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The Therapeutic Potential and efficacy determination of "*Kushmanda Rasayanam*" ayurvedic formulation by chromatographic techniques

Research Article

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Abstract

Background: The present study deals with the GC MS analysis of one Ayurvedic medicine, *Kushmanda Rasayanam*. *K. rasayanam* is a medicine prescribed to treat cough, breathing problems, chest injuries, bleeding disorders and depletive diseases. It is also claimed that its use increases memory power, intellect and is a good heart tonic. Results: The medicine was procured from standard Ayurvedic vendor at Chennai, India and was subjected to GC MS analysis following proper protocol. It was observed that some important biomolecules such as beta.-1, 5-O-Dibenzoyl-ribofuranose, n-Hexadecanoic acid, 11-Octadecenoic acid, methyl ester, n-Propyl cinnamate, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, Ethyl iso-allocholate, Cholesterol, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester do have far reaching medicinal values. Conclusion: The antioxidant, membrane stabilizing, and anti-inflammatory assays have indicated poor potential of *K. rasayanam* as compared to the respective standards.

Key Words: GC-MS, *Kushmanda Rasayanam*, Ayurvedic, Antioxidant, Anti-inflammatory, Beta.-1,5-O-Dibenzoyl-ribofuranose, n-Hexadecanoic acid, Cholesterol.

Introduction

Kushmanda rasayana otherwise called *Kushmanda avaleha* is an ayurvedic supplement very much used in Indian continental which is full of nourishment and provides strength to the body. It is used in case of an aphrodisiac, strengthening the body, nervine tonic, and as hemostatic medicine. K. rasayanam is a medicine prescribed to treat cough, breathing problems, chest injuries, bleeding disorders, hemorrhages, fever, phthisis, dry mouth (xerostomia), bronchitis, asthma, excessive thirst (polydipsia), vomiting, and physical weakness or debility in old age and depletive diseases. It is also used to increases memory power, intellect and is a good heart tonic (1, 2).

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In basic *K. Rasayana* is a mixture of various herbs and used in jam form. The ingredients are: *Benincase hispida*; Honey; Crystalized Rock Sugar; Cow Ghee; Powders of *Piper longum*, *Zingiber officinale*; *Cuminum cyminum*; *Cinnamomum zeylanica*; *Elattaria cardamomum*; *Cinnamomum tamala*; *Coriandum sativum*; *Piper nigrum*. For making *K. Rasayana*, take *B. Hispida* (Petha) fruit slices are boiled until water reduces to half. Separated boiled fruit residue are taken in a cotton cloth, squeezed to remove the water, and then spread on a clean cloth under the sun for a few hours. It is then dried in Ghee till the colour become honey like followed by addition of sugar and other ingredients. It becomes like Jam which is then consumed at a dose of 10 to 20 g once or twice a day as per the prescription of a recognized medical professional. The reference of this medicine is found in Ayurvdic treatise, Ashtanga Hrudaya Chikitsa shtanan 3/114-117 (1, 2, 3, 4).

Some work with regards to the medicinal role of *K. Ghrita* is available. Chandra *et al*, 2011 have clinically evaluated the role of this medicine in the management of depressive illness (1). Similarly, Kale *et al*, 2018 have reported the management of bronchitis by the treatment with Kushmanda ghrita (2). Sengupta *et*

al, 2019 have indicated the pharmaceutical standardization of this medicine.[3] The present study is in continuation of our endeavour to establish the efficacy of Ayurvedic medicines in line of modern scientific perspective (3, 4).

K. Rasayana is made of most of ingredients already known to be safe for consumption and hence likely to be safe to be consumed by lactating mothers. There are no adverse effects reported with the use of *K. Avaleha* in lactating mothers and breastfeeding babies. In ayurveda, *K. Rasayana* is used during pregnancy for nourishing the mother and developing a baby. However, the safety of *K. rasayana* is not fully clear. Consult an ayurvedic physician before using *Kushmanda avaleha* during pregnancy (4, 5).

Considering its easy availability and to explore a new combination of Ayurvedic herbs in modern era of ever-growing Hypertension, an attempt was made in our work to determine the therapeutic potential and efficacy of "*K. rasayanam*" ayurvedic formulation by various techniques. Here aim of study was to explore *K. Rasayanam* ayurvedic formulation based on chemical composition and look for its major chemical factors.

