



## Original Research Article

# Antibacterial and Antioxidant Activities of Biotransformed Resveratrol by *Geotrichum histeridarum*

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Abstract	Keywords
This study has been performed in biotransformation of resveratrol by <i>Geotrichum histeridarum</i> isolated from bark of <i>Saraca asoca</i> and study also focused the antioxidant and antimicrobial activities of trans-resveratrol by transforming to bis-resveratrol. Resveratrol presented antibacterial activity against the bacteria, <i>Escherichia coli</i> , <i>Proteus mirabilis</i> and <i>Klebsiella pneumoniae</i> . However, bis-resveratrol the transformed product of resveratrol has 1.7 fold increased activity than parent substrate. Resveratrol also showed bacteriostatic activity against Gram positive bacteria since their growth was prevented as compared to the positive control. The DPPH scavenging activity of the transformed product, bis-resveratrol was higher than parent compound.	Antibacterial activity Antioxidant activity Biotransformation Bis-resveratrol <i>Geotrichum histeridarum</i> <i>Saraca asoc</i>

## Introduction

Food intake modulates metabolism and health, and supplies a variety of bioactive molecules necessary for the life. In the 1800s vitamin deficiencies caused diseases such as scurvy. Whereas today a low intake of phytooxidants in association with an excess intake of saturated fats in the western diet is linked to cardiovascular diseases and cancer (Signorelli and Ghidoni, 2005). Polyphenols comprise a large class of antioxidants and include flavonoids, anthocyanins, phenolic acids, lignans and stilbenes. These compounds are all derived from phenylalanine and contain an aromatic ring with a reactive hydroxyl group. Within the subclass of stilbenes, resveratrol is the common term for 3, 4,5-hydroxy stilbene and exist both in trans and cis isomeric forms. Resveratrol is

found in several edible natural products such as grapes, peanuts, berries and rhubarbs (Sanders, 2000). The major studies on biological activities of resveratrol have considered only trans-resveratrol. It has been found that resveratrol modulates vascular cell function, inhibits LDL oxidation, suppresses platelet aggregation and reduces myocardial damage during ischemia reperfusion (Bradamante et al., 2004).

Antioxidants are generally compounds that are capable even in small quantities to prevent or reduce the oxidative destruction of biologically important compounds such as lipids, proteins and nucleic acids (Halliwell, 1990; Madhavi et al., 1996). Resveratrol being an antioxidant is biologically important and

selected for the present study due to its promising medicinal values in oxidative stress. Biotransformation is a process by which organic compounds are transformed from one form to another aided by organisms such as bacteria, fungi and enzymes. Biotransformations are used as a valuable strategy to build molecules similar to parent drug in the drug discovery programme. Microbial biotransformation or molecular biotechnology is extensively used to generate metabolism in bulk amounts (Ravindran et al., 2012).

Nature has bestowed our country with an enormous wealth of medicinal plants therefore India has often been referred to as the medicinal garden of the world. So stand the medicinal plant *Saraca asoca* as one of the foremost plants utilized from antiquity till to date. Asoka or ashoka is a Sanskrit word which means “without sorrow” or one that gives no grief. Asoka is one of the most legendary and sacred trees of India. Asoka tree is universally known by its binomial Latin name *Saraca asoca* belonging to the family *Caesalpiniaceae*.

Fungi are member of a large group of eukaryotic microorganisms are important sources of bioactive natural products with enormous potential for the discovery of new molecules for drug discovery, industrial use and agricultural applications. Several reports in the recent years show that the endophytic fungi from host produce several bioactive compounds (Wu et al., 2008 and Kharwar, 2009). Mycotoxins are secondary fungal metabolites same as antibiotics and alkaloids and are produced by filamentous fungi. Fungi produce a variety of secondary metabolites as products of their metabolism (Simone et al., 2009).

Over the past years consumer demand for natural ingredient has widely increased. Thus alternative natural sources of ingredient have been considered and production processes based on microbial biotransformation of natural precursors have become increasingly attractive (Alvarado et al., 2003). Till date no research has been performed in biotransformation of resveratrol by *Geotrichum histeridarum* isolated from bark of *Saraca asoca*. Moreover *Saraca asoca* is known well for its medicinal values. The present study focused the antioxidant and antimicrobial activities of trans-resveratrol by transforming to bis-resveratrol. The phenomenon has added the necessary functional groups responsible for the pharmacological properties.

## Materials and methods

Fungi were isolated from woody branches of *Saraca asoca* tree. The sampling was taken from the *Saraca asoca* trees present in and around Anna Nagar, Chennai. Plant samples were collected randomly from the branches up to the height of 10ft and their age was 20 years.

