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RESEARCH ARTICLE

Evaluation of Phytochemical and Biological Activities of Siddha-Based Formulation - Kalarchi Chooranam

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Abstract

Siddha-based formulations and nutraceuticals are attracting interest due to their versatile biological activities. Many plant-based phytochemicals have been reported to have various pharmacological activities such as antimicrobial, anti-insecticidal, anti-ulcer and anticancer properties. The evaluation of traditional siddha-based formulations will open new windows in the treatment of different pathogenesis. In the present study, we have formulated Kalarchi Chooranam (Kalarchi kaai and Milagu) in 8 different ratios (1:1, 2:1, 3:1, 4:1, 1:2, 2:2, 3:2, 4:2) and profiled their phytoconstituents and evaluated their biological activities. The herbal mixture was first extracted using Soxhlet's apparatus using an aqueous phase, followed by their qualitative and quantitative phytochemical analysis. In addition to this, the antioxidant and antimicrobial potential of these formulations were evaluated and the results revealed that the 4:1 ratio of Kalarchi Chooranam has significant antioxidant and antimicrobial activities compared to the other ratios. This formulation ratio could be evaluated for its versatile biological activities in the near future.

Keywords: Antimicrobial, Antioxidant, Kalarchi Chooranam, Phytochemicals, Siddha Medicine

1. Introduction

Ayurveda is an Indian traditional system of medicine practiced for thousands of years in south Asian regions[1,2]. It is considered complementary and alternative medicine which has three main pillars such as ethical, philosophical and spiritual for the treatment and healing of ailments[3–6]. Like Ayurveda, herbal based medicines were practised in Greek, Chinese, and Egyptian countries with ancient knowledge[7,8]. These herbal medicines and plant-based ayurvedic formulations are acquiring more interest due to their versatile pharmacological activities such as antimicrobial, anti-insecticidal, antioxidant, anti-inflammatory, anti-ulcer, and antiproliferative anticancer properties[9–11]. A report from WHO states that India is a country of medicinal plants which has nearly 13000 medicinal plants and has clearly studied 7000 plants for the treatment of various diseases[12]. In herbal medicine, both single herbal and multiple herbal medicines (polyherbal) can be used to treat disorders[13,14]. Herbal medicines and phytoconstituents derived from plants are used to treat severe pathogenesis and infections with greater efficacy and fewer side effects[15,16]. Polyherbal formulations such as nutraceuticals and chooranas containing versatile phytoconstituents such as alkaloids, phenols, and terpenes would possess combinatorial effects on the treatment with increased efficiency over the therapy[17,18].

The Kalarchi Chooranam is a polyherbal composition of Kalarchi paruppu (*Caesalpinia bonduc*) and Milagu (*Piper nigram*) prepared using distilled water. This composition was reported to possess significant pharmacological activities such as antimicrobial, antioxidant, anti-inflammatory, and anticancer properties[19–21]. Even though it has versatile phytoconstituents in the formulations, it

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might have some side effects at an observable level due to the presence of tannin content. In the present study, we have formulated Kalarchi Chooranam (Kalarchi kaai and Milagu) in 8 different ratios (1:1, 2:1, 3:1, 4:1, 1:2, 2:2, 3:2, 4:2) respectively, and extracted them through Soxhlet's apparatus. Then their phytoconstituents by both qualitative and quantitative analysis and found that the 4:1 ratio of Kalarchi Chooranam had a higher number of alkaloids and flavonoids compared to the other ratios, so this particular formulation was taken for further study. Because an increase in reactive oxygen species inside the cells can lead to disease pathogenesis such as cancer and cardiovascular disease, reducing these ROS in the cells is necessary to prevent oxidative stress[22–24]. The DPPH free radical scavenging assay was performed for the 4:1 ratio of Kalarchi Chooranam and found that at 100 µg/ml concentration it showed potent antioxidant activity in comparison with ascorbic acid. Pathogenic microorganisms pose a serious threat to humans and animals due to their severity and the complexity of treatment[18]. The antibacterial activity of the 4:1 ratio of Kalarchi Chooranam was tested against *Escherichia coli, Azospirillium bracilense, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae* using paper disc method and the antifungal activity was tested against *Candida albicans, Aspergillus niger, Candida auris,* and *Fusarium sporotrichioides, Streptococcus agalactiae* using well plate method respectively. The clear zone of inhibition indicates the antimicrobial potential of Kalarchi Chooranam against the tested microorganisms. In the future, this Kalarchi Chooranam might be deeply evaluated for its significant biological activities.

