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Qualitative estimation of bioactive compounds and evaluation of antimicrobial activity of *Strychnos nux-vomica* L. leaf extracts

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ABSTRACT

Strychnos nux-vomica, an important wild medicinal plant of Odisha, was evaluated for the possible bioactive compounds through qualitative screening and thin layer chromatography (TLC). The Minimum Inhibitory Concentration (MIC) was determination for the anti-microbial activity of its leaf extracts (n-hexane, acetone, methanol and aqueous) against five bacterial strains (*Streptococcus pyogenes, Streptococcus mutans*, *Shigella flexnerii*, *Salmonella enteric-typhii* and *Vibrio cholerae*) and two fungal strains (*Candida parapsilosis* and *Aspergillus tubingensis*). The results of phytochemical screening revealed the presence of saponin, tannin, alakaloids, flavonoids, phenolic compounds, steroids and terpenoids in the leaf extracts. With n-hexane and aqueous extracts, the MIC of 400 µg/ml was found effective against *Streptococcus pyogenes*, *Shigella flexnerii* and *Candida parapsilosis*. TLC showed visible bands with aqueous and n-hexane extracts. The results of the present study corroborates the claims of tribals and traditional healers about the use of *S. nuxvomica* for the treatment of bacterial and fungal infections.

1. Introduction

An impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine (Abraham and Thomas, 2012). Traditional medicines are used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system . The herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries; more than 65% of the global population uses medicinal plants in their primary health care needs (Kamaraj *et al.,* 2012). In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial and fungal.

Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates. Numerous investigations have proved that medicininal plants as well as microorganisms contain diverse classes of bioactive compounds such as tannins, alkaloids, flavonoids, terpenoids, phenols, etc (Chitemerere and Mukanganyama, 2011). Plants have been a major focus of investigations for novel biologically active compounds and the searches for new anti-microbial agents from medicinal plants are even more urgent in the countries like India where infectious diseases of bacterial origin are not only rampant, but the causative agents are also developing an increasing resistance against many of the commonly used antibiotics (Kamaraj *et al.,* 2012).

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Strychnos nux-vomica (Poison nut) belongs to family Loganiaceae and is locally known as Kochila. It is a medium-sized, deciduous tree with a straight trunk, leathery leaves, funnel-shaped, greenish-white flowers and orange coloured (when matured), globose fruits. Leaves are used for treating chronic wounds and ulcers (Arivoli and Tennyso, 2012). Fruit are used as appetizer, tonic and useful in the treatment of leucoderma, blood disorders, piles, ulcers, pneumonia, haemoptysis, occipital headache, anemia, jaundice, itching, and urinary infection (Gamble and Fischer, 1957; Han *et al.,* 2008; Ghosh, 1935). Bark is used as tonic to cure epilepsy (Pattanaik, 2006; Gruenwald, 2000; Singh *et al.,* 2009).

Thus the main aim of this work was to detect the various bioactive components present in leaf extracts of *Strychnos nux-vomica* and to determine the antibacterial activity of the species to prove its use as a safe and potent antibacterial agent.

2. Materials and methods

2.1. Collection of plant samples and preparation of extracts

Leaves of Strychnos nux-vomica plants were collected from Chandaka forest, Khordha, Odisha, India. The collected plant parts were dried at room temperature under shade and powdered using mechanical devices after drying. The leaf powder was kept in thimble and extraction was carried out using the Soxhlet apparatus (Tiwari *et al.,* 2011). The residues were collected and left for air drying later and the dried crude extracts were stored in refrigerator for further experimental work.

2.2. Phytochemical screening

Five grams of powered leaf samples were soaked in 55 ml test tube (Borosil, India) containing 30 ml each of aqueous (distilled water), acetone, chloroform, n-hexane, methanol and toluene. All these were kept at room temperature for overnight. Then the solvent extracts were filtered through Whatman No. 1 filter paper (Himedia, India) and were used for the preliminary qualitative phytochemical analysis following standard procedures (Harborne, 1973; Sofowara, 1993; Trease and Evans, 1989).

