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SYNERGETIC EFFICACY OF THREE GYMNOSPERMS AGAINST *Klebsiella pneumoniea*

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ABSTRACT

The present investigation were focused on the synergetic efficacy of the essential oil of the three gymnospermous foliages *i.e.*, *Pinus roxburghii* Sarg., *Taxodium distichum* L. and *Thuja occidentalis* L., against *Klebsiella pneumoniea* (MTCC-4032). The oils were extracted from the needles and foliages of aforementioned plant species using hydro-distillation method. The antibacterial activity of the extracted essential oils was evaluated against *Klebsiella pneumoniea* (MTCC- 4032) using broth micro-dilution method recommended by Clinical Laboratory Standards Institute (CLSI). The Inhibition Concentration *i.e.* IC_{50} and Minimum Inhibition concentrations (MIC) using SpectramaxPlus[384], of Molecular Devices Corporation, USA were recorded while Streptomycin as standard was taken. The IC_{50} value of *P. roxburghii, T. distichum* and *T. occidentalis* were showed 0.278, 0.093 and 0.161 mg/ml respectively. The *T. distichum* was found most effective with their MIC 0.277 mg/ml while *P. roxburghii* found least effective with their MIC 0.616 mg/ml against *K. pneumoniea*. Hence, essential oil from foliages and needles of gymnosperms exhibit great potential for the development of eco-friendly, non-toxic, cost effective anti-bacterial formulations after undergoing detailed investigation which is in progress.

KEYWORDS: Gymnosperms, Essential Oil, Synergetic Efficacy, Broth Micro-dilution

P. roxburghii Sarg. is also known as chir pine, belongs to family Pinaceae, is a native of Himalayas and distributed throughout India, Nepal, Bhutan and Pakistan. It is widely distributed throughout Himalayan range of India.*P. roxburghii* is a large tree attaining up to 30-60 m in height with a trunk diameter reaching up to 2 m (figure 1). Ovoid and conic cones of *P. roxburghii*usually open up to 18-20 cm to release the seeds (Press *et al.,* 2000). *P. roxburghii* oil has been traditionally used to treat cuts, wounds, boils and blisters (Wu and Raven, 1999). In addition, phytochemical screening of Pinus needles and stems have pound abundant amounts of vitamin C, tannins, and alkaloids while the stem has been primarily used as a source of turpentine oil (Gewali, 2008) (Vallejo *et al.,* 1994) (Asta *et al.,* 2006). Previous research on microbes suggests that the essential oil on *P. roxburghii* has shown significant anti-fungal and anti-bacterial activity (Hassan and Amjid, 2009) while alcoholic extract of the needle, stem, and cones are reported to exhibit strong anti-bacterial activity.

Taxodium distichum. (L.) L. C. is commonly known as bald cypress, is an unusual and interesting tree, belongs to Taxodiaceae. It may grow upto 25 m in height and over 300 cm in diameter (figure 2). The leaves are small, 5–15 mm long, green to yellow-green and appearing two-ranked. The fruits are cones and are composed of scales forming a woody, brown sphere with rough surface 1.5 to 4 cm in diameter. *Taxodium distichum* (L.) has three extant taxa ranging from the eastern United States through Mexico to Guatemala (Adams, 2001). The trees are used for their wood because heartwood is extremely rot and termite resistant (Tantawy *et al.,* 1999). Leaves and cones are rich in essential oils and used traditionally to treat gastro-intestinal, skin, respiratory, inflammation, and infections (Flamini *et al.,* 2000) (Geiger and de Groot-Pfleiderer, 1979).Flavonoids and diterpenoids are the main secondary metabolites (Adams, 2001). *T. distichum* trees can grow on rivers, lake margins, swamps, wet poorly drained habitats and are tolerant to various soil conditions and air pollution (Denny, 2007). In many countries these are widely used for landscape. The heartwood of bald cypress is used for building materials, and has been reported to resist the attacks of the subterranean termite (Kusumoto *et al., 2010*).

