SIDDHA PAPERS
A PEER REVIEWED ONLINE JOURNAL
ISSN: 0974-2522

Siddha Papers 2019 (14) (1)
RESEARCH ARTICLE
https://www.siddhapapers.com

Antimicrobial potency and phytochemical analysis of Vembu (Azadirachta indica) Chooranam

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ABSTRACT

Purpose

Siddha Medicine, traditional system of healing that originated in South India and is considered to be one of the India's oldest system of medicine among AYUSH systems of medicine. There is a need for novel anti microbial agent to combat the existing antibiotic resistance and that is from a herbal origin would help to ease the treatment procedure without any adverse event and antibiotic resistance. The test drug *vembu chooranam*, consists of chief ingredients *Azadiracta indica* and *Abrus precatorious*.

Objective

This study was carried out to determine the antimicrobial sensitivity and Qualitative phytochemical estimation of *vembu chooranam.*

Methodology

The *vembu chooranam* referenced from *Gunapadam Mooligai vaguppu* (Plants and vegetable section), primarily indicated for the management of *Ammai noi (*Chickenpox). Primarily carried out phytochemical estimation to access the possible constituents' helps to evade the microorganisms. Further antimicrobial activity was done through Disc diffusion method for common six microorganisms (includes gram positive and gram negative bacteria).

Results

The *vembu chooranam* is sensitive to *Staphylococcus aureus, Streptococcus mutans, Bacillus subtilis, Klebsiella pneumoniae, Proteus vulgaris*. The preliminary phytochemical

shows the presence of Carbohydrate, alkaloids, flavonoids, saponin, phenol, tannin, terpenoids.

Conclusion

Thus the phytochemicals present in the Siddha formulation *'Vembu Chooranam'* probably helpful to evade the microorganism.

KEYWORDS

Vembu (Azadirachta indica) chooranam, Antimicrobial analysis, Phytochemicals, Siddha Medicine

INTRODUCTION

Microscopic organisms, commonly known as microorganisms or microbes are found around us and even inside our bodies. The microbes includes a massive range of bacteria, fungi, virus etc. An antimicrobial is an agent that kills microorganism or stop their growth. These are simply called as antibiotics. The overuse of antibiotics against infectious microbes caused mutations in the microbes leads to antimicrobial resistance. So, the need for alternative antimicrobial agents arises .

In this study, Siddha herbal formulation of *vembu chooranam* indicated mainly *ammai noi* (chicken pox). *Vembu chooranam* contains neem leaves and jequirity (Indian Liquorice) root. The leaves of *vembu* contains chemical constitutents such as Azardiractin, β-sitosterol, polyphenolic flavonoids which are well known to have antibacterial and antifungal activities. Based on this, we have choosed qualitative phytochemical estimation for this test drug. *Vembu chooranam* is tested against gram positive *Staphylococcus aureus, Streptococcus mutans, Bacillus subtilis* and gram negative bacteria *Klebsiella pneumoniae, Proteus vulgaris, E-coli.* The infections caused by selected micro-organism are discussed in Table. 1.

Table .1 Charting of infection caused by selected microorganism.

Bacteria	Infection
Staphylococcus aureus	Skin infection, pneumonia, Heart valve infection
Streptococcus mutans	Dental caries, ulcerative colitis, crohn's disease, major inflammatory bone disease.
Klebsiella pneumonia	Pneumonia, Atophic rhinitis, rhinoscleroma
Proteus Vulgaris	UTI, Haematuria, Nosocomical infection
Bacillus subtilis	Endocarditis, musculoskeletal infection, septicemia.

MATERIALS AND METHODS

Collection

The tender neem leaves (*Kozhunthu*) were collected from Neem tree around Tirunelveli, Tamil Nadu, India. Jequirity (*Kundri ver*) root was collected from a climber in Krishnagiri, Tamil Nadu, South India.

