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Synergistic Effect of Essential Oils of Three Gymnosperms Against Vibrio Cholerae

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Abstract: The present investigation focused on the determination of antibacterial efficacy of essential oils obtained from leaves of three gymnosperms i.e. Pinus roxburghii Sarg., Taxodium distichum L. and Thuja occidentalis L. The oils were extracted from the leaves of aforementioned plant species using Clevenger type apparatus by hydro-distillation method. The antibacterial activity of the extracted essential oils was evaluated against Vibrio cholerae (MTCC No. 3906) using broth micro-dilution method recommended by Clinical Laboratory Standard Institute (CLSI). The Inhibition Concentration i.e. IC50 and Minimum Inhibition concentrations (MIC) using SpectramaxPlus384, of Molecular Devices Corporation, USA were recorded while Streptomycin as standard was taken. The IC50 value of P. roxburghii Sarg., T. distichum L. and T. occidentalis L., were showed 1.294, 0.914 and 1.277 mg/ml respectively. The P. roxburghii, was found most effective with their MIC 1.403 mg/ml while T. distichum L., showed least effective with their MIC 1.995 mg/ml against V. cholerae. Hence, essential oil from leaves of gymnosperms exhibit great potential for the development of eco-friendly, non-toxic, cost-effective anti-bacterial formulations.
Key words: Gymnosperms, Essential oil, Synergistic effect, Broth Micro-dilution assay

I. INTRODUCTION

P. roxburghii Sarg. belongs to Pinaceae and commonly known as Chir pine. P. roxburghii is the native of Himalayas and distributed throughout India, Nepal, Bhutan and Pakistan. It is widely distributed from lower to midrange of Himalaya in India. P. roxburghii is a large tree attaining up to 55 m in height with a trunk diameter reaching up to 2 m (figure 1). The cones of P. roxburghii are ovoid, conic and usually open up to 20 cm to release the seeds (1). P. roxburghii oil has been traditionally used to treat blisters, cuts, boils and wounds (2). Phytochemical screening of Pinus needles revealed the abundant amounts of tannins, vitamin C and alkaloids while the stem has been primarily used as a source of turpentine oil (3-5). Some microbiological research suggests that the essential oil on P. roxburghii has shown significant anti-fungal activity (6) as well as antibacterial activity (7) while alcoholic extract of the needle, stem, and cones are reported to exhibit strong anti-bacterial activity. P. roxburghii grows in the region of forests of 1200-1850 m altitude at a temperature between 5-15ºC (8).

Taxodium distichum. (L.) belongs to Taxodiaceae and commonly referred as bald cypress. It is an unusual but interesting tree. It can grow over 25 m in height and over 3 m in diameter (figure 2). The leaves are small, green to yellow-green, 5–20 mm long and appeared in two-ranks. Young trees have a pyramid shape but eventually form an irregular flattened canopy. Cones are the fruits and are composed of scales forming a woody, brown sphere with rough surface. T. distichum has three extant taxa ranging from the eastern United States through Mexico to Guatemala (9). Heartwood of bald cypress is extremely rot and termite resistant (10). Leaves and cones are rich in essential oils and used traditionally to treat respiratory, skin, gastro-intestinal, inflammation, and infections (11, 12). Diterpenoids and Flavonoids are the main secondary metabolites (13). T. distichum trees can grow near lake margins, rivers, swamps, wet poorly drained habitats and are tolerant to various soil conditions and air pollution (14). These long-lived conifers have been widely used for landscape in many countries. The heartwood is used for building materials, and has been reported to resist the attacks of the subterranean termite (15).

