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REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ESTIMATION OF BIOACTIVE COMPOUNDS FROM DIFFERENT FRACTIONS OF NEW POLYHERBAL FORMULATION OF *CLINACANTHUS NUTANS* AND *ELEPHANTOPUS SCABER*

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Keywords:

Clinacanthus nutans, *Elephantopus scaber*, Polyherbal formulation, Herb-Herb combination, Flavonoids, Apigenin, luteolin, b-sitosterol, vanillin, gallic acid, tetra methoxy flavone, quercetin, rutin, benzoic acid, gallic acid, HPLC

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ABSTRACT: Background: Polyherbal formulation is the trend of modern pharmacognosy and holistic approach in the treatment of uncurable diseases such as cancer. Medicinal plants contain many bioactive compounds that give different pharmacological activities. Therefore, it is important to investigate the quality of new polyherbal formulation by identification of bioactive markers. **Objective:** To identify apigenin (1) , luteolin (2), b-sitosterol (9), 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone (8), quercetin (3), vanillin(7), gallic acid (5), benzoic acid (6) and rutin (4) as biomarker from different fractions of the new polyherbal formulation of *Clinacanthus nutans* and *Elephantopus scaber* by RP-HPLC gradient method. **Methods:** A Shimadzu HPLC was utilized to perform the analysis which was equipped with an autosampler, column oven, and UV/VIS detector. An HPLC column used was Merck Licrochart Purospher Start RP 18 column (250mm, 4.6 mm i.d, 5µm pore size). The temperature was maintained at 40.0 ºC throughout the study. The mobile phase isocratic method with acetonitrile 100%. The flow rate was 0.5 mL min^{-1}. Absorbance was observed at λ = 360 nm. **Results:** After identification of bioactive compounds it was established that 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone, gallic acid, benzoic acid and vanillin was found in a polyherbal formulation that was not present before in *Clinacanthus nutans* and *Elephantopus scaber*. Other bioactive compounds such as Apigenin. Luteolin, b-sitosterol, quercetin, and rutin were present in both medicinal herbs as well as inside polyherbal fractions. Vanillic acid was the oxidative product of vanillin that was found in *Elephantopus scaber*. In the polyherbal formulation, vanillin was found that shows the transformation of vanillic acid into vanillin. **Conclusion:** Formation of new bioactive compounds from a polyherbal formulation that was not present before in both medicinal plant. Identification of biomarkers helps to increase the quality of the new product for future.

INTRODUCTION: The use of medicinal plants in human health has been documented since ancient times and they provide a useful source of new therapeutics [1]. Knowledge of Traditional medicinal plant leads to the discovery of new medicines [2].

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These plants can now be found in herbal products and as part of the traditional Malaysian health care system because of their therapeutic efficacy [3]. Natural phenolic compounds play an important role in cancer prevention and treatment. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others [4].

Elephantopus scaber and *Clinacanthus nutans* is a well-known medicinal plant in Malaysia and enriched with numerous bioactive compounds [5], [6].

The objective of our study is the identification of biomarkers from the polyherbal formulation to improve the quality of the new product and compare the bioactive compounds of individual herb (i.e. *Clinacanthus nutans* and *Elephantopus scaber*) from the literature to find similarities and differences from polyherbal fractions.

METHOD AND MATERIAL:

Plant material: The leaves of *Clinacanthus nutans and Elephantopus scaber* were collected from Institute Of Sustainable Agrotechnology, Sg. Chuchuh, Universiti Malaysia Perlis (UniMAP) and washed using clean water. After that, the leaves were dried in a dryer at the temperature of 35-40ºC for two days. Once dried, the leaves were ground into a fine powder by using a mechanical grinder.

Preparation of Plant Extract: Soxhlet extraction was used in this experiment to extract the herbs. For Soxhlet extraction, a powder sample is weighted approximately. A powdered mixture containing equal proportions of two herbs (5 g each) was extracted with100 ml of aqueous ethanol 50% for 12-hour extraction. The extract solution was then evaporated by using a rotary evaporator to remove the solvent in the extract solution and dried in an oven at 35-40 ºC for 12 hours.

The extract was fractionated using different solvents viz. ethyl acetate, n-butanol, and water. The supernatant was filtered using Whatman No. 1 sheet, pooled and concentrated using vacuum rotary evaporator. The concentrated solutions were then dried in an oven at 35 ºC to get the dry form of respective fractions.

Chemicals: Methanol (Fischer scientific, USA), ethanol, Benzoic acid (HmbG, Germany). Rutin, b-sitosterol, vanillin, gallic acid (Sigma-Aldrich, USA). Apigenin, luteolin, quercetin, gallic acid, Merck (Darmstadt, Germany) whereas 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone (Indofine Chemical Company, New Jersey, USA).

The sample was filtered through a 0.45µm nylon membrane filter into an HPLC vial prior to HPLC analysis. Solvent mixtures were filtered through a 0.45µm nylon membrane filter and degassed before use.

Instrumentation: A Shimadzu HPLC was utilized to perform the analysis which was equipped with an autosampler, column oven, and UV/VIS detector.

