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Optimization of Conditions for Flavonoid Extraction from the Leaves of *Cocculus hirsutus* (L.) And its Antibacterial Activity

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Abstract: Natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs. In green plants, flavonoid compounds are the active constituents which have medicinal properties. *Cocculus hirsutus* is widely used medicinal plant in famine food and pharmaceutical industries. It is a potent detoxifier and curator for many diseases. This study was attempted to obtain maximum yield of flavonoids and the various conditions are optimized in *Cocculus hirsutus* leaf using different solvents like ethanol, methanol and water at different concentrations (0.1:10, 0.1:20, 0.1:30) under different temperatures (5, 37, 50 °C) and various incubation times (1 h and 24 h). It was found that ethanolic leaf extract possesed high concentration of flavonoids compared to other extracts tested. The antimicrobial assay, using the same ethanolic leaf extract recorded maximum inhibition against disease causing microorganisms like *E. coli, Salmonella typhi, Micrococcus luteus, Staphylococcus aureus, Acetobacter laffi, Proteus mirabilis* and *Bacillus cereus* suggesting that flavonoids present in the ethanol extract may possess the ability to inhibit pathogenic organisms and same may be formulated as an antimicrobial agent in near future.

Key words: *Cocculus hirsutus* · Flavonoids · Optimization · Antimicrobial Activity.

INTRODUCTION

Plants are useful and essential for animal and human life. Directly and indirectly most of the plants are helpful to human kind because of its medicinal properties. Herbal remedy is chosen for many diseases because of its minimized side effects, natural way of treating disease and it is cost-effective compared to modern drugs [1]. It is very important to study the actual principle lies behind it [2]. The lack of scientific information and its action is a big disadvantage in using traditional drugs [3]. Now-a-days, most of the people depend on herbs for their ailments. In this regard, plants have wide variety of uses such as foods, woods, fireworks and rainfalls. By using herbal drugs, one can get cure without any side effect. Tamil Nadu is known for its ancient history in siddha. In Ayurveda, *C. hirsutus* is known as Patalagarudi in Sanskrit. It is used in curing sickness of urinary system. On the basis of the Unani system of medicine, this plant contains numerous phytoconstituents to treat diseases. It has antipyretic properties, acts as tonic, lessens thirsty, Treating fractures and useful in tubercular glands related problems. It is the commonly used herb in treating injuries and acts as first aid in Indian herbal pharmacopoeia [4].

Cocculus hirsutus belongs to the family Menispermaceae, a perennial climber that can form a dense cover on top of other plants mainly found in tropical and subtropical climatic conditions [5]. In India, it is almost found throughout the open habits and dry localities including Karnataka, Uttar Pradesh, Gujarat, Orrisa, Rajasthan, Tamil Nadu, Bihar, West Bengal, Maharashtra [6]. Common name of the plant is broom creeper.

C. hirsutus is widely distributed in Central Asia, India, China and Pakistan. It is a vine climbing up to 3 m with white to yellowish color flower and dark purple fruits of 4 to 8 mm in diameter. The Vernacular name of this plant is Kattukodi. The leaves are simple and dark green in

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color. The leaves and root system of this plant has great value in treating diseases both internally and externally. It acts as diuretic agent, treats skin and kidney problems, heals joint pains, relieves constipation, used as tonic, helps as alterative laxative, demulcent, diuretic and as antiperiodic in fever [7].

Cocculus hirsutus has a special potency as a detoxifier. The leaves are useful in treating Gonorrhoea, cough, ophthalmia, cephalalgia, neuralgia and also used to treat skin infections and itchy skin including rheumatism [7]. The root extract possess analgesic and anti-inflammatory effect [8]. *Cocculus hirsutus* contains numerous phytochemicals like Triobine, isotrilobine, coclurine, alkaloids, flavonoids, carbohydrates, terpenoids, phenols, glycosides, conirsine, conirstinine, etc. The biologically active compounds derived from various parts of plant are known to posses the therapeutic properties. This plant can be used in carrying out certain pharmacological activities such as antidiabetic, acute toxicity, antimicrobial, anti-inflammatory and analgesic, cardiotonic, laxative and diuretic, immune stimulant, spermatogenic and so on [9].

