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PHYSICO-CHEMICAL AND PHYTOCHEMICAL EVALUATION
OF *ACORUS CALAMUS.LINN* – BEFORE AND AFTER
PURIFICATION

R. Karpagambal ¹, M. Raghavi ², B. Abarna ³, P. Shanmugapriya ^{4*}, R. Madhavan ⁵

1,2,3 – PG Scholar, 4- Associate Professor, 5- Associate Professor, Head of the Department,
Department of Nanju Maruthuvam, National Institute of Siddha, Tambaram Sanatorium,
Chennai 47, affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai
600032.

Corresponding Author:

P. Shanmugapriya
Associate Professor
Department of Nanju Maruthuvam,
National Institute of Siddha,
Tambaram Sanatorium, Chennai.
Email ID: spriyaatham@gmail.com
Phone No: 9962513101

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ABSTRACT:

Introduction: The rhizome of *Acorus calamus. Linn* is an emergent aquatic macrophyte admired for its medicinal properties due to its bioactive components. *AC* naturally containing β -asarone and α -asarone, has been researched for many pharmacological benefits while consumed in high doses or over extended periods can cause toxic effects. *AC* is highly valued in Siddha medicine for its role in treating children's health issues as an ingredient in polyherbal formulations.

Objectives: This study aims to evaluate the physicochemical and phytochemical analysis before and after purification and highlights the importance of the purification process of *AC* rhizome.

Materials and methods: *AC* rhizome was subjected to the Siddha purification procedure and a sample was collected. The physicochemical parameters such as loss on drying at 105°C, total ash, acid insoluble ash, water, and alcohol soluble extractive values before and after purification were measured according to PLIM guidelines. The phytochemical studies aim to

evaluate the changes in chemical composition that take place in three stages of the purification process: before (sample A), intermediate (sample B), and after purification (sample C) of *AC* using a Shimadzu GC 2010 plus gas chromatograph equipped with MS operating in electronic ionization mode.

Results: The physicochemical results determine the purity and quality of the purified sample. The phytochemical analysis showed that the fraction of harmful substances like asarone decreased and beneficial components were enhanced following purification.

Conclusion: This study concludes by comparing the results of both analyses before and after purification unequivocally underscoring the significance of the *AC* purification process.

Keywords: *Acorus calamus*, Asarone, Physicochemical, Phytochemical, Purification.

INTRODUCTION:

Acorus calamus. Linn, commonly called Sweet Flag, is prominently featured in Siddha medicine due to its diverse pharmacological applications. Its essential oil is a revered component of traditional medicine practices, renowned for its multifaceted therapeutic attributes.^[1] *AC* rhizome plays a substantial role in Siddha polyherbal formulations such as "*Urai Mathirai*,^[2] *Uthamani Kudineer*,^[3] *Uthamani Nei*,^[3] *Vembaathy Kudineer*,^[3] and *Vidathaari Chooranam*"^[4] which are frequently utilized to address gastrointestinal issues, respiratory problems, and other common ailments in children. *AC* exerts a gastroprotective role due to its antidiarrheal and anti-ulcer activity, making it effective for treating chronic diarrhea and dyspepsia.^[5,6] After the purification process, *AC* rhizome used in these formulations is believed to offer therapeutic benefits for digestive health through interactions with the gut microbiome. Owing to the immunomodulatory properties of *AC*^[7], "*Urai mathirai*" an important formulation of *Acorus*, is an esteemed Siddha immuno-supplement, aimed at boosting children's immunity against infectious diseases such as fever, indigestion, recurrent cough, and primary complex. This supplement is extensively administered in all government Siddha hospitals.^[8] Every formulation probably has a different composition and specific therapeutic purpose, but *AC* plays a pivotal role in its effectiveness. According to Siddha philosophy, everything in nature has positive (beneficial benefits) and negative (adverse effects) characteristics. In the same way, unprocessed drugs exhibit both harmful and beneficial characteristics.^[9]

