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Research Paper

GC-MS analysis of phytocomponents and *in-vitro* inhibitory effects of *Calanthe triplicata*

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ABSTRACT

The present investigation is focused to find out phytocomponents which are effective on anti-inflammatory and anti-arthritic activities of various extracts of entire plant (shrub) of *Calanthe triplicata* (Williemet) Ames. Gas Chromatography-Mass Spectrometry (GC-MS) was used for the analysis of phytocomponents in ethyl acetate and methanol extracts. *In-vitro* activities of anti-inflammatory and anti-arthritic effects were carried out in petroleum ether, chloroform, ethyl acetate and methanol extracts by HRBC (human red blood cell) membrane stabilization and inhibition of protein denaturation method respectively. The concentration level of 100, 200, 400, 600, 800, 1000, 2000µg/ml were used to screen *in- vitro* activities with the standard drug of diclofenac sodium (400 and 600µg/ml). GC-MS analysis of ethyl acetate extract revealed that the presence of a flavanoid compound 4H-Pyran-4one, 2, 3-dihydro-3,5 dihydroxy-6-methyl which has already been reported as an anti-inflammatory effect. The results of *in- vitro* activities showed that percentage inhibition of ethyl acetate extract had significant effects.

Keywords: Phytocomponents; In vitro; GC-MS.

INTRODUCTION

GC-MS is a technique used for screening/identification/quantification of many susceptible compounds in plant extracts. Gas chromatography (GC) is used to separate drugs that might be present in the sample. The retention time (RT) is an identifying characteristic of a drug. The detector for the GC is the mass spectrometry (MS). The fragmentation pattern for a drug is unique and therefore is an identifying characteristic of a drug. The identification of a drug by its retention time (GC) and fragmentation pattern (MS), along with sample specific information afforded to make GC-MS the foremost confirmation method for analyzing herbal extract. A number of drugs from plant sources are known to cause anti-inflammatory effects and used in conditions like rheumatoid arthritis, gouts etc. *In-vitro* models will minimize the animal usage and also provide a much safer mechanism for investigating new drugs and therapies. Various *in-vitro* models have been processed as being able to detect anti-inflammatory and anti-arthritic effects that have been found effective in

modifying the process in the development of numerous methods of assay for detecting anti- inflammatory substances.

Calanthe triplicata is a low growing evergreen terrestrial orchid, snow white flowers with yellow or red callus found in the hilly parts of south India like Coimbatore, Dindigul, Namakkal, Nilgiri and Salem districts. It belongs to the family of orchidaceae. The flowers and leaves contain a glycoside, indican which on hydrolysis yields indigo-blue. The herbs are reported to be used in diseases of stomach and intestine; and the root is chewed along with betel nuts or other aromatic substances, in diarrhoea. A poultice of the flowers is used to reduce pain due to ulcers. A paste of bulbs of other species of *Calanthe* is applied to painful joints (The wealth of India, 1992). As there is no scientific literature on anti-inflammatory and anti-arthritic activities of *Calanthe triplicata*, this plant has been selected for the present study. The folklore claim revealed that the selected plant is explored for anti-inflammatory and anti-arthritic effects. Hence in this study, it is proposed to establish the anti-inflammatory and anti-arthritic activities by *in-vitro* models and also to find out the bio active constituents in the selected plant using GC-MS analysis.

MATERIALS AND METHODS

Plant material: The selected plant was collected from Kolli hills, Namakkal district, Tamil Nadu in the month of November and authenticated by Dr. Sasikala Ethirajulu, Assistant Director (Pharmacognosy), Siddha Central Research Institute Arignar Anna Govt. Hospital Campus Arumbakkam, Chennai- 600 106 Tamil Nadu, India.

Chemicals: HRBC suspension, Alsever's solution, Dextrose, Sodium citrate, Citric acid, Sodium Chloride isosaline pH 7.2, Phosphate buffer, Hyposaline, Dimethyl sulphoxide (DMSO) bovine serum albumin, diclofenac sodium, hydrochloric acid.

Instrument: GC-MS-5975C [AGILENT]

Gas Chromatography Condition: Column Oven temperature 70°C, Injector temperature 250°C, Helium 99.9995% purity-carrier gas, Column Flow 1.51ml/min and Injection volume 1microlitre were used.

Column (DB-5ms Agilent): Length of the column 30.0m, Diameter of the column 0.25mm and film thickness 0.25um were used.

MS Condition: Ion source temperature 230°C , Interface temperature 240°C , Scan range 40-700m/z, Solvent cut time 5mins, MS start time 5(min), MS end time 35 (min), Ionization EI (-70ev), Scan speed : 2000.

Preparation of Extracts: Freshly collected plants were dried in shade and then powdered. The dried powder was extracted successively with petroleum ether, chloroform, ethyl acetate and methanol by cold maceration method. The solvent was removed by distillation. Then the extracts were evaporated and concentrated.

