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TOXICOLOGICAL EVALUATION OF NEERZHIVU CHOORNAM-A SIDDHA ANTI-DIABETIC POLYHERBAL FORMULATION

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ABSTRACT

Herbal formulations are getting popularity throughout the world and commercialized extensively for various medicinal properties. WHO has emphasized the need for quality assurance of herbal products, including testing of heavy metals and pesticides residues. "Neerzhivu choornam" is a popular Siddha anti-diabetic herbal formulation, which consists of seven different drugs. In view of WHO guidelines, single herbal drugs used in " Neerzhivu choornam" were collected from different places of markets in India for testing heavy metals, microbial load and persistent pesticides residue. Pesticide residues, heavy metals and microbial contamination were produce toxic effects like irritation of eye, blurring of eye, colic, breathlessness etc. The systemic effect includes hypotension, vascular collapse, respiratory paralysis, atoxia and convulsion. Hence the presence of these contaminants in medicinal plants must be avoided. The present study highlights the methods and procedure for these toxic contaminants and also an attempt has been made to evaluate the Neerzhivu choornam- a Siddha polyherbal anti-diabetic formulation in respect with toxicological contaminants to ensure the safety and efficacy.

Keywords: Neerzhivu choornam, Toxicological contaminants, Pesticide residues, Heavy metal contamination, Microbial load.

INTRODUCTION

The history of Siddha medicine is as old as the history of the Tamil culture and civilization. The Siddha system of medicine with greater antiquity is serving the society mainly in the southern peninsular India and also amongst the Tamil Diaspora who have spread out throughout the world. The Siddha practioner used to prepare medicines by themselves for the patient as per their individual needs. Once mass production of Siddha medicine for commercialization came into existence, the quality and purity of the drugs tender to vary from manufacturer to manufacturer. In order to ensure safety to the public, the government of India, considered it expedient to extend the provision of drug and cosmetic act 1940 and exercise supervisory control over manufacture of Siddha, Ayurveda and Unani drugs to enhance uniform standards [1-2].

Diabetes and Siddha

Diabetes Mellitus is a condition which can be compared with Neerizhivu in Siddha. The other names described in the texts are the Madhumegham and Inippuneer. The signs and symptoms explained are increased urination both in frequency and quantity, there will be flies surrounding the urine voided place, weight loss, dryness of the skin, etc. Various diagnostic methods in Siddha system like Naadi (The Pulse reading), Moothira parisothanai (Urine Examination) etc are used to diagnose the condition. The disease comes under the Mega diseases [3].

Neerzhivu choornam

Ingredients: Kadukkai thol, kariveppilai, nellivatral (each 2 parts), naval kottai, seenthil, keezhkkai nelli, korai kizhangu (each one parts) are separately collected, cleaned, crushed them individually and sifted. Then the ingredients are mixed well in a mortar to get uniform mixer for better use.

Dosage: 1gm to 2gm two times a day orally with milk.[4].

MATERIALS AND METHODS

Determination of specific organochlorine, organophosphorus and pyrethroid insecticide residues [5-9]

Extraction

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To 10 g of the substance being examined, coarsely powdered, add 100 ml of acetone and allow to stand for 20 min. Add 1 ml of a solution containing 1.8 µg/ml of carbophenothion in toluene. Homogenize using a high-speed blender for 3 min. Filter and wash the filter cake with two quantities, each of 25 ml, of acetone. Combine the filtrate and the washings and heat using a rotary evaporator at a temperature not exceeding 40°C until the solvent has almost completely evaporated. To the residue add a few milliliters of toluene and heat again until the acetone is completely removed. Dissolve the residue in 8 ml of toluene. Filter through a membrane filter (45µm), rinse the flask and the filter with toluene and dilute to 10.0 ml with the same solvent (*solution A*).

Purification

Organochlorine, organophosphorus and pyrethroid insecticides Examine by size-exclusion chromatography. The chromatographic procedure may be carried out using:
-a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with styrene-divinyl benzene copolymer (5 µm); -as mobile phase toluene at a flow rate of 1 ml/min.

Performance of the column. Inject 100 µl of a solution containing 0.5 g/l of methyl red and 0.5 g/l of oracet blue 2 in toluene and proceed with the chromatography. The column is not suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary calibrate the column using a solution containing, in toluene , at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determine which fraction of the eluate contains both insecticides.

Purification of the test solution. Inject a suitable volume of solution A (100µl to 500µl) and proceed with the chromatography. Collect the fraction as determined above (*solution B*). Organophosphorus insecticides are usually eluted between 8.8 ml and 10.9 ml. Organochlorine and pyrethroid insecticides are usually eluted between 8.5 ml and 10.3 ml.