AC, for instance, contains both bioactive and potentially toxic compounds thus, acting as a mild co-carcinogen. One of the primary bioactive compounds, β -asarone, has been researched for its possible pharmacological benefits. However, β -asarone is also linked to potential toxic effects, especially when consumed in high doses or over extended periods. Researchers investigated the impact of β -asarone on chromosomes in human lymphocyte cultures, finding significant structural chromosome aberrations after metabolic activation, which led to cellular damage. These results highlight the genotoxic nature of β -asarone, implying that *Acorus* with low β -asarone levels is preferable for use. Additionally, another study revealed β -asarone's

mutagenicity in the Salmonella-mammalian microsome test, reinforcing that only commercial drugs lacking or containing minimal β -asarone should be employed in human phytotherapy. Conversely, α -asarone displayed mutagenic effects on *Salmonella typhimurium* in a concentration-dependent fashion, necessitating a premutagenic mix with liver S-9 fraction and NADPH for its mutagenicity to manifest. The mutagenic strength of α -asarone was comparable to aflatoxin, confirming its status as a mutagen.^[10] The Indian variety of AC has high levels of α and β asarone, with the triploid and tetraploid variety containing 75-96% beta asarone respectively. The beta asarone content in AC depends on the plant's ploidy level, due to its high concentration that cannot be used in medicines or food supplements.^[11] Thus, this toxicity may be reduced through a purification process as per Siddha literature.

This purification of herbal ingredients is a crucial step to reduce the toxic compounds and enhance their therapeutic benefits. The purification is carried out using various pharmaceutical techniques such as boiling, frying, washing, triturating with various plant juices, pudam (calcination) method, grinding with specific organic or inorganic materials, and soaking in specific media. These processes reduce toxicity and enhance the drug's efficacy, making purification, or suddhi murai, a vital aspect of preparing Siddha medicines.^[12] Therefore, the lack of scientific evaluation of before and after purification of the AC necessitates this study to comprehensively evaluate the physicochemical and phytochemical analysis of before and after purification.

MATERIALS AND METHODS:

DRUG PROFILE:

The sample dried rhizomes of AC and turmeric were collected from the local market in Chennai. The collected drug was identified and authenticated (**code A02022301C**) at the Department of Pharmacognosy, Siddha Central Research Institute (SCRI), Arumbakkam, Chennai.

METHODS OF PURIFICATION:

250gm of turmeric rhizome were made into a paste. This paste was applied to the 250 g of dried rhizome of AC and left for shade drying. After that, the turmeric-coated rhizome was burned using a castor lamp flame until the outer coat was burned. Then the turmeric paste was applied over the burnt AC and left to dry to burn the outercoat again. This process was repeated about six times, and then the burnt rhizome was powdered to get the purified form of AC.^[13] The obtained samples were:

Sample A – The dried rhizome of AC was made into powder and taken as sample A which is an unpurified sample.

Sample B – It is the intermediate sample which is processed about 3 times.

Sample C – It is the purified sample which is processed about 6 times.

METHODOLOGY:

Physicochemical analysis – Qualitative studies:

Before and after purification, organoleptic evaluation and solubility profile were performed. Physicochemical analysis typically examines parameters such as loss on drying at 105°C, total ash, acid insoluble ash, and water and alcohol soluble extractive values before and after purification were measured according to PLIM guidelines, which helps to determine the purity and quality of the raw and purified material.^[14]

Phytochemical analysis – Quantitative studies:

Phytochemical analysis focuses on identifying and quantifying the various bioactive compounds present in the rhizome and also aims to evaluate the changes in chemical composition that take place in three stages: before (sample A), intermediate (sample B), and after purification (sample C) of AC using a Shimadzu GC 2010 plus gas chromatograph equipped with MS operating in electronic ionization mode. This device was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). Sample introduction utilized a split injection with a split ratio set to 10:1. The oven temperature started at 35°C, held for 2 minutes, then increased at a rate of 20°C per minute to 450°C, holding for an additional 5 minutes. The helium carrier gas maintained a flow rate of 2 ml/minute in constant flow mode. Analyses were conducted using a direct connection with a capillary column metal quadrupole mass spectrometer operating in electron ionization (EI) mode, with software GCMS solution ver. 2.6. Component identification was achieved by matching their recorded spectra with the NIST library V 11 mass spectra provided by the instrument's software. A GC/MS metabolomics database was used for similarity searches based on the retention index.

