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Research Article

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GC-MS analysis, antimicrobial, antioxidant activity of an Ayurvedic medicine, Nimbapatradi Choornam

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ABSTRACT

Nimbapatradi Choornam is a known Ayurvedic medicine for Leprosy, eczema, gout, leukoderma, skin eruptions and psoriasis. The ingradiaents of Nimbapatradi choornam are neem leaves, sulphur and turmeric, which are known for their antiseptic properties. The present study is to understand the medicinal efficacy of Nimbadipatra choornam undertakig phytochemical analysis, antimicrobial activity effect, antioxidant effect and GC MS analysis. The phytochemicals present were saponins, tannins, triterpenoids, cardiac glycosides, phytosterol, coumarin and phenolic compounds. Strong antimicrobial activity of this medicine was onbserved against the microorganisms such as, Klebsiella pneumoniae, Staphylococcus aureus, E coli and Candida albicans. The antioxidant activity of Nimbapatradi also was found in three types of asays namely, reducing power assay, peroxidase assay and catalase assay. The GC MS patterns have shown important peaks which represented Cyclic octatomic sulfur S8, Phenol, 2,4-bis(1,1-dimethylethyl)-derivative, Ar-tumerone, Isopropyl myristate, Eicosane, 2-methyl-derivative, Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, Hexadecanoic acid, ethyl ester, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, 3-Hexadecanol, Ergosterol etc. The presence of these biocactive compounds correspond well with the activity of Nimbadipatra choornam as a stong medicine for skin related diseases.

Keywords: Nimbapatradi choornam, saponins, cardiac glycosides, phytosterol, *Candida albicans*, *E. coli*, Peroxidase, Catalase, Cyclic octatomic sulfur S8, Phenol, Ar-tumerone

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INTRODUCTION

Nimbapatradi choornam is an Ayurvedic formulation which contains Neem (*Azadiracta indica*.Juss), Turmeric (*Curcuma longa*) and Sulphur as its ingredients. Neem is described as *Krimighna* in ayurvedic literature meaning thereby destroyer of worms. As the name suggests, it is made out of neem leaves which is an antimicrobial herb. It is rubbed over the body mixed with sour butter milk after application of suitable ayurvedic oil such as Dinesavalyadi or Eladi to get antiseptic effect on the skin. The dosage is between 5 to 10 grams of the leaf powder in hot water. Nimbapatradi choornam is heals chronic non healing wounds, as in case of diabetic's wounds and also used in spleen disorders. It relieves joint inflammation caused due to rheumatoid arthiritis.

Neem
Subapriya and Nagini, 2005 have reviewed the various medicinal properties of Neem leaves. [1] Hashmat *et al*, 2012, reviewed the medicnal role of neem. [2] Biswas *et al*, 2002 have elaborately discussed the biological activities and medicinal properties of neem (*Azadirachta indica*). [3] Anyaehie reported the medicinal value of leaf extarcts of *Azadirachta*. Parida *et al*, 2002, have shown the inhibitory potential of neems leaves against dengue fever. [4] This plant is known to have medicinal values such as antibacterial, antiviral, immunomodulatrory, antinflammatory, antioxidant and anticarcinogenic. [5-12]The plant is also used against digestive disrorders and parasitic diseases. [13-14]

Turmeric
Turmeric is another important wonder drug with its wide application as food, medicine and as preservative. Many workers have worked on this plant on various aspects. Turmeric is anti-inflammatory, antimicrobial, preservative, antifungal, anticancer, cardioprotective, hyporglycemic and antidiabetic. [15-23]

Sulphur
Sulphur is an important element in our system mainly being a part of many amino acids. Its role in the physiology is immense. Sulphur is used in various medicines due to its antisecptic properties. It was reported by Duan *et al* 2015 that sulphur is anticancer. [24]

The knowledge of the phytoconstituents like alkaloids, tannins, flavonoids etc. present in plants as well as in plant based medicines could be very helpful in drug discovery and new drug formulation. [25, 26, 27, 28, 29]

The present study envisages corelating the medicinal properties of each constituent plant to the bioactive molecules that are present in Nimbapatradi choornam as found by GC MS analysis.

EXPERIMENTAL SECTION

COLLECTION OF SAMPLES
Medicines were purchased from ayurvedic shop, Chennai. Nimbapatradi chooernam is prepared by standard pharmaceutical companies like Ashoka pharmaceuticals, Arya Vaidya Sala Kottakkal, Arya Vaidya Pharmacy.

NIMBAPATRADI CHOORNAM
Each 100g is prepared out of:
- Nimbapatram (Neem Leaves) – 75 grams
- Gandhaka (Sulphur) – 12.5 grams
- Nisa (Turmeric)- 12.5 grams

The three constituents are ground and mixed. This this mixed with sour butter mik along with some ayurvedic oils and rubbed over the skin parts.

COLLECTION OF MICRO ORGANISM
Culture (micro organism) collected for antimicrobial sensitivity test were *Klebsiella pueumoniae, Staphylococcus aureus, Escherichia coli* and *Candida albicans.*These microorganisms were collected from King Institute of preventive medicine and Research, Chennai. Samples were collected in slants and were subcultured for antimicrobial tests after three sub culture and the fourth sub culture was used for the anti microbial tests.