An HPLC column used was Merck Licrochart Purospher Start RP 18 column (250mm, 4.6 mm i.d, 5µm pore size). The temperature was maintained at 40°C throughout the study.

Chromatography conditions: The mobile phase was an isocratic system of Acetonitrile 100% (v/v), The flow rate was 0.5 mL min^{-1}. Absorbance was monitored at $\lambda = 360$ nm.

Preparation of stock solution: A standard stock solution was prepared by dissolving 5 mg of standard in ethanol, yielding 12.25 mL of a concentration stock = 0.41 mg mL^{-1}. Dilution of 5.0 mL was prepared by aliquoting 5.0 mL of the standard stock solution and diluted with the ethanol to yield 10 mL of standard solutions containing 200µg mL^{-1} of the standard.

RESULT AND DISCUSSION:

Method development: The main objective of the chromatographic method was to identify different bioactive compound inside a new polyherbal formulation such as Apigenin, luteolin, b-sitosterol, vanillin, gallic acid, tetra methoxy flavone, quercetin, rutin, benzoic acid, gallic acid.

This new polyherbal formulation contains an equal amount of *Clinacanthus nutans* and *Elephantopus scaber* in the treatment of the wound.

After identification of compounds from polyherbal formulation it was found that apigenin, luteolin, b-sitosterol, quercetin, rutin was found in *Elephantopus scaber* [7, 8] whereas *Clinacanthus nutans* contains b-sitosterol [9], apigenin [10]. 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone, gallic acid, benzoic acid and vanillin was not found in both medicinal plants.