RESULTS:

Morphological Examination:

Table 1: Organoleptic evaluation of before and after purification

Organoleptic characters	SAMPLE A (Raw sample)	SAMPLE C (purified sample)
State	Solid	Solid
Nature	Moderately Coarse powder	Fine powder
Odor	Characteristic	Characteristic
Touch	Rough	Soft
Flow Property	Non-free flowing	Free-flowing
Appearance	Pale Brownish	Black

Table 2: Solubility profile of before and after purification

S.No	Solvent Used	Solubility / Dispersibility	Solubility /Dispersibility of
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		of raw sample	purified sample
1	Chloroform	Insoluble	Insoluble
2	Ethanol	Soluble	Soluble
3	Water	Soluble	Soluble
4	Ethyl acetate	Insoluble	Insoluble
5	DMSO	Soluble	Soluble

Table 3: Comparison of physicochemical analysis before and after purification

S. No	Parameter	Raw sample Mean (n=3) SD	Purified sample Mean (n=3) SD
1.	<i>Loss on Drying at 105 °C (%)</i>	4.567 ± 0.351	2.73 ± 0.35
2.	<i>Total Ash (%)</i>	7.333 ± 0.321	8.3 ± 0.62
3.	<i>Acid insoluble Ash (%)</i>	0.023 ± 0.01	0.06 ± 0.01
4.	<i>Water soluble Extractive (%)</i>	13.7 ± 1.04	17.53 ± 1.25
5.	<i>Alcohol Soluble Extractive (%)</i>	10.17 ± 1.66	12.13 ± 0.65

Phytochemical GCMS analysis of samples A, B, and C

The detailed reports of compound name, retention time, peak intensity rank, and area percentage are tabulated below (table 4). The Peak intensity of the three samples is shown in (Figures 1, 2, 3).

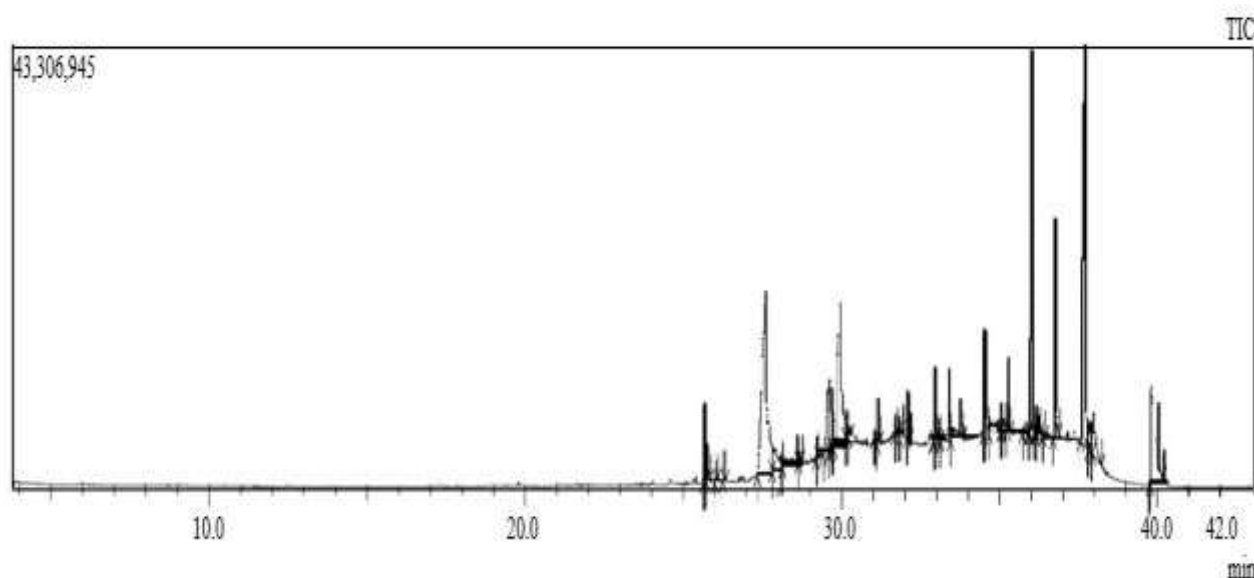


Figure 1: chromatographic assessment of Unpurified (sample A)