2.3. Antimicrobial study

Four different solvents (n-hexane, acetone, methanol and aqueous) as per their polarity index were used for antibacterial activity testing. The extracts of experimental plant were screened for antibacterial activity against five bacterial strains [*Streptococcus pyogenes* (MTCC-1926), *Streptococcus mutans* (MTCC-497), *Shigella flexnerii* (MTCC-1457), *Salmonella enteric-typhii* (MTCC-1252) and *Vibrio cholera* (MTCC-3906)] and two fungal strains [*Candida parapsilosis* (MTCC-2513) and *Aspergillus tubingensis* (MTCC-4285)] collected from the IMTECH, Chandigarh, India. Nutrient broth (Hi-Media, India) was used to maintain broth cultures. An additional 1.8 gm of agar (Hi-Media, India) per 100 ml made up the nutrient agar medium. The medium was autoclaved at 15 psi pressure in a temperature of 121^0 C for 20 min to ensure sterilization. The media used for fungal culture was Sabouraud's dextrose agar/ broth of (Hi media, India).

Antibacterial activity was assessed by Minimum Inhibitory Concentration (MIC) using two fold serial dilution methods (CLSI, 2002; CLSI, 2009). Selected colonies of aforesaid microbes were picked off from a fresh isolation plate and inoculated in corresponding tubes containing 5 ml of nutrient broth (Hi-media, India) and Sabouraud's dextrose broth. The broth was incubated for 6±1 hours at 35±2 ^{0}C for bacteria and 24-48 hours at 28±2 ^{0}C for fungal strain or until there was visible growth appeared. McFarland 0.5 standard was used to adjust the turbidity to get 10^5 colony forming units (CFU)/ml. McFarland standard was prepared by standard methods (Chapin, 2003) using barium chloride and sulphuric acid (1.17 % of $BaCl_2$. $2H_2O$ with 1 % of H_2SO_4) and visual comparison was carried out (Carlberg, 1985; CLSI, 2009; Versalovic *et al.,* 2011) using Wickerham Card (B005R43DK8, Carolina Biological Supp. Comp.) or white card with black lines (Jiang, 2011). Each crude leaf extracts of 16 mg extract dissolved in 10 ml of DMSO to get desired drug concentrations.

MIC was calculated by two fold serial broth dilution method for leaf extracts/solvents with standard Ampicilin (Hi-media, Mumbai, India) for bacterial strains and Amphotericine B (Hi-media, India) for fungal strains. The method includes 24 tubes of 5 ml capacity were arranged in 3 rows/replications with each row containing 8 tubes. Nutrient broth of 1.9 ml for bacteria and Sabouraud's dextrose of 1.9 ml for fungal strain was taken to first tube and 1ml to other 7 tubes was added in each row or to replication. Crude extract (16 mg in 10 ml of DMSO) of 100 µl was added to the first tube in each row and after mixing the content; 1 ml was serially transferred from first tube to the second tube, then 1ml from second to third, third to four, four to five, five to six then six to seven in each of the rows. This provide extract concentrations of 1600, 800, 400, 200, 100, 50, 25 and 12.5 µg/ml in the first to seventh tube respectively in each row. Finally, 1 ml (10^5 CFU/ml) of bacterial suspension and fungal suspension were added to first, second and third rows of tubes respectively. All the test samples and control/standard tubes were then incubated

for 12-18 hours at 35±2 °C for bacteria and 48 hours at 30 ± 1 ^{0}C for fungal strain (Bayati and Mola, 2008). After the incubation, the tubes of lowest concentration showing no visible growth after 8 hours till 12 hours were considered to be inhibition of bacteria and 24 hours till 48 hours for fungus (Liete *et al.,* 2014**)** which represent MIC values. Inoculums control showed visible growth due to no antimicrobial agents whereas, the negative control DMSO showed no growth due to absence of microbes. Triplicates were maintained and the experiment was repeated thrice, for each replicates the average readings were taken for all the experiments designed. Data mentioned for MIC values is mean ± SD for all readings.

2.4. Thin Layer Chromatography(TLC)

For TLC, the readymade aluminum sheets (20×20 cm) of TLC silica gel 60 F254 (Merck, Germany) were used. The samples were applied on the silica gel by capillary made up of glass. Thin layer chromatography of different solvent systems was prepared for leaf extracts. Out of which Toluene/Glacial acetic acid (3:1) showed higher band separation in both the extract (hexane, acetone, methanol and aqueous) of *S. nux-vomica*. All the solvents used are of laboratory grade (Merck, India). The R_f was calculated for different compounds by dividing the distance of the compound travelled from the original position by the solvent travelled from the original position (the solvent position). All the experiments were repeated three times and the mean data recorded for all the observations.

3. Results and discussion

Plants are known as the "chemical factories" of nature as they provide the richest source of organic chemicals on earth (Prabha *et al.,* 2014). The results of qualitative phytochemical screening of experimental plants (leaf extracts) showed the presence of seven different phytochemical like saponin, tannin, alkaloids, flavonoids, phenolic compounds, steroids and terpenoid. It was observed that methanol and acetone extracts showed highest number of bioactive compounds followed by aqueous and n-hexane extracts (Table 1). No bioactive compounds were detected in toluene extract of *S. nux-vomica* leaf. Similar results have been reported in *S. nux-vomica* extracts by Magdalin Joy and Reginald Appavoo (2014).