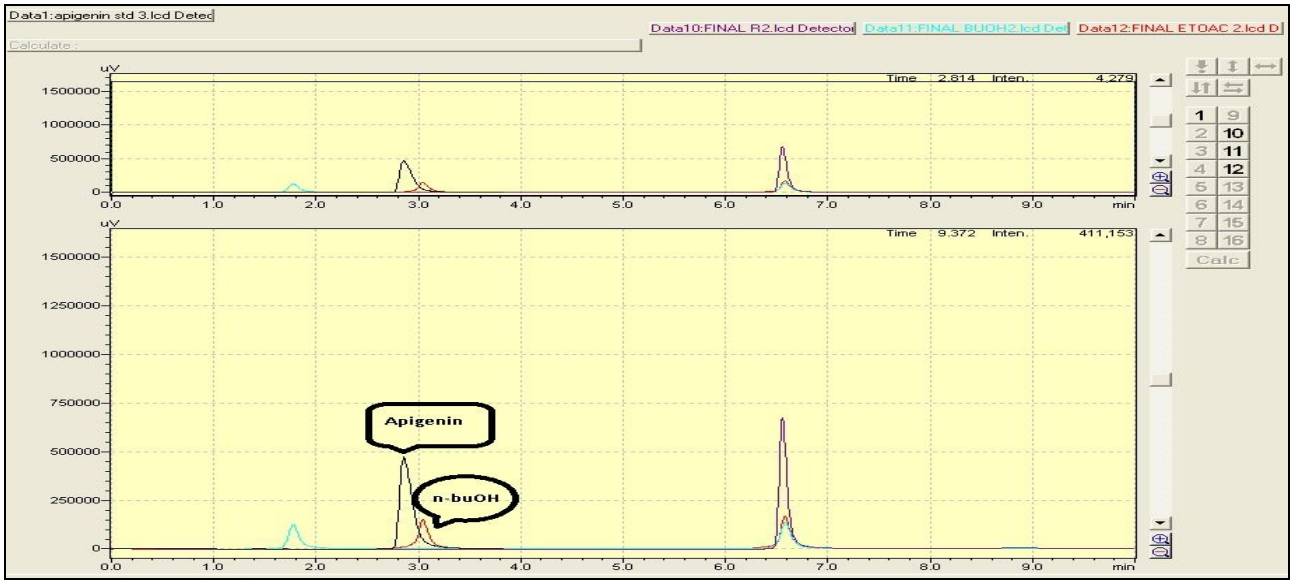

FIG. 1: CHROMATOGRAM FOR STANDARD APIGENIN VS DIFFERENT FRACTIONS

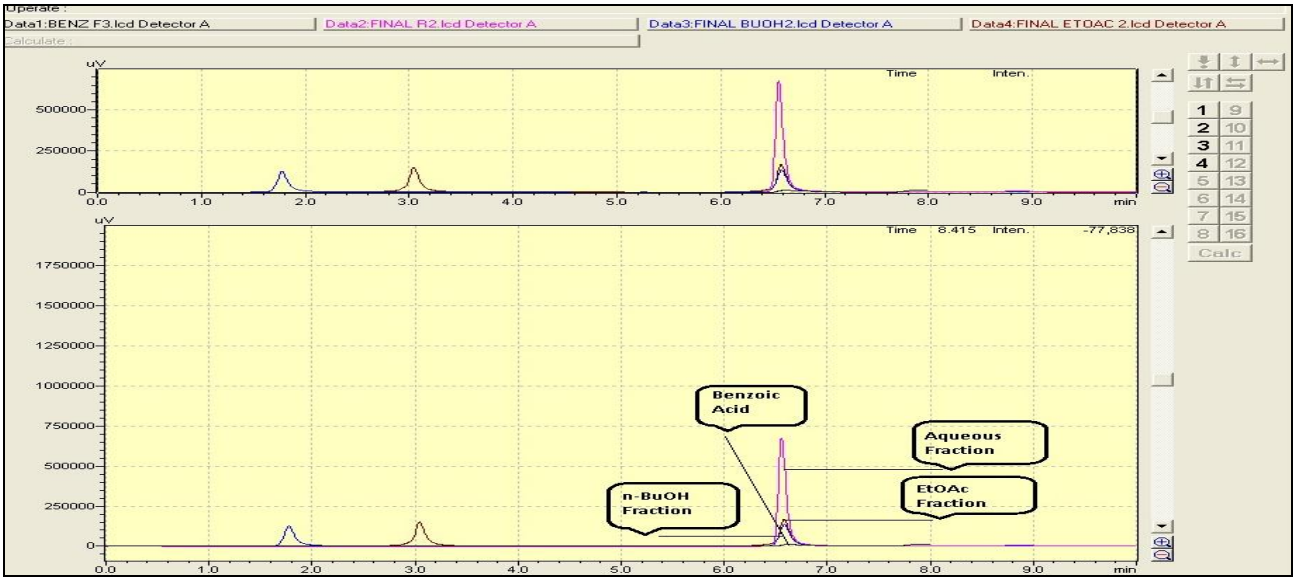

FIG. 2: CHROMATOGRAM FOR STANDARD BENZOIC ACID VS DIFFERENT FRACTIONS

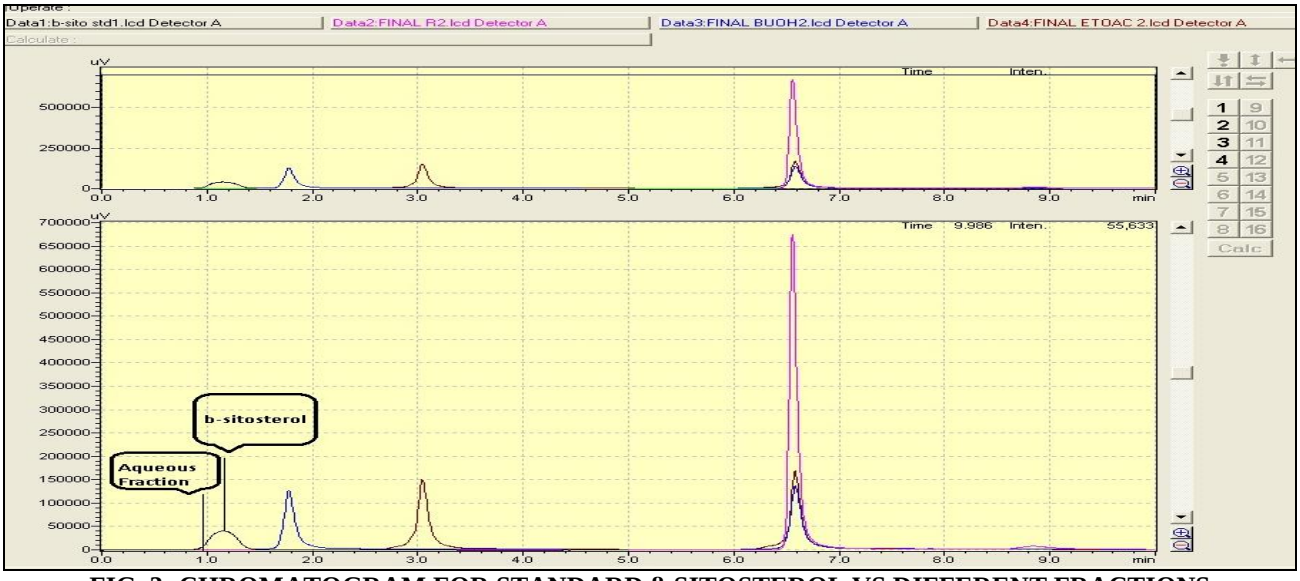

FIG. 3: CHROMATOGRAM FOR STANDARD β-SITOSTEROL VS DIFFERENT FRACTIONS

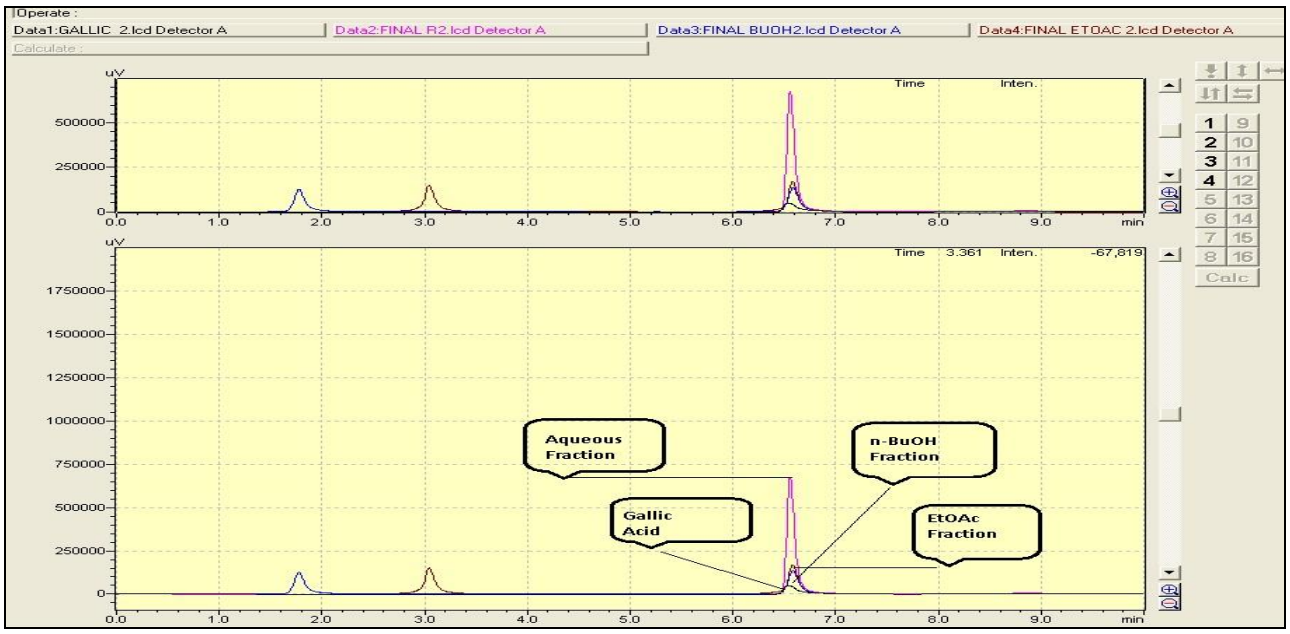

FIG. 4: CHROMATOGRAM FOR STANDARD GALLIC ACID VS DIFFERENT FRACTIONS

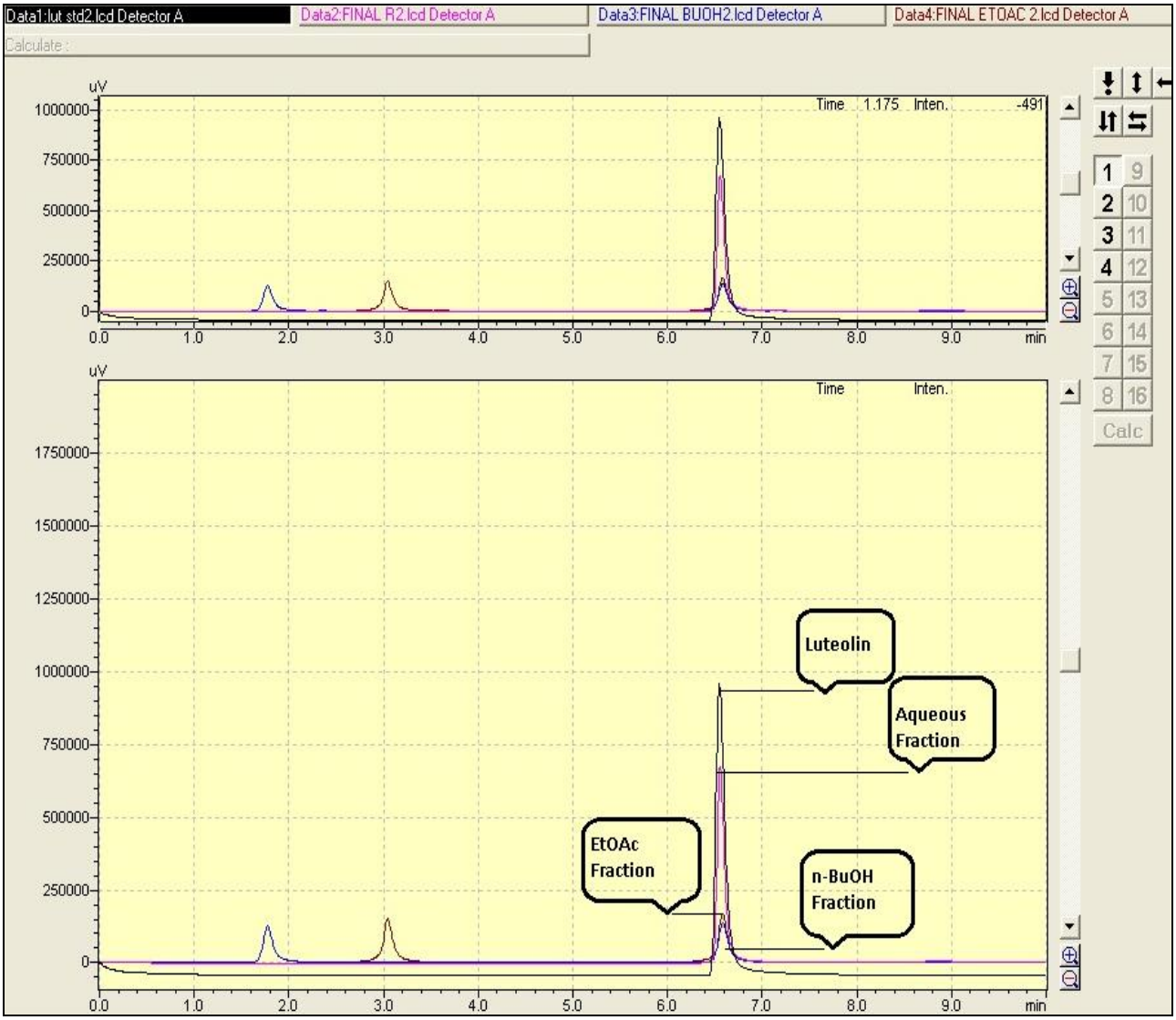