Plants are widely used for its medicinal properties. *C. hirsutus* contains most of the secondary metabolites, especially flavonoids are highly present [10]. Flavonoids, have a broad range of activity against a different group of microorganisms. In pharmacological studies, flavonoids show different activities such as diuretics, anti hypertensiveness, antiviral, insecticidal, antifungal, antihistamines, antioxidant and antimicrobial [11]. The influence of flavonoids in plants, is an important reason for being used in Ayurvedic system of medicine. Taxonomically related plants produced same types of flavonoids [12]. Many researchers suggest to incorporate flavonoid in daily diet. In drugs (Alkaloids, terpenoids and flavonoids) are the compounds used to prevent various diseases and also in preventing and inhibiting various types of cancer [13]. Generally, flavonoids are identified by functional group and their relative positions at the 15th carbon atom. Flavonoids are classified into several groups and subgroup including flavonol, anthocyanidin, flavone, flavanone, isoflavonoids and chalcones.

Considering the above points in view, the main focus of the present study is to optimize the conditions to extract flavonoids from *C. hirsutus* using solvents like water and ethanol at different concentration in different temperature at different time intervals. The condition which yields maximum flavonoid is followed further in the study. The antibacterial activity of the obtained flavonoid is allowed to expose against different organisms which cause diseases in animals. Already it is reported that crude plant extract has effect on animal diseases [14]. This study is executed with the hypothesis, of expecting increased effect of flavonoid alone against the microorganism. So, isolation of phytochemical especially flavonoid known to possess antimicrobial activity can give new approach for newer drugs.

MATERIALS AND METHODS

Collection of Plant: *Cocculus hirsutus* was collected from Jolarpet, Vellore district, Tamil Nadu during the month of May, 2017 and the temperature was between 37 to 41°C during the time of collection.

Sample Preparation: The healthy leaves of *Cocculus hirsutus* were collected and taken to the laboratory and surface sterilized. The surface sterilized leaves were shade dried for a week and powdered and bottled for further analysis.

Optimization of Conditions: Two types of solvent system were used. Ethanol and water at different concentrations was used for the extraction of flavonoids, i.e. 0.1:10, 0.1:20 and 0.1:30 at different temperatures (5, 37 and 50 °C). Flavonoid extraction was calculated under the basis of time (1st h and 24th h).

Preparation of Calibration Curve Standard Solutions Quercetin: Preparation of calibration curve by introducing 10 ml of standard solution of quercetin (10, 20, 30.40, 50 and 60 ppm) to a 25 ml flask, then added 1 ml of 2 % aluminum chloride solution (In 5 % glacial acetic acid) and glacial acetic acid (In 5 % methanol) upto the mark. Settling for optimum incubation time and absorbance measured at a wavelength of maximum, after which a calibration curve is made between the concentrations of the solution to the uptake value obtained and sought a curve equation.

Determination of Flavonoid Content: Flavonoids levels was determined by adding 10 ml of ethanolic extract of *Cocculus hirsutus* in a 25 ml flask and 1 ml of 2 % aluminum chloride (in 5 % glacial acetic acid) was added and matched to mark boundaries with glacial acetic acid (in n 5% methanol). The solution was allowed to stand for optimum incubation time and measured at a wavelength of maximum absorption, the next level of flavonoid ethanol extract of *Cocculus hirsutus* is determined by the

equation of the calibration curve with the standard solution of quercetin. Then the total flavonoid content was calculated using the formula: flavonoid contents (%, w/w) = [value (ppm) × dilution factor × volume of titrant (ml)/1.000.000 × weight of sample (g)] × 100 % [15].

Preparation of Methanol Extract: The shade dried leaves of *Cocculus hirsutus* were ground well in a mixer grinder and made into powder form of about 30 g. The powdered leaves of about 20 g were extracted with methanol in a soxhlet apparatus. Then, the extract was evaporated in a rotary vacuum evaporator at 40°C under reduced pressure. The crude extract of about 3 g was obtained which is equivalent to about 20% of total extraction [18].

Preparation of Aqueous Extract: The aqueous extract for wound healing assay was prepared by mixing 10 g of powdered leaves of this plant with sterile distilled water and boiled to slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and the procedure was repeated twice. The extracted supernatant was concentrated to make the final volume one-fourth of the original volume [15]. It was then autoclaved at 121°C and at15 lbs pressure and stored at 40°C.

Preparation of Ethanol Extract: The shade dried leaves of *Cocculus hirsutus* were ground well in a mixer grinder and made into powder form of about 30 g. The powdered leaves of about 20 g were extracted with methanol in a soxhlet apparatus. Then, the extract was evaporated in a rotary vacuum evaporator at 60°C under reduced pressure. The crude extract of about 3 g was obtained which is equivalent to about 20 % of total extraction [18].