2. Materials and Methods

2.1 Sample Preparation

The Kalarchi paruppu (*Caesalpinia bonduc*) and Milagu (*Piper nigram*) were purchased from a local vendor in Thanjavur, Tamil Nadu, India. The samples were duly washed and impurities and other materials were removed, powdered with mortal and pistol and properly stored for further usage. The Kalarchi Chooranam was prepared in in 8 different ratios such as 1:1, 2:1, 3:1, 4:1, 1:2, 2:2, 3:2, 4:2 by mixing the finely powdered *Caesalpinia bonduc* and *Piper nigram* respectively.

2.2 Extraction

For the extraction purpose, about 25g of Kalarchi chooraman powder samples were taken. The samples were extracted with 125 mL of D.H_2O (1:5 W/W composition) through Soxhlet's apparatus until the most efficient extract was obtained[25]. Then the extract was collected in a separate vessel and further filtered by Whatman No. 1 filter paper to remove the excess debris present in the extract. Then the excess solvent present in the filtrate was evaporated in a rotary vacuum evaporator and properly stored until further use. The same procedure was followed for all the 8 ratio formulations.

2.3 Qualitative Testing of Phytoconstituents

The qualitative determination of the phytoconstituents of the Kalarchi chooraman formulations was performed as described earlier[26].

2.3.1 Wagner's Test

To 4 Ml of extract, 3 drops of Wagner's reagent were added and kept for 10 minutes undisturbed. The appearance of a reddish-brown precipitate indicates the alkaloid presence.

2.3.2 Sodium Hydroxide Test

To dissolve 0.6 g of the extract formulation, the cold and diluted NaOH and distilled water were added to the samples. The flavonoid presence was confirmed by the absence of the yellow color.

2.3.3 Copper Acetate Test

To the 1.5 mL extract formulation, 5 drops of Copper (II) acetate solution were carefully added and incubated at room temperature. The terpenoids' presence was confirmed by the formation of a beryl green color.

2.3.4 Salkowski Test

To the 1.5 mL of extract formulation, 1 mL of chloroform and 1 mL of concentrated sulphuric acid were added. The appearance of greenish yellow and red fluorescence in the sulphuric acid layer and chloroform layer confirms the steroids' presence in the samples.

2.3.5 Foam Test

To the 6 mL of the extract formulation, 5 mL of distilled water was added and rapidly mixed for 8-10 min. Saponin's presence was confirmed by the appearance of stable foam in the sample.

2.3.6 Ferric Chloride Test

To the 2 mL of the extract formulation, 10 mL of distilled water was added and incubated for 5-10 min. To 3 mL of the filtrated, 2-3 drops of 5% $FeCl_3$ solution were added, and the appearance of greenish violet confirms the presence of phenolics in the sample.

2.3.7 Lead Acetate Test

To the 1 mL of the extract formulation, 2.5 mL of lead acetate were added and mixed well. The tannins and phenols were confirmed by the appearance of a white precipitate in the sample.

2.3.8 Borntrager's Test

About 5 mL of the extract formulation was mixed and boiled with H_2SO_4 and to the filtrate $CHCl_3$ was added, followed by the addition of ammonia. The presence of anthraquinone glycosides was confirmed by the color change from pink to red in the ammonia layer of the sample.

2.3.9 Fluorescence Test

In addition to the 2 mL of the extract formulation, 4 mL of NaOH solution was mixed and incubated at room temperature. The coumarin glycosides' presence was confirmed by the appearance of bluish green fluorescence.

2.3.10 Kellar Killani's Test

To 3-5 mL of the extract formulation, CH_3COOH, $FeCl_3$ and conc. H_2SO_4 mixture was added and mixed thoroughly. The cardiac glycosides' presence was confirmed by the formation of a brown ring.

2.3.11 Spot Test

About 2 mL of the extract formulation was kept in between two Whatman No. 1 filter papers and pressed tightly for 5 min. The oil in the paper confirms the presence of fixed oils in the sample.

