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Antibacterial activity and Phytochemical Analysis of *Morinda tinctoria* leaf extracts

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Abstract
The objective of the present work is to evaluate the presence of Phytochemical constituents and antimicrobial activity of different extracts of leaves of *Morinda tinctoria*. The serial exhaustive extraction was done with a series of solvents: Hexane, Ethylacetate and Methanol with increasing polarity using soxhlet apparatus. The Phytochemical analysis was done by using the standard procedures. Antimicrobial activity was evaluated by Agar well diffusion method against human pathogens. The results revealed that the leaf extracts contain a broad spectrum of secondary metabolites: Alkaloids, Flavonoids, Phenols and in major proportion. Methanol extract was shown to be more effective against all the organisms followed by Ethylacetate and Hexane extracts. *Proteus vulgaris* (35mm) was found to be most sensitive organism followed by *Klebsiella pneumonia* (33mm) and *Staphylococcus aureus* (34mm). The present study concludes that the different extracts of *M. tinctoria* leaves contain a broad spectrum of secondary metabolites and also exhibit antimicrobial activity against all the tested microorganisms. It can also be concluded that *Morinda tinctoria* plant can be exploited to discover the bioactive natural products that may serve as leads in the development of new pharmaceuticals.

Keywords: *Morinda tinctoria,* phytochemical, ethylacetate and antimicrobial

Introduction
Due to alarming increase in the rate of infections with antibiotic resistant microorganism and due to side effects (Rekha bisht *el al*., 2009) [1] of some synthetic antibiotics there is an increasing interest in medicinal plants as a natural alternate to synthetic drugs (SEyyedednejad and Motamedi 2010) [2]. Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as pharmaceuticals. Species of higher plants were less much surveyed for antibacterial activity (Mohana *et al.,* 2008) [3].
Morinda tinctoria belongs to the family Rubiaceae grows wildly and distributed throughout Southeast Asia, commercially known as Nunaa and locally known as "Togaru", is a small tree with immense medicinal properties. It is indigenous to tropical countries and is considered as an important folklore medicine. In the traditional system of medicine, leaves and the roots of *M. tinctoria* are used as astringent, Deobstrent, Emmengogue and to relive pain in the gout (Thirupathy Kumaresan and Saravanan, 2009) [4]. There is a greater demand for fruit extract of morinda species in treatment for different kinds of illness such as arthritis, cancer, gastric ulcer and other heart disease (Sivaraman and Muralidharan, 2010) [5].
The various parts of this tree has been extensively used as eczema, fever due to primary complex, ulcer, glandular swellings, digestive disorder especially in children, in venereal diseases (Jancy Mery and Inbathamizh, 2005) [17]. Antioxtidant, cytotoxic activities, anticancer activity (Jaya kumar and Jaya Santhi, 2012) [18], Antimicrobial activity and phytochemical screening (Goyal *el al.,* 2013) [19]. The ashes of M.tinctoria leaves are also reported to act as biosorbents in controlling ammonia pollution in waste waters (Suneetha and Ravindhranath, 2012) [6]. The present study aims in exploring the phytochemical constituents, antibacterial properties of the crude leaf extracts of *Morinda tinctoria* (L).

Materials and Methods
Plant Collection
Morinda tinctoria plant was identified and collected from STET Women's College Herbal garden, Mannargudi, Thiruvarur Dt, India. Fresh plant material was washed under running tap water, air dried and then homogenized to fine powder. The powder was stored in airtight bottles at -20 °C until further use.

Crude Extraction
The dried plant material of 1kg was extracted with 2 lit of Hexane in a Soxhlet apparatus for 72h at 50 °C. After the extraction the solvent was removed with the help of rotatory evaporator. The same process was carried out to get, ethyl acetate & methanol extracts.
The total yield of the extracts obtained after removing the solvents was Hexane-15g, Ethyl acetate- 20g and Methanol-43.6 g. All the solvents and chemicals used and obtained from Madras Scientific Supplies, Trichy, India. The nutrient agar was obtained from Hi-media (Mumbai, India). Streptomycin and Tetracycline were used as the reference antibiotics.