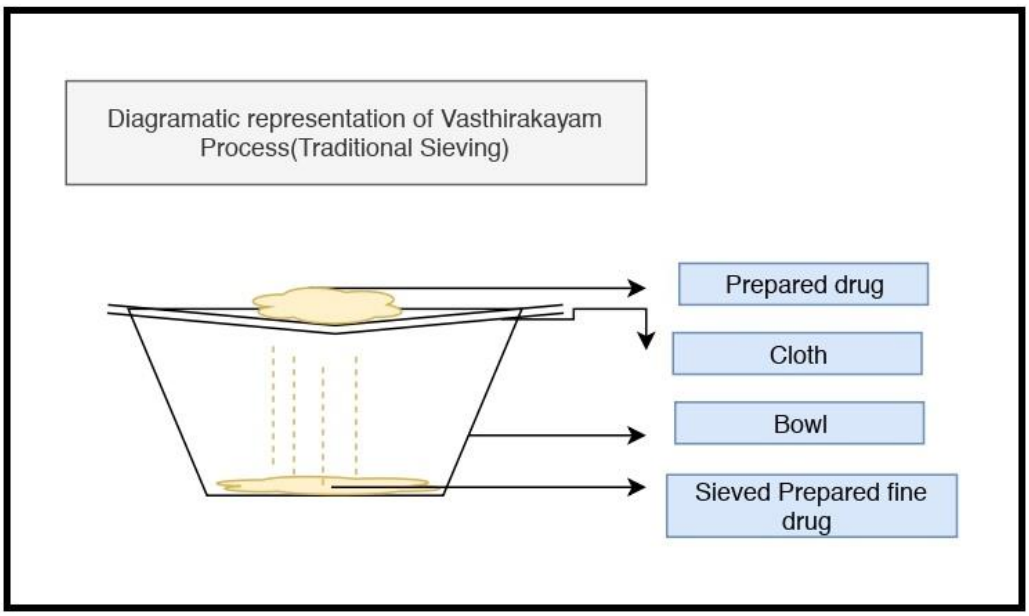
Purification

The tender neem leaves & jequirity root was collected and removed the dust particles and subject to fresh water wash. Later it was dried in shade and carried the preparation process.

Preparation

The ingredient Neem leaves(Dried) of 15*g* and Jequirity root(Dried) of 15*g* were powdered separately, mixed and subject to *Vasthirakayam* Process(powder is sieved by using cloth) where the powdered *chooranam* becomes more finer in this process (in Figure 1). Thus the fine was tested for antimicrobial analysis and phytochemicals analysis.

Figure 1. Diagramatic representation of traditional particle size reduction method *(Vasthirakayam)*



The mouth of an empty bowl is covered using cotton cloth. *Chooranam* is placed over it, by tapping it gently for few times, the fine powder of *chooranam* is collected inside the bowl and stored for use. This is the process of *vasthirakayam.* It is done to reduce the particle size of *chooranam* thereby facilitating its absorption into the body.

Ingredients of *vembu chooranam*

1. *Azadirachta indica - vembu kolundhu.*
2. *Abrus precatorius - Kundrimani root*

They are clearly explained in table no: 2.

Table. 2 List of ingredients of *Vembu Chooranam*.

S. No.	Botanical Name	Family	Part used	Chemical constituent	Action	Disease
1	*Azadirachta indica*	Meliaceae	Tender neem leaf	Azadirachtin	Antiseptic, cathartic, anthelmintic, astringent, diuretic, spermicidal	Skin disorder, Scrofula, splenic enlargement, rheumatism, leprosy hysteria
2	*Abrus precatorius*	Fabaceae	Root	Triterpenoids, Saponins, oledinic acids, glycyrrhizin	Emetic, expectorant	Conjunctivitis, Epithelioma, Ulcers,, granular opthalmia keratitis, inflammation which spread to face and neck.

MICROBIAL ANALYSIS

Dilution

0.1*g* in 1*ml*

Test Organism

The test micro organisms used for anti microbial analysis micro organisms name were purchased from Microbial Type Culture Collection and Gene Bank (MTCC) chandigarh. The bacterial strains were maintained on Nutrient agar (NA).