Table 1
Qualitative estimation of bioactive compounds of *Strychnos nux-vomica* leaf extracts

Phytochemicals	Extracts					
	Aqueous	n-hexane	Toluene	Acetone	Chloroform	Methanol
Saponin	+	-	-	-	-	+
Tannin	+	+	-	+	-	+
Alkaloids	+	+	+	+	-	+
Flavonoids	-	-	-	+	-	+
Phenolic compounds	+	-	-	+	-	+
Steroids	-	-	-	-	-	-
Terpenoids	-	-	-	-	-	-

(+ = presence, - = absence)

Since no information was available in published literature with respect to phenolic content of the leaves of *S. nux-vomica* (Eldahshan and Abdel-Daim, 2015), an attempt was made to find out potential bioactive compounds in the leaves. Only few workers studied the effect of different solvent based leaf extracts on different microbial strains like *Staphylococcus aureus, Salmonella, Klebsiella pneumonia, Aspergillus niger*, *Aspergillus terreus, Aspergillus flavus* (Gnanavel *et al.,* 2012); *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Aeromonas hydrophyla, Pseudomonas aeruginosa* (Senthilkumar *et al.,* 2005). In our work, leaf extracts of four different solvent systems tested showed anti-microbial activity (MIC values) against all pathogenic bacterial and fungal strains at different levels. Among the four solvents screened, n-hexane and aqueous leaf extract showed excellent (MIC = 400µg/ml) antimicrobial activity against 3 pathogenic strains and MIC value of 800µg/ml) for other 4 strains. While highest antimicrobial activity (MIC = 800µg/ml) was observed in acetone and methanol solvent extracts of leaf against 3 pathogenic strains, the lowest antibacterial activity (MIC = 1600 µg/ml) was recorded against the other 4 strains. Similar results have been also obtained earlier in medicinal plants like *S. nux-vomica* (Prabha *et al.,* 2014; Magdalin Joy and Reginald Appavoo, 2014; Thambi and Cherian, 2015), *Lawsonia inermis* (Moharana *et al.,* 2014), *Tinospora*

cordifolia (Kumari, 2012) and *Tylophora indica* (Jahan *et al.,* 2013). Of the seven microbial strains evaluated, the best MIC value (400 µg/ml) was observed in n-hexane and aqueous leaf extract against *Streptococcus pyogenes*, *Shigella flexnerii* and *Candida parapsilosis* but the least MIC value (1600 µg/ml) was noticed in acetone and methanol leaf extract against *Streptococcus mutans*, *Salmonella enteric-typhii*, *Vibrio cholera* and *Aspergillus tubingensis* (Fig. 1). Ampicilin was used as standard for bacterial strains with highest MIC at 25µg/ml and a lowest MIC 12.5µg/ml while Amphotericine B was used as standard for fungal strains with a MIC of 25µg/ml (Fig. 1). Similar experiment was conducted by Kalaivanan *et al.,* (2014), in which they prepared leaf extracts of different solvents like hexane, chloroform, ethyl acetate and methanol and treated them against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli, Salmonella typhimurium, Shigella flexneri, Proteus mirabilis, P. vulgaris* and *Vibrio cholera*. Among these extracts, they found methanol extract having higher efficacy against *S. flexnerii* and *S. aureus*.

As the results of Table 1 revealed the presence of bioactive compounds, TLC screening was carried out. The TLC of *S. nux-vomica* showed the visible spot in aqueous extract at mean R_f 0.50 (lemon yellow), R_f 0.55 (lemon green) and visible spot of n-hexane extract at mean R_f 0.40 (light bottle green), R_f 0.44 (light bottle green), R_f 0.48 (light bottle green) using mobile phase Toluene/acetic acid (3:1 v/v) (Fig. 2). Rathi *et al.* (2008) optimized TLC procedure of methyl extract of fruit pulp of Kochila (*S. nux-vomica*) in the mobile phase chloroform / ethyl acetate / diethyl amine (0.5:8.5:1, v/v/v) and found bands at R_f 0.42 and 0.55, of the two R_f values one was coinciding with our R_f value i.e. 0.55 in aqueous extract. In such an experiment, Mathivanan *et al.* (2014) optimised TLC procedure of methanol leaf extract of *S. nux-vomica* in the mobile phase methanol/chloroform (1:9, v/v) and found R_f 0.48, 0.60,