Antimicrobial activity

Microbes and Media: The clinical bacterial isolates such as *E. coli, Salmonella typhi, Micrococcus, Staphylococcus aureus, Acetobacter laffi, Proteus mirabilis, Bacillus cereus* and clinical fungal isolates such as *Candida albicans,* were used. The bacterial and fungal isolates were obtained from Department of Microbiology, Sacred Heart College (Autonomous), Tirupattur.

Antimicrobial Activity: The extracts were screened for their antimicrobial activity *in vitro* agar well cut method. The clinical bacterial isolates such as *Escherichia coli, Salmonella typhi, Micrococcus luteus, Staphylococcus aureus, Acetobacter laffi, Proteus mirabilis* and *Bacillus cereus* and were used. Three to five similar colonies were selected and transferred to 5 ml broth with a loop and the broth cultures were incubated at 37°C for 24 h. After incubation the suspension was checked for 0.7 Mc Farland value and was used as the seeding culture. For screening, Muller-Hinton agar and potato dextrose agar was prepared and seeded with respective bacterial pathogens respectively. The wells were made using a sterile cork borer and added with different volumes (25, 50, 75 and 100 µl) of the crude extract of *Cocculus hirsutus* (1 g /10 ml D.W) and kept for incubation at 37°C for 24 h for the bacterial isolates. After incubation at 24 h and 48 h, the results were recorded for the zone of inhibition. Streptomycin (10 µg/5 ml) and gentamycin (10 µg/5 ml) were used as standards for bacteria [16].

RESULTS AND DISCUSSION

The main focus on plant research is found in all parts of world. Biologically dynamic constituents from natural origin have always been of great interest to scientists working on various infectious diseases. Modern researchers are focusing their attention towards folk medicine. Keeping this in mind, this study is executed and the findings are discussed. From the present study, qualitative analysis of phytoconstituents revealed the presence of flavonoids, which was found to contain many biological activities. This report was also supported by Kalirajan *et al.* [18].

In order to obtain maximum yield of flavonoids, the condition is optimized. The results obtained using standard quercetin, followed by aqueous extract and ethanolic extract (Table 1). Among the different solvents *viz*., ethanol, methanol and water at different concentration (0.1:10, 0.1:20, 0.1:30), different temperature (5, 37 and 50 °C) and different incubation time (1 h and 24 h) used. It was found that ethanol at 1 h and 37 °C incubation has recorded optimum flavonoid production (Table 4). The same trend was observed by Hasan *et al*. [15].

In recent years, scientists have turned towards various flavonoids present in fruits and vegetables to explain their health benefits associated with disease curing potential. Flavonoids acts as powerful antioxidants with anti-inflammatory efficient and boost the immune system. Diets rich in flavonoid plays major role in prevention of cancer, neurodegenerative and cardiovascular diseases.

Table 1. Optimization of various conditions for flavonoid on different extraction.

S.No	Criteria	24 h at 37 °C	1 h at 37 °C	1 h at 50 °C	1 h at 5 °C
1	Aqueous Extract (10 mg/ml)	0.13	0.24	0.21	0.11
2	Aqueous Extract (20 mg/ml)	0.16	0.24	0.31	0.32
3	Aqueous Extract (30 mg/ml)	0.21	0.21	0.21	0.05
4	99.9% Ethanolic Extract (10 mg/ml)	1.18	0.72	1.26	0.69
5	99.9% Ethanolic Extract (20 mg/ml)	1.26	1.53	1.48	1.17
6	99.9% Ethanolic Extract (30 mg/ml)	1.23	1.03	1.42	1.05

Table 2: Optimization of percentage of ethanol for maximum yield

S.NO	Criteria	10ml (mg/ml)	20ml (mg/ml)	30ml (mg/ml)
1	1 hour at 37 °C in 70 % ethanol.	0.78	0.90	1.20
2	1 hour at 37 °C in 80 % ethanol.	1.13	1.70	1.49
3	1 hour at 37 °C in 90 % ethanol.	0.69	0.90	0.69
4	1 hour at 37 °C in 99.9% ethanol.	0.72	1.48	1.03