### Sampling of plant materials

The plant barks were collected using ethanol wiped scissors and the samples were then placed in sterile transparent plastic zip-lock bags to seal the moisture and transported to lab. These collected samples were stored below 4°C and processed within 4 h of their collection for the isolation of fungi.

### Effect of culture medium

The culture media tested for the fungal growth were PDA medium, Malt extract, Czapek's medium. The samples were placed in the Petri plates containing the different medium. All culture plates were incubated at 25°C in a shaking incubator.

### Preparation of agar plates

The isolation of fungal isolates was done using solidified culture medium (2% agar). The medium was sterilized in autoclave at 121°C at 15kPa for 15minutes. It was then cooled down to 40-45°C to avoid condensation of moisture during solidification of agar. 100mg/l of antibacterial agent Streptomycin was added to avoid bacterial contamination. Twenty milliliter of medium was poured in Petridish, presterilized in laminar air flow using UV. To avoid contamination, moisture around the flasks was whipped off and mouth was sterilized in flame while pouring the medium. After pouring the medium, Petri plates were kept undisturbed for a few minutes so that the medium got solidified.

### Isolation of fungal strains from source

Fungal strain was isolated from the woody branches and twigs of *Saraca asoca* according to Santamaria and Bayman (2005). Samples were cut into small pieces of 5 × 5 mm<sup>2</sup> with a sterile blade and placed on different media with its adaxial surface facing down. Four pieces for each sample was placed in each Petri

plate. Plates with samples were incubated at  $25 \pm 2^\circ\text{C}$  in BOD incubator and observed every day for growth of fungal colonies. A total of 12 Segments, four segments per Petri plate for each sample were placed on media amended with streptomycin (100mg/l).

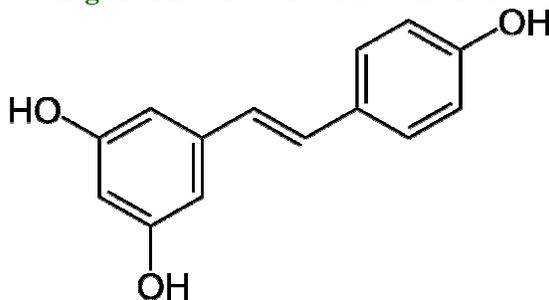
### Biotransformation

*Organic molecules for biotransformation:* The organic molecules present in natural sources were used as the lead compound for biotransformation. Resveratrol was purchased from SD Fine Chemicals, Chennai.

### Resveratrol

Resveratrol (3,5,4'-hydroxy-trans-stilbene) is a stilbenoid with the molecular formula  $\text{C}_{14}\text{H}_{12}\text{O}_3$ , a type of natural phenol and a phytoalexin produced naturally in the skin of grapes and in other fruits (Fig. 1). Resveratrol is oxidised into metabolites having higher efficacy in pharmaceuticals.

Fig. 1: The structure of resveratrol.



### Culture medium for fungal biotransformation

Five fungal strains identified from woody branches of *Saraca asoca* tree were used. Cultured suspension cells of fungal isolates were prepared as described by (Furuya et al., 1987). The fungus was cultured in the PDA plate and incubated for one week and the marginal area culture was taken, because there will be active, young cells in the marginal area. The cultures were cultivated and conserved by periodic replications, every 2 weeks on optimised culture medium.

Each solid culture was changed to a suspension culture by transferring the solid mycelia to a liquid medium broth (100ml) in 250ml conical flasks. The fungal culture was grown in the culture medium with continuous shaking for a week using a shaking incubator under illumination at  $25^\circ\text{C}$ .

### Biotransformation of organic compounds with different fungus

The selection of fungi for biotransformation was done in the screening media. The 50mg of substrate was dissolved in 1ml of solvent and individually administered into 5 conical flasks containing 100ml suspension of pre-cultured fungal suspension for a week in a 250ml Erlenmeyer flask.

Control sample was the 50mg of substrate dissolved in 1ml of solvent added to 100ml of culture medium without fungi and 5 conical flasks containing 100ml suspension of pre-cultured fungal suspension for a week in a 250ml Erlenmeyer flask was the fungal control sample. The control was subjected to the same sampling procedure as the fungal cultures. The flasks were incubated at  $25^\circ\text{C}$  for a week under illuminator and kept for shaking in a shaking incubator.

After 7 days incubation time, the fungal mycelium and the culture medium were separated by filtration using Whatmann filter No.3. The filtered reaction mixture was extracted thrice with equal volume of ethyl acetate (100 ml  $\times$  3). The combined extract was concentrated under reduced pressure to dryness to obtain the crude extract.

### Screening of organic compounds by TLC

The transformation of the product was obtained using TLC to monitor the formation of transformed product in each fungus. The parent compound and the extract of the transformed products were spotted on TLC silica plates and the transformation of the product was noted at regular intervals until complete transformation was monitored. The samples were eluted in a TLC chamber with ethyl acetate: hexane as the mobile phase in the ratio 3:7. The plates were then air dried and the plates were visualized under UV light chamber at 254nm and 363nm.