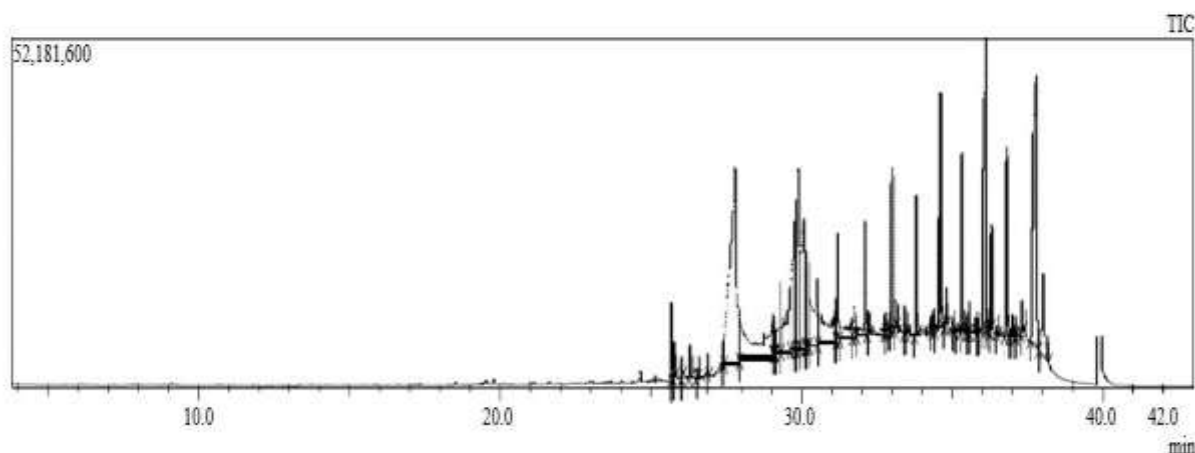


Figure 2. Chromatographic assessment of Intermediate (Sample B)

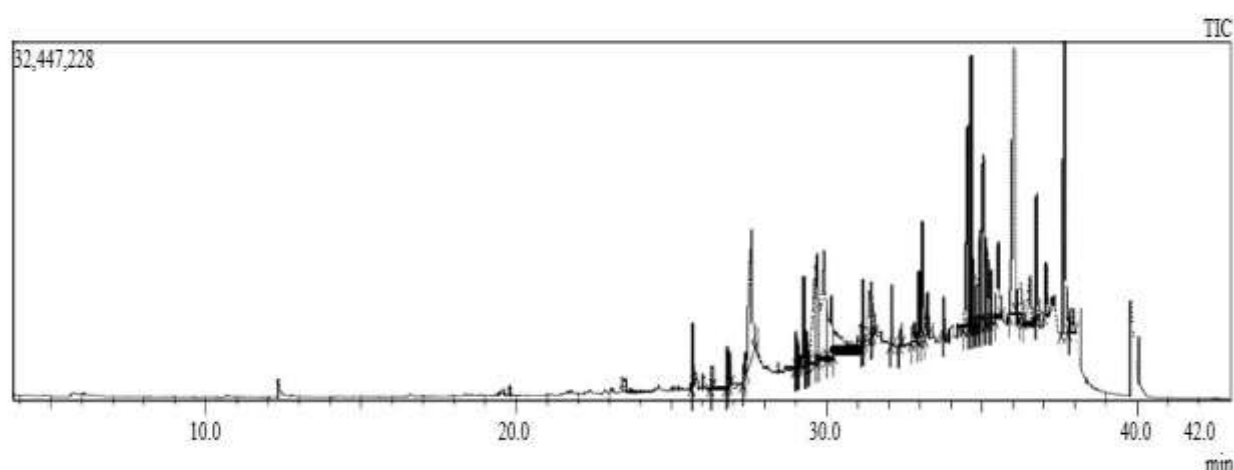


Figure 3. Chromatographic assessment of Purified (Sample C)

Table 4: Phytochemical analysis of three samples (A, B, C)

Name of the compound	R.T. Time			Area%			Properties of the compound
	sample A	sample B	Sample C	sample A	sample B	Sample C	
Phytol, acetate	25.685	25.69	25.688	1.34	0.81	0.85	Anticancer, antimicrobial, anti-inflammatory, antioxidant, and diuretic ^[15,16]
3,7,11,15-Tetramethyl-2-hexadecen-1-o	26.288	26.293	26.293	0.54	0.34	0.29	-
Asarone	27.605	27.791	27.574	17.12	14.55	6.66	Genotoxicity, carcinogenicity, hepatomas, and mutagenicity ^[17]
Phytol	29.233	29.252	29.24	0.36	1.68	1.32	Anti-inflammatory, antimicrobial, antioxidant, diuretic, anticancer ^[15,16]
Tetracosane	32.097	33.495	33.78	0.72	0.07	0.46	Inhibitor of beta-amyloid (anti-Alzheimer) ^[18]
n-Nonadecanol-1	32.905	31.093	31.075	0.08	3.8	3.57	Anti-microbial, cytotoxic ^[16]