Fig1. P. roxburghii

Fig 2. T. distichum

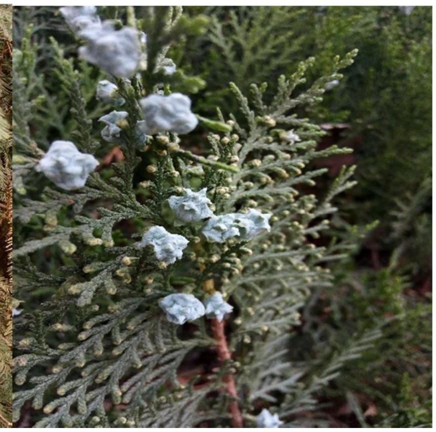

Fig 3. T. occidentalis

Thuja occidentalis L. belongs to Cupressaceae and commonly known as White Cedar and Morpankhi in Hindi. It is native to Eastern Canada and other regions on United State; widely cultivated as an ornamental plant throughout the planet (figure 3). T. occidentalis has been used to treat uterine carcinomas, bronchial catarrh, psoriasis and rheumatism (16). The essential oil of the plant has been used for soft soaps, room sprays, disinfectants and insecticides. Cedar leaf oil can be obtained by hydro distillation of the foliage and is used for the production of perfumes, insecticides, soaps and deodorants (17, 18). The essential oil is an active ingredient in the production of antibacterial herbal formulations, cough suppressants, soap and perfumes, while many cultivars are grown for ornamental purposes (7, 19). The oil of eastern cedar leaves (T .occidentalis) has been independently investigated by Shaw (20) and Rudloff (20), who reported the thujone fraction as a mixture of Z-thujone and E-isothujone, while Keita et al. (21) in their analyses, reported twenty-two compounds including α-thujone (= Z-thujone) (49.64%), fenchone (14.06%) and β-thujone (= E-isothujone) (8.98%) as the most abundant compounds.

II. MATERIAL AND METHOD:

A. Extraction of essential oil

The plant materials of P. roxburghii Sarg., T. distichum L., and T. occidentalis L., were collected from Roxburgh Garden, Department of Botany, University of Allahabad. Plant were identified at Department of Botany, University of Allahabad(7). Leaves were crushed and loaded into a Clevenger type Apparatus for hydrodistilation for 4-5 hours at 45 degree Celsius (7). Essential oils of T. distichum (bald cypress) appears as dark yellow, T. occidentalis (white Cedar) as yellow in colour followed by P. roxburghii (chir pine) i.e., pale yellow. Oil content was stored at 4°C until analysis (7, 22).

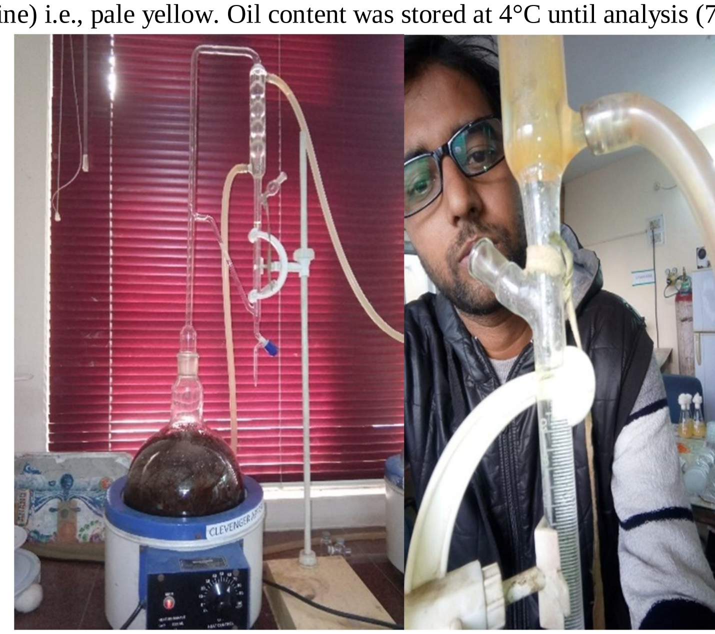

Fig4 and 5. Clevenger type apparatus.

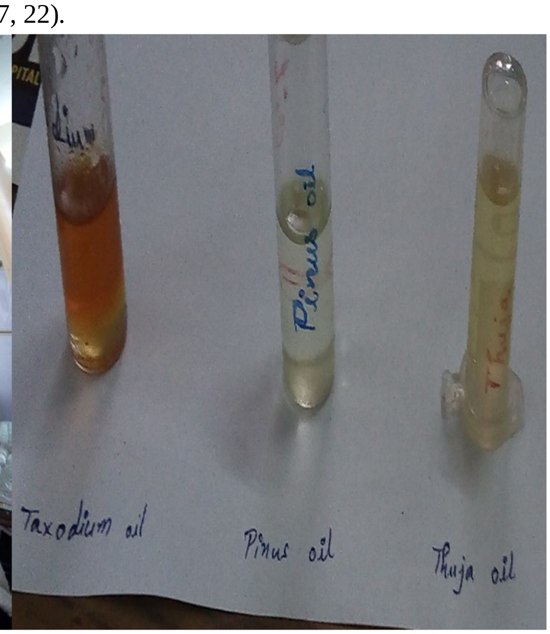

Fig6. Extracted oils

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B. Preparation Of 0.5 Mcfarland Solution And Saline Media

On adding 1% of $BaCl_2$ to the freshly prepared solution of 200 ml of 1% H_2SO_4, 0.5 McFarland solution is formed (7, 23). Now prepare saline media by dissolving 1 gm of NaCl into 100 ml of DDW (7). Take the O.D. of this saline media and McFarland solution.