### Quantification of transformed product by HPLC

Analyses were performed on Shimadzu liquid chromatographic system equipped with LC 20AD solvent delivery system, SPD 20A UV-VIS detector. Column used for separation was Phenomenex Luna C18 column (250 $\times$ 4.6 mm id) 5 micron. Mobile phase was filtered through 0.2 micron membrane filter and

degassed using sonicator for 8mins. Sample was injected using a Hamilton syringe. System suitability was confirmed by performing all analysis.

Detector : UV-VIS detector  
Mode : Reverse phase  
Column : Phenomenex C-18, 250\*4.60 mm  
Mobile phase : Acetonitrile: water (60:40)  
Flow rate : 0.5ml/min  
Wavelength : 272nm  
Injection volume: 20µl

### Isolation of transformed product

The crude extract obtained was subjected to column chromatography to isolate the transformed products. Depending on the TLC and product yield obtained by HPLC, the crude extract was applied to a silica column and eluted with increasing concentrations of ethyl acetate (from 10 to 100%) in n-hexane.

### In vitro bioactivity studies

#### Antibacterial activity

*Growth and maintenance of test microorganism:* Bacterial cultures of *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* were obtained from Department of Microbiology, Ethiraj College, Chennai, India and were used as antimicrobial test organisms. These bacteria are among the most important pathogenic bacteria of human diseases and it was chosen to evaluate the antibacterial activities of the parent molecules and the transformed products. The bacteria were maintained on nutrient broth media at 37°C.

*Preparation of inoculums:* The nutrient broth medium was prepared for 50 ml and sterilized in an autoclave at pressure 15 lbs, 121°C for 15min. The bacteria, *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* were inoculated into tubes of nutrient broth separately and incubated at 37°C for 24 h, then it was centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically ( $A_{610}$  nm). All the microbial cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately  $1.5 \times 10^8$  CFU/ml (Andrews, 2001).

*Antibacterial activity by agar well diffusion method:* In determining the antimicrobial activity of the compounds was evaluated by agar well diffusion method (Ahmad and Beg, 2001). The substrate and products (500µg) were freshly reconstituted with Dimethylsulphoxide. 20ml of Mueller-Hinton agar media was poured into each Petri plates and plates were swabbed with 100µl inocula of the test microorganisms. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60°C each time to ensure even distribution of the inoculums.

The plates were allowed to dry for 30 min. to allow adsorption. Using sterile cork borer of 8mm diameter, wells were bored into the seeded agar plates and these were loaded with a 100µl volume of extracts. All the plates were incubated at 37°C for 24 h for Pre-diffusion. Antimicrobial activities of samples were evaluated by measuring the zone of bacterial growth inhibition against the test organisms with zone reader (Hi Antibiotic zone scale). The medium with DMSO as solvent was used as a negative control and streptomycin (100µg) was taken as positive control for bacteria. The experiments were performed in triplicates.

#### Anti-oxidant activity

The quantitative estimation of radical scavenging activity was determined according to the methods described by (Blois, 1958). The free radical scavenging activity was measured using DPPH assay. Five milliliters of 0.04% DPPH radical solution was added to sample solutions at 5mg/mL. The mixtures were vortex-mixed and kept under darkroom conditions for 30 min. The optical density (OD) was measured at 517 nm. Methanol was used as baseline control. Ascorbic acid at 1mg/ml was used as positive control. The experiments were performed in triplicates. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{DPPH radical scavenging effect (\%)} = [(A_0 - A_1 / A_0) \times 100]$$

Where,  $A_0$  was the absorbance of the control;  $A_1$  the absorbance of the sample after incubation. The radical scavenging activity is represented as percentage inhibition of DPPH radical scavenging effect.

## Results and discussion

The transformation of isolated compound was found in the fungal isolate, *Geotrichum histeridarum* which were then used for antimicrobial and antioxidant assay (The identification of fungal strains is not shown). From the results of agar well diffusion assay had led to the observation that resveratrol presented antibacterial activity against different bacteria, but bis-resveratrol the transformed product of resveratrol has 1.7 fold increased activity than parent substrate (Tables 1, 2 and 3). Paulo et al. (2010) have reported that resveratrol has antibacterial activity against both Gram positive and Gram negative bacteria. The zone of inhibition for various concentration 250, 500, 1000 $\mu$ g were determined in mm. Resveratrol shows

bacteriostatic activity against Gram positive bacteria since their growth was prevented as compared to the positive control. Docherty et al. (2007) have shown that resveratrol inhibited *Propionibacterium acnes* and presented highest tested concentration (200  $\mu$ g/ml).