Hexadecanoic acid, 2-hydroxy-1-(hydro	33.08	33.1	33.095	0.49	0.27	2.32	Anti-inflammatory ^[19]
Octacosanoic acid, methyl ester	37.979	38.014	37.985	2	2.27	1.28	-
Tetratetracontane	33.778	32.999	32.968	1.89	2.34	1.1	Antioxidant, cytoprotective, anti-inflammatory ^[16]
Tetratriacontane	34.552	35.105	36.075	2.57	0.16	5.94	-
Octadecanoic acid, 2,3-di-hydroxypropyl ester	34.72	34.741	34.731	1.17	0.11	1.32	anticancer, antimicrobial, arachidonic acid inhibitor, acidifier ^[16]
Octadecanoic acid	29.94	30.079	29.923	10.63	5.44	6.55	Anti-bacterial, anticancer, anti-asthmatic ^[19]
Heneicosane	30.161	26.529	30.166	0.61	0.08	2.92	Anti-bacterial ^[20]
2-Pentadecanone, 6,10,14-trimethyl-	25.77	25.775		0.76	0.43		-
Butyl 2-(2-(2-butoxy ethoxy) ethoxy)	29.708	29.74		1.47	3.88		-
Eicosane	35.287	36.103		1.14	2.06		Antitumor, bronchodilator, antifungal ^[16]
Phthalic acid, di (2-propyl pentyl) ester	33.41	33.421		1.87	0.26		Mutagenicity, reproductive toxicity ^[21]
Nonadecanoic acid, methyl ester	36.25	37.067		0.31	0.13		-
cis-9-Hexadecenal	36.397	31.655		0.1	1.48		-
Hexadecanoic acid, methyl ester		26.885	26.887		0.19	0.48	Anti-inflammatory ^[19]
Oxirane, heptadecyl-		34.364	32.376		0.18	0.18	-
Tetracosanoic acid, methyl ester		34.806	34.8		0.32	0.91	-
9,12,15-Octadecatrienoic acid, methyl ester		29.095	29.09		0.43	0.6	-
Methyl stearate		29.375	29.378		3.39	0.66	Antimicrobial ^[22]
2-methylhexacosane	36.77		34.554	4.84		5.52	-
.beta.-Amyrin	37.869		35.134	1.29		1.53	Hepatoprotective, gastroprotective, anti-inflammatory ^[23]
Z-2-Octadecen-1-ol	28.11			2.31			-
2-Hydroxy-1,8-naphthyridine	28.425			0.54			-
l-(+)-Ascorbic acid 2,6-dihexadecanoate	28.67			0.21			antibacterial, antitumor, and wound healing properties ^[24]
Z,E-3,13-Octadecadien-1-ol	29.57			2.47			-
Oleyl alcohol, trifluoroacetate	29.627			2.68			-
Octadecane, 1-(ethenyl oxy)-	30.255			0.21			Anti-bacterial, anticancer, anti-asthmatic ^[19]
1-Heneicosanol	31.062			0.17			-
4,8,12,16-Tetramethylheptadecan-4-ol	31.724			0.37			-
Octatriacontyl pentafluoro propionate	32.972			1.68			-
Bicyclo[5.3.0]decane	35.041			0.17			-
2H-Pyran-2-one, tetrahydro-6-tridecyl-	35.184			0.11			-
1-Pyrrolidinebutanoic acid, 2-[(1,1-dim	35.755			0.08			-
2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8	35.915			0.13			-
Hexatriacontane	36.037			10.04			-
15-Hydroxypentadecanoic acid	36.125			0.16			-
Pentane, 2,4-dimethyl-	39.803			7.42			-
n-Propyl 9,12-octadecadienoate		29.79			2.76		-
Phthalic acid, butyl 2-chloropropyl este		27.385			0.35		-
Ethyl 9,12-hexadecadienoate		29.01			6.36		-
Dichloroacetic acid, trident-2-vinyl ester		29.872			4.96		Anticancer, antimicrobial, antioxidant ^[16]
Tetradecanoic acid, ethyl ester		30.145			1.07		Anti-inflammatory ^[19]
Lanostane-3.beta.,11.beta.-diol, 3-acetat		30.484			3.4		-
17-Acetyl-14-hydroxy-16-methoxy-10,1		31.737			0.65		-
4,8,13-Cyclotetradecatriene-1,3-diol, 1,		32.214			0.27		-
(R)-(-)-14-Methyl-8-hexadecyn-1-ol		32.769			0.2		-
Trichloroacetic acid, undecyl ester		32.828			0.31		-
Tetracosanoic acid		35.467			0.15		-
Pentacosanoic acid, methyl ester		35.538			0.31		-
9-Octadecen-1-ol, (Z)-		35.845			0.19		-
Isopropyl stearate		36.2			0.07		-
Hexacosanoic acid, methyl ester		36.286			1.07		-
Hexacosanoic acid		36.975			0.26		-
Hexadecanal			26.795			0.63	-