FIG.5: CHROMATOGRAM FOR STANDARD LUTEOLIN VS DIFFERENT FRACTIONS

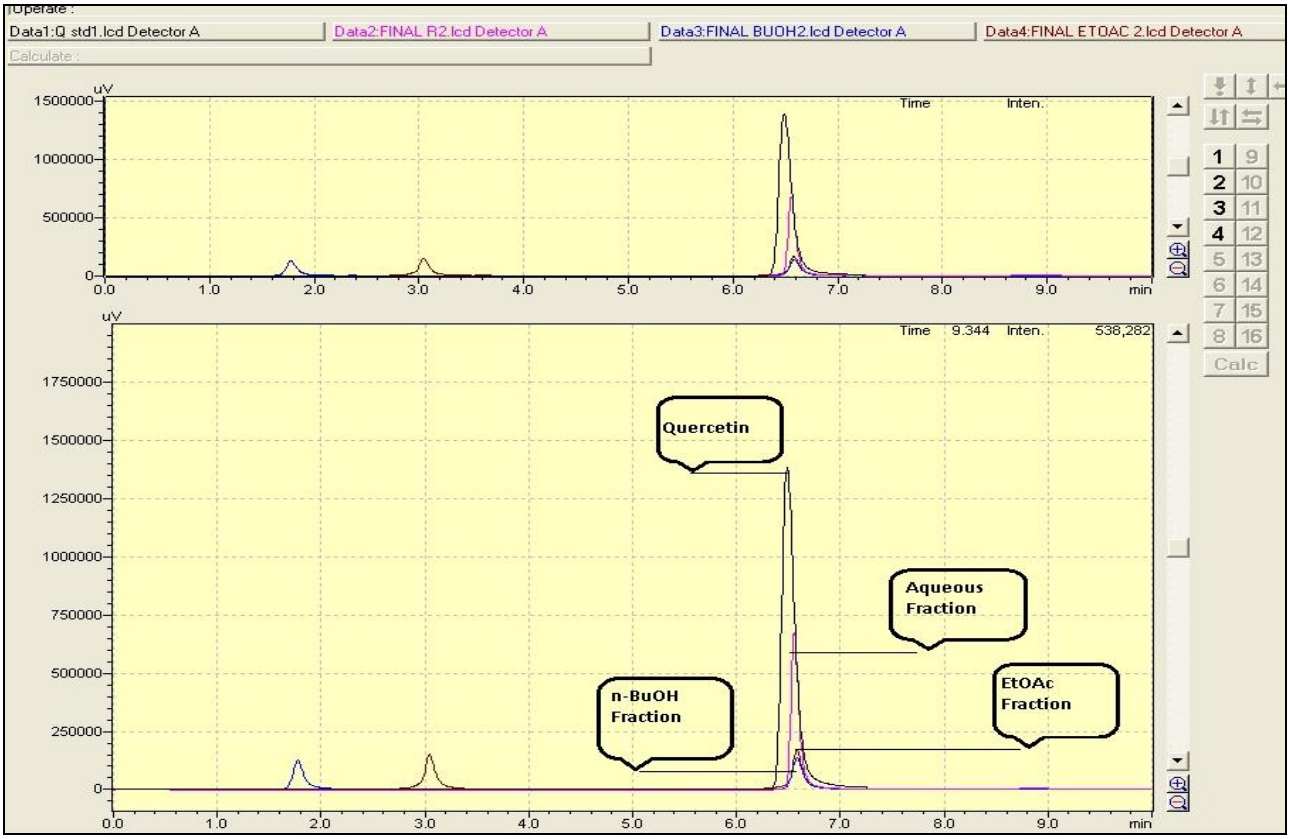

FIG.6: CHROMATOGRAM FOR STANDARD QUERCETIN VS DIFFERENT FRACTIONS

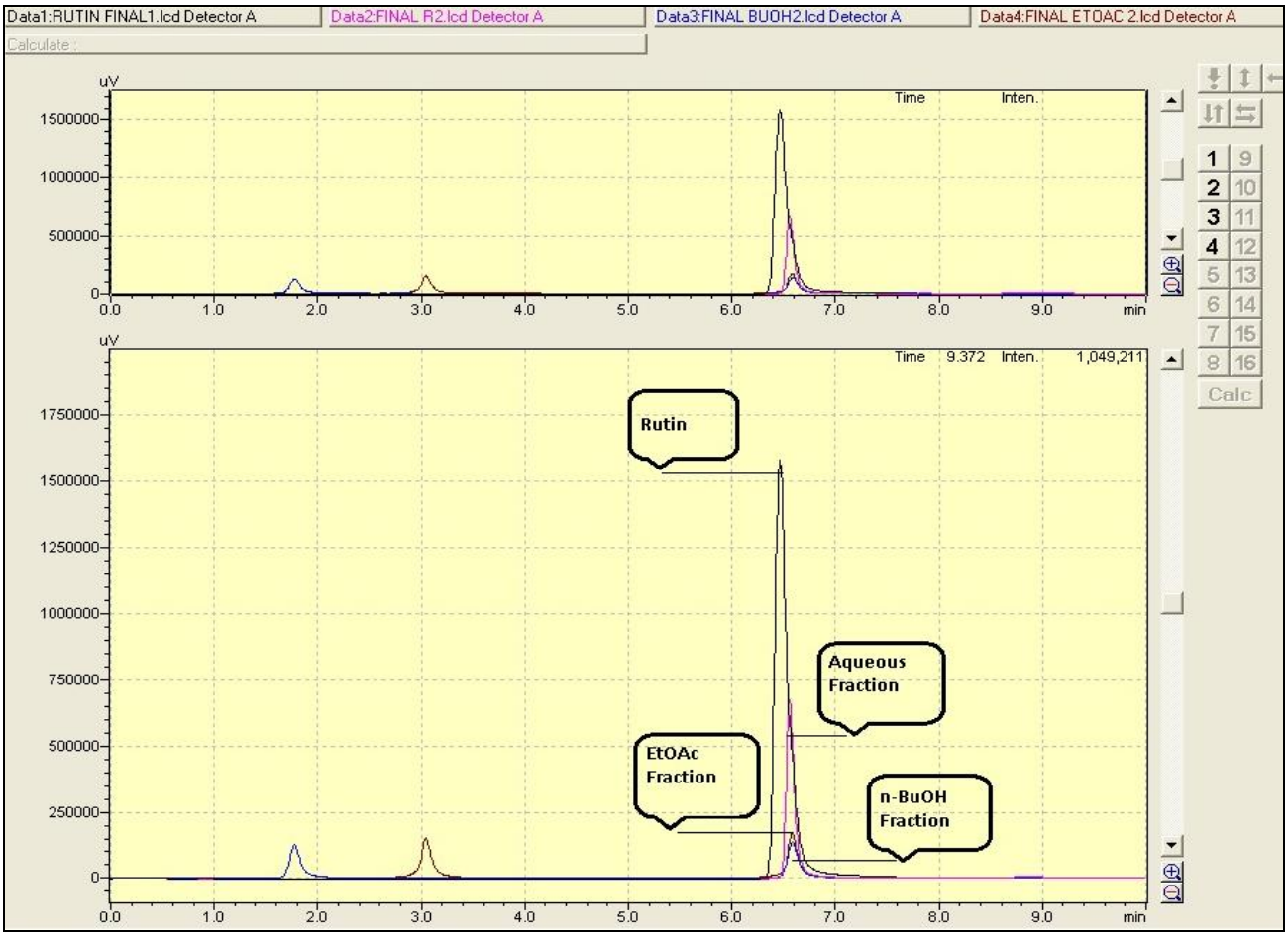

FIG. 7: CHROMATOGRAM FOR STANDARD RUTIN VS DIFFERENT FRACTIONS

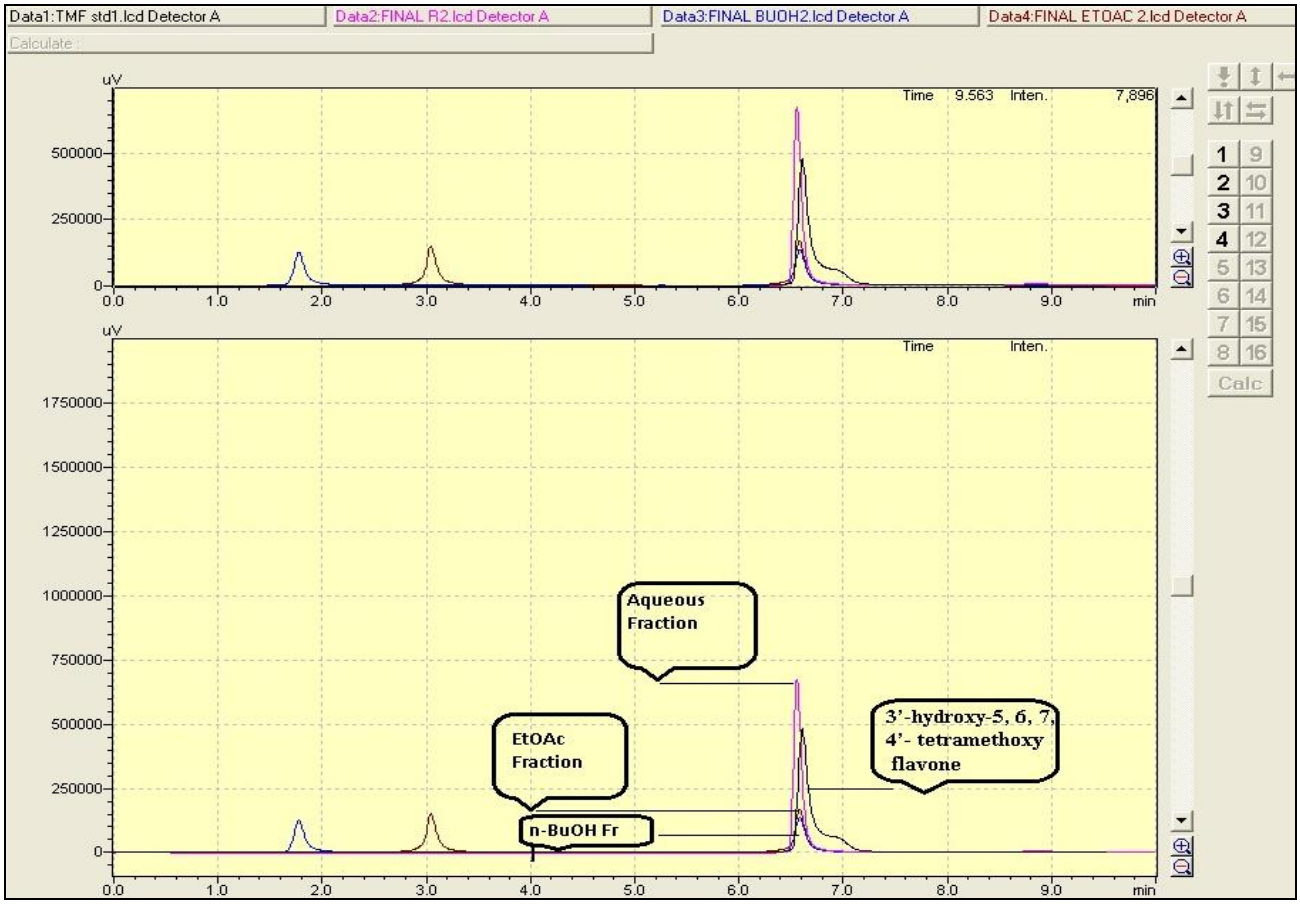

FIG. 8: CHROMATOGRAM FOR STANDARD 3'-HYDROXY-5, 6, 7, 4'- TETRAMETHOXY FLAVONE VS DIFFERENT FRACTIONS

TABLE 1: RETENTION TIME FOR STANDARDS

Bioactive compounds	Retention tim (Rt)	Area	Height	%Area	% Height