Zoran et al. (2006) has reported that resveratrol has inhibited more Gram positive than Gram negative bacteria which may be due to the outer lipopolysaccharide protein cover in *Escherichia coli* that protects the cells from the action of tested compound. The fungus *Geotrichum histeridarum* has transformed the structure a s bis-resveratrol which was characterized by NMR and MS values. The antibacterial activity of bis-resveratrol is increased than parent compound.

**Table 1. Antibacterial activity of resveratrol and biotransformed product against *Escherichia coli*.**

Name of compound	250 $\mu$ g					500 $\mu$ g					1000 $\mu$ g				
	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD
Streptomycin	27.4	27.0	26.8	27.1	0.31	28.8	29.4	29.6	29.3	0.42	31.9	32.3	31.5	31.9	0.40
Cinnamic acid	12.8	13.1	13.5	13.1	0.35	15.6	14.9	15.2	15.2	0.35	18.8	19.5	19.2	19.2	0.35
TP of cinnamic acid	17.4	16.9	16.4	16.9	0.50	19.3	19.1	18.6	19.0	0.36	22.1	21.2	21.9	21.7	0.47

**Table 2. Antibacterial activity of resveratrol and biotransformed product against *Klebsiella pneumonia*.**

Name of compound	250 $\mu$ g					500 $\mu$ g					1000 $\mu$ g				
	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD
Streptomycin	15.8	15.2	14.6	15.2	0.60	17.2	17.4	16.1	16.9	0.70	21.8	21.3	20.5	21.2	0.66
Cinnamic acid	6.6	5.9	6.8	6.4	0.47	9.3	8.7	8.4	8.8	0.46	13.2	12.6	12.4	12.7	0.42
TP of cinnamic acid	9.1	8.4	8.6	8.7	0.36	11.2	11.5	10.7	11.1	0.40	15.4	14.7	14.7		0.65

**Table 3. Antibacterial activity of resveratrol and biotransformed product against *Proteus mirabilis*.**

Name of compound	250 $\mu$ g					500 $\mu$ g					1000 $\mu$ g				
	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD	R1	R2	R3	Mean	SD
Streptomycin	13.5	13.1	12.6	13.1	0.45	15.7	15.2	14.8	15.2	0.45	19.7	19.5	18.4	19.2	0.70
Cinnamic acid	4.2	4.5	4.1	4.3	0.21	6.5	6.1	5.7	6.1	0.40	8.1	7.6	7.4	7.7	0.36
TP of cinnamic acid	7.8	7.5	7.0	7.4	0.40	9.6	9.1	9.7	9.5	0.32	11.8	11.5	11.0	11.4	0.40

Balik et al. (2009) has reported that higher concentration of polyphenols and flavonoids are correlated with higher antioxidant activity. The DPPH scavenging activity of the transformed product, bis-resveratrol was higher than parent compound (Table 4). This may be due to the increase in the phenolic compounds, hydroxyl groups during biotransformation. Chochkova et al. (2012) has reported that the absence of phenol group on N-trans cinnamoyl-L-cysteine ethyl ester decreases the radical

scavenging activity. Conversely the presence of phenolic compounds and methoxy groups in the phenyl propenoyl part of the molecules increases significantly RSA.

The DPPH free radical when it reacts with hydrogen donors initially DPPH radical is purple and upon reaction with hydrogen donors become colorless. It is a discoloration assay which is evaluated by addition of the antioxidant to a DPPH solution in methanol and

the ability to scavenge the stable free radical of DPPH (Shirwaikar et al., 2006).

**Table 4. Anti-oxidant activity (DPPH) of resveratrol and biotransformed product.**

Components	R1	R2	R3	Mean	Sd
Ascorbic acid positive control	89.43	88.85	88.32	88.90	0.56
Resveratrol	65.49	64.33	65.55	65.1	0.69
Bis-resveratrol	70.54	69.22	70.29	70.0	0.70

## Conclusion

The value of antioxidants in foods has long been recognized for their health benefits. It is believed that higher intake of antioxidants rich fruits are associated with decreased risk of degenerative diseases like CHD and cancer. Antioxidants are compounds which can inhibit or delay the oxidative damage preventing many diseases. Resveratrol is having the capacity to inhibit LDL oxidation and its transformed product exhibit higher efficacy. Also the antibacterial activity of resveratrol is high towards Gram positive bacteria *Staphylococcus aureus* than Gram negative bacteria. In future the development of new drug with high antioxidant activity and antibacterial activity would be of great importance to mankind. Further the fungal biotransformation lead to mass production of medicines. Resveratrol has draught attend not only for being a possible natural antimicrobial but also for its potential functional and therapeutic application. Therefore its significant potential for use both in clinical practice and large scale production by fungal biotransformation has been performed in this work.

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