Figure 1: ***P. roxburghii***

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Figure 2: ***T. distichum***

Figure 3: ***T. occidentalis***

Thuja occidentalis L. is the native to Eastern Canada and other regions on United State; widely cultivated as an ornamental plant (figure 3). *T. occidentalis* has been used to treat bronchial catarrh, psoriasis, rheumatism and uterine carcinomas (Peng and Wang, 2008). The essential oil of the plant has been used for disinfectants, insecticides, room sprays, and soft soaps. Cedar leaf oil can be obtained by steam distillation or hydro-distillation of the foliage and is used for the production of perfumes, insecticides, soaps and deodorants (Kamden and Hanover, 1993) (Duke, 1985). The essential oil is an active ingredient in the production of cough suppressants, perfumes and soaps, while many cultivars are grown for ornamental purposes (FAO, 1995).

MATERIALS AND METHODS

Extraction of Essential Oil

The plant materials of *P. roxburghii*, *T. distichum*, and *T. occidentalis* were collected from Roxburgh Garden, Department of Botany, University of Allahabad, in the month of October. Plant were identified at Department of Botany, University of Allahabad. Leaves (needles, foliages) and branchlets were crushed and hydrolyzed using a Clevenger type Apparatus for 4 hours (figure 4). Secondary metabolites of *T. distichum* (bald cypress) appears as dark yellow, yellow coloured oil were extracted from *T. occidentalis* followed by *P. roxburghii* (chir pine) i.e., pale yellow (figure 5). Oil content was stored at 4°C until analysis (Isiaka *et al.,* 2007).

Figure 4: Clevenger Type Apparatus.

Figure 5: Extracted Oils

Preparation of 0.5 McFarland Solution and Saline Media

Standard method was slightly modified for our study. Dissolve 2.04 ml of H_2SO_4in double distilled water (DDW). Now add 1% $BaCl_2$ to the freshly prepared solution (McFarland, 1907). 0.5 McFarland solution is ready. To prepare saline media, dissolve 1 gm of NaCl into 100 ml of DDW (Singh *et al.,* 2018). Take the O.D. of this saline media.

Preparation of Mueller-Hinton Broth (MHB)

Take 250 ml of DDW in a beaker. Add 5.25 gms of MHB powder. Shake well and boil up to 100 ºC. Close the mouth with cotton plug. Place the solution inside autoclave. After this, MHB is ready to use.

Preparation of Inocula

Saline media was taken in a culture tube. Add bacterium in the same tube. Final volume in the culture tube is 1000 µL. Now take 500µL of this solution and add it into another culture tube containing MHB, so that the final volume becomes 20 ml. Now inocula will be ready for use.

