Phytochemical, Antioxidant, Antibacterial and Mutagenic property (AMES) in vitro analysis of *Strobilanthes barbatus*

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***Abstract* - *Strobilanthes barbatus* (SB) was analyzed for its phytochemical, antioxidant, antibacterial, and mutagenic properties. The shade-dried powdered material of leaves was evaluated by phytochemical analysis. The antibacterial activity of a test compound extract of (*Strobilanthes barbatus*) against Streptococcus pyogenes was assessed by an automated BD Phoenix system and compared to a Resazurin microtitre assay (REMA) plate using left-over throat swab samples of arthritis patients. The antioxidant activity of the extract was evaluated by DPPH assay, phosphomolybdenum assay, and Ferric reducing assay. The results of the preliminary phytochemical analysis revealed the detection of alkaloids, flavonoids, tannins, saponins, sterols, and triterpenoids. The Strobilanthes extract was shown to exhibit marked dose-dependent radical scavenging, ferric reducing potential and antibacterial effect against Streptococcus pyogenes and have not provided any mutagenic effect. Thus, extract of SB was concluded as promising in terms of antibacterial and antioxidant potential with no mutagenic property. Further studies are to be undertaken to investigate their biological activities in animal studies.**

***Index Terms* - *Strobilanthes barbatus*, antioxidant activity, AMES assay, and antibacterial activity.**

I.INTRODUCTION

Plants have been extensively used since time immemorial for various purposes such as medicine, food, fodder, spices, and construction tools (Nambiar et al., 1985). In various systems of medicine, plants have been used in certain formulations to treat several ailments or disorders (Rani UK et al.,2013). The majority of the population, especially those living in remote places and having no access to modern medicines, rely on plant-based traditional medicine for primary healthcare (Sasidharan N, 2016). It is well known that drugs such as vincristine, vinblastine, artemisinin, quinine, digoxin, reserpine, and morphine are of plant origin (Koay et al., 2013). Herbal medicines are given more important even in the Western population owing to their negligible or no side effects. Advancement in bioanalytical techniques resulted in the isolation and identification of several bioactive metabolites from higher plants. Crude extracts and isolated components from plants exhibit a range of bioactivities including antioxidant and anticancer activity.

Strobilanthes belong to Acanthaceae and the second largest genus of this family (Jayaraman et al., 2013). It comprises approximately 300 species in tropical Asia (Koay et al., 2013). The Indian subcontinent has nearly 150 species, out of which 59 are seen in peninsular India. The genus is not greatly explored for economic utility. Strobilanthes spp is one of the endemic and potential medicinal plants. It is widely used in Ayurveda as a source of the drug 'Sahacharya' (Samal, 2013). Western Ghats of India serves as a rich repository of medicinal plants. Strobilanthes species are known to exhibit various biological activities such as antiviral, antifungal, acetylcholine esterase inhibitory, antiobesity, anti-inflammatory, anticancer, antioxidant, antidiabetic, antinociceptive, and hypoglycemic activity. In the present study, we evaluated the antibacterial, antioxidant, and mutagenic potential of *Strobilanthes barbatus.*

II.MATERIAL AND METHODS

2.1 Collection, identification, and extraction of plant material

The plant *Strobilanthes barbatus* collected in June 2017 from Amruth Vana – FRLHT's Ethanomedicinal Garden, Attur, Yelahanka, Bangaluru. The plant was identified and authenticated by an Ethanobotanist, (Dr. Ganesh Babu, Head, Department of Herbal Gardens Sciences, Bangalore, Karnataka. The fresh green aerial parts of the plant were collected and were dried at room temperature under shade for five days. This dried powder was subjected to various extraction using a different solvent in Soxhlet apparatus. Then the extract was evaporated to dryness using Bauchi Rotavapor (Switzerland) and ultimately dried in an oven.

2.2 Chemicals

All solvents and chemicals employed for the present work were of analytical grade.

2.3 Phytochemical analysis:

Qualitative Phytochemicals

Test for Carbohydrates:

200µl of plant extract, 100µl of Molisch's reagent, and few drops of concentrated sulphuric acid were added. The presence of reddish rings indicates the presence of carbohydrates.