Nutrient Broth Preparation

Pure culture from the plate were innoculated into Nutrient Agar plate and sub cultured at 37◦C for 24hr. Inoculum was prepared by aseptically adding the fresh culture into 2*ml* of sterile 0.145 *mol / L* saline tube and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a bacterial suspension of 1.5 x 108 *cfu / ml*. Standardized inoculum used for Anti microbial test.

Anti microbial test

The medium was prepared by dissolving 38*g* of Muller Hinton Agar medium (Hi media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 *Lbs* pressure at 121◦C for 15 min (pH 7.3). The autoclaved medium was cooled, mixed well and poured petriplates (25*ml* / plates) the plates were swabbed with pathogenic Bacteria culture viz. Microorganisms name finally, the sample or sample loaded Disc was then placed on the surface of Muller-Hinton medium and the plates were kept for incubation at 37◦C for 24 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimeters. The size of the zone of inhibition (including disc) was measured in millimeters. The absence of zone of inhibition was interpreted as the absence of activity (kohner et al., 1994; mathabe et al., 2006). The activities are expressed as resistant, if the zone of inhibition was less than 7*mm*, intermediate (8-10*mm*) and sensitive if more than 11*mm*, (Assam et al., 2010).The zone of inhibition for each microorganism is given in the following figures.

PHYTOCHEMICAL ANALYSIS

Qualitative Analysis

Test for Carbohydrates - Benedict's test (brain & Turner, 1975)

To 0.5*ml* of test drug about 0.5*ml* of Benedict's reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar.

Glycosides (Ansari 2006)

Keller-Killani Test

To 2 *ml* of the extract, glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4 was added. Reddish brown colour appreared at junction of two liquid layers and upper layer turned bluish green indicating the presence of glycosides.

Steroids (IP1996)

Salkowski Test

To 2 *ml* of extract, 2*ml* of chloroform and 2*ml* of conc. H_2SO_4 was added. The solution was shaken well. As a result chloroform layer turned red and acid layer showed greenish yellow fluorescence.

Alkaloids (Ansari, 2006)

The extract was evaporated in a test tube. To the residue dilute HCL was added, shaken well and filtered.

Mayer's Test

To the 2-3 *ml* of filtrate mayer's reagent was added. Formation of yellow precipitate showed the presence of alkaloids.

Flavonoids (kokate , 1994)

Shinoda test

To the extract, 5*ml* of 95% of ethanol and few drops of concentrated hydrochloric acid was added. To this solution 0.5*g* of magnesium turnings were added. Pink colouration indicated the presence of flavonoids.

Tannins (Mukherjee, 2002)

Lead Acetate Test: On addition of lead acetate solution to the extract white precipitate appeared.

Saponin (Ansari 2006)

Foam Test

Drug extract was shaken vigorously with water. No persistent foam was formed.

Protein (Ansari, 2006)

Biuret Test

With 3*ml* of test solution, few drops of 4% NaOH and 1% CuSO4 solution were added. The tubes were observed for violet or pink colour formation.

Phenol(Mukhergee,2002)

Ferric chloride test

The extract was diluted to 5 *ml* with distilled water. To that a few drop of neutral 5% ferric chloride solution was added. A dark green color indicates the presences of phenolic compounds.

Test for Glycosides (Horbone, 1984)

0.5*mg* of extract was dissolved in 1*ml* of water and then aqueous NaOH solution was added. Formation of yellow colour indicates the presence of glycosides.

Test for Triterepnoids (Horbone, 1984)

To the test solution 2*ml* chloroform was added with few drops of conc. Sulphuric acid (3*ml*) at the side of the test tube. An interface with a reddish brown coloration is formed if terpenoids consistent is present.

Quantitative Procedure

Quantitave Estimation of carbohydate

The total sugar content was estimated by Anthrone method (Roe, 1955). A known amount of the sample was taken, ground well with 80% ethanol and was centrifuged at 4000 *rpm*. From the supernatant, 0.5*ml* was taken and 5*ml* of anthrone reagent was added. The tubes were kept in a boiling water bath for 15 min. After that, they were kept in a dark room for another 15 mins. The colour intensity developed was read in a spectrophotometer at 650 *nm*.