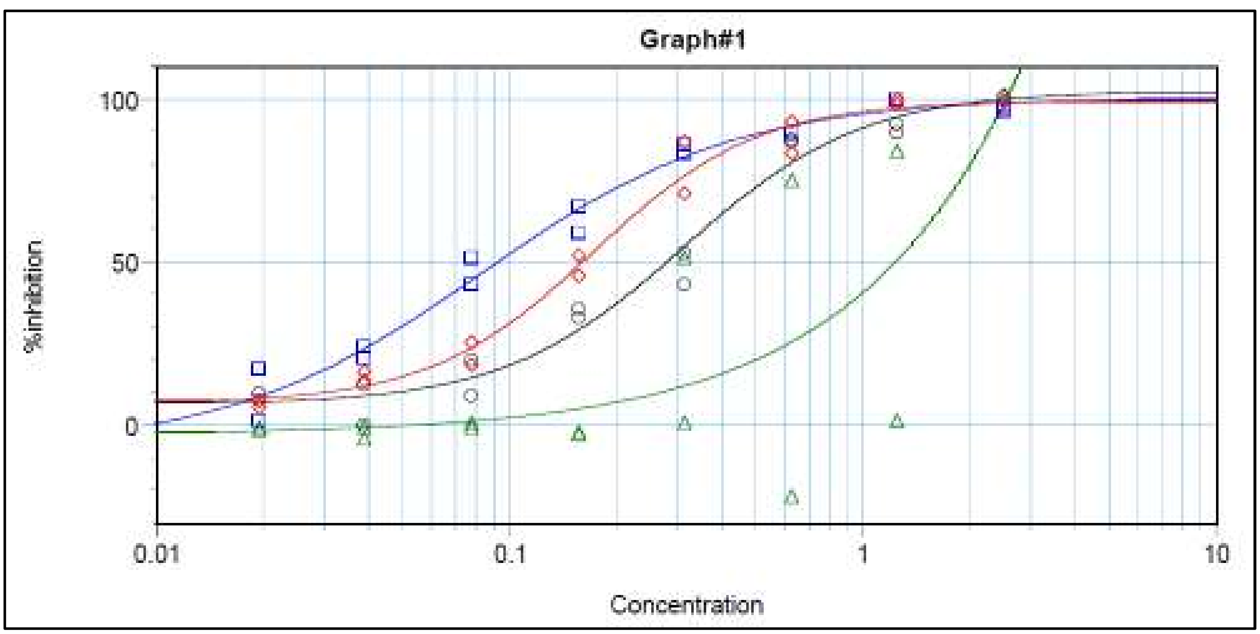
Antibacterial Screening

Essential oils were screened for antibacterial activity against *K. pneumoniea*. Minimum Inhibitory Concentrations (MIC) were determined using Broth Micro-dilution method recommended by Clinical Laboratory Standard Institute (CLSI). 96 well plate was used for microdilutions. Column-1 contains 190µL and 10 µL of formaldehyde (added after the completion of microdilution). Column-2 contains 200µL of MHB. Column-3 is the drug control. Row A and B of column-3 contains streptomycin. Row C and D contains*Pinus* oil. Row E and F contains *Taxodium* oil whereas row G and H contains *Thuja* oil. 100 µL of broth were added from column-4 to column-12. In column-4, we add drugs in each row one by one as described previously for column-3. Now dilute the drugs horizontally from column-4 to column-11. Now add 100µL inocula to each well from column-4 to column-11. Final volume of each well were 200µL.The extract solutions over horizontally diluted 1:1 in MHB in a 96 well plates were incubated at 37 °Cfor 24 hours (Satyal *et al.,* 2012) (Singh *et al.,* 2018). The final minimum inhibitory concentration and it was determined as the lowest concentration without turbidity. Streptomycin used as positive control. Formaldehyde was used as a negative control.

RESULTS

Percent yield: % yield = weight of oil / weight of sample x 100.

P.roxburghii = 0.100 %, *T. distichum* = 0.260% and *T. occidentalis* = 0.350 %.



Graph 1: Graph Showing Percent Inhibition against Concentration of Different Drugs.

IC50 Determinations

IC-50 Of the **four** drugs is as follows :-

IC50-1 = 1.232

IC50-2 = 0.278

IC50-3 = 0.093

IC50-4 = 0.161

MIC Of the **four** drugs is as follows :-

MIC-1 = 2.008

MIC-2 = 0.616

MIC-3 = 0.277

MIC-4 = 0.350

Figure 6: IC50 and MIC values obtained from Spectra Max Plus 384.

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 0.278, 0.093 and 0.161 mg/ml respectively (Figure 6). The minimum inhibition concentrations (MIC) of *P. roxburghii, T. distichum* and *Thuja occidentalis* were recorded 0.616, 0.277 and 0.350 mg/ml respectively (Figure 6). *T. distichum* was found to be most effective with their MIC 0.277 mg/m1 whereas *P. roxburghii* was found to be least effective with their MIC 0.616 mg/m1 against *K. pneumoniea.*

CONCLUSION

It can be concluded from the present study that all the three Gymnospermous essential oil have some activity against *K. pneumoniea*. Taxodium oil shows remarkable efficiency over Pinus oil and Thuja oil against bacteria. Taxodium oil shows great efficiency against *K. pneumoniea* and other microbes (Von Rudloff, 1975). The components of essential oil of *P. roxburghii* needles are highly active against microbes (Singh *et al.,* 2018). As this oil significantly inhibited the growth of certain bacteria and fungi 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 (Gulten *et al.,* 2012). The essential oil from the leaves and cones of baldcypress trees grown exhibited potent antimicrobial activities against bacteria (Tantawy *et al.,* 1999).So, essential oils of these gymnosperms exhibit great potential eco-friendly, non-toxic, cost-efficient and antibacterial herbal formulations (Singh *et al.,* 2018).

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