Test for Tannins:

100µl of plant extract, 200µl of 5% ferric chloride were added. The presence of dark blue indicates the presence of tannins.

Test for Saponins:

200µl of plant extract, 200µl of distilled water were added and shaken in a graduated cylinder for 15mins lengthwise. The presence of formation of 1cm layer foam indicates the presence of saponins.

Test for Flavonoids:

200µl of plant extract, 100µl of 2N sodium hydroxide were added. The presence of yellow color indicates the presence of flavonoids

Test for Alkaloids:

200µl of plant extract, 200µl of concentrated hydrochloric acid were added. Then few drops of Mayer's reagent were added. The presence of Green or white precipitate indicates the presence of alkaloids.

Test for Quinones:

100µl of plant extract, 100µl of concentrated sulphuric acid were added. The presence of Red color indicates quinones.

Test for Glycosides:

200µl of plant extract, 300µl of chloroform, and 10% ammonia solution were added. The presence of pink color indicates glycosides.

Test for Cardiac glycosides:

50µl of plant extract, 200µl of glacial acetic acid, and few drops of 5% ferric chloride were added. This was under layered with 100µl of concentrated sulphuric acid. Presence of a brown ring at the interface.

Test for Terpenoids:

50µl of plant extract, 200µl of chloroform was added, and concentrated sulphuric acid was added carefully. Absence of red-brown color.

Test for Triterpenoids:

150µl of plant extract, 100µl of chloroform shaken with concentrated sulphuric acid were added. The absence of the lower layer turns yellow.

Test for Phenols:

100µl of plant extract, 200µl of distilled water followed by few drops of 10% ferric chloride was added. Presence of dark blue color.

Test for Coumarins:

100µl of plant extract, 100µl of 10% NaOH was added. Absence of yellow color.

Test for Steroids:

100µl of plant extract, an equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid. Presence of a brown ring.

Test for Phlobatannins:

100µl of plant extract few drops of 2% HCL were added. Absence of red color precipitate.

Test for Anthraquinones:

100µl of plant extract few drops of 10% ammonia solution was added. Absence of pink color precipitate.

Quantification of Phytochemicals

Estimation of Carbohydrates

The sample was weighed 100mg and hydrolyzed in a boiling water bath with 5ml of 2.5N HCl for 3 hours. This was cooled at room temperature and solid sodium carbonate was added until effervescence ceases. Then the contents were centrifuged, the pellet was discarded and the supernatant was made to 100 ml by using distilled water. From this 200µl of the sample was pipetted out and made up the volume to one ml with distilled water. 1ml of phenol reagent with 5ml of sulphuric acid was also added. The tubes were kept at 25-30 C for 20 min. The absorbance was read at 490 nm (Krishnaveni et al., 1984).

Estimation of Total phenolic content (TPC):
Total phenolic content in the extracts determined by the Folin Ciocalteau colorimetric method (Slinkard and Singleton, 1977). For the analysis, 0.5 ml of dry powdered ethanolic leaf extracts were added to 0.1 ml of Folin- Ciocalteau reagent (0.5N), and the contents of the flask were mixed thoroughly. Later 2.5 ml of Sodium carbonate ($Na_2 CO_3$) 2% (wv) was added. The blend was incubated in the dark at room temperature for 15 min. The absorbance of the blue-colored solution of all samples was measured at 765 nm. The results were expressed in mg of gallic acid equivalent (GAE) per g of the dry weight of plant powders.

Estimation of Total Flavonoid Content
Total flavonoids content in the extracts was determined by the aluminium chloride colorimetric method (Mervat et al., 2009). 0.5 ml of leaf extracts at a concentration of 1mg/ ml were taken and the volume was made up to 3ml with methanol. Then 0.1ml aluminium chloride- AlCl 3 (10%), 0.1 mL of 1M potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. A standard calibration plot was generated at 415nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