C. Preparation of Mueller-Hinton broth (MHB) and Inocula

Take 1000 ml of DDW in a beaker. Add 21 gms of MHB powder. Shake well and boil up to 100 ºC. Close the mouth with sterilized synthetic plug (7). Inocula were prepared by using saline media and bacteria (7).

D. Antibacterial Screening

Essential oils were screened for antibacterial activity against S. typhimurium. Minimum Inhibitory Concentrations (MIC) were determined using Broth Micro-dilution method recommended by Clinical Laboratory Standard Institute (CLSI). 96 well plate were used for broth microdilutions. Column-1 contains 190 µL and 10 µL of formaldehyde and known as negative control (7). Column-2 contains 200 µL of MHB and known as broth control (7). Column-3 contains 180 µL broth and 20 µL drug in each row and known as drug control. Row A and B of column-3 contains 20 µL of Streptomycin. Row C and D contains 20 µL of chir oil. Row E and F contains bald cypress oil whereas row G and H contains cedar oil. Now add 100 µL of broth from column-4 to column-12. In column-4, add 80 µL broth and 20 µL drugs in each row as mentioned previously. Now homogenize and dilute the drugs 1:1 in MHB horizontally from column-4 to column-11. Finally add 100 µL inocula from column-4 to column-12 (7) (figure 7). Final volume of each well were 200 µL. The solutions were incubated at 37 °C for 24 hours (7, 24). *Streptomycin* used as positive control. Formaldehyde was used as a negative control.