Methods

Gas chromatography/Mass spectrometry Study

Kushmanda Ghritha was obtained from standard Ayurvedic vendor at Chennai and was subjected to GC MS analysis by standard procedure using instrument Gas chromatography (Agilent: GC: (G3440A) 7890A. MS MS: 7000 Triple Quad GCMS,) which was equipped with Mass spectrometry detector.

Sample Preparation

5 mg sample dissolved in 1 ml of suitable solvents. The solution stirred vigorously using vortex stirrer for 10 seconds. The clear extract was determined using gas-chromatography for analysis.

GC-MS protocol

The GC MS Column consisted of DB5 MS (30mm×0.25mm ID ×0.25 µm, composed of 5% phenyl 95% methyl poly siloxane), Electron impact mode at 70 eV; Helium (99.999%) was used as carrier gas at a Constant flow of 1ml/min Injector temperature 280 °C; Auxilary Temperature: 290°C Ion-source temperature 280°C. The oven Temperature was programmed from 50°C (isothermal for 1.0 min), with an increase of 40°C/min, to 170°C (isothermal for 4.0 min), then 10°C/min to 310°C (isothermal for 10min) fragments from 45 to 450 Da. Total GC running time is 32.02 min. The compounds are identified by GC-MS Library (NIST & WILEY) (6, 7).

Therapeutic Potential and efficacy determination

Protein denaturation assay by Membrane stabilization assay

Ferric reducing antioxidant potential (FRAP) assay

The antioxidant capacity of the test samples *K. Rasayanam* to reduce of Fe^{3+} TPTZ (2,4,6-tri (2-pyridyl)-1,3,5-triazine) complex (colourless complex) to Fe^{2+}-tripyridyltriazine (blue colored complex) formed by the action of electron donating antioxidants at low pH was estimated spectrophotometrically. It was estimated spectrophotometrically following the modified procedure of Benzie and Strain et al., 1999 (8). This reaction is monitored by measuring the change in absorbance at 593 nm. The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM acetate buffer, 10 ml TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the proportion of 10:1:1 at 37°C. 3.995 ml of freshly prepared working FRAP reagent was pipetted and mixed with various concentrations (5-320 µg/ml) of with 5µl test samples and standard, Ascorbic acid (5-320 µg/ml) were mixed thoroughly. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml FRAP reagent + 5µl distilled water) after 30 min incubation at 37°C. All the determinations were performed in triplicates.

Anti-inflammatory assay

The anti-inflammatory capacity of the test samples *K. rasayanam* was carried out by protein denaturation assay. The experiment was carried out with minor modification Gnana *et al.* 2011 (9). The standard drug, Diclofenac sodium and sample was dissolved in Dimethyl sulfoxide (DMSO) and diluted with phosphate buffer (0.2 M, PH 7.4). Final concentration of DMSO in all solution was less than 2.5%. Test Solution (4ml) containing different concentrations of drug (5-320 µg/ml) was mixed with 1 ml of 1mM albumin solution in phosphate buffer and incubated at 37°C in incubator for 15 min. Denaturation was induced by keeping the reaction mixture at 60°C in water bath for 15 min. After cooling, the turbidity was measured at 660 nm. The same concentration was taken for standard drug and its turbidity was recorded. Percentage of inhibition of denaturation was calculated from control where no drug was added. The diclofenac sodium was used as standard drug. The percentage inhibition of denaturation was calculated by using following formula.

% Inhibition = [(OD of test - OD of control)/OD of test] x 100

Membrane stabilisation assay

Fresh whole human blood (5 ml) was collected in a heparinized tube and transferred to the centrifuge tubes. It was centrifuged at 3000 rpm for 10 min and washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 40% v/v suspension with isotonic solution (10 mM sodium phosphate buffer). In 0.1 ml of 40% RBCs suspension are mixed with varying concentration form 50 -1600 µg/ml of *K. Rasayanam* and standard Diclofenac sodium, respectively. The control consisted of 0.1 ml of RBC mixed with isotonic solution alone. The reaction mixture was incubated in water bath at 56 ˚C for 30 min. At the end of incubation, the tubes were cooled to room temperature. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was measured at 560 nm. Percent

membrane stabilization activity was calculated by using the following formula.