Determination of Total alkaloids:
With some variations, the alkaloids quantification system (Harborne,1998) was used. 100 ml of 10% acetic acid in ethanol was added to 1 gram of dry powdered plant and then the extracts were covered and allowed to stand for 4 hours. After that, the extracts have been filtrated and concentrated on a water bath to 25 ml of the original volume. The droplets of concentrated ammonium hydroxide were added to the extract until the precipitation of the whole solution was allowed to settle, and then the precipitates were washed with dilute ammonium hydroxide and then filtered using a Whatman filter paper. The residue was dried in the oven at 40 °C and weighed. The alkaloid content was determined using the following Formula:
% Alkaloid = [final weight of the sample / initial weight of the extract] × 100

Determination of Terpenoids:
100 mg (Initial weight wi) of plant powder were taken separately and soaked in 9ml of ethanol for 24 hours. Then filtered, the filtrate was extracted with petroleum ether using a separating funnel (Sun et al 2005). The ether extract was separated into preweighed glass vials and waited for its complete drying (final weight wf). Then the ether was evaporated and the yield (%) of total Terpenoids content was calculated using the formula below
% Terpenoid = [Initial weight(wi) – Final weight(wf) / Initial weight(wi)] X 100

Determination of Saponin content:
The determination of total saponin was done according to the method used by (Obadoni and Ochullo 2001) with minor modifications. 1 g of powdered plant has been added to 100 ml of 20% aqueous ethanol and kept in a flask on a stirrer for half-hour and then heated over a for 4 h at 45 °C with mixing. The mixture was filtered by using filter paper Whatman and the residue was again extracted with another 100 ml of 25% aqueous ethanol. The combined extracts were concentrated by using a rotary evaporator at 40 °C to gets 40 ml approximately. The concentrate was transferred into a separator funnel and extracted twice with 20 ml diethyl ether. The ether layer was discarded while the aqueous layer was kept and then re-extracted with 30 ml n-butanol was added. The n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated. After evaporation, the samples were dried in the oven at 40°C to a constant weight and the saponin content was calculated.

Estimation of Steroids:

1ml of acetone extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water bath maintained at 70±20C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank (Devanaboyina N et al., 2013).

2.4 Antioxidant activity

DPPH radical scavenging method

DPPH radical scavenging assay the potential of various concentrations (20-100 mg/ml) of extract and ascorbic acid to scavenge free radicals was evaluated by DPPH radical scavenging assay. The extent of scavenging of DPPH radicals by extract and ascorbic acid (reference antioxidant) was determined

Ferric reducing assay

The reducing potential of various concentrations (5-100 µM/ml) of and ascorbic acid was evaluated by ferric reducing assay. The absorbance of the reaction mixture was measured at 700nm.

Phosphomolybdenum Assay

Total antioxidant capacity of extract was determined by phosphomolybdate method and expressed as equivalents of ascorbic acid (mg/g of extract) at (0.62-10mg/ml).

2.5 Antibacterial Activity

The throat swab of arthritis confirmed the patient's leftover sample collected from VRR diagnostic, T.Nagar Chennai India. Eleven *Streptococcus Pyogenes* samples are taken for the study to evaluate the potential of antibacterial activity in Arthritis patients. The antibacterial activity was performed by BD Phoenix automated instrument and the test compound (*Strobilanthes barbatus*) compared with the Resazurin microtitre assay (REMA) plate method (Satyajit et al., 2007).

2.6 Mutagenicity Assay

The mutagenicity assay with S. *typhimurium* was performed as described by (Maron et al., 1993). The test is based on the plate incorporation method, using S. *typhimurium* test strains TA98). The test strains from frozen cultures were grown overnight for 12–16h at 37 °C in the oxoid nutrient broth No. 2. All concentrations studied of aqueous extracts were added to 2 ml of top agar, supplemented with 0.5 mM L-histidine and 0.5 mM D-biotin, mixed with 100 µ l of bacteria culture, and then poured onto a plate containing minimum agar. The plates were incubated at 37◦C for 48 h and revertant colonies were counted after 2 days of incubation.