Sub culturing of microorganism for the anti microbial test:
Media used for sub culturing of microorganisms were, for *Klebsiella pueumoniae*- Simon citrate, for *Staphylococcus aureus* - Blood Agar, for *Escherichia coli*- Nutrient Agar and for *Candida albicans*- (YEPD).

Phytochemical analysis
The preliminary phytochemicals present in the herbal medicine were analyzed for the presence of alkaloids, saponins, tannins, steroids, flavonoids, anthraquinones, triterpinoids, cardiac glycosides, Amino acids, phytosterol, carbonyl, quinones, coumarines, phlobatanin, phenolic compounds based on the protocols available in the literature (Eazhisaivallabi *et al*, 2012; Adetuyi *et al*, 2007; Trease and Evans, 1989). [30-32]

Preparation of sample
About 5g of sample was taken and dissolved in 50ml of distilled water and it was kept undisturbed for 10 hours. Another 5gm of sample was taken and dissolved in mixture of ethanol and water in the ratio of 1:1.

Antimicrobial activity test
The assay was carried out according to the method of Natarajan *et al* (2005) with some modifications. [33]

Preparation of sample:
About 500mg of drug were weighed and dissolved in 100ml of distilled water and and filterted. The filtrate was used for to test the efficacy of antimicrobial activity.

Test Organisms:
Gram positive & Gram negative bacteria were used as test organism for this study. Gram positive bacteria such as *Staphylococcus aureus*, Gram negative bacteria such as *Escherichia coli, Klebsiella pneumonia* and fungus like *Candida ablicans.* The organisms were sub cultured on to agar plate in order to determine their viability. Stock cultures were maintained on agar slants at 4 ºC and then sub-cultured in agar plates at 37 ºC prior to each antimicrobial test.

Activity by agar well diffusion assay:
Antimicrobial susceptibility testing was done using the well diffusion method to detect the presence of anti bacterial activity of the samples corresponding Agar (Hi-media) for bacteria and for fungus were prepared according to the manufacturer's instructions. The antibacterial activity of drug was determined by agar well diffusion method. Agar in the Petri plate after solidification was inoculated with the test microorganisms, by spreading the bacterial inoculums under aseptic conditions. Wells of 5mm diameter were punched in the agar medium with sterile cork borer and filled with drug in the particular concentration. The antibiotic Penecillin weas used in the test system as positive controls. The plates were incubated at 37 ºC for 24 hrs. The antibacterial activities were assessed by measuring the diameter of the zone of inhibition for the drug and antibiotic.

ANTIOXIDANT TEST
Preparation of sample:
1gm of sample were weighed and dissolved and dissolved in 10 ml of distilled water and after sometime it was filtered. The concentrations of drug were varied from 0.2 to 1ml.

Determination of Reducing Property (Reducing power assay)
The reducing power of the herbal medicine extract was determined by a slightly modified method (Oyaizu, 1986.) [34] The reducing ability of the drug extract was measured by the transformation of Fe^{3+} toFe^{2+} in the presence of the extract at 700nm. Increased absorbance of the reaction mixture indicates increased reducing power.1 ml of extract concentration (0.1, 0.5 and 1 mg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN_6)] (2.5 ml, 1 %). The mixtures were then incubated at 50 ºC for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10 %) were added to each mixture, which were then centrifuged for 10 min at 1036 x g. The upper layer of the solutions (2.5 ml) were mixed separately with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %), and the absorbance levels were measured at 700 nm using a spectrophotometer.

Guaiacol Peroxidase (POD)
Peroxidase activity was determined according to Panda *et al* (2003). [35] Each solution was treated with 2ml of a solution containing Guaiacol, H_2O_2 and phosphate buffer (pH 7) in the concentrations of 1%, 40mM and 100mM, respectively. The enzyme produced a colourful product by using H_2O_2 and Guaiacol as substrates. The absorbance of the product was monitored at 470 nm and peroxidise activity was indicated in the form of graph.

Catalase assay
Catalase activity was determined according to (Aebi and Lester, 1984). [36] The decomposition H_2O_2 of was followed as a decrease in absorbance at 240 nm in a UV/Vis spectrophotometer. 50 mM potassium phosphate buffer, pH 7.0) and 10 mM H_2O_2.The extinction coefficient of H_2O_2 (40 mM−1 cm−1 at 240 nm) was used to calculate the enzyme activity that was expressed in terms of millimoles of H_2O_2 per minute per gram fresh weight).

GC MS Analysis

The GC MS analysis was carried out by standard method after preparing the sample suitably.

RESULTS AND DISCUSSION

The phytochemical analysis results are tabulated in Table 1**.**

Table 1.The phytochemical analysis of various extracts of Nimbapatradi choornam

Serial number	Phytochemicals	(raw sample)	(sample in distilled water)	(ethanol:water)
1	Saponins	+	+	_
2	Tannins	_	_	+
3	Triterpinoids	_	+	+
4	Quinones	+	_	_
5	Steroids	_	_	_
6	Amino acids	_	_	_
7	Cardiac glycosides	_	+	+
8	Anthroquinones	_	_	_
9	Flavonoids	_	_	_
10	Alkaloids	_	_	_
11	Phytosterol	+	_	+
12	Coumarines	_	_	+
13	Phenolic compounds	+	+	+
14	Phlobatannin	_	_	_

(+) = Present, (-) = Absent

The antibacterial and antifungal activities of Nimbapatradi choornam is indictaed in Figure 1 and Table 2. The comparative acitivity with standards is shown in Figure 2.

