**SCREENING METHODS TO DETERMINE THE ANTIBACTERIAL ACTIVITY Dated:**

**Evaluation of the antibacterial activity**

**Collection of microorganisms**

Five pathogenic bacterial strains were used as the test organisms for antibacterial screening of the test compound. Among them both gram positive and gram negative strains are used.

Preparation of media and maintenance of bacteria

All the bacterial strains were grown and maintained on Muller Hilton agar (Hi media, India) media at 37°C and pH (7.3±0.2). The bacteria were sub-cultured overnight using Muller Hilton broth medium

**Antibacterial assay**

**Disc diffusion technique**

The test organisms were transferred to the test tubes containing about 5 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial suspension was immediately transferred to the sterilized Petri dishes. The Petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

Sterilized Whatman® paper discs (6 mm in diameter) were treated with desired concentration (400 µg/disc) of previously prepared solution of the test compound using a micropipette and dried in air under aseptic condition and placed at the equal distance in a circle on the plate. These plates were kept at low temperature for 4-6 h and by this time the test materials diffuse from disc to surrounding medium. The same process was conducted for the negative control \_\_\_\_\_ and the positive control \_\_\_\_\_\_\_\_. All the experiments were conducted in triplicates. Then the plates were incubated for 24 h at 37°C. At the end of the period, the inhibition zone against each microorganism by the test compound was measured and analyzed by using one way ANOVA followed by paired t-test in SPSS version 18.0.

**Other Evaluation methods**

In order to suggest methodologies for screening the natural products antimicrobial activity, two different qualitative methods for evaluation are as follows: agar diffusion tests, employing two different types of reservoirs (filter paper disc impregnated with compound-test and wells in dishes) and bioautographic method (agar diffusion and chromatogram layer).

microdilution methods are used for the determination of minimum inhibitory concentration (MIC).

1. **Agar diffusion well-variant**
2. **Agar diffusion disc-variant**
3. **Bioautographic method direct-variant (chromatogram layer)**

 (1) Preparation and application of natural products on thin layer chromatography plates (TLC) (silica gel G60 F254, Merck);

 (2) Preparation and application of the bacterial inoculum to TLC plates;

 (3) Incubation; and

 (4) Growth detection by colorimetric assay (INT) and measurement of growth inhibition diameters.

1. **Bioautographic method indirect-variant (agar diffusion)**

 In this procedure, first step corresponded to bioautographic variant-direct step 1. In step 2, TLC plates were covered with Müeller-Hinton agar layer (9 mL of the medium on 81 cm2 petri plate area). However, contact between bacterial suspension and natural products were performed by two distinct procedures: mixing with agar (100 mL test-bacterial suspensions were mixed with 9 mL of agar and carefully poured on TLC plate) and swabbing with a cotton swab (inoculum was spread on the agar surface).