2.4 Qualitative Testing of Phytoconstituents

The quantification of total alkaloids, total flavonoids, total phenolics, total saponins and total tannins of the Kalarchi chooraman extracts (all 8 ratios) was performed as prescribed earlier[27].

2.4.1 Total alkaloids Content

About 2 mg/mL of the extract formulation was dissolved in dimethyl sulfoxide, to which 1 mL of 1N hydrochloric acid was also added, and the filtrate was collected. To this, 3 mL of bromo-cresol green dye and 3 mL of PBS solution were also added. To the mixture, 3 mL of $CHCl_3$ was added and shaken rapidly. 20-100 mg/ml concentrations of Atropine were used as standards, and the absorbance of the samples was measured at 470 nm.

2.4.2 Total Flavonoid Content

The total flavonoid content of the extract formulation was estimated by an aluminum chloride assay. To the 2 mL of the extract formulation, 5 mL of distilled water and 0.5 mL of 3% sodium nitrite solutions were added and incubated at room temperature. After 10 min, 0.5 mL of aluminum chloride and 3 mL of 1M sodium hydroxide solutions were added. 20-100 mg/ml concentrations of Quercetin were used as standards, and the absorbance of the samples was measured at 510 nm.

2.4.3 Total Saponin Content

About 10 g of the extract formulation was mixed with 100 mL of 25% ethanol and boiled for 1 hr at 70°C. Then the filtrate was collected and again 250 mL of 25% ethanol was mixed and boiled as per the previous condition. To about 20 mL of the filtrate, 10 mL of 25% ethanol was added again, and the aqueous layer was collected. Then the filtrate was washed twice with 10% NaOH. 20-100 mg/ml concentrations of Diosgenin were used as standards, and the absorbance of the samples was measured at 550 nm.

2.4.4 Total Tannins Content

To the 7 mL of the extract formulation, 5 mL of $D.H_2O$, 1 mL of Folin-Ciocalteu, and 5 mL of 15% sodium

carbonate solutions were added and kept incubated for 25-30 min at room temperature. 20-100 mg/ml concentrations of Gallic acid were used as standards, and the absorbance of the samples was measured at 725 nm.

2.4.5 Total Phenolics Content

To the 5 mL of the extract formulation, 3 mL of distilled water and 10 mL of Folin-Ciocalteu were added and incubated at room temperature for 15 min. After 10-12 min, 8 mL of Na_2CO_3 was added to the filtrate and incubated at room temperature for 60-90 min. 20-100 mg/ml concentrations of Gallic acid were used as standards, and the absorbance of the samples was measured at 550 nm.

2.5 DPPH Assay

The antioxidant potential of the Kalarchi chooraman formulation extracts was evaluated by the DPPH free radical scavenging assay as described previously[28]. The 0.1 mM DPPH solution was prepared by mixing 3.94 mg of DPPH in 100 mL of methanol and keeping it incubated in the dark for 30-45 minutes for the proper solubilization. Ascorbic acid is a known free radical scavenger with potent antioxidant potential and is used as a standard for this assay. The various concentrations, such as 20, 40, 60, 80, 100 µg/mL of samples and the ascorbic acid (standard) were added to 1 mL of 0.1 mM DPPH solution and kept undisturbed for 1 hr in the dark. Finally, the absorbance was measured at 517 nm and the free radical scavenging activity was calculated as follows:

$$\% \text{ DPPH scavenging} = \frac{\text{Control absorbance - Sample absorbance}}{\text{Control absorbance}} \times 100$$

2.6 Antibacterial Activity

The antibacterial activity of Kalarchi chooraman formulation extracts was tested using the paper disk method against *Escherichia coli, Azospirillium bracilense, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus agalactiae*[29]. The bacteria inoculum was plated on petri dishes and paper discs with 25µg, 50µg, 75µg, and 100µg of the extracts along with distilled water as a negative control and streptomycin as a standard, and incubated at growth conditions overnight. The appearance of a clear zone of inhibition shows the antibacterial potential of the Kalarchi chooraman formulation extracts.