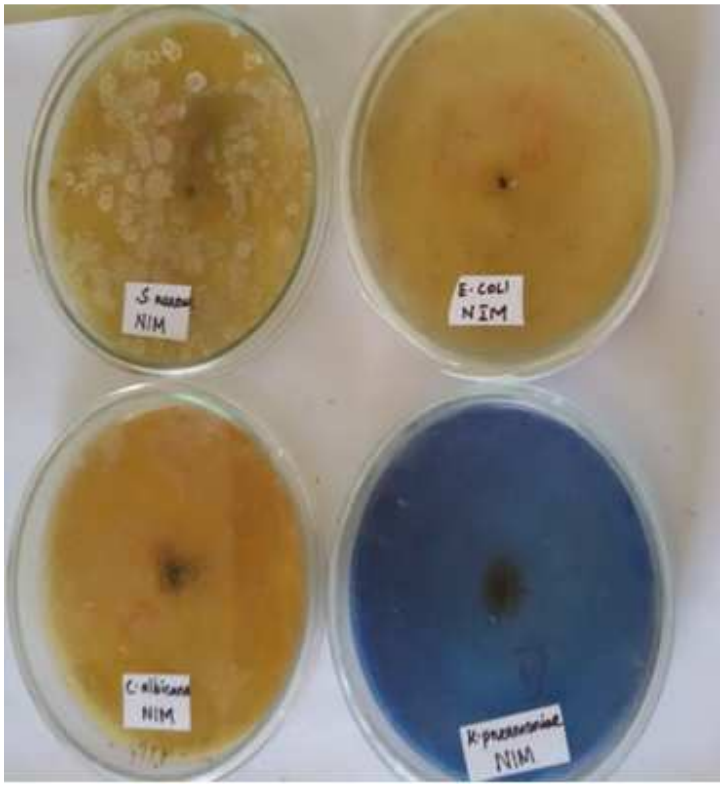


Figure 1. Zone of inhibition of different microorganism with a specific concentration of Nimbapatradi Choornam as medicine

Table 2 Microorganisms with different zones of inhibition

Concentration of extract(g/ml)	Microorganism	Zone of inhibition (mm)	Zone of inhibition (Penicillin)
0.5	*Staphylococcus aureus*	4.8	5
	Escherichia coli	15.2	16
	Candida albicans	7.2	0
	Klebsiellapneumoniae	5.2	8

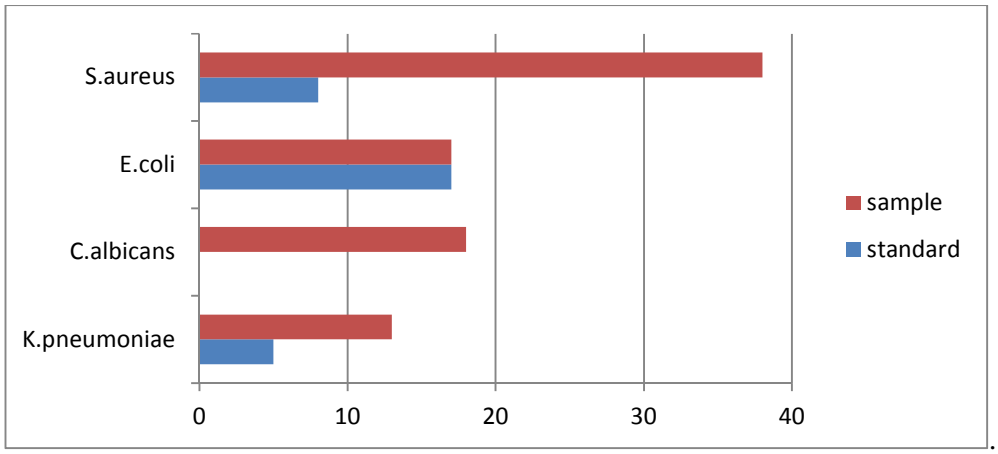
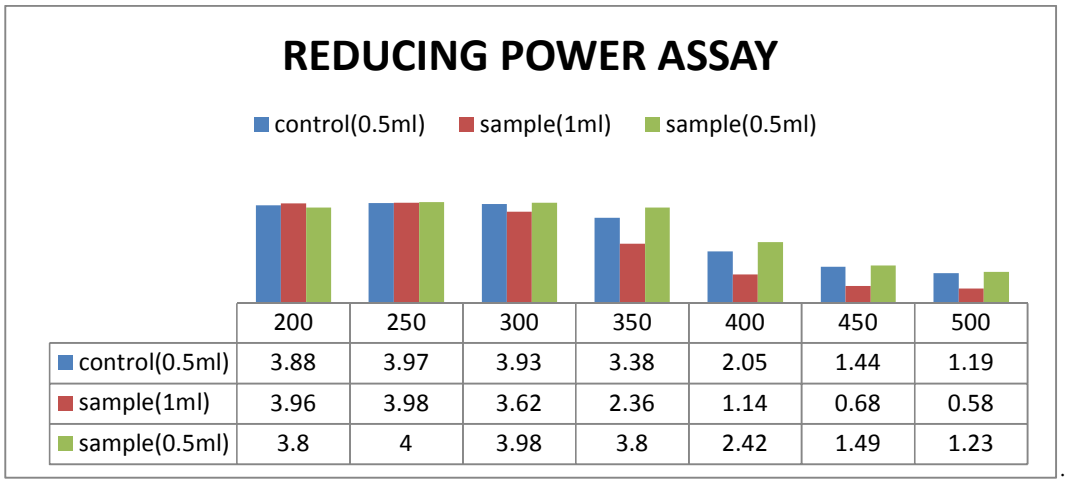