III.RESULTS AND DISCUSSION

Table 1: Phytochemical analysis (Qualitative Tests)

Phytochemical Test	Samples		
	Chloroform	Ethanol	Aqueous
Carbohydrates	+	+	+
Tannins	-	-	-
Saponins	-	+	+
Flavonoids	+	+	+
Alkaloids	+	+	-
Quinones	-	+	+
Glycosides	-	-	-
Cardiac Glycosides	-	+	+
Terpenoids	+	+	+
Triterpenoids	+	+	+
Phenols	+	+	-
Coumarins	+	+	+
Steroids	+	+	+
Phytosteroids	+	-	-
Phlobatannins	-	-	-
Anthraquinones	-	-	-

+ indicates Presence; - indicates the absence

The ethanolic extracts elute all the different phytoconstituents of the plant, hence ethanolic extracts were majorly used for *in vitro* experiments also chloroform extracts were used to compare the quantitative presence of phytoconstituents tests alone.

Figure 1: Carbohydrate content

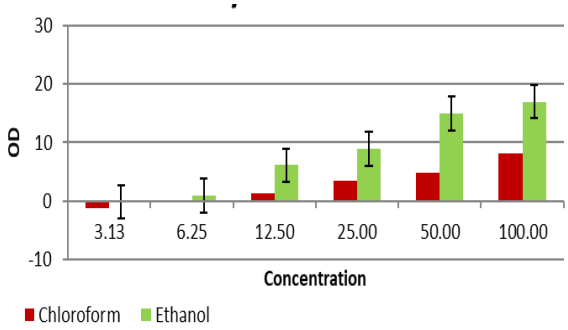


Figure 2: Total Phenol content

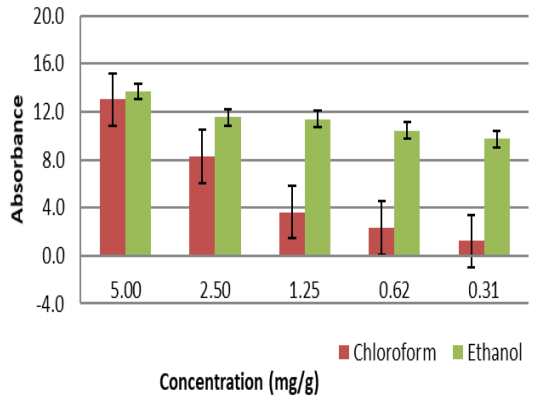


Figure 3: Total Flavonoid content

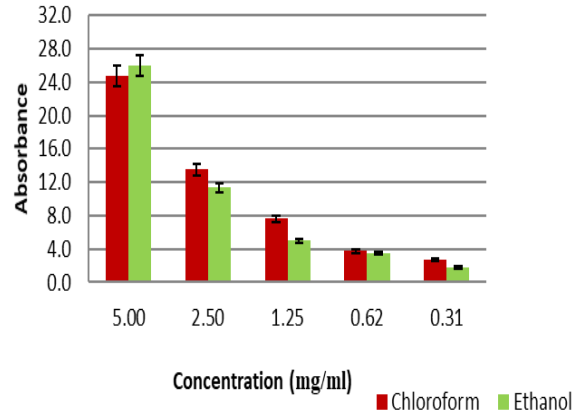
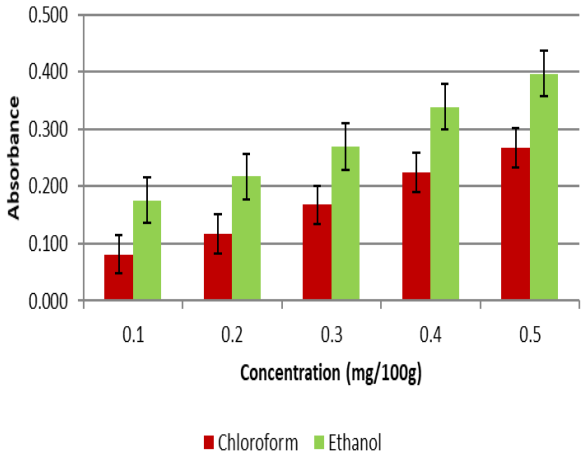


Figure 4: Total Steroid content



The phytochemical study of the extracts revealed the presence of different bioactive compounds such as alkaloids, saponins, steroids, flavonoids, phenols. Bioactive compounds stored in plants possess biological and antibacterial activities that can be used as an alternative medicine for the treatment of bacterial infections in man.