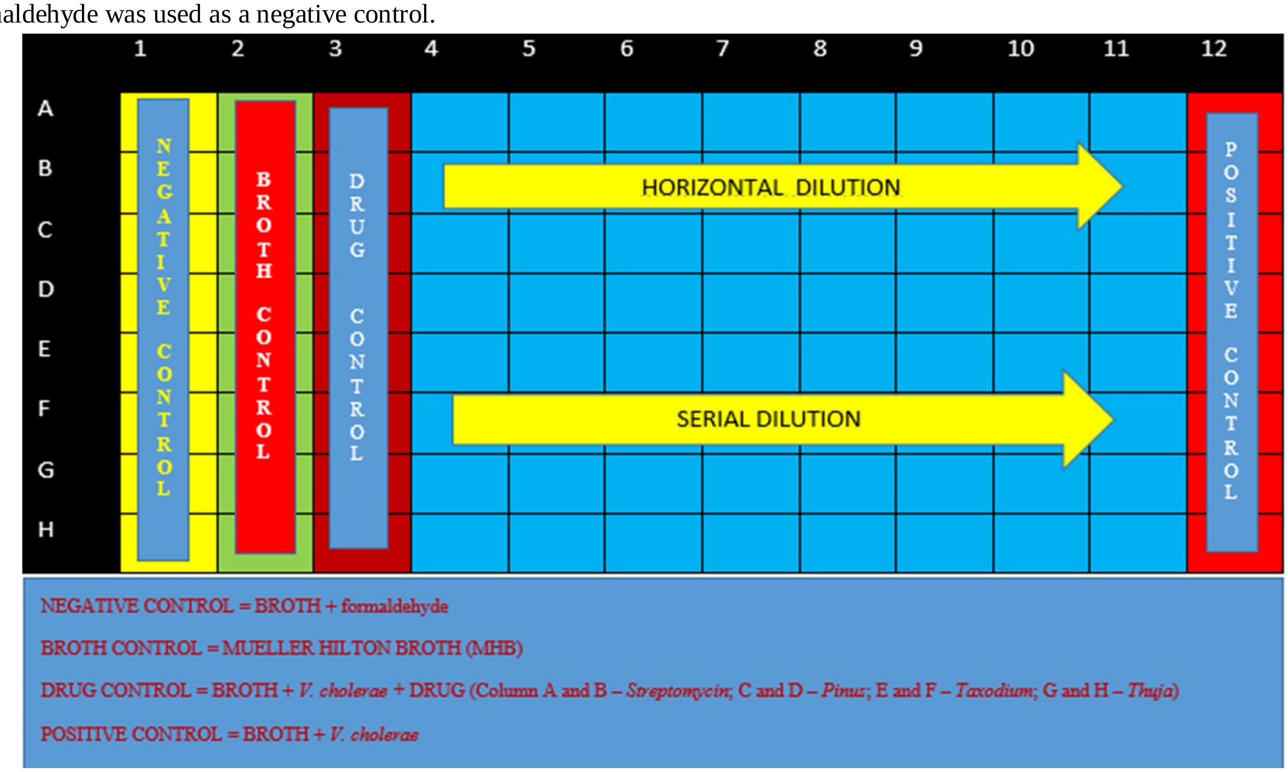

Fig7. – Diagrammatic representation of horizontal broth microdilution.

III. RESULTS

The results were recorded in terms oil Inhibition Concentrations (IC50) and Minimum Inhibition Concentrations (MICs) via SpectramaxPlus384, Molecular Devices Corporation, USA. IC50 value of P. roxburghii, T. distichum and T. occidentalis were showed 1.294, 0.914 and 1.277 mg/ml respectively (Figure 8, 9). The minimum inhibition concentrations (MIC) of P. roxburghii, T. distichum and Thuja occidentalis were recorded 1.403, 1.995 and 1.864 mg/ml respectively (Figure 8,9). P. roxburghii was found to be most effective with their MIC 1.403 mg/m1 whereas T. distichum was found to be least effective with their MIC 1.995 mg/m1 against V. cholerae (Figure 9).

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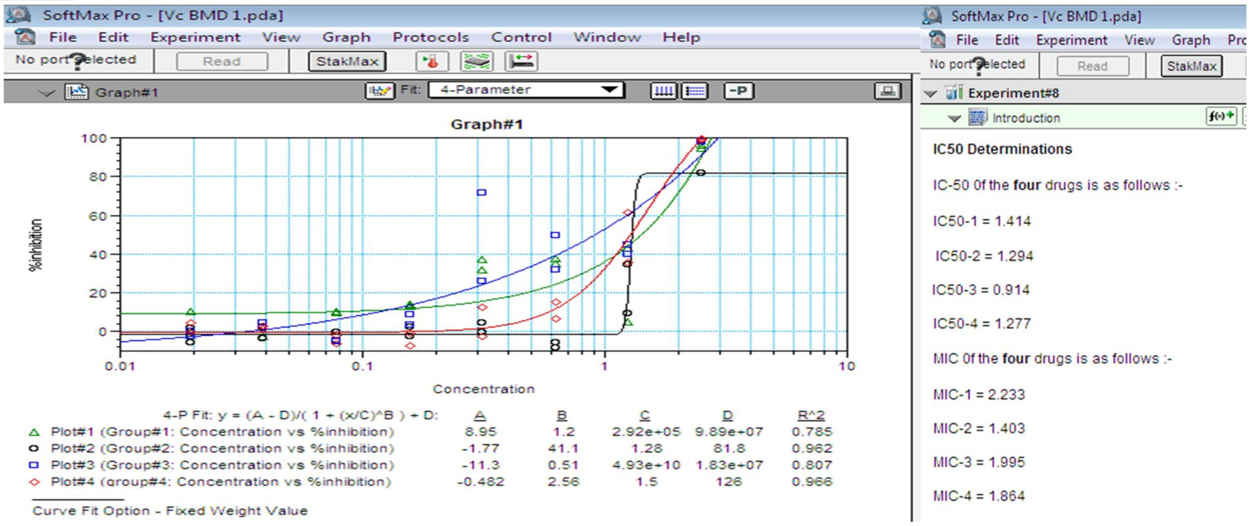

Fig8. Graph obtained for antibacterial activity of essential oils and values of IC50 and MIC.