Organochlorine and Pyrethroid insecticides

In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduce a piece of defatted cotton and 0.5 g of silica gel treated as follows: heat silica gel for chromatography in an oven at 150 °C for at least 4 h. Allow to cool and add dropwise a quantity of water corresponding to 1.5% of the mass of silica gel used; shake vigorously until agglomerates have disappeared and continue shaking for 2 h using a mechanical shaker. Condition the column using 1.5 ml of hexane. Prepacked columns containing about 0.5 g of a suitable silica gel may also be used provided they have been validated beforehand. Concentrate solution B in a current of helium for chromatography or oxygen- free nitrogen almost to dryness and dilute to a suitable volume with toluene (200µl to 1 ml according to the volume injected in the preparation of solution B). Transfer quantitatively on to the column and proceed with the chromatography using 1.8 ml of toluene as the mobile phase. Collect the eluate (*solution* C).

Quantitative analysis

Organophosphorus insecticides

Examine by gas chromatography, using carbophenothion as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbo phenothion.

Test solution. Concentrate solution B in a current of helium for chromatography almost to dryness and dilute to 100 µl with toluene.

Reference solution. Prepare at least 3 solutions in toluene containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:
- a fused-silica column 30 m long and 0.32 mm in internal diameter, the internal wall of which is covered with a layer 0.25 µm thick of poly(dimethyl)siloxane;
- hydrogen for chromatography as the carrier gas (other gases such as helium for chromatography or nitrogen for chromatography may also be used provided the chromatography is suitably validated);
- a phosphorus-nitrogen flame-ionization detector or an atomic emission spectrometry detector, maintaining the temperature of the column at 80 °C for 1 minute, then raising it at a rate of 30 °C/minute to 150 °C, maintaining it at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/minute to 280 °C and maintaining at this temperature for 1 minute, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 1. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

Organochlorine and pyrethroid insecticides

Examine by gas chromatography, using carbophenothion as the internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

Test solution. Concentrate solution C in a current of helium for chromatography or oxygen-free nitrogen R almost to dryness and dilute to 500 µl with toluene.

Reference solution. Prepare at least three solutions in toluene containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:

- a fused silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a 0.25 µm thick layer of poly (dimethyl) (diphenyl) siloxane;
- hydrogen for chromatography as the carrier gas (other gases such as helium for chromatography or nitrogen for chromatography may also be used, provided the chromatography is suitably validated);
- an electron-capture detector;
- a device allowing direct cold on-column injection,maintaining the temperature of the column at 80 °C for 1 minute, then raising it at a rate of 30 °C/minute to 150 °C, maintaining it at 150 °C for 3 minutes, then raising the temperature at a rate of 4 °C/minute to 280 °C and maintaining at this temperature for 1 minute, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 2. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

DETERMINATION OF HEAVY METAL CONTAMINATION

Limit test for total toxic metals as lead

In this method, the heavy metals are the metallic inclusions that are darkened with sodium sulfide in acidic solution; their quantity is expressed in terms of the quantity of lead (Pb).

Preparation of sample solution and blank solution

Test solution. Place an amount of the sample, as directed in the monograph, in a quartz or porcelain crucible, cover loosely with a lid, and carbonize by gentle ignition. After cooling, add 2 ml of nitric acid and 5 drops of sulfuric acid, heat cautiously until white fumes are evolved, and incinerate by ignition between 500 °C and 600 °C. Cool, add 2 ml of hydrochloric acid, evaporate to dryness in a water-bath, moisten the residue with 3 drops of hydrochloric acid, add 10 ml of hot water, and warm for 2 minutes. Then add 1 drop of phenolphthalein, add ammonia drop by drop until the solution develops a pale red colour, add 2 ml of dilute acetic acid, filter, if necessary, and wash with 10 ml of water. Transfer the filtrate and washings to a Nessler tube, and add water to make 50 ml. Designate this as the test solution.

Control solution. Evaporate a mixture of 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid on a water-bath, further evaporate to dryness on a sand-bath, and moisten the residue with 3 drops of hydrochloric acid. Hereinafter, proceed as directed above for the test solution, and then add the volume of standard lead solution as directed in the monograph and sufficient water to make 50 ml.

Procedure

Add 1 drop of sodium sulfide both to the test solution and to the control solution, mix thoroughly, and allow to stand for 5 minutes. Then compare the colours of the two solutions by viewing the tubes downwards or transversely against a white background. The test solution has no more colour than the control solution.

Limit test for total toxic metals as lead in extracts

Test solution. Ignite 0.3 g of extracts to ash, warm with 3 ml of dilute hydrochloric acid, and filter. Wash the residue with two 5 ml portions of water. Neutralize the combined filtrate and washings by adding ammonia, filter, if necessary, and add 2 ml of dilute acetic acid and water to make 50 ml. Perform the heavy metals limit test using this solution as the test solution.