cis-13,16-Docasadienoic acid			27.325			0.47	-
9,12-Octadecadienoic acid (Z,Z)-			29.62			4.51	-
7-Tetradecenal, (Z)-			29.692			3.32	-
Tetrapentacontane, 1,54-dibromo-			31.365			2.02	-
Octadecanoic acid, 3-hydroxy-2-tetradecanoic			31.446			1.19	Anti-bacterial, anticancer, anti-asthmatic [19]
Squalane			31.51			0.39	Anti-inflammatory, antioxidant, anticancer [19,25]
Ethanol,2-(9,12-octadecadienyloxy)			32.75			0.66	-
9,12,15-Octadecatrienoic acid			34.62			5.37	antioxidant, anti-inflammatory, anticancer, antimicrobial, Chemo preventive properties used in vaccine formulations, [16]
Chondrillasterol			34.936			3.1	Anti-bacterial [26]
Stigmasta-7,25-dien-3-ol			35.059			6.26	-
4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one			35.205			2.09	-
7,22-Ergostadienone			35.534			2.52	Anti-bacterial [27]
9,19-Cyclolanostan-3-ol, 24-methylene-			37.073			1.34	-

DISCUSSION:

AC with low asarone levels should be used for medicinal purposes due to its carcinogenic, genotoxic, and mutagenicity properties.^[11] As Siddhar Agathiyar stated, "*Marunthu suddhi gunam nigandu kanngal moondram*", medicines prepared from purified raw materials should be safe for use in treating diseases.^[28] After purification, AC can be safely consumed as a medicine, free from allergic reactions or adverse side effects.

The organoleptic evaluation showed that the unpurified sample was solid with a pale brownish color, coarse texture, and rough surface, exhibiting non-free-flowing properties. However, after purification, the sample turned black, became fine in texture, soft to the touch, and demonstrated free-flowing properties. Its increased fineness indicates that it will absorb quickly, enhancing bioavailability. Both samples had a distinctive odor. Solubility plays a crucial role in the bioavailability of a drug substance, as it helps determine the drug's form and the processing of its dosage form. Both the unpurified and purified samples were soluble in ethanol, water, and dimethyl sulfoxide (DMSO), but insoluble in chloroform and ethyl acetate. This solubility profile suggests effective solubility in the stomach, thereby indirectly increasing bioavailability.^[29]

Physico-chemical analysis confirmed the quality, purity, and nature of adulterants in the drug sample. Both unpurified and purified samples showed a reduction in loss on drying from 4.56% to 2.73%, indicating that the purified sample has a longer shelf life and is free from microbial contamination. Ash value was found to be 7.3% before purification and 8.3% after purification. High ash content in a purified sample typically indicates the presence of a significant amount of inorganic residue, such as minerals and salts, after the organic material has been burned off. However, AC naturally contains high levels of minerals and inorganic compounds, contributing to a higher ash content even after purification.^[30] The acid-insoluble ash value, which indicates inorganic adulterants, showed 0% silica in both samples. Alcohol-soluble extractive values showed an increase from 10.17% to 12.13% in the purified sample, suggesting a higher concentration of alcohol-soluble constituents like steroids, phenolics, flavonoids, alkaloids,

and glycosides. Water-soluble extractive values also showed an increase from 13.7% to 17.53%, indicating a higher presence of water-soluble secondary metabolites.^[29] These higher alcohol and water-soluble extractive values in the purified sample suggest a greater concentration of bioactive compounds, enhancing its potential therapeutic effects. (fig 4)

Overall, the physicochemical analysis confirms that the purification process has effectively improved the quality and stability of the AC sample while concentrating its bioactive constituents, making it more potent for medicinal use.