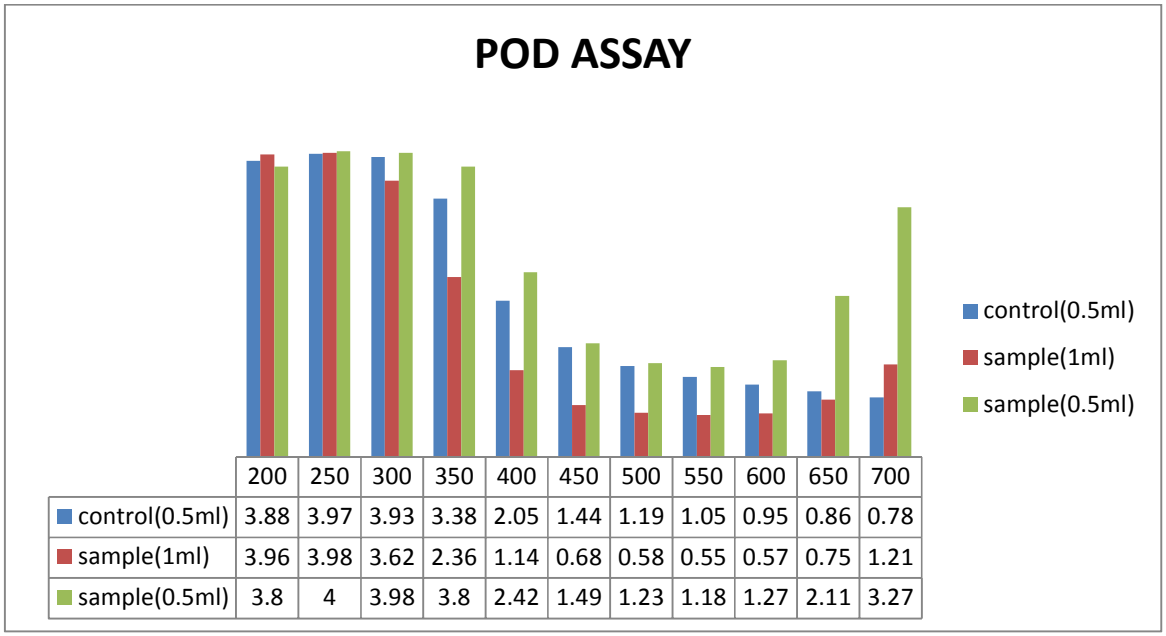
Figure 2. Zone of inhibition of microorganism with concentration of sample compared with the standard

The antioxidant activities, i. Reducing power assay, POD assay and Catalase assay results are shown in Figres 3, 4 ans 5 respectivly



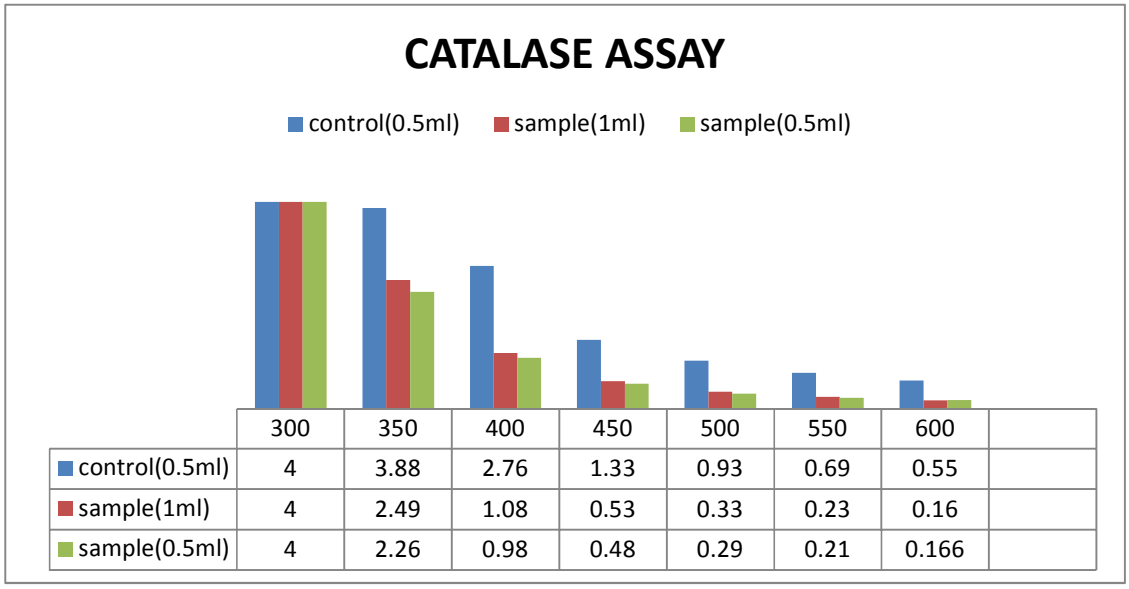
	200	250	300	350	400	450	500
control(0.5ml)	3.88	3.97	3.93	3.38	2.05	1.44	1.19
sample(1ml)	3.96	3.98	3.62	2.36	1.14	0.68	0.58
sample(0.5ml)	3.8	4	3.98	3.8	2.42	1.49	1.23