Control solution. Proceed with 3 ml of dilute hydrochloric acid in the same manner as directed above for the preparation of the test solution, and add 3 ml of standard lead solution 1 ppm, and water to make 50 ml.

Procedure

Add 1 drop of sodium sulfide to both the test solution and to the control solution, mix thoroughly, and allow to stand for 5 minutes. Then compare the colours of the two solutions by viewing the tubes downwards or transversely against a white background. The test solution has no more colour than the control solution.

Determination of specific toxic metals

Atomic absorption spectrometry (AA) is used for the determination of the amount or concentration of specific heavy metals. AA uses the phenomenon that atoms in the ground state absorb light of a specific wavelength, characteristic of the particular atom, when the light passes through an atomic vapour layer of the element to be determined.Caution must be exercised when using the recommended closed high-pressure digestion vessels and microwave laboratory equipment, and the operators should be fully familiar with the safety and operating instructions given by the manufacturer.

Detection of cadmium, copper, iron, lead, nickel and zinc

Measure the content of cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), nickel (Ni) and zinc (Zn) by the standard additions method using reference solutions of each heavy metal. Suitable instrumental parameters are listed in Table 3.

The absorbance value of the compensation liquid (*blank solution*) is subtracted from the value obtained with the test solution. [10-16]

ffectiveness of the culture medium, confirmation of antimicrobial substances and validity of the counting method [17-19]

The following strains are normally used

Staphylococcus aureus NCIMB 8625 (ATCC 6538-P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83, IFO

13276)
Bacillus subtilis NCIMB 8054 (ATCC 6633, CIP 52.62, IFO 3134)
Escherichia coli NCIMB 8545 (ATCC 8739, CIP 53.126, IFO 3972)
Candida albicans ATCC 2091 (CIP 1180.79, IFO 1393) or ATCC 10 231(NCPF 3179, CIP 48.72, IFO 1594)
Clostridia botulinum ATCC 19297 (NCTC 7272)
Clostridium perfringens ATCC 13124 (NCTC 8239)
Clostridium tetani ATCC e19406 (NCTC 279)

Allow the test strains to grow separately in tubes containing *soybean-casein digest medium* at 30–35 °C for 18–24 hours for aerobic bacteria and between 20–25 °C for *Candida albicans*, for 48 hours (Antibiotics are often added to the culture medium to attain a particular selectivity).

Dilute portions of each of the cultures using buffered sodium chloride-peptone solution, pH 7.0 or phosphate buffer, pH 7.2 to prepare test suspensions containing 50–200 viable colony forming units (cfu) (microorganisms) per ml. Growth- promoting qualities are tested by inoculating 1 ml of each microorganism into each medium. The test media are satisfactory if clear evidence of growth appears in all the inoculated media after incubation at the indicated temperature for 5 days. When a count of test organisms with a test specimen is less than one fifth of that without the test specimen, any such effect must be eliminated by dilution, filtration, neutralization or inactivation.

To confirm the sterility of the medium and of the diluent and the aseptic performance of the test, follow the TVC method using sterile buffered sodium chloride-peptone solution, pH 7.0, or phosphate buffer, pH 7.2, as a control. There should be no growth of microorganisms.

To validate the method, a count for the test organism should be obtained differing by not more than a factor of 10 from the calculated value for the inoculum.

Validation of the tests for specific microorganisms

If necessary, grow separately the test strains listed in Table 17 on the culture media indicated, at 30–35 °C for 18–24 hours. Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 so that the test suspensions contain about 103 microorganisms per ml. Mix equal volumes of each suspension and use 0.4 ml (approximately 102 microorganisms of each strain) as an inoculum in tests for *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in the presence of the material being examined, if necessary. The test method should give a positive result for the respective strain of microorganism.

Table 1. Relative retention times of organophosphorus insecticides

Substances	Relative retention times	Substances	Relative retention times
Dichlorvos	0.20	Parathion	0.69
Fonofos	0.50	Chlorpyrifos	0.70
Diazinon	0.52	Methidathion	0.78
Parathion-methyl	0.59	Ethion	0.96
Chlorpyrifos-methyl	0.60	Carbophenothion	1.00
Pirimiphos-methyl	0.66	Azinphos-methyl	1.17
Malathion	0.67	Phosalon	1.18

Table 2. Relative retention times of organochlorine and pyrethroid insecticides

Substances	Relative retention times	Substances	Relative retention times
α- hexachlorocyclohexane	0.44	*p,p'*-DDE	0.87
hexachlorobenzene	0.45	*o,p'*-DDD	0.89
β- hexachlorocyclohexane	0.49	endrin	0.91
lindane	0 49	β-endosulfan	0.92
δ- hexachlorocyclohexane	0.54	*o,p'*-DDT	0.95
ε- hexachlorocyclohexane	0.56	carbophenothion	1.00
heptachlor	0.61	*p,p'*- DDT	1.02
aldrin	0.68	*cis*-permethrin	1.29
cis-heptachlor-epoxide	0.76	*trans*-permethrin	1.31
o,p'-DDE	0.81	cypermethrin[a]	1.40

[a] The substance shows several peaks.