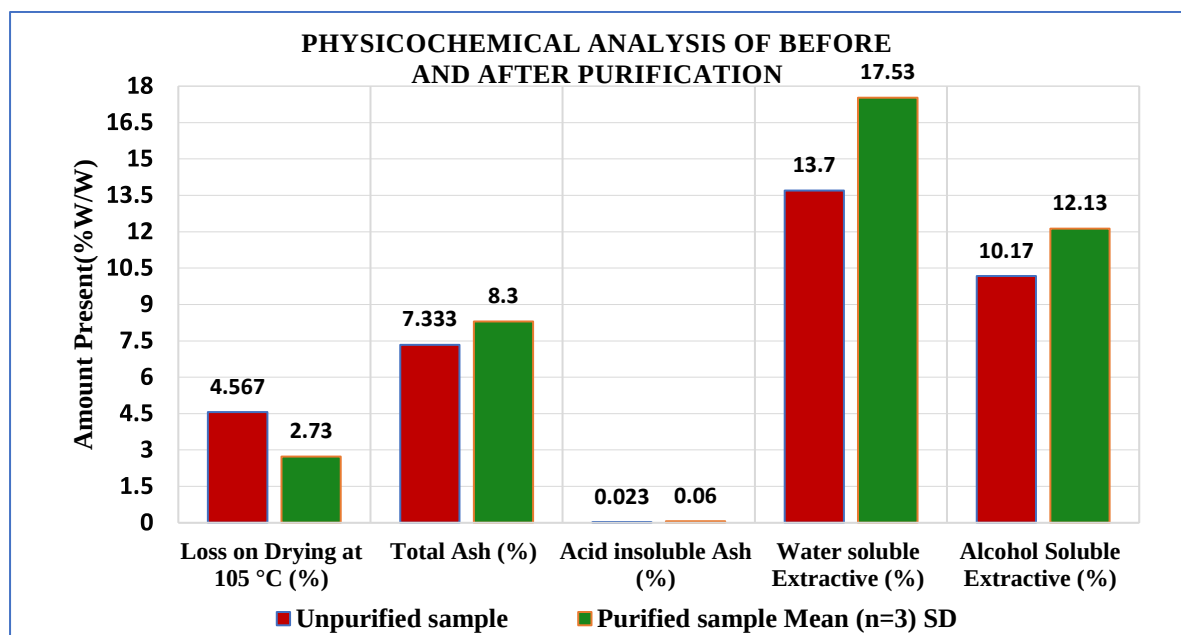


Figure 4: Physicochemical analysis of before and after purification

There were notable variations between the phytochemical assessments of the unpurified and purified samples following partial purification. The unpurified sample changes composition after the purification process. The phytochemical analysis of the three samples revealed the existence of asarone (isomers of α , β asarones) along with an additional 41 constituents in sample A, 57 constituents in sample B, and 44 constituents in sample C. Out of these asarone showed the highest peak.