Table 2: Quantitative estimation

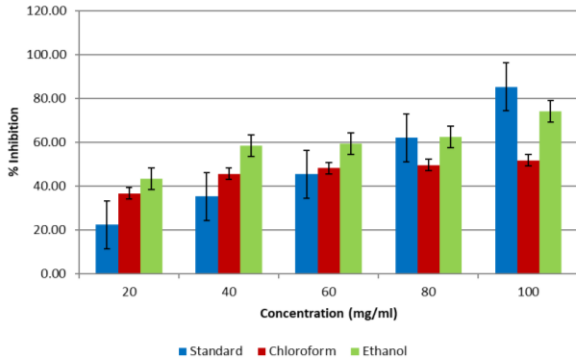
Quantitative Estimation @ Maximum conc extracts

Test	Chloroform	Ethanol	in units
Carbohydrates	8.15	17	mg/ml
Flavanoids	24.727	26	mg quercetin equivalent/g
Saponin	6.5	14.5	%
Alkaloid	26.5	31.25	%
Terpenoid	68.7	51.8	%
Phenol	13.02	13.691	mg/g of GAE
Steroids	0.267	0.397	mg/100g

These compounds have been reported to bestow resistance in opposition to microbial pathogens and this could be accountable for the exhibition of antibacterial activity by both extracts in this present study Also, secondary metabolites like terpenoids have been reported to have pharmacological potentials.

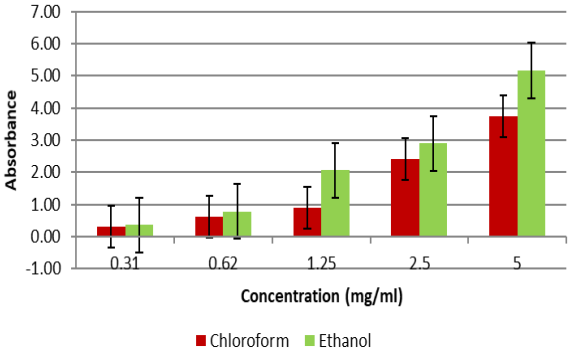
Antioxidant activity

Figure 5: DPPH assay



The method of scavenging of stable, organic, nitrogen centered DPPH radicals is widely used to evaluate the antiradical activity of extracts Both extract and ascorbic acid scavenged radicals dose-dependently with percentage of inhibition, the reduction capability of DPPH radical showed 43.33% of ethanol, and 36.69 % of chloroform inhibition at 5mg/ml concentration.

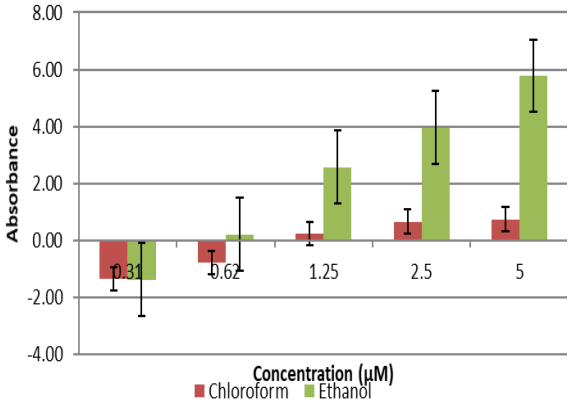
Figure 6: Phosphomolybdenum assay



Since free radicals, and hence oxidative stress, represent important stimuli for inflammatory response, the antioxidant activities of the extracts were also examined. In the phosphomolybdenum assay, extracts showed similar total antioxidant activities. A similar trend was observed in the DPPH radical scavenging assay. The promising antioxidant activities of extract may contribute to their ability to reduce inflammation. Antioxidants in the extracts could mop up free radicals and thus maintain a redox balance within the cells of the tissues.

Figure 7: FRAP assay

The reducing ability is due to the presence of



reductions in the samples and the reducing potential is considered as a significant indicator of antioxidant activity. The result of the ferric reducing activity of the extract and ascorbic acid was observed. The reducing potential of the extract was lesser than that of ascorbic acid.