Quantitative Estimation of Saponins (Evans, 1996)

Methanolic and water extract was dissolved in 80% methanol, 2*ml* of Vanilin in ethanol was added, mixed well and the 2*ml* of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 600◦C for 10min, absorbance was measured at 544*nm* against reagent blank. Diosgeninis used as standard material and compared the assay with Diosgening equivalents.

Quantitative Estimation of Tannins (Robert, E.B. 1971. Agro. J.63p.511)

1*ml* of the extract was mixed with 5*ml* of vanillin hydrochloride reagent (mix equal volumes of 8% HCL in methanol and 4% vanillin in methanol). The mixed was allowed to stand for 20 mins and measure the absorbance at 500*nm*. The standard graph was plotted for working standard catechin solution. (0 to 25 $\mu g/\mu l$)

Quantitative Estimation of flavonoids (Evans, 1996)

Total flavonoid content was determined by Aluminium chloride method using catechin as a standard. 1*ml* of test sample and 4*ml* of water were added to a volumetric flask (10*ml* volume). After 5min 0.3*ml* of 5% Sodium nitrite, 0.3*ml* of 10% Aluminium chloride was added, After 6 min incubation at room temperature, 2*ml* of 1M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10*ml* with distilled water. The absorbance of the reaction mixture was measured at 510*nm* against a blank spectrophotometrically. Results were expressed as catehin equivalents (mg catechin/g dried extract)

Quantitative Estimation of Alkaloids (Evans, 1996)

To 1*ml* of Methanolic extract 5*ml* pH 4.7 phosphate Buffer was added and 5*ml* BCG solution and shake a mixture with 4*ml* of chloroform. The extracts were collected in a 10*ml* volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470*nm* against blank prepared as above but without extract. Atropine is used as a standard material and compared the assay with Atropine equivalents.

Quantitative Estimation of Steroid

0.1*ml* and 0.2*ml* of triple acid extract is taken and a set of standards (0.5 to 2.5*ml*) were taken and made up to 5 *ml* with ferric chloride diluting reagent. A blank was prepared simultaneosly by taking 5.0*ml* diluting reagent. Then add 4.0*ml* of concentrated sulphuric acid to each tube. After 30 minutes incubation, intensity of the colour developed was read at 540*nm*.

Quantitative Estimation of Phenolic Compounds (Evans 1996)

The total phenolics content in different solvent extracts was determined with the Folin-Ciocalteu's reagent (FCR). In the procedure, different concentrations of the extracts were mixed with 0.4 *ml* FCR (diluted 1:10 v/v) After 5 min 4*ml* of sodium carbonate solution was added. The final volume of the tubes were made upto 10*ml* with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750*nm* using a spectrophotometer. A calibration curve was constructed using Gallic acid solutions as standard (0 to 250 $\mu g/\mu l$).

Quantitative Estimation of Terpenoid

Total terpenoid content was determined by the method of Ghorai et al (2012)17. To 1 *ml*, of the plant extract, 3 *ml* of chloroform was added. The sample mixture was thoroughly vortexed and left for 3 min and then 200 μl of concentrated sulfuric acid (H_2SO_4) was added. Then it was incubated at room temperature for 1.5h-2h in dark condition and during incubation a reddish brown precipitate was formed. Then carefully and gently, all supernatant of reaction mixture was decanted without disturbing the precipitation. 3 *ml* of 95% (v/v) methanol was added vortexed thoroughly until all the precipitation dissolve in methanol completely. The absorbance was read at 538 *nm* using UV / visible spectrophotometer.

RESULTS AND DISCUSSIONS

In phytochemical analysis, carbohydrate, alkaloid, flavonoids, saponin, phenol, tannin and terpenoid are present in *vembu chooranam*, whereas steroid, glycoside, protein are absent. The drug is not effective against *E-coli*.