% Inhibition of Haemolysis = (OD of test - OD of control) /OD of test x 100

Statistical analysis

Results will be expressed as mean ± S.E.M. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by a Dunnett's multiple-comparison test with 95% confidence intervals. P values less than 0.05 was considered significant.

Results

GC MS Study

The GC MS profile of *K. Ghritha* is represented in **Figure 1**. Table1 indicates the retentions values, types of possible compound, their molecular formulae, molecular mass, peak area and their medicinal roles of each compound as shown in the GC MS profile of *K. Ghritha*. The identification of metabolites was accomplished by comparison of retention time and fragmentation pattern with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS along with the possible pharmaceutical roles of each bio molecule as per Dr. Duke's Phytochemical and ethnobotanical data base (National Agriculture Library, USA) and others as shown in **Table 1**.

Antioxidant Assay

The standard drug Ascorbic acid were used as standard for FRAP antioxidant assay. In case of anti-inflammatory assay like Protein denaturation assay and Membrane stabilisation assay, Diclofenac sodium is used as standard. It was carried out to have better comparative study with test sample, *K. rasayanam*. The concentration of sample *K. rasayanam* and standard are 5, 10, 20, 40, 80,160 and 320 µg/ml for all antioxidant assay, where 50, 100, 200, 400, 800 and 1600 µg/ml concentration was taken for anti-inflammatory assay. The average value of the reactions performed in triplicate was obtained and plotted against the different concentrations of *K. rasayanam* and its standards. The IC50 value, that is half maximal inhibitory concentration was calculated from R^2 equation obtained from linear thread line from the respective graph of concentration of *Kushmanda rasayanam* standard against % inhibition and activity values.

The FRAP assay result are indicated in Table 2, the increase in value is noted in increase in concentration in both test and standard. The results indicate that our *K. rasayanam* shows comparatively more FRAP activity than standard ascorbic acid indicating fair amount of antioxidant activity of *K. rasayanam*. The IC_{50} value for Protein denaturation activity of *K. rasayanam* and standard, Diclofenac sodium was found to be **733.88 µg/ml** and **221.23 µg/ml,** respectively as shown in Table 3 indicating poor protein denaturation activity of *K. rasayanam*. The IC_{50} value for Membrane Stabilisation activity obtained from Table 4, where **986.75 µg/ml** and **378.83 µg/ml** are the IC_{50} of *K. rasayanam* and Diclofenac sodium, respectively.

Discussion

Some molecules such as beta.-1,5-O-Dibenzoyl-ribofuranose, n-Hexadecanoic acid, 11-Octadecenoic acid, methyl ester, n-Propyl cinnamate, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, Ethyl iso-allocholate, Cholesterol, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester were identified and they do have far reaching medicinal value which corroborate well with the medicinal role of *K. Ghrita*. *K. rasayanam* and Diclofenac sodium, respectively thus indicating not appreciable response of *K. rasayanam*. Thus, from the above studies *K. rasayanam* has low antioxidant, low protein stabilizing and low membrane stabilising roles (10, 11, 12, 13).

Overall effect of Ayurvedic formulation can be summarized as Tridosha Shamaka (mainly Vata), Manasa Doshahara, Hridya, Medhya and Mutrala. Due to wider range of action, the Ayurvedic formulation thus prepared has shown better results in relieving the symptoms of Hypertension (14, 15). In lowering the Blood Pressure, satisfactory result was obtained from the preparation. Moreover, no side effects were observed in patients during and after the treatment so, it can be concluded that the patients of Hypertension can be managed effectively by Ayurveda without fear of side effects.

Conclusion

The result of antioxidant, membrane stabilizing, and anti- inflammatory assays have indicated that *Kushmanda rasayanam* did not show appreciable activity when compare to the respective standards this indicate that the medicinal role of *Kushmanda rasayanam* has to be further probed to understand its medical role as claimed by Ayurveda for treating the desease asignd to it.

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Figure Legends

Figure 1. Represents the GC MS graph of Kushmanda Ghritha.