Figure 3. The reducing activity of sample of different concentration with respect to control



	200	250	300	350	400	450	500	550	600	650	700
control(0.5ml)	3.88	3.97	3.93	3.38	2.05	1.44	1.19	1.05	0.95	0.86	0.78
sample(1ml)	3.96	3.98	3.62	2.36	1.14	0.68	0.58	0.55	0.57	0.75	1.21
sample(0.5ml)	3.8	4	3.98	3.8	2.42	1.49	1.23	1.18	1.27	2.11	3.27

Figure 4. The peroxidise activity of sample of different concentration with respect to control



	300	350	400	450	500	550	600	
control(0.5ml)	4	3.88	2.76	1.33	0.93	0.69	0.55	
sample(1ml)	4	2.49	1.08	0.53	0.33	0.23	0.16	
sample(0.5ml)	4	2.26	0.98	0.48	0.29	0.21	0.166	

Figure 5. The catalase activity of sample of different concentration with respect to control

The GC MS patterns graphs are shown Figure 6 and the details of GC MS analysis of compounds is detailed in Table 3.

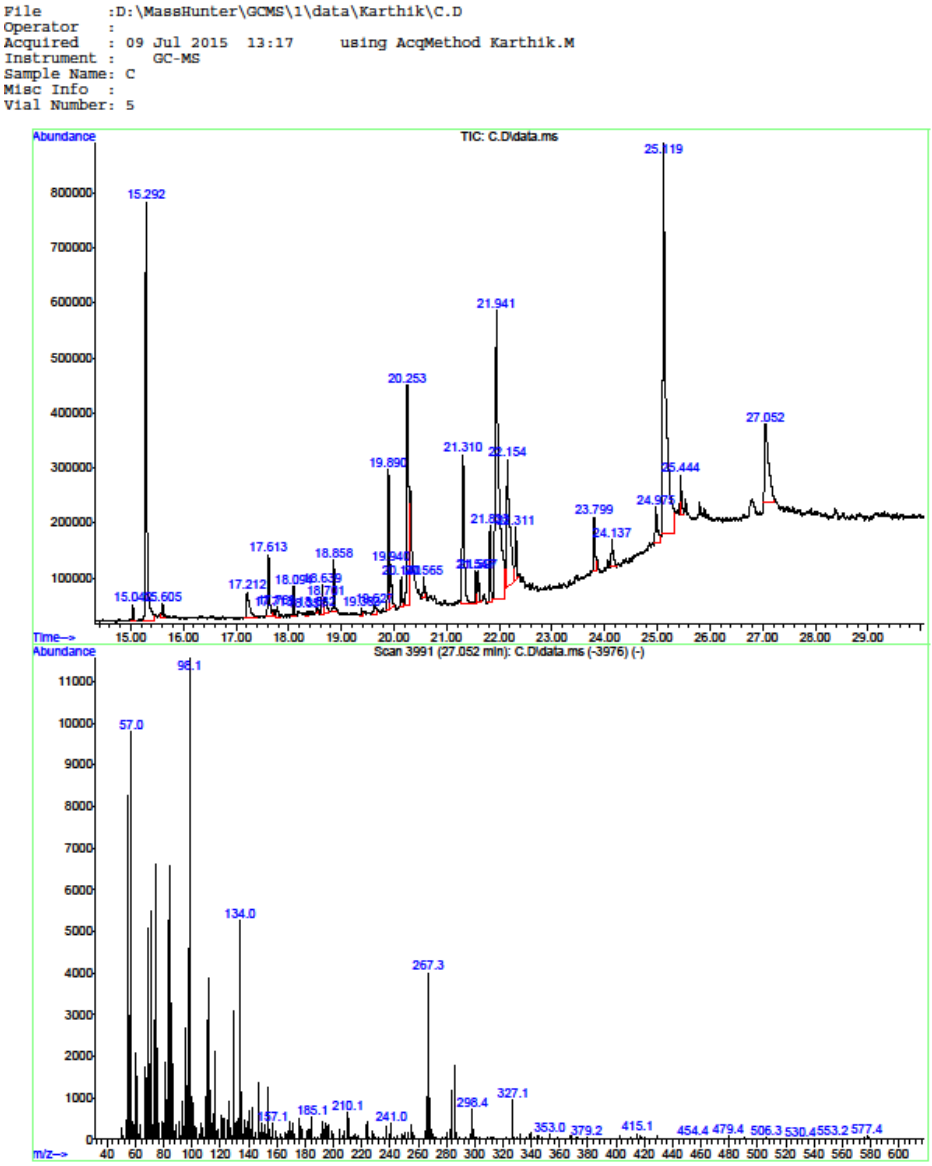


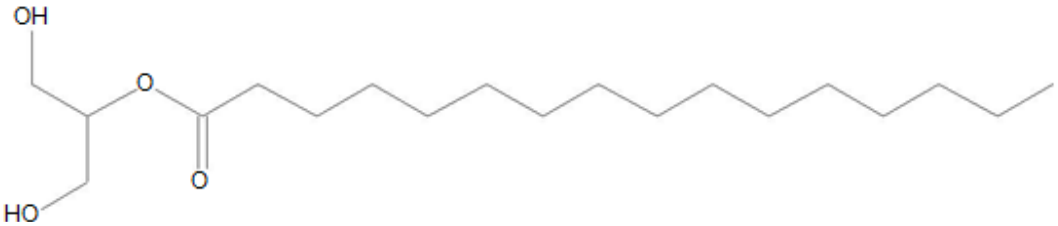
Figure 6. GC MS Graphs of Nimbapatradi Choornam

Table 3. Indicating the various results of the GC MS analysis of Nimbapatradi Choornam

Sl No.	R T Value (In Min.)	Compound	Mol. Formula	Mol. Wt.	Peak area (%)	Probability %
1.	15.042	Eicosane, 2-methyl-	C21H44	296	0.356	4.44
2.	15.292	Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O	206	11.122	62.1
3.	15.605	Heptadecane, 2,6,10,15-tetramethyl-	C21H44	296	0.342	8.02
4.	17.212	Ar-tumerone	C15H20O	216	1.543	97.4
5.	17.613	Hexadecanoic acid, 14-methyl-, methyl ester,	C18H36O2	284	1.764	15.9
6.	17.713	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	C26H54	266	0.174	21.1
7.	17.769	Hexadecanoic acid, 14-methyl-, methyl ester.	C18H36O2	284	0.342	15.9
8.	18.094	Eicosane, 2-methyl-	C21H44	296	0.651	6.67
9.	18.351	Fumaric acid, cyclohexyl pentadecyl ester	C25H44O4	408	0.107	14.7