Antibacterial Activity

Table 3: Antibiotic Sensitivity (MBC)

ATB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
CH	R	S	S	S	S	R	R	R	S	S	S
CEF	S	S	S	S	S	S	S	S	R	S	R
LF	S	S	S	S	S	S	S	S	S	S	S
TC	R	R	R	R	R	S	R	S	R	S	R
TM/SF	S	S	S	S	S	S	S	R	R	S	R
BP	S	S	S	S	S	S	S	S	IS	S	IS
AMP	S	IS	S	S	S	S	S	S	IS	S	IS
MOR	IS	S	S	S	IS	S	S	S	S	S	S
ERY	R	R	IS	R	IS	R	R	R	R	S	R
CLD	R	R	IS	R	R	S	R	R	R	S	R
LN	S	S	S	S	S	S	S	S	S	S	S
VA	S	S	S	S	S	S	S	S	S	S	S
CFT	S	S	S	S	S	S	S	S	R	S	R

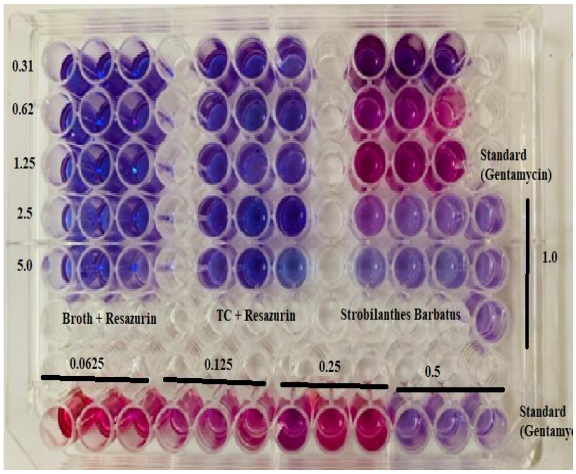
The bacterial isolates: *Streptococcus pyogenes* from throat swab were used for studying the antibacterial activity of the compound (Mackie et al., 2004). The isolates were identified using BD Phoenix™ Automated Microbiology System. AST (MIC) (Judith Beuving et al., 2011) were performed and provided in Table 4 below.

Table 4: Minimal I inhibitory concentration (MIC)

ATB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
CH	>=16	4	4	2	2	>=16	>=16	>=16	2	2	2
CEF	<=0.12	<=0.12	<=0.12	0.25	<=0.1	<=0.12	<=0.12	<=0.12	2	<=0.12	4
LF	>=16	1	1	1	2	0.5	1	0.5	2	1	2
TC	>=16	>=16	>=16	>=16	>=16	<=0.25	>=16	<=0.25	>=16	<=0.25	>=16
TM/SF	<=10	<=8	<=10	<=10	<=16	<=10	<=10	160	<=10	<=10	<=10
BP	<=0.06	<=0.12	0.12	0.12	<=0.12	<=0.06	<=0.06	<=0.06	2	<=0.06	2
AMP	<=0.25	4	<=0.25	<=0.25	<=0.25	<=0.25	<=0.25	<=0.25	2	<=0.25	2
MOR	2	2	0.25	0.25	0.5	0.12	0.25	0.12	0.12	0.25	0.12
ERY	2	>=8	0.5	>=8	0.5	2	>=8	>=8	>=8	<=0.12	>=8
CLD	<=0.25	>=1	0.5	<=0.25	>=4	<=0.25	>=1	0.5	>=1	<=0.25	>=1
LN	<=2	2	<=2	<=2	2	<=2	<=2	<=2	<=2	<=2	<=2
VA	0.5	<=4	0.5	0.25	<=0.5	<=0.12	<=0.12	<=0.12	0.5	<=0.12	0.5
CFT	<=0.12	0.5	<=0.12	<=0.12	4	<=0.12	<=0.12	<=0.12	>=8	<=0.12	>=8