Table 3. Zone of inhibition produced in the test Bacterial Strain Name

	Bacterial Strain Name					
Sample code	***Staphylococcus aureus* (G+)**	***Streptococcus mutans* (G+ve)**	***Bacillus subtilis* (G+ve)**	***Klebsiella pneumoniae* (G-ve)**	***Proteus vulgaris* (G-ve)**	***E-coli* (G-ve)**
AA	13	12	12	10	9	8
Positive Control	28	13	23	22	28	20
Negative control	NZ	NZ	NZ	NZ	NZ	NZ

Note: NZ –No zone, mm-millimetre, G+ve - Gram positive, G-ve- Gram negative organism

Figure. 2 Percentage representation of Microbial sensitivity of drug.

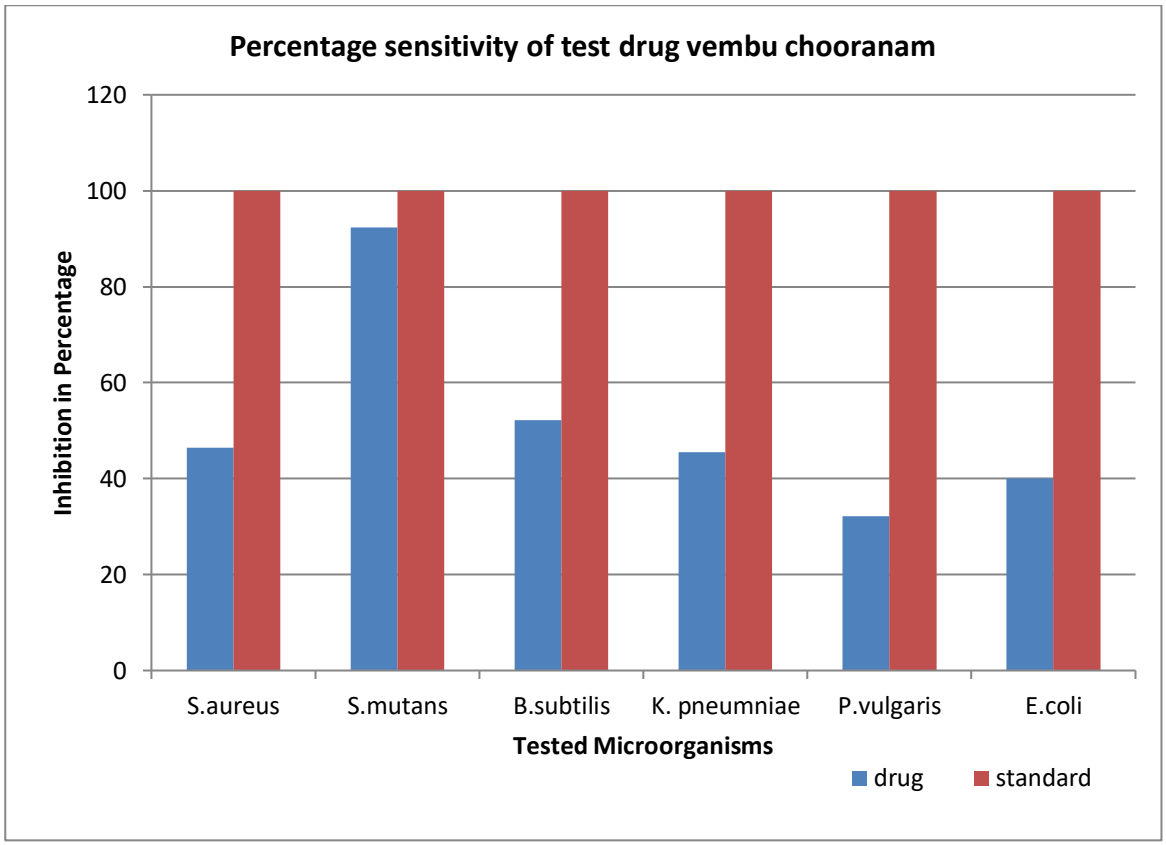


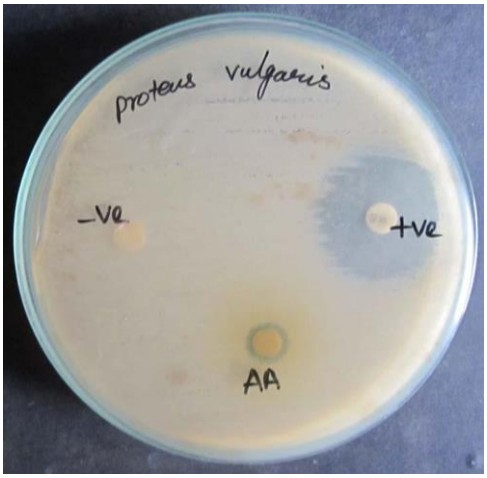
Figure 3. Zone of inhibition of test drug against tested organisms

Zone of inhibition for *Staphylococcus aureus* (G+)

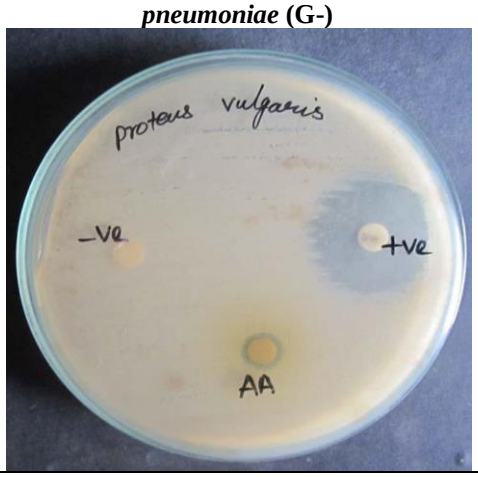
Zone of inhibition for *Streptococus mutans* (G+)

Zone of inhibition for *Bacillus subtilis*(G+)

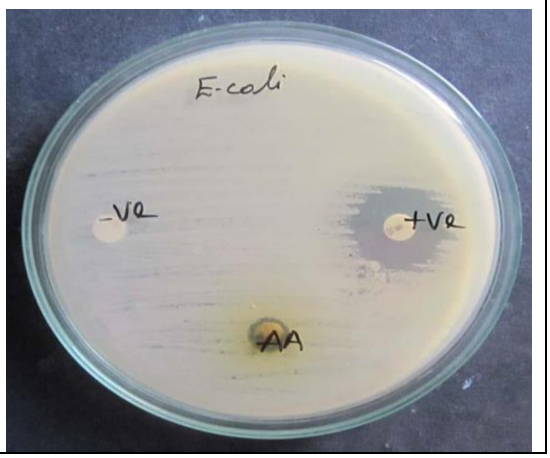
Zone of inhibition of *Proteus vulgaris (*G-)



Zone of inhibition for *Klebsiella pneumoniae* (G-)



Zone of inhibition of *E.coli*(G-)



