-100	Sodium azide (5 µg/plate)	2- Aminoanthracene (2 µg/plate)
TA-102	Mitomycin C (2.5 µg/plate)	2- Aminoanthracene (2 µg/plate)
TA-1535	Sodium azide (5 µg/plate)	2- Aminoanthracene (2 µg/plate)
TA-1537	9-Aminoacridine (20 µg/plate)	2- Aminoanthracene (2 µg/plate)

All experiments are performed in triplicate to ensure the efficacy of results. Further, results were demonstrated in terms of mutagenicity of the extract based on the number of revertants as compared to negative control and positive controls.

RESULTS

Bacterial Reverse Mutation

The results of reverse mutation test are summarized in Table 2. The data showed no significant increase in the number of revertant colonies as a result of *E. globulus* treatment in the five strains of *S. typhimurium*(TA98, TA102, TA100, TA1535 and TA1537) at any tested concentration with those of the corresponding solvent controls in either the absence or presence of S9 mix ($P>0.05$). However, the number of revertant colonies in positive controls increased remarkably (>2 folds) with or without S9 mix ($P<0.001$). Hence, the result for *E. globulus* is negative.

Table 2: Bacterial Reverse Mutation Test (Ames Test)

***Salmonella typhimurium* Strains**	**TA - 98**		**TA – 100**		**TA – 102**		**TA – 1535**		**TA - 1537**	
Metabolic Activation System	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**
Negative Control (DMSO 0.1 ml / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Plant Extract (1000 µg / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Plant Extract (2000 µg / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Plant Extract (3000 µg / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Plant Extract (4000 µg / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Plant Extract (5000 µg / plate)	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
Positive Controls	Mt	Mt	Mt	Mt	Mt	Mt	Mt	Mt	Mt	Mt

NM=Non-mutagenic; Mt=Mutagenic

DISCUSSION

Phytochemicals derived from plants or microbes serve as valuable sources for isolating and characterizing lead molecules with specific functions. This approach assists in identifying compounds that show specific bioactivity. *Eucalyptus* is commonly used in remedies to treat coughs and the common cold. It can be found in many lozenges, cough syrups, rubs, and vapor baths. Herbalists recommend the use of fresh leaves in teas and gargles to soothe sore throats and treat bronchitis and sinusitis. Ointments containing eucalyptus leaves are also applied to the nose and chest to relieve congestion and treat bronchitis, coughs, and the flu. On the skin, eucalyptus oil has been used to treat arthritis, boils, sores and wounds. Eucalyptus oil contains 70%-85% 1,8-cineole (eucalyptol), which significantly reduces the myeloperoxidase activity, and causes repletion of glutathione which confirmed the anti-inflammatory action of 1,8-cineole (Chaudhary and Payasi, 2013).

The multipurpose use of this plant attracts the scientists across the world to evaluate its potential mutagenic activity so as to ensure the safety of using this plant as medicines for the humans. Therefore, the present study has been designed to evaluate *in vitro* mutagenicity effects in terms of bacterial reverse mutation test of this plant's extracts. The results obtained show that *E. globulus*extract is non-mutagenic

up to dose of 5mg/plate both in the presence and absence of S9 (Table 1). The maximum dose of 80 µl/plate *Eucalyptus* tincture showed a toxicity that was reduced in the presence of S9 mix. However, mutagenicity could not be detected (European Medicines Agency, 2012). The absence of mutagenicity is not characteristic of all natural products in use, since other medicinal plants assayed with the Ames test, with or without the S9, have yielded positive results for mutagenicity (Issazadeh et al., 2012). The results of anti-mutagenic activities showed that the extract was highly effective in reducing the mutagenicity caused by themutagen 4-nitroquinolene-1-oxide, sodium azide and 2-aminoflourene. The positive response obtained in TA102 strain for A. satureoides extract can be either due to oxidative reactions of caffeic acid and quercetin originating quinines and generating hydrogen peroxide, or by alkylating action of quercetin metabolization products (quinone-methides) in the bacterial DNA bases (Vargas et al., 1991). The mutagen 2-Aminoflurone works by causing framshift mutation by forming adducts on the C8 position of guanine in DNA in presence of microsomal activation. In contrast, sodium Azide requires no activation by hepatic microsomal enzymes to damage DNA and induce mutagenicity. This suggests that this extract may inhibit microsomal enzymes activation or that they may directly protect DNA strands from the electrophilic metabolites of the mutagen. Many triterpenoids have antioxidants depending on the redox potential, either accept or donate electrons, which may alternatively reduce them protective against mutagen. Our results from these experiments on antimutagenicity, suggest that triterpenoids might contain antioxidants which protects from the mutagens. Olive leaf extract with the inhibition of 54.21% sodium azide and 51.62% 2-nitrofluorene showed high potential in decreasing mutagenic agents (Issazadeh et al., 2012). The mutagenic potential of *C. asiatica* aerial parts (leaves and stems) and roots in aqueous extracts were determined using the Ames test. The method involved was pre-incubation on *Salmonella typhimurium* TA 98 and TA 100 bacterial strains in the presence and absence of metabolic activator S9 system. All aqueous extracts of the aerial parts and roots of *C.asiatica* were non mutagenic because it shown no significance difference ($p<0.05$) when compared to negative control towards TA98 and TA100 strain with and without S9 metabolic activation for all concentration studied (Florinsiah et al., 2013).

Ultimately, the present study indicates that *E. globulus*leaf extract is found to be non-mutagenic when analyzed by bacterial reverse mutation test i.e. Ames test, exhibits protection against the mutagenicity induced by 4-nitroquinolene-1-oxide, sodium azide and 2-aminoflourene in TA98 and TA100 strain.

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