Apigenin	2.854	4361364	471520	100	100
Luteolin	6.545	7717110	1008205	99.959	99.976
b-sitosterol	1.147	724840	40330	96.567	97.375
Quercetin	6.484	12649427	1383209	99.987	99.991
Rutin	6.460	14405905	1578157	99.931	99.980
3'-hydroxy-5, 6, 7, 4'- tetramethoxy flavone	6.602	4887896	483674	100	100
Vanillin	6.581	1349524	109808	99.30	99.321
Benzoic acid	6.613	220638	11270	100	100
Gallic acid	6.540	577490	49780	100	100

TABLE 2: RETENTION TIME FOR ETHYL ACETATE FRACTION OF POLYHERBAL FORMULATION

Sr. No	Retention time (Rt)	Area	Height	%Area	% Height	Compound identified
1	3.040	1504358	151270	47.39	46.879	Apigenin
2	6.577	1635270	168071	51.51	51.086	Rutin, Quercetin, Gallic acid, Luteolin, Vanillin, 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone, Benzoic acid (Rt=6.460-6.613)

TABLE 3: RETENTION TIME FOR N-BUTANOL FRACTION OF POLYHERBAL FORMULATION

Sr. No	Retention time (Rt)	Area	Height	%Area	% Height	Compound identified
1	2.822	11455	755	0.458	0.282	Apigenin
2	6.580	1311170	136695	52.439	51.062	Rutin, Quercetin, Gallic acid, Luteolin, Vanillin, 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone, Benzoic acid (Rt=6.460-6.613)

TABLE 4: RETENTION TIME FOR AQUEOUS FRACTION OF POLYHERBAL FORMULATION

Sr. No	Retention time (Rt)	Area	Height	%Area	% Height	Compound identified
1	0.952	2655	195	0.074	0.034	b-sitoterol
2	6.561	3414202	558580	94.746	98.472	Rutin, Quercetin, Gallic acid, Luteolin, Vanillin, 3'-hydroxy-5, 6, 7, 4'- tetra methoxy flavone, Benzoic acid (Rt=6.460-6.613)