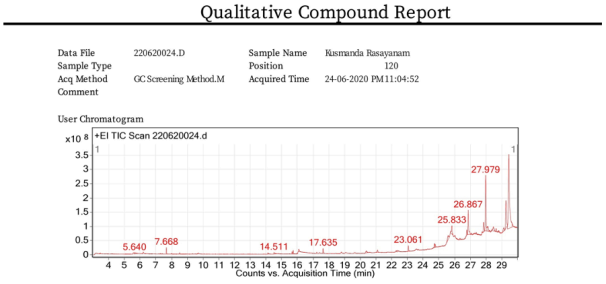

Figure 2: Comparative graphical representation of FRAP assay of Kushmanda Rasayanam and Ascorbic acid

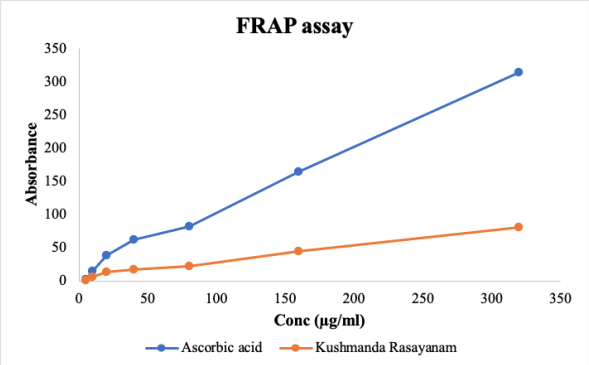

Figure 3: Comparative graphical representation of Protein denaturation activity of Kushmanda Rasayanam and Diclofenac sodium

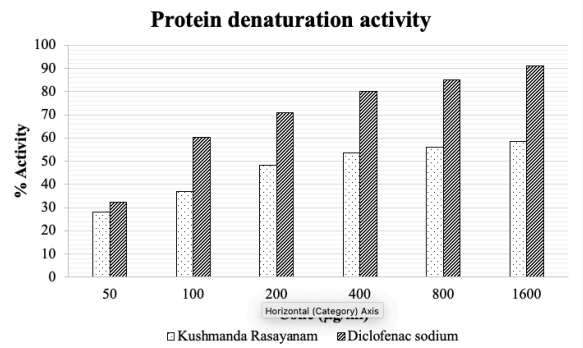

Figure 4: Comparative graphical representation of Membrane Stabilisation activity of Kushmanda Rasayanam and Diclofenac sodium

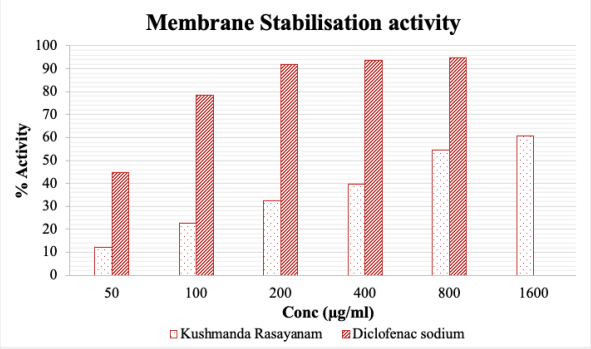

Table 1: Indicates the retentions values, types of possible compound, their molecular formulae, molecular mass, peak area and their medicinal roles of each compound as shown in the GC MS profile of Kushmanda Rasayanam