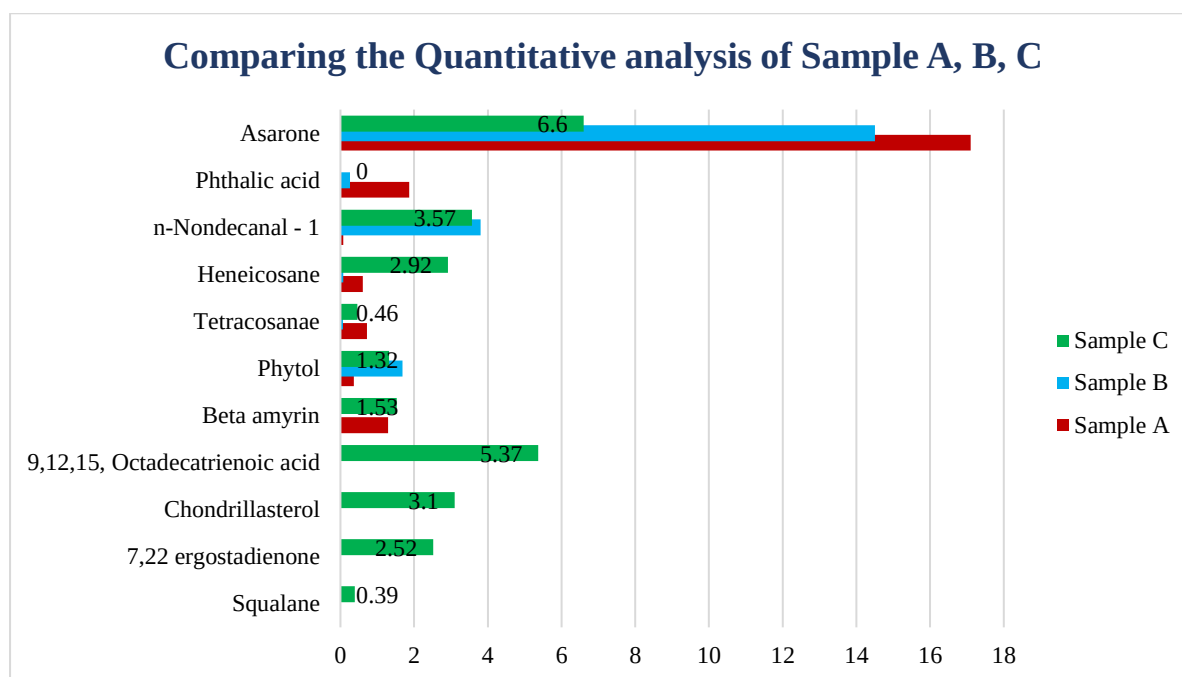


Figure 5: Comparing the Quantitative analysis of Samples A, B, C

The possible role of toxic compound and bioactive compound changes in the three samples corresponding to the area % identified is explained in detail. (fig.5)

I Role of decreasing nature of toxic compounds in purified sample

Asarone

Asarone isomers certainly occurring in *Acorus* and *Asarum*, belong to the class of phenylpropanoid. The percentage of asarone in the raw sample A was significantly lower in the purified sample C and relatively lower in the intermediate sample B. Based on available data, asarones may be hazardous substances, and their elevated levels in herbal remedies may pose certain dangers. Considering different exposure scenarios with herbal food supplements, the margin of exposure is between 60 and 7000. Modeling of the benchmark dose was based on the tumor incidence of leiomyosarcomas of the small intestine.^[17] Asarones have the potential to cause oxidative stress in hepatocytes leading to cytotoxicity and genotoxicity.^[31,32] Asarone has a variety of pharmacological activity at lower dosages (<50 mg/kg), whereas at higher levels (≥ 50 mg/kg), it can cause hypomotility, reduced motor coordination, and other side effects.^[33] A clinical study of seven consumers experienced protracted vomiting after being exposed to high amounts of asarone in the herb *Acorus calamus*.^[34]

Phthalic acid

Phthalic acid is a germ-cell mutagen that shows mutagenicity, developmental toxicity, and reproductive toxicity.^[21] The purified sample did not include phthalic acid.

II Role of increasing nature of bioactive compounds in the purified sample

Beta amyryl, Phytol, Tetracosane, Heneicosane, and n-Nonadecanol-1 which were found to be increasing in nature in the purified sample.

III Role of bioactive compounds detected only in the purified sample

Squalane, 7,22-ergostadienone, Chondrillasterol, and 9,12,15 Octadecatrienoic acid were found only in sample C.

IV Trivial changes were observed in all three samples that have been scientifically proven to have distinct pharmacological actions, as mentioned in Table 4.

The significance of the *Acorus calamus* purification process is thus shown by the results, which indicate that some of the beneficial components were enhanced, and the fraction of harmful substances was decreased following purification.

CONCLUSION:

From the study, the results of before and after purification from both analyses depict that this purification process reduces the toxic compound and formation of new organic compounds, unequivocally underscoring its significance. Siddha's classical texts highlight the importance of purifying the *Acorus calamus* with turmeric to neutralize its harmful effects.

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