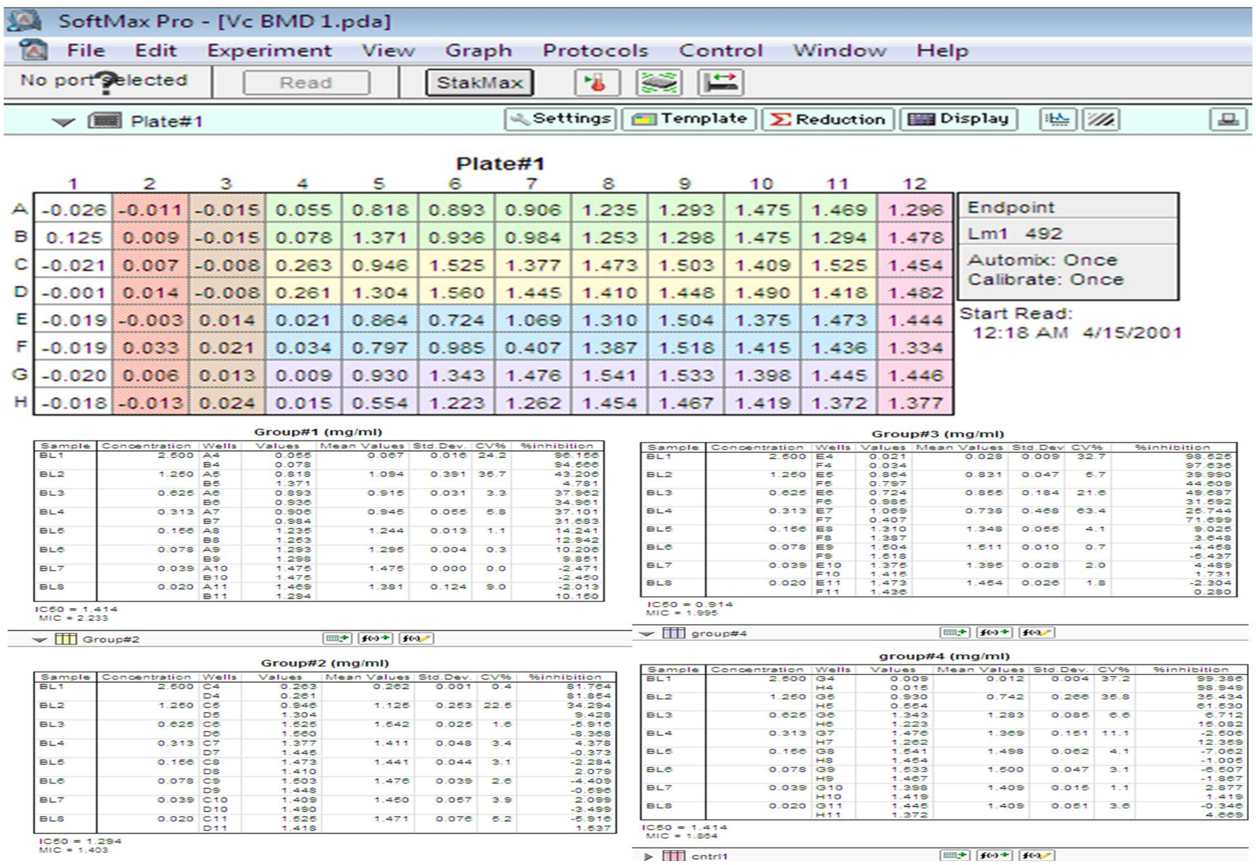

Fig9. Different values of different drugs against V. cholerae obtained from SpectramaxPlus 384 molecular Device USA.

V. CONCLUSION

It can be concluded from the present study that all the three Gymnospermous essential oil have some synergistic activity against V. cholerae. It was first time in our best knowledge that essential oil of P. roxburghii shows remarkable effect against V. cholerae. Chir oil shows remarkable efficiency over bald cypress oil and white cedar oil against bacteria. Pinus oil shows great efficiency against V. cholerae and other microbes (25). The components (terpenes) of essential oil of P. roxburghii needles are highly active against microbes. As this oil significantly inhibited the growth of certain bacteria tested. The main oil component of P. roxburghii essential oil are monoterpene and sequiterpene hydrocarbons and their derivatives. These derivatives act as antibacterial and antifungal substance, the most well-known of which being terpenes and phenolics in general (26). The essential oil from the leaves and cones of bald cypress trees grown exhibited potent antimicrobial activities against bacteria (27). Essential oils from needles and foliages of these gymnosperms plants viz., P. roxburghii, T. distichum and T. occidentalis, exhibit great potential eco-friendly, non-toxic, cost-efficient and antibacterial herbal formulations.

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