Table 3: Antibacterial activity of aqueous extract of *C. hirsustus*

	Zone of Inhibition in (mm)			
Organisms	25µl (mm)	50µl (mm)	75µl (mm)	100µl (mm)
Escherichia coli	6	9	12	14
Acetobacter laffi	7	NIL	12	15
Bacillus cereus	10	12	16	20
Proteus mirabilis	10	15	18	20
Micrococcus luteus	NIL	NIL	12	14
Staphylococcus aureus	NIL	NIL	8	10
Staphylococcus typhi	NIL	6	8	12

Table 4: Antibacterial activity of methanol extract of *C. hirsustus*

	Zone of Inhibition (in mm)			
Organisms	25µl (mm)	50µl (mm)	75µl (mm)	100µl (mm)
Escherichia coli	7	10	12	14
Acetobacter laffi	15	17	16	21
Bacillus cereus	9	12	15	15
Proteus mirabilis	8	NIL	12	14
Micrococcus luteus	8	11	13	15
Staphylococcus aureus	9	10	13	15
Staphylococcus typhi	7	NIL	11	11

From Table 2 it was very clear that ethanolic extract contain comparatively increased amount of flavonoids than aqueous extract. It is being concluded that ethanol was found to posses high concentration of flavonoids. Further the percentage of ethanol is optimized at different concentrations viz., 70, 80, 90, 99.9 respectively. From Table 1, it was evident that 80 % of ethanol has produced 1.70 mg/ml of flavonoids and recorded as higher yield.

The results obtained from the aqueous extract of *Cocculus hirsutus* showed that it has the ability to kill pathogens which has the ability to cause diseases to humans. Flavonoid extracted from *C. hirsutus* clearly showed the inhibition of microbial growth at all concentrations tested. At certain concentrations *Acetobacter laffi, Micrococcus luteus, Staphylococcus aureus* and *Staphylococcus typhi* are not being inhibited. Among all the microorganisms *Proteus mirabilis* and *Bacillus cereus* has recorded maximum inhibition of 20 mm (Table 3).

The results of methanol extract of *Cocculus hirsutus* revealed that *Acetobacter laffi* showed maximum inhibition of 16 mm at the concentration of 75 µl, except *Proteus mirabilis* and S*almonella typhi* which was not affected at the concentration of 50 µl (Table 4).

Table 5: Antibacterial activity of ethanol extract of *C. hirsustus*

Organisms	Zone of Inhibition (in mm)			
	25µl (mm)	50µl (mm)	75µl (mm)	100µl (mm)
Escherichia coli	9	11	12	13
Acetobacter laffi	11	11	12	13
Bacillus cereus	7	9	11	13
Proteus mirabilis	NIL	8	9	11
Micrococcus luteus	11	11	12	13
Staphylococcus aureus	9	11	12	13
Salmonella typhi	12	15	16	13

Antimicrobial efficacy of the optimized flavonoids from the ethanolic extract of *Cocculus hirsutus* clearly shows the inhibition of microbial growth at a concentration of 25, 50, 75, 100 µl against *Escherichia coli*, *Acetobacter laffi, Bacillus cereus, Micrococcus luteus, Staphylococcus aureus* and *Salmonella typhi.* The inhibition of microbial growth at a concentration of 25µl (Table 5) was not observed in *Proteus mirabilis.* The maximum inhibition of 16 mm was recorded against *Salmonella. typhi* at 75 µl. Previously it is reported that antimicrobial activity of *C.hirsutus* has reported against *Staphylococcus aureus*, *Pseudomonas aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* using agar disc diffusion methods using petroleum ether and ethanolic extract crude alkaloid fractions is screened at various concentrations and zone of inhibitions were recorded so by this result suggest that ethanolic extract has significant antimicrobial activity and used to treat various disease. Similarly here again, the ethanolic extract has shown major activity when compared with other extracts.

CONCLUSION

From the study it was concluded that ethanol of 80% at 37 °C for 1 h incubation had registered maximum yield of flavonoids when compared to other conditions and other solvents. The antibacterial efficacy of ethanol, methanol and aqueous extract of leaves of *Cocculus hirsutus* against different Gram positive and Gram negative pathogens showed maximum inhibition against disease causing microorganisms. This experimental result clearly shows that flavonoids present in the ethanol extract have the ability to inhibit pathogenic organisms. So it is recommended to use *Cocculus hirsutus* as a potent remedy for diseases caused by pathogens. It is still unclear that the exact component present in *Cocculus hirsutus* responsible for antimicrobial activity against pathogens. So in future by isolating and identifying those compounds we can formulate an effective drug which can be administered efficiently against challenging and dreadful pathogens.

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