2.7 Antifungal Activity

The antibacterial activity of the Kalarchi chooraman formulation extracts was evaluated against *Candida albicans, Aspergillus niger, Candida auris,* and *Fusarium sporotrichioides, Streptococcus agalactiae* using the well diffusion method[29]. The fungal cultures were cultured with potato dextrose agar and punched a hole with a well puncher, followed by the addition of 25µg, 50µg, 75µg, and 100µg of the extracts along with distilled water as a negative control and Clotrimazole as a standard, and incubated at growth conditions overnight. The appearance of a clear zone of inhibition shows the antifungal potential of the Kalarchi chooraman formulation extracts.

3. Results and Discussion

3.1 Qualitative Screening

Table 1 indicates the qualitative analysis of phytochemicals present in the eight different ratios (1:1, 2:1, 3:1, 4:1, 1:2, 2:2, 3:2, 4:2) of Kalarchi Chooranam distilled water extract. The results show that the Kalarchi Chooranam has high phytoconstituent content. Comparatively, we can observe that the 4:1 ratio Kalarchi Chooranam formulation has high alkaloids, flavonoids, glycosides, saponins, and fats and oils. Though it has high alkaloid and flavonoid content, the 4:1 might possess better pharmacological activities than the other ratios of Kalarchi Chooranam.

3.2 Quantitative Determination

Table 2 represents the qualitative analysis of the substances present in the eight different ratios (1:1, 2:1, 3:1, 4:1, 1:2, 2:2, 3:2, 4:2) of Kalarchi Chooranam distilled water extract. The Total Alkaloid Content (TAC), Total Flavonoid Content (TFC), Total Saponin Content (TSC), Total Tannin Content (TTC) and Total Phenolic Content (TPC) values of the samples were given as atropine, quecertin, diosgenin, gallic acid, and gallic acid standard equivalents, respectively. All the experiments were performed in triplicates, and the results were given as mean±SD. These results

Table 1. Qualitative screening of Kalarchi Chooranam phytoconstituents

S.No	Chemical constituents	Test Name	1:1	2:1	3:1	4:1	1:2	2:2	3:2	4:2
1.	Alkaloids	Mayer's test	++	+	++	++	+	++	+	+
2.	Flavonoids	Sodium hydroxide test	++	++	+	++	+	+	+	+
3.	Terpenoids	Copper acetate test	++	+	+	+	-	+	-	-
4.	Carbohydrates	Molisch's test	+	+	+	++	-	+	+	+
5.	Proteins	Millon's test	+	+	+	++	-	-	+	+
6.	Amino acids	Ninhydrin test	+	-	-	++	+	+	+	-
7.	Fats and oils (Fixed)	Saponification	+	-	-	-	-	+	-	+
8.	Steroids	Salkowski Tests	-	+	-	+	+	-	+	-
9.	Cardiac glycosides	KellarKillani's test	+	+	+	+	+	+	+	+
10.	Phenolics	Ferric chloride test	-	+	+	++	+	-	+	-
11.	Saponins	Foam test	+	+	+	+	+	+	+	+
12.	Tannins	Ferric chloride test	+	+	+	+	+	+	+	+

Note: (++) denotes highly present, (+) denotes present, (-) denotes not detected.

Table 2. Quantitative screening of Kalarchi Chooranam phytoconstituents

S.No	Ratio	Quantitative determination of chemical constituents (%)				
		Alkaloids / Atropine equivalent	Flavonoids / Quercetin equivalent	Saponins / Diosgenin Equivalent	Tannins / Tannic acid equivalent	Phenols / Gallic acid equivalent
1.	1:1	12.30±0.30	16.05±0.5	10.15±0.5	11.30±0.15	6.50±0.5
2.	2:1	18.20±0.05	10.20±0.15	12.55±0.15	14.50±0.05	11.40±0.05
3.	3:1	24.10±0.5	22.60±0.05	18.60±0.05	21.10±0.15	12.20±0.15
4.	4:1	32.05±0.5	36.50±0.05	16.05±0.15	25.22±1.5	15.05±0.5
5.	1:2	10.65±0.05	6.05±0.15	12.80±0.5	16.10±0.05	5.20±0.15
6.	2:2	15.10±0.5	20.15±0.5	10.50±0.5	11.20±0.05	3.20±0.5
7.	3:2	22.40±1.50	15.15±1.5	13.10±0.5	17.50±0.25	4.55±2.5
8.	4:2	9.20±0.15	12.50±0.5	8.70±1.5	12.15±0.20	8.30±1.15