Table 3. Instrumental parameters for heavy metals

		Cd	Cu	Fe	Ni	Pb	Zn
Wavelength	nm	228.8	324.8	248.3	232	283.5	213.9

Slit width	nm	0.5	0.5	0.2	0.2	0.5	0.5
Hollow-cathode lamp current	mA	6	7	5	10	5	7
Ignition temperature	°C	800	800	800	800	800	800
Atomization temperature	°C	1800	2300	2300	2500	2200	2000
Background corrector		on	on	on	on	on	on
Nitrogen flow	Litre/min	3	3	3	3	3	3

Table 4. Validation of tests for detection of specific microorganisms in the herbal sample

Microorganism	Strain numbera	Medium
Escherichia coli	e.g. NCIMB 8545	lactose broth
Pseudomonas aeruginosa	e.g. NCIMB 8626	soybean-casein digest medium
Salmonella typhimurium	No strain number is recommended. Species not pathogenic for humans, such as Salmonella abony (NCTC 6017, CIP 80.39), may be used	lactose broth
Clostridium botulinum	e.g. ATCC 19297 (NCTC 7272)	cooked-meat medium
Clostridium perfringens	e.g. ATCC 13124 (NCTC 8239)	cooked-meat medium
Clostridium tetani	e.g. ATCC e19406 (NCTC 279)	cooked-meat medium
Staphylococcus aureus	e.g. NCIMB 8625 (ATCC 6538 P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83, IFO 13276)	soybean-casein digest medium

RESULTS

Table 5. Results for organophosphorus insecticides

Sr. No	Substances	Observed in ppm
1	Dichlorvos	Not detected
2	Fonofos	Not detected
3	Diazinon	Not detected
4	Parathion-methyl	Not detected
5	Chlorpyrifos-methyl	Not detected
6	Pirimiphos-methyl	Not detected
7	Malathion	Not detected
8	Parathion	Not detected
9	Chlorpyrifos	Not detected
10	Methidathion	Not detected
11	Ethion	Not detected
12	Carbophenothion	Not detected
13	Azinphos-methyl	Not detected
14	Phosalon	Not detected

Table 6. Results of organochlorine and pyrethroid insecticides

Sr. No	Substances	Observed in ppm
1	α- hexachlorocyclohexane	Not detected
2	hexachlorobenzene	Not detected
3	β- hexachlorocyclohexane	Not detected
4	lindane	Not detected
5	δ- hexachlorocyclohexane	Not detected
6	ε- hexachlorocyclohexane	Not detected
7	heptachlor	Not detected
8	aldrin	Not detected
9	*cis*-heptachlor-epoxide	Not detected
10	*o,p'*-DDE	Not detected
11	*p,p'*-DDE	Not detected
12	*o,p'*-DDD	Not detected
13	endrin	Not detected

14	β-endosulfan	Not detected
15	*o,p'*-DDT	Not detected
16	carbophenothion	Not detected
17	*p,p'*- DDT	Not detected
18	*cis*-permethrin	Not detected
19	*trans*-permethrin	Not detected
20	cypermethrin	Not detected
21	fenvalerate	Not detected
22	deltamethrin	Not detected

Table 7. Results for heavy metal contamination

Sr. No	Substances	Observed value in ppm	Limit
1	Lead	1.3 ppm	Not more than 5ppm
2	Cadmium	0.01ppm	Not more than 0.2 ppm
3	Mercury	0.01ppm	Not more than 0.1 ppm

Table 8. Results of microbial contamination

Sr. No	Test	Observation	Limit
1	Total viable aerobic count (bacteria)	1,22,000 cfu/gm	Not more than 10^7 cfu/gm
2	Total viable aerobic count (fungi)	3000 cfu/gm	Not more than 10^5 cfu/gm
3	Escherichia coli	<100 cfu/gm	Not more than 10^2 cfu/gm

CONCLUSION

The toxicological evaluation of herbal drugs and their formulation was a recent advanced analytical tool developed by the scientist in keen interest of uplifting the herbal drug to the global markets. In the current research the anti-diabetic Siddha poly herbal formulation was subjected to various toxicological evaluation to ascertain its safety for safe use of the diabetic patients. The results obtained from the above research might give a definite assurance for better use of "Neerzhivu choornam" for diabetic management and also to create a good demand in the market.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest for this research work on Neerzhivu choornam.

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