Phytochemical Screening
The freshly prepared leaf extracts of *Morinda tinctoria* were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extracts was performed using the following reagents and chemicals: Alkaloids with Mayer's, Wagner's, Hager's and Dragendorffs reagent;

carbohydrates with Molish's, Fehling's, Barfoerd's and Benedict's reagents; Glycosides with Modified Brontragers test and Legal's test; Saponins were tested with Froth test and Foam test; Fixed oils and fats with Stain test and acetone-water test; Phenols with Ferric chloride test; Tannins with Gelatin test and L ead acetate test; Flavonoids with Lead acetate test, Alkaline reagent test, Shinoda test and Zinc hydrochloric acid reduction test; Proteins and Amino acids with Xanthoproteic test, Biuret test and Ninhydrin test; They were identified by characteristic color changes and precipitation reactions using standard procedures (Trease and Evans,1989, Jeffrey Harborne, 1998) [8, 9].

Isolation of test pathogens

Sample collection

The test bacterial pathogens were isolated from wound samples were collected from Government Hospital, Mannargudi, Thiruvarur District, India. These are using sterile cotton swabs (fresh pus) but small screw capped bottle a firmly stopper tube or syringe or a sealed capillary tube it must be bearing the patients name, age (Koneman *et al*., 2005) [14]. The appearance of a specimen of pus and that of any appreciable amount of pus on a swab was observed.

Characterization of Bacterial Isolates

The pus specimen was inoculated on blood and MacConkey agar plates. The streaked plates were incubated at 37°C for 24 hr. Bacterial colonies on blood agar plates were later Gram stained. Characterization of bacterial isolates was based on standard microbiological methods. Identification of isolates were done based on colony morphology, motility, catalase test, oxidase test, coagulase test and biochemical tests like Tripal sugar iron agar, Methyl red test, Urease test, Voges proskauer, Citrate utilization test and Indole test (Koneman *et al*., 2005) [14].

Antimicrobial Activity

The antimicrobial assay was carried out using agar well diffusion method (Saravanna *et al.*, 2010, Orla Sherlock Anthony Dolan Rahma Athman, 2010) [10, 11]. Streptomycin and Tetracyclin (30µg/ml each) are used as reference drugs and the corresponding solvents (Hexane, Ethylacetate & Methanol) are used as positive controls. About 20 ml of Muller-Hinton agar medium for bacteria was poured in the sterilized Petri dishes and allowed to solidify. The agar medium was spread with 24 hrs cultured 10^8 CFU/ ml of microbial strains by a sterilized rod. Wells of 6 mm in diameter were made in the culture medium using sterile cork borers. About 50µl of the plant extracts (1mg/ml) was added to the wells. Plates were then incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the inhibition zone diameters in mm formed around the well. The assay was carried out in triplicates and the result thus obtained is taken as the mean of the three readings.

Results

Phytochemical screening

Phytochemical evaluation of the various extracts of the leaf of *M. tinctoria* were done for the presence of alkaloids, saponins, carbohydrates, phenols, proteins, amino acids, tannins, flavonoids, fats& oils and glycosides and the results are presented in Table 1. Compare to all the extract the hexane extract contain alkaloids, glycosides, saponins, fats and oil. But in the ethyl acetate extract contain phenol and flavonoids. The methanol extract contain phenols and tannins.

Identification of test pathogen

After incubation the plates contain rod, bacilli and cocci shaped organisms. In the Gram staining techniques both Gram positive rod and Gram negative cocci shaped organisms were observed. These organisms were confirmed by various biochemical tests. The results were tabled in Table 2. Finally the isolated organisms were confirmed by Bergeys manual determinative bacteriology. The isolated organisms are *Proteus vulgaris, Bacillus subtilis, E.coli, Staphyloccus aureus, Pseudomonas aeuroginosa* and *Klebsiella pneumoniea*.

Antimicrobial activity

The Antimicrobial activity was examined by agar well diffusion method. The methanol extract of *M. tinctoria* leaf exhibited potent antimicrobial activity towards all the microbes. The Zones of inhibition values are presented in Table 3. *Proteus vulgaris* was found to be more susceptible towards the Methanol extract of leaf with a maximum inhibitory zone (28 mm) followed by Hexane (17 mm), and Ethylacetate extract (6mm). *Klebsiella pneumonia* was found to be more sensitive to the Methanol extract with a maximum inhibitory zone (22 mm) followed by Hexane (NA) and Ethylacetate (8 mm). *Staphylococcus aureus* was found to be sensitive to Ethylacetate extract with a maximum inhibitory zone (22 mm), followed by Hexane (20 mm), Methanol (15 mm) *Bacillus subtilis* was found to be more susceptible to Ethylacetate extract (23 mm). followed by Methanol (19 mm), Hexane (12 mm). *E.coli* was more susceptible to Methanol extract (18 mm) followed by Hexane (15 mm), and Ethylacetate extract (10 mm). *Pseudomonas aeuroginosa* was sensitive followed by Methanol (15 mm), Hexane (11 mm) and Ethylacetate (6 mm). *K.pneumoniae* was more susceptible towards Methanol extract (22 mm) followed by Hexane extract (NA) and Ethylacetate extract (8 mm). The results obtained shows that all the extracts showed very significant antimicrobial activity against the tested organisms.