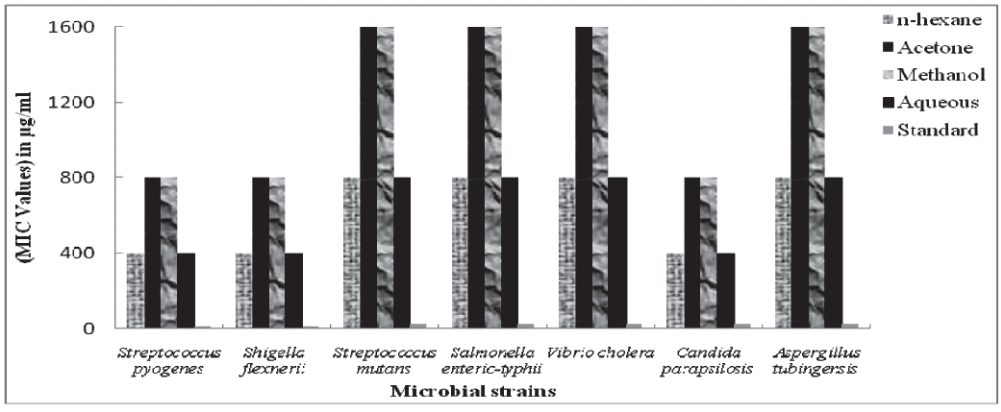

Fig. 1 Antimicrobial activity of *S. nux-vomica* leaf extracts against pathogenic strains

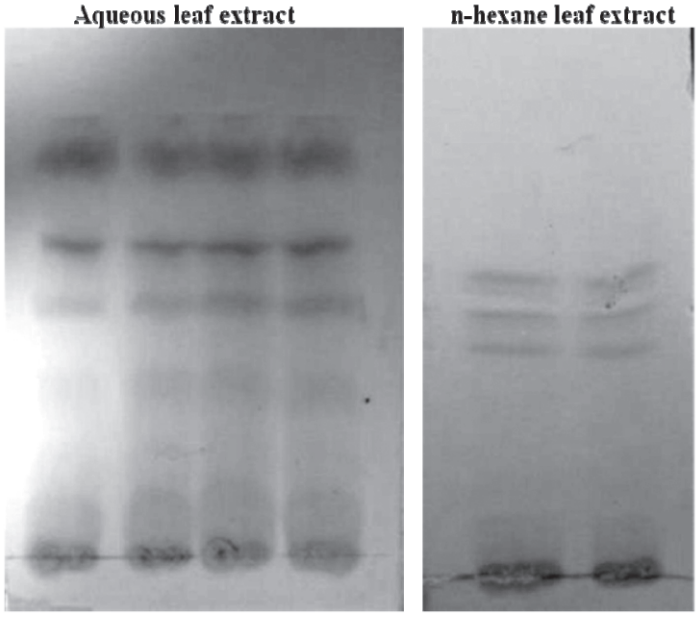

Fig. 2. TLC Fingerprinting of *S. nux-vomica*

0.71 and 0.83. From these four values, one was coinciding with our R_f value i.e. 0.48 in n-hexane extract.

Recent research indicated that the antibiotics are becoming ineffective against common pathogens such as *S. pyogenes* (Feng *et al.,* 2010; Gracia *et al.,* 2009). In addition, currently fungal infections are ranked fourth of nosocomial infections. The yeast candida is implicated in more than 75% of invasive or systemic fungal infections (Richardson, 2005). About 25% of all medicine available in the market have been derived directly or indirectly from plants (De Smet, 1997; WHO 2005). Herbal medicines are generally believed to be safe, but it is important to evaluate their biological safety aspect before use so that harmful consequences could be avoided (Kunle *et al.,* 2012).The present study validates medicinal uses of *S. nux-vomica* against bacterial infections. The antimicrobial activity of the plant may be attributed to the various phytochemical constituents present in the crude extract of leaf. The work reported here is of preliminary nature and aimed at finding out the antimicrobial activity of this medicinal plant and the result of the study established good antibacterial and antifungal activity of *S. nux-vomica* leaf extracts. The study indicates the plants could be a potential source of newer antimicrobial agents. Further work on the types of phytoconstituents and purification of individual groups of bioactive compounds can reveal the potential of the plant extract to control microbial infections and as an effective application for control of a broad spectrum microbes causing severe skin problems, upper respiratory tract infections, eye infections, onychomycosis, nosocomial infection etc.

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