Table 3. Anti-Oxidant activity of Kalarchi Chooranam

Concentration (µg/ml)	Ascorbic acid (standard)	Kalarchi Chooranam Extract
20	210.25 ± 0.5	185.50±1.5
40	222.50 ± 1.0	192.10±2.0
60	235.40 ± 2.0	215.30±0.5
80	310.60 ± 1.5	232.10±1.5
100	345.25 ± 1.5	258.40±2.5

also suggest that the 4:1 ratio of Kalarchi Chooranam formulation has high alkaloid and flavonoid content compared to other ratios, and this particular ratio was selected for further biological studies.

3.3 Antioxidant Activity

Table 3 represents the DPPH free radical scavenging activity of Kalarchi Chooranam extract (4:1 formulation). The DPPH measures the antioxidant potential of the samples by the scavenging activity of

Table 4. Antibacterial activity of Kalarchi Chooranam extract

Organisms Name	Zone of inhibition (mm)				
	25µg	50µg	75µg	100µg	Streptomycin (standard)
Escherichia coli	8	14	18	29	10
Azospirillium bracilense	13	16	17	19	10
Pseudomonas aeroginosa	11	14	15	22	11
Streptococcus agalactiae	12	14	15	24	10
Staphylococcus aureus	10	12	13	17	15

Table 5. Antifungal activity of Kalarchi Chooranam extract

Organisms Name	Zone of inhibition (mm)				
	25µg	50µg	75µg	100µg	Control (Clotrimazole)
C. albicana	12	14	18	20	11
A. niger	12	15	19	22	10
Candida auris	09	11	17	25	-
Fusarium sporotrichioides	11	15	19	24	09

the free radicals. The various concentrations 20-100 µg/ml of Kalarchi Chooranam extract and ascorbic acid were measured for their scavenging activity and the results revealed that at 100 µg/ml of Kalarchi Chooranam extract, the maximum scavenging activity was observed at 345.25 ± 1.5. This confirms the 4:1 ratio of Kalarchi Chooranam formulation has significant antioxidant activity.

3.4 Anti-bacterial Activity

Table 4 represents the antibacterial activity of the 4:1 ratio of Kalarchi Chooranam extract against the tested bacteria. The various concentrations such as 25 µg, 50 µg, 75 µg, 100 µg of the samples and the Streptomycin (standard) were evaluated against *Escherichia coli, Azospirillium bracilense, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus agalactiae*. The results showed that the 4:1 ratio of Kalarchi Chooranam extract had significant antibacterial activity against *Escherichia coli* and *Streptococcus agalactiae* with a clear zone of inhibition (without deduction of the disc size).

3.5 Antifungal Activity

Table 5 represents the antifungal activity of the 4:1 ratio of Kalarchi Chooranam extract against the tested fungal species. The various concentrations such as 25 µg, 50 µg, 75 µg, 100 µg of the samples and the Clotrimazole (standard) were evaluated against *Candida albicans, Aspergillus niger, Candida auris, Fusarium sporotrichioides* and *Streptococcus agalactiae*. The results showed that the 4:1 ratio of Kalarchi Chooranam extract had significant antibacterial activity against *Candida auris* and *Fusarium sporotrichioides* with a clear zone of inhibition (without deduction of the well size).

4. Conclusion

Siddha-based formulations and nutraceuticals are attracting interest due to their versatile pharmacological activities such as antimicrobial, anti-insecticidal, anti-ulcer and anticancer properties. The evaluation of traditional siddha-based formulations will open new windows in the treatment of different pathogenesis. Here in this study, we prepared Kalarchi Chooranam extracts of 8 different ratios and profiled their phytoconstituents and found out that the 4:1 ratio has high flavonoid and flavonoid content, and this particular extract was taken for further studies. The 4:1 ratio of Kalarchi Chooranam extract showed significant free radical scavenging activity, confirmed by the DPPH assay. It also showed potent antibacterial and antifungal activities against the tested human pathogens. This

Kalarchi Chooranam has to be explored more in the future to predict its various pharmacological activities.

5. References

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