In-Vitro Anti-Inflammatory

HRBC membrane stabilization method

Preparation of HRBC suspension: The approval was granted by Institutional Ethics Committee (IEC) to collect blood for the experiment. IEC reference number: IEC-NI/13/FEB/32/12. About 4 ml of venous blood was collected from healthy volunteers and mixed with equal volumes of Alsever's solution and centrifuged at 3000rpm and the packed cells were washed with isosaline and a 10% v/v suspension was made with isosaline.

Preparation of Assay mixture: The assay mixture contains 1 ml of phosphate buffer (pH 7.4, 0.15M), 2ml hyposaline (0.36%), 0.5 ml HRBC suspension (10% v/v) and

0.5ml of various extracts of different concentration (100, 200, 400, 600, 800, 1000, 2000µg/ml) were prepared in DMSO.

Standard: The middle concentration (400 and 600µg/ml) was selected from the assay mixture concentration (100, 200, 400, 600, 800, 1000, 2000µg/ml) for the preparation of a standard drug diclofenac sodium.

Procedure:

- Test solution (4.5ml): It consists of 2ml of hypotonic saline (0.25% w/v), 1 ml of phosphate buffer (pH 7.4), and 1 ml of test extract (100, 200, 400, 600, 800, 1000, 2000µg/ml) in normal saline and 0.5ml of 10% w/v human red blood cells in isotonic saline.
- Product control (4.5ml): It consists of 2ml of hypotonic saline (0.25%, w/v), 1ml of phosphate buffer (pH 7.4), and 1ml of test extract (200 and 400µg/ml) in normal saline and 0.5ml of isotonic saline.
- Test control (4.5ml): It consists of 2ml of hypotonic saline (0.25% w/v), 1ml of phosphate buffer (pH 7.4), 1ml of isotonic saline and 0.5ml of 10% w/v human red blood cells in isotonic saline.
- Standard solution (4.5ml): It consists of 2ml of hypotonic saline (0.25% w/v), 1ml of phosphate buffer (pH 7.4), and 1ml of diclofenac sodium (400 and 600µg/ml) in normal saline and 0.5ml of 10% w/v human red blood cells in isotonic saline.

  The above four solution were incubated at 56°C for 30 min. The tubes were then cooled under running tap water for 30 min. After that they were centrifuged and supernant liquid was separated. Absorbance was measured at 560 nm by UV spectrophotometer. The percentage membrane stabilization was calculated as follows:

$$\text{Percentage stabilization} = \frac{100 - (\text{OD of test} - \text{OD of control})}{\text{OD of test control}} \times 100$$

In- Vitro Anti-arthritic Activity

Inhibition of Protein Denaturation Method: To determine the anti arthritic activity by inhibition of protein denaturation method, the following four solutions are used. Test solution consists of 0.45ml of bovine serum albumin (5 % w/v aqueous solution), test control solution consists of 0.05ml of distilled water, product control solution consists of 0.45ml of distilled water and 0.05ml of test solution (100, 200, 400, 600, 800, 1000, 2000µg/ml) and standard solution consists of 0.45ml of bovine serum albumin (5% w/v aqueous solution) and 0.05ml diclofenac sodium solution (400 & 600µg/ml). The pH of all the solutions was adjusted to 6.3 by using a small amount of 1N hydrochloric acid. All the samples were incubated at 37°C for 20 minutes and heated at 57°C for 3 minutes. After cooling, 2.5ml of phosphate buffer (pH 6.3) was added to each tube. Turbidity was measured using spectrophotometrically at 660 nm. The percentage inhibition of protein denaturation is calculated as follows:

$$\text{Percentage inhibition} = \frac{100 - (\text{OD of test} - \text{OD of control})}{\text{OD of test control}} \times 100$$

RESULTS AND DISCUSSION

GC-MS analysis was carried out on ethyl acetate and methanolic extracts of *Calanthe triplicata.* Ethyl acetate extract showed that the presence different constituents. Among those, 4H-Pyran-4one, 2, 3-dihydro-3,5 dihydroxy-6-methyl was found to be a bio active compound.

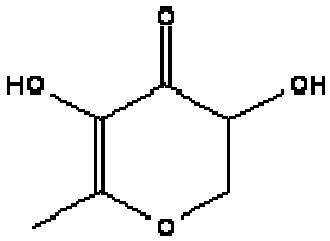

Figure: Structure of 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl

The literature of this compound showed that it is a flavonoid compound and also has an anti-inflammatory effect. Its retention time and molecular weight was found to be 7.975 and 144 respectively. Retention time, peak area and molecular formula were made known in the Table 1, 2.

Anti-inflammatory and anti-arthritic activities were carried out on petroleum ether, chloroform, ethyl acetate and methanol extracts of *Calanthe triplicata* by the *in-vitro* model of HRBC membrane stabilization and inhibition of protein denaturation method respectively. The results were compared to the reference standard diclofenac sodium (400 and 600µg/ml). The results confirmed that the ethyl acetate extract had good effects as compared to petroleum ether, chloroform and methanol extracts of *Calanthe triplicata.* The percentage inhibition of ethyl acetate extract was found to be 62.34±0.5 (400µg/ml), 64.19±0.4 (600µg/ml) for anti-inflammatory effect and 69.20±0.7 (400µg/ml), 70.35±0.5 (600µg/ml) for anti-arthritic effect. The results were tabulated in Table 3, 4.