Retention Time	Molecule	Mol. Formula	Mol. Wt.	% Peak Value	Possible medicinal role
5.64	Beta.-1,5-O-Dibenzoyl-ribofuranose	C19H18O7	358.1	4.79	17 beta dehydrogenase inhibitor, androgen blocker, anti-amyloid beta, anticancer, Anti TGF beta, Beta 2-receptor, beta blocker, beta-galactosidase inhibitor, beta-glucuronidase inhibitor, anticancer, Catechol-O-methyl tranferase inhibitorm Decreses glutamate oxaloacetate transaminase, decreases oxalate excretion
7.67	2-Methylbicyclo [4.3.0] non-1(6)-ene	C10H16	136.1	4.77	Not Known
14.51	n-Hexadecanoic acid	C16H32O2	256.2	3.29	Acidifier, Arachidonic acid inhibitor, Increase Aromatic Amino acid Decarboxylase activity, Inhibit Production of Tumor necrosis factor, Antidote, antitumor, Arylamine-N-Acetyltransferase Inhibitor, Decrease Norepinephrine Production, Gaba-ergic, Increase NK cell Activity, Myoneuro stimulant, NADH oxidase inhibitor
15.67	9,12-Octadecadienoic acid (Z,Z)-	C18H32O2	280.2	5.00	Not Known
15.73	11-Octadecenoic acid, methyl ester	C19H36O2	296.3	3.43	Catechol-O-methyl-Transferase Inhibitor, methyl Donar, Methyl Guanidine Inhibitor, Acidifier, Arachidonic acid inhibitor, Increase Aromatic Amino acid Decarboxylase activity
17.64	n-Propyl cinnamate	C12H14O2	190.1	4.99	Anaphylactic, Arylamine-N-acetyltransferase inhibitor, decreases epinephrine production, down regulates nuclear and cytolsol androgen reuptake, GABA-nergic, Increases Natural Liller cell activity, inhibits production of Tumor necrosis factors, Myo-neuro stimulant, NADH oxidase inhibitor, NADH biquinone-oxidoreductase inhibitor
20.39	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C19H34O2	294.3	4.97	Anticancer, Cytochrome-P450-2E1 inhibitor, decreases deoxypyridinoline excretion, decreases endothelial leukocyte adhesion, decrease endothelial platelet adhesion, decrease epinephrine production, decreases oxalate excretion, EDRF promoter
21.10	Estra-1,3,5(10)-trien-17.beta.-ol	C18H24O	256.2	3.84	Not known
22.37	Ethyl iso-allocholate	C26H44O5	436.3	2.35	Anti-coagluant, antidyspeptic, anti-inflammatory, mucolyte, proteolytic
23.06	Cholesterol	C27H46O	386.4	6.54	Precursor of steroid metabolism
24.77	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	C35H68O5	568.5	3.10	Acidifier, acidulant, Arachidonic acid inhibitor, Increase aromatic amino acid decarboxylase activity, inhibits production of Uric acid
26.87	Eicosanoic acid, 2-[(1-oxohexadecyl)oxy]-1-[[(1-oxohexadecyl)oxy]methyl]ethyl ester	C55H106O6	862.8	35.79	Not known

28.48	5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one, 3,9,9a-tris(acetyloxy)-3-[(acetyloxy)methyl]-2-chloro-1,1a,1b,2,3,4,4a,7a,7b,8,9,9a-dodecahydro-4a,7b-dihydroxy- 1,1,6,8-tetramethyl-, [1aR-(1a.alpha.,1b.beta.,2.alpha.,3.beta.,4a.beta.,7a.alpha.,7b.alpha., 8.alpha.,9.beta.,9a.alpha.)]-	C28H37ClO11	584.2	11.22	Not known
28.65	3,19:14,15-Diepoxypregnan-20-one, 3,11,18-triacetoxy-	C27H36O9	504.2	3.61	Not known
28.81	1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-, 5,9,9a-triacetate, [1aR-(1a.alpha.,1b.beta.,4a.beta.,5.beta.,7a.alpha.,7b.alpha.,8.alpha., 9.beta.,9a.alpha.)]	C26H36O8	476.2	2.31	Not known

Table 2: FRAP assay of Kushmanda Rasayanam and Ascorbic acid

SL.No	Conc (µg/ml)	FRAP assay	
		Kushmanda Rasayanam	Ascorbic acid
1	5	0.79738587	1.67391
2	10	5.4885745	14.2101
3	20	13.3754087	38.6304
4	40	17.5220559	62.1087
5	80	22.5075487	81.8913
6	160	44.4740984	164.645
7	320	81.3012156	314.138

Table 3: Percentage Protein denaturation activity of Kushmanda Rasayanam and Diclofenac sodium

SL.No	Conc (µg/ml)	% Protein denaturation activity	
		Kushmanda Rasayanam	Diclofenac sodium
1	50	28.08191083	32.25501284
2	100	36.9404756	60.36903754
3	200	48.17004226	71.00695774
4	400	53.45052475	80.30738871
5	800	56.00832678	85.12479699
6	1600	58.36341432	91.06446792
IC50 value(µg/ml)		**733.88**	**221.23**

Table 4: Percentage Membrane Stabilisation activity of Kushmanda Rasayanam and Diclofenac sodium

SL.No	Conc (µg/ml)	% Membrane Stabilisation activity	
		Kushmanda Rasayanam	Diclofenac sodium
1	50	12.3464499	44.7745152
2	100	22.7924054	78.57957387
3	200	32.3341767	91.77985111
4	400	39.6226382	93.85498837
5	800	54.6676673	94.69464567
6	1600	60.6846415	-
IC50 value(µg/ml)		**986.75**	**378.83**

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