10.	18.532	Picrotoxinin	C15H16O6	292	0.118	10.4
11.	18.639	3-Hexadecanol	C16H34O	242	0.906	81.5
12.	18.701	Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-	C21H38O2	322	0.573	23.8
13.	18.858	Isopropyl myristate	C17H34O2	270	1.164	75.5
14.	19.383	Phthalic acid, isobutyl octadecyl ester	C30H50O4	474	0.193	6.55
15.	19.627	Ergocalciferol	28H44O	396	0.420	4.70
16.	19.890	Eicosane, 2-methyl-	C21H44	296	3.159	4.32
17.	19.940	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	C17H24O3	276	1.120	92.9
18.	20.140	-[5-Nitro-2-thienylmethylideneamino]-2-oxazolidinone	C8H7N3O4S	241	0.688	16.4
19.	20.253	n-Hexadecanoic acid,	C16H32O2	256	8.056	70.6
20.	20.565	Hexadecanoic acid, ethyl ester	C18H36O2	284	0.461	69.5
21.	21.310	Cyclic octaatomic sulfur S8;	sulfur S8	256	5.684	98.8
22.	21.547	Methyl 9-cis,11-trans-octadecadienoate	C19H34O2	294	0.708	7.69
23.	21.597	trans-13-Octadecenoic acid, methyl ester	C19H36O2	296	0.766	7.72
24.	21.823	Methyl stearate	C19H38O2;	298	1.798	42.1
25.	21.941	Oleic Acid C18H34O	C18H34O2	282	18.221	17.0
26.	22.154	Octadecanoic acid	C18H36O2	284	7.170	52.9
27.	22.311	Heneicosane	C21H44	296	1.251	6.05
28.	23.799	Heneicosane	C21H44	296	1.397	6.22
29.	24.137	Octadecane, 3-ethyl-5-(2-ethylbutyl)	C26H54	366	0.969	8.31
30.	24.975	Hexadecanoic acid, 3-[(trimethylsilyl)oxy]propyl ester	C22H46O3Si	386	1.424	40.5
31.	25.119	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	C19H38O4	330	20.651	57.2
32.	25.444	Diisooctyl phthalate	C24H38O4	390	0.973	9.71
33.	27.052	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	C21H42O4	358	5.671	59.1

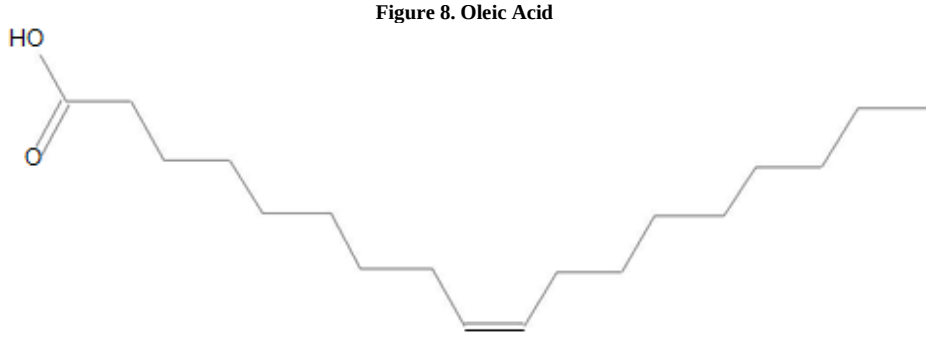
The detailed individual GC MS graph along with the structure of the molecules are depicted in figures 7 to Figure 18.