Table 1: Phytochemical screening of leaf extracts of *Morinda tinctoria*

Chemical Constituents	Hexane	Ethylacetate	Methanol
Alkaloids	+	-	-
Carbohydrates	-	-	-
Glycosides	+	-	-
Saponins	+	-	-
Fats & Oils	+	-	-
Resins	-	-	-
Phenols	-	+	+
Tannins	-	-	+
Flavonoids	-	+	-
Proteins & Aminoacids	-	-	-

Note: (+) Presence; (-) Absence

Table 2: Morphological and Biochemical characters of test bacterial pathogens

S. No	Tests	*P.vulgaris*	*B.subtilis*	*K.pneumoniae*	*E.coli*	*S.aureus*	*B.aeuroginosa*	*K.pneumonia*
1	Colony morphology	rod	rod	rod	bacilli	cocci	circular	rod
2	Gram stain	G -ve	G +ve	G -ve	G +ve	G +ve	G -ve	G -ve
3	Motility test	Motile	Motile Or Non-motile	Non-motile	Motile	Non-motile	Motile	Non-motile
4	Catalase test	+	+	+	+S	-	+	+
5	Oxidase test	+	+	-	-	-	+	+
6	Coagulase test	-	+	-	-	+	+	-
7	Triple Sugar iron test	+	A/A	A/A	+	-	-	+
8	MR test	-	-	-	+	+	-	-
9	Urease test	+	-	+	-	-	-	+
10	VP test	-	+	+	-	+	-	+
11	Citrate Utilization test	+	+	+	-	-	-	+
12	Indole test	+	-	-	+	-	-	-

Note: (+) Positive; (-) Negative; (A/A) Acid/ Alkaline; (S) Slant

Table 3: Antimicrobial activity of various leaf extracts of *Morinda tinctoria* (Zone of inhibition in mm)

S. No	Name of the organism	Hexane extract	Ethyl acetate extract	Methanol extract	Streptomycin	Tetracyclin
1	*Proteus vulgaris*	17	6	28	27	23
2	*Bacillus subtilis*	12	23	19	NA	NA
4	*E. coli*	15	10	18	30	25
5	*Staphylococcus aureus*	20	22	15	32	22
6	*Pseudomonas aeuroginosa*	11	6	15	22	22
7	*Klebsiella pneumoniea*	NA	8	22	30	23

Discussion

Morinda tinctoria leaf extracts have a significant antimicrobial activity against broad spectrum of microorganisms. The antibacterial activity of the extracts against *Proteus vulgaris, Klebsiella pneumonia, Pseudomonas aeuroginosa* were reported for the first time. The microbial studies of the extracts showed the most promising antimicrobial properties indicating the potential for the discovery of novel drugs from plants. Pus infection has been a major concern among health care practitioners not only in terms of increased trauma to the patient but also in view of its burden on financial resources and the increasing requirement for cost- effective management within the health care system (Alexander, 1994) [12]. The microbiological analysis reveals that *S. aureus* is the leading etiologic agent of infection in these health institutions. (Emele *et al.*, 1999 and Mashita *et al.*, 2000) [13, 16]. Extracts containing Phenols and Methanol were shown to be more efficient in the antibacterial efficacy than the other extracts. Methanol extract was shown to be as potent as Tetracyclin (Zone of inhibition -34 mm). The order of the antimicrobial efficacy is Methanol Extract> Ethylacetate extract> Hexane extract. The results clearly shows that Phenols, Phytosterols, Flavonoids which were abundantly found in Methanol, Ethylacetate extracts were responsible for the antimicrobial activity of *M.tinctoria* leaves.The present study through light on the antibacterial efficacy of *M.tinctoria* leaves this study offers a valuable source for the discovery of alternatives to the present antibacterial drugs. The study also concludes that *M. tinctoria* leaves contain a number of pharmaceutically important phytochemicals like alkaloids, saponins, carbohydrates, phenols, tannins, flavonoids, fats& oils, A further study of the extracts is in progress to isolate, characterize and elucidate the structure of the bioactive compounds present which were responsible for potent antimicrobial activity.

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