Abbreviations are CH-Chloramphenicol, CEF-ceftriaxone, LF-Levofloxacin, TC-Tetracycline, TM/SF-Trimethoprim/ Sulfamethoxazole, BP-Benzylpenicillin, AMP- Ampicillin, MOR-Moxifloxacin, ERY-Erythromycin, CLD-Clindamycin, LN-Linezolid, VA-Vancomycin, CFT-Cefotaxime. S-Sensitive, R-Resistance, IS-Intermediate Sensitive, ATB- antibiotic

Figure 8: Resazurin Microtiter Assay Plate Testing



Plates after incubation in resazurin assay [pink color indicates growth and blue means inhibition of growth; the test organism was *Streptococcus pyogenes*; Broth + resazurin, test compound + resazurin, *Strobilanthes*

barbatus, and standard Gentamycin in serial dilution) with concentration mention in the above Figure. 8.

The results of the *Strobilanthes barbatus* showed potentially significant inhibition compared with the standard drug (gentamycin). This compound can be used specifically for the arthritic patient having streptococcal infection because like most arthritis comorbidities are clinically correlating with the Streptococcal infection.

Mutagenicity Assay

Figure 9 AMES test (Sample and Control plates)

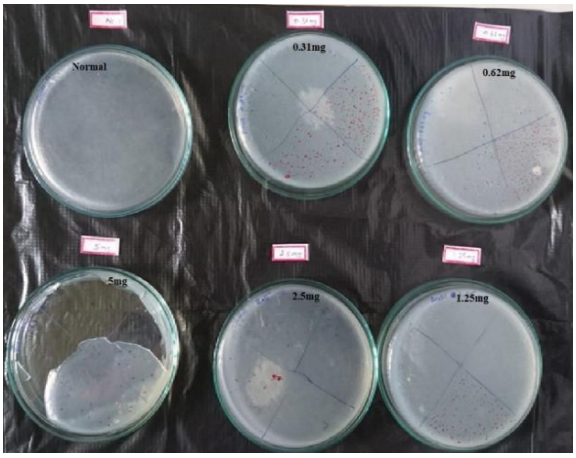


Figure 10: Positive control Plate (Sodium Azide)

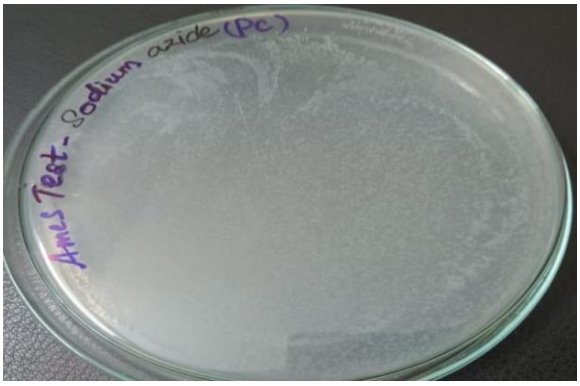


Table 5: Colony forming units- TA 98

Concentration (mg/ml)	Ethanolic Extract
	MEAN CFU
5	47
2.5	57
1.25	39
0.62	59
0.31	78
NC	112
PC	354

(Basaran et al., 1996) Histidine (-) auxotrophic Salmonella typhimurium is an indicator organism of the Ames test, which gets mutated to Histidine (+) auxotroph if the sample is mutagenic. The ethanolic extract of the plant proved to be non – mutagenic as the CFU was not more than the mutagenic sodium azide (positive control) indicating there's no mutation.

IV.CONCLUSIONS

An in-depth study of the *Strobilanthes barbatus* extract revealed the sources of bioactive compounds with potent *in vitro* activities. According to the obtained results, *Strobilanthes barbatus* EtOH extracts represent important sources of flavonoids. Moreover, the biological properties of these *Strobilanthes barbatus* indicate their potential usage in oxidative stress-related disorders. Particularly, even though the investigated *Strobilanthes barbatus* is traditionally used as herbal remedies with antimicrobial potential, the results obtained for the current investigated samples are modest. However, further studies are necessary to elucidate the mechanisms of *in vivo* pharmacological action and involved metabolic pathways. In conclusion, the aqueous extracts showed the non-mutagenic effect on TA98 strains are safely consumed and can be used as part of traditional medicine.

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