Figure 7. Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester



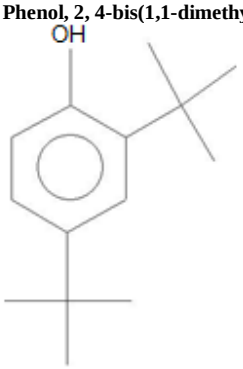
Name: Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Formula: $C_{19}H_{38}O_4$
MW: 330 Exact Mass: 330.27701 CAS#: 23470-00-0 NIST#: 15400 ID#: 7272 DB: mainlib

Figure 8. Oleic Acid



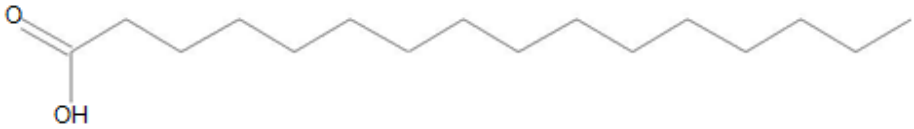
Name: Oleic Acid
Formula: $C_{18}H_{34}O_2$
MW: 282 Exact Mass: 282.25588 CAS#: 112-80-1 NIST#: 134027 ID#: 2532 DB: mainlib

Figure 9. Phenol, 2, 4-bis(1,1-dimethylethyl)-



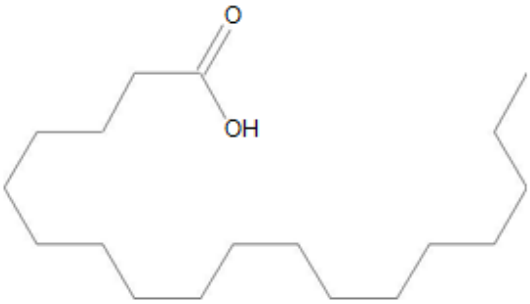
Name: Phenol, 2,4-bis(1,1-dimethylethyl)-
Formula: $C_{14}H_{22}O$
MW: 206 Exact Mass: 206.167066 CAS#: 96-76-4 NIST#: 228966 ID#: 156338 DB: mainlib

Figure 10. n-Hexadecanoic acid



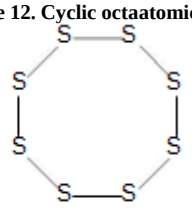
Name: n-Hexadecanoic acid
Formula: $C_{16}H_{32}O_2$
MW: 256 Exact Mass: 256.24023 CAS#: 57-10-3 NIST#: 151973 ID#: 8689 DB: mainlib

Figure 11. Octadecanoic acid



Name: Octadecanoic acid
Formula: $C_{18}H_{36}O_2$
MW: 284 Exact Mass: 284.27153 CAS#: 57-11-4 NIST#: 290961 ID#: 8691 DB: mainlib

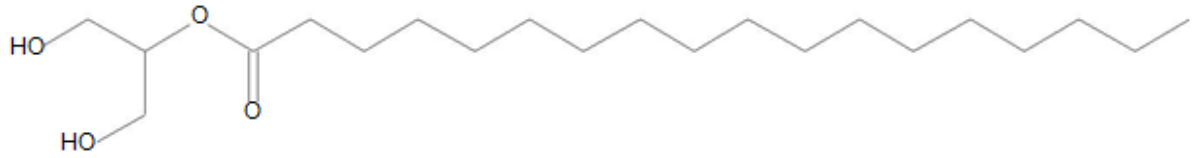
Figure 12. Cyclic octaatomic sulfur



Formula: S_8
S8; MF: 886; RMF: 918; Prob 98.8%; CAS: 10544-50-0; Lib: mainlib; ID: 29209.

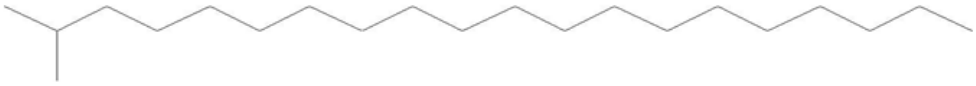
Figure . 13.

Name: Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester



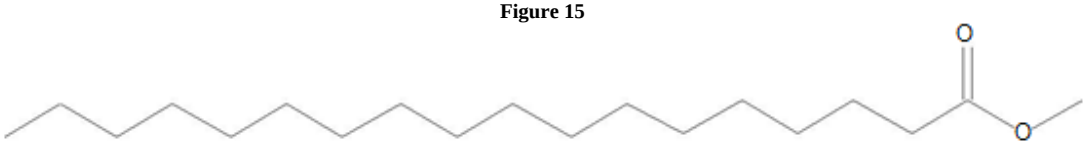
Name: Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
Formula: $C_{21}H_{42}O_4$
MW: 358 Exact Mass: 358.30831 CAS#: 621-61-4 NIST#: 16116 ID#: 7334 DB: mainlib

Figure 14.