PHYTOCHEMICAL TEST

Table. 4 Qualitative result of *Vembu chooranam*

S.No.	TEST NAME	AQUEOUS EXTRACT
1	Carbohydrates	Present
2	Protein	Absent
3	Alkaloid	Present
4	Flavonoid	Present
5	Glycoside	Absent
6	Steroid	Absent
7	Saponin	Present
8	Phenol	Present
9	Tannin	Present
10	Terpenoid	Present

Table. 5 Quantitative results of *Vembu chooranm*

S.No.	TEST	AQUEOUS
1	Carbohydrates	38
2	Alkaloid *mg / G*	60
3	Flavonoid *mg / G*	60
4	Tannin *mg / G*	125
5	Saponin *mg / G*	6
6	Phenol *mg / G*	90
7	Terpenoid *mg / G*	45

CONCLUSION

This study shows that the trial drug is more sensitive to *Staphylococcus aureus, Bacillus subtilis, Streptococus mutans*. It is sensitive to *Klebsiella pneumoniae* and *Proteus vulgaris*. In phytochemical studies, carbohydrate, alkaloid, flavonoid, saponin, phenol, tannin and terpenoid whereas protein, glycoside and steroid are absent. Hence, *vembu chooranam* is used to treat the diseases caused by the test microorganisms and *ammai noi* (Chicken pox).

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