Statistical analysis: Values are reported as three evaluations of mean±standard deviation and statistical analyses of data were assessed using Student's t-test and obtained significance level of $P<0.05$.

CONCLUSIONS

In the present work, GC-MS analysis of ethyl acetate extract showed the presence of a flavonoid compound 4H-Pyran-4one, 2, 3-dihydro-3,5 dihydroxy-6-methyl. This compound had good retention time and peak area and also anti-inflammatory effect. With the evidence of GC-MS analysis, *in- vitro* activities of petroleum ether, chloroform, ethyl acetate and methanolic extracts of *Calanthe triplicata* were investigated. Ethyl acetate extract showed high percentage of stabilization of anti-inflammatory effects and high percentage inhibition of anti-arthritic effects as compared to petroleum ether, chloroform extract. So it can be concluded that the selected plant *Calanthe triplicata* possess anti-inflammatory and anti-arthritic activities.

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Table -1: GC-MS spectra data of ethyl acetate extract.

Compound	Retention time	Peak area	Molecular Formula	Molecular Weight
D-Alanine, N-propargyloxycarbonyl- dodecyl ester	6.732	3.89	$C_{19}H_{33}NO_4$	339.47
1, 2, 3-Propanetriol, 1-acetate	7.066	3.30	$C_5H_{10}O_4$	134.13
2, 5-dihydro-1H-pyrrole	7.357	7.78	C_4H_7N	69.11
Glycerin	7.778	10.40	$C_3H_8O_3$	43.10
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	5.149 7.981 9.956	1.47 9.38 1.75	$C_6H_8O_4$	144.13
4-Methyl-cyclohex-2-en-1-ol	9.216	10.08	$C_7H_{12}O$	112.17
DL-Proline, 5-oxo-, methyl ester	11.423	4.59	$C_6H_9NO_3$	143.14
5-Methoxy-4-methyl-2,1,3-benzothiadiazole	13.848	5.82	$C_8H_8N_2OS$	180.23
2, 4(1H, 3H) - Quinazolinedione	17.566	4.25	$C_8H_6N_2O_2$	162.15

Table -2: GC-MS spectra data of methanolic extract.

Compound	Retention time	Peak area	Molecular Formula	Molecular Weight
Triacetin	9.492	10.00	$C_9H_{14}O_6$	*218.21*
Phytol, acetate	16.927	36.65	$C_{22}H_{42}O_2$	338.57
6-Octen-1-ol, 3,7-dimethyl-,propanoate	17.378	12.33	$C_{13}H_{24}O_2$	212.33

Table - 3: HRBC membrane stabilization method.

Concentration (µg/ml)	Percentage Inhibition				
	Pet ether	Chloroform	Ethyl acetate	Methanol	Standard
100	33.20 ± 1.0	32.20 ± 0.2	60.71 ± 0.2	39.68 ± 0.4	-
200	37.23 ± 0.3	35.78 ± 0.5	62.29 ± 0.4	41.25 ± 0.3	-
400	38.03 ± 0.5	36.29 ± 0.6	62.34 ± 0.5	43.20 ± 0.7	71.87 ± 0.6
600	38.24 ± 0.4	40.87 ± 0.6	64.19 ± 0.4	43.61 ± 0.4	75.15 ± 0.7
800	42.12 ± 0.5	42.47 ± 0.4	65.11 ± 0.3	45.93 ± 0.5	-
1000	44.34 ± 0.6	45.24 ± 0.4	66.91 ± 0.3	46.34 ± 0.5	-
2000	45.46 ± 0.4	47.48 ± 0.4	68.43 ± 0.4	48.88 ± 0.7	-

- Values are reported as three evaluations of mean ± Standard deviation

Table - 4: Inhibition of protein denaturation method.

Concentration (µg/ml)	Percentage Inhibition				
	Pet ether	Chloroform	Ethyl acetate	Methanol	Standard
100	37.91 ± 0.8	32.27 ± 0.5	65.05 ± 0.1	45.20 ± 0.8	-
200	40.38 ± 0.7	34.46 ± 0.5	67.25 ± 0.4	47.51 ± 0.6	-
400	42.94 ± 0.7	34.56 ± 0.7	69.20 ± 0.7	47.82 ± 0.6	75.17± 03
600	44.51 ± 0.5	35.45 ± 0.6	70.35 ± 0.5	48.09 ± 0.2	78.16 ± 0.3
800	47.3 ± 0.6	40.62 ± 0.7	72.52 ± 0.5	50.72 ± 0.4	-
1000	47.14 ± 0.1	45.22 ± 0.6	72.61 ± 0.5	51.18 ± 0.3	-
2000	48.91 ± 0.5	46.30 ± 0.5	73.08 ± 0.4	51.91 ± 0.6	-

- Values are reported as three evaluations of mean ± Standard deviation