Name: Eicosane, 2-methyl-
Formula: $C_{21}H_{44}$
MW: 296 Exact Mass: 296.344301 CAS#: 1560-84-5 NIST#: 113884 ID#: 22547 DB: mainlib

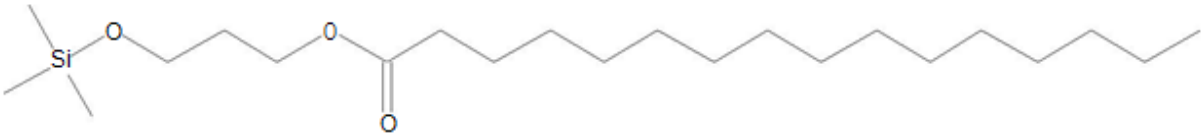
Figure 15



Name: Methyl stearate
Formula: $C_{19}H_{38}O_2$
MW: 298 Exact Mass: 298.28718 CAS#: 112-61-8 NIST#: 291013 ID#: 40712 DB: mainlib

Figure 16.

Hexadecanoic acid, 3-[(trimethylsilyl)oxy]propyl ester



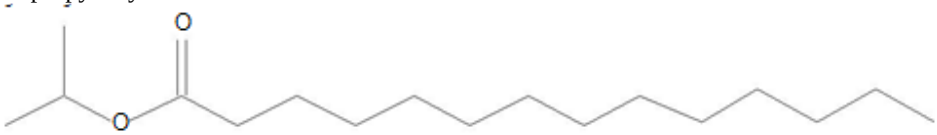
Name: Hexadecanoic acid, 3-[(trimethylsilyl)oxy]propyl ester
Formula $C_{22}H_{46}O_3Si$ MW: 386 Exact Mass: 386.32162 CAS#: 56630-48-9 NIST#: 79111 ID#: 22855 DB: mainlib

Figure 17.

Name: Heneicosane
Formula: $C_{21}H_{44}$
MW: 296 Exact Mass: 296.344301 CAS#: 629-94-7 NIST#: 230947 ID#: 23607 DB: mainlib

Figure 18.

Isopropyl Myristate



Formula $C_{17}H_{34}O_2$
MW: 270 Exact Mass: 270.25588 CAS#: 110-27-0 NIST#: 107374 ID#: 10204 DB: mainlib

The photochemical study results revealed that Nimbapatrdi choornam contains some very important phytochemicals like tannins, saponins, terpenoids, cardac glycosides and phenol compounds whichcould be responsible for its antimicrobial as well as antioxidant properties.

The GC MS patterns have shown important peaks which represented Cyclic octaatomic sulfur S8, Phenol, 2,4-bis(1,1-dimethylethyl)- derivative, Ar-tumerone, Isopropyl myristate, Eicosane, 2-methyl-derivative, Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, Hexadecanoic acid ethyl ester, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, 3-Hexadecanol, n-hexadeconoic acid, Methyl stearate, Picrotoxinin, Fumaric acid, 3-[5-Nitro-2-thienylmethylidene amino]-2-oxazolidinone and Ergosterol etc. among some minor components as listed in Table 3.

The medicinal properties of the important component indicate the veracity of the use of this medicine. Cyclic octatomic sulfur S8 is an essential element as a constituent of many amino acids and also in other metabolic processes. Phenol, 2, 4-bis (1, 1-dimethylethyl)- derivative_is present in various plants and is known for its antibacterial and anti-inflammatory activities. [37, 38] Ar-tumerone has been shown as having antileishmanial and antiplatlet activity. [39, 40, 41] Isopropyl myristate is a known compound used as skin care lotion and emmolient. [42] Eicosane, 2-methyl- derivative is a good antioxidant. Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl) methyl) is used a potential antidimentia drug. [43] Octadecanoic acid esters are reported to be antiviral, antibacterial and antioxidant activities. [44, 45] Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester is an antioxidant. 4. 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione is also as antioxidant. [46, 47] Ergocalciferol is Vitamin D. - [5-Nitro-2-thienylmethylideneamino]-2- oxazolidinones are class of antibacterial compounds. [48] Fumaric acid esters are proven skin treatment formulations. [49, 50, 51] Tridecane, 2-methyl-derivative is a good antimicrobial. [52].

The findings from the above results indicate that neem, turmeric and sulphur are known for their skin care properties. Nimbapatradi choornam, which is made from these three components, obviously should show similar results. The results of antibacterial, antifungal, antioxidant and GC MS analysis clearly indicate that the components present in Nimbapatradi choornam have important properties such as anti viral, antibacterial, antifungal, antioxidant, anti-inflammatory etc. These properties of Nimbapatradi choornam augures well with the claim by Ayurvedic proponents about its efficacy as a potential skin care medicinal formulation. Further studies on the pharmacological and toxicological aspects of Nimbapatradi choornam are under way which is necessary to standardize and validate this medicine.

CONCLUSION

The medicine, Nimbapatradichoornam is a standard Ayurvedic medicine for skin diseases and the present study has revealed its efficacy by the standard techniques like antimicrobial, antioxidant and GC MS analysis study. Further work is going on to establish the mechanism of action of this medicine.

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