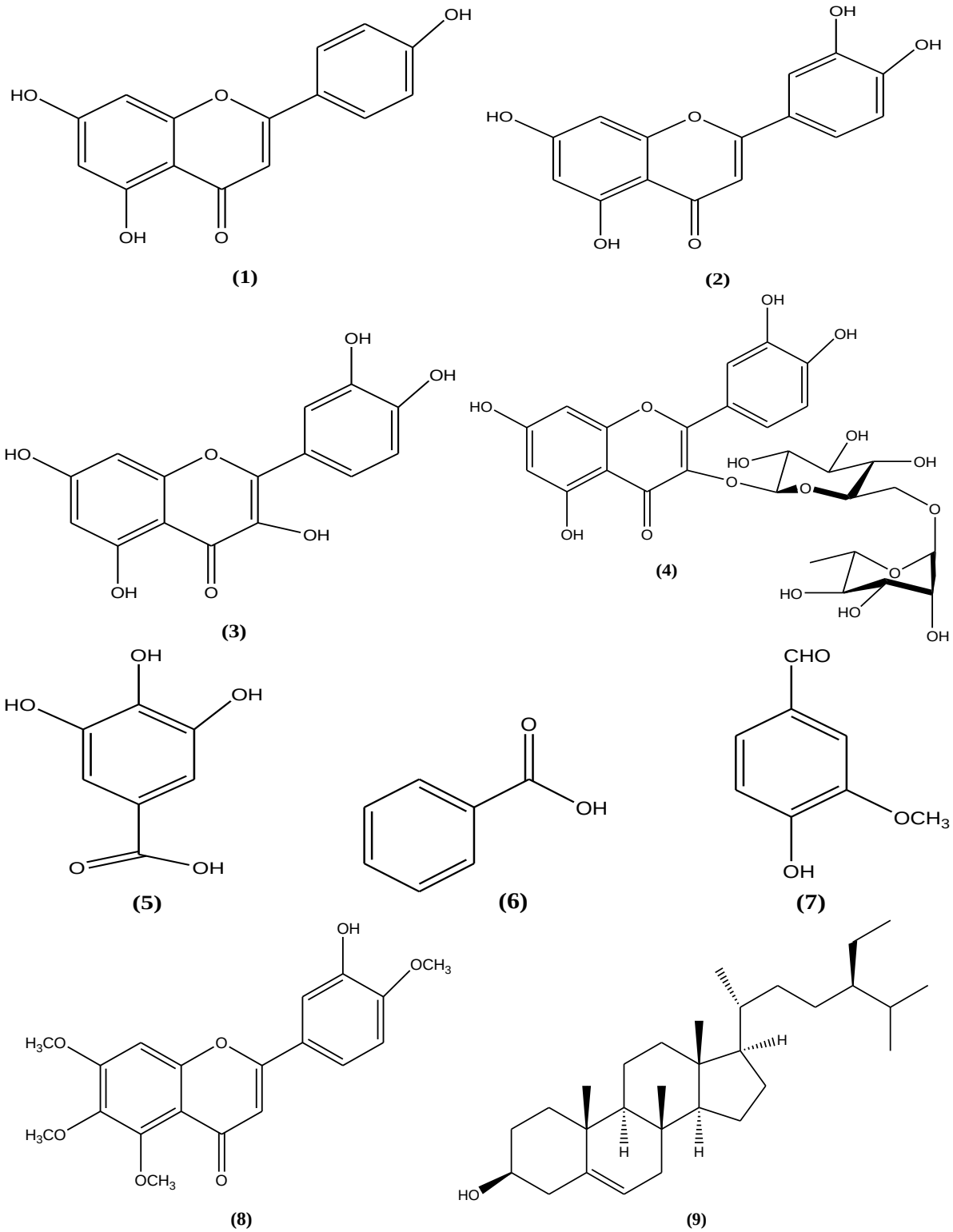

FIG. 9: LIST OF BIOMARKERS IDENTIFIED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

TABLE 5: LIST OF PHARMACOLOGICAL ACTIVITIES REPORTED IN IDENTIFIED COMPOUND

Bioactive compounds	*In-vitro* studies/ *In-vivo* studies	References
Apigenin	Anti-inflammatory, Anti-cancer (human prostate cancer, breast cancer), anti-leukemic activity, Wound healing activities, Alzheimer's Disease	11, 12, 13, 14, 15
Luteolin	Anti-tumor activity (breast cancer), Improves Cardiac Function, Oxidative stress suppression, Wound healing activity, mast cell inhibition	16, 17, 18, 19, 20
b-sitosterol	Anti-inflammatory, heart disease, hypercholesterolemia, modulating the immune system, prevention of cancer, as well as for rheumatoid arthritis, tuberculosis, cervical cancer, hair loss and benign prostatic hyperplasia.	21, 22
Quercetin	Anti-proliferation, apoptosis, Antiangiogenic activity, Wound healing activity	23, 24
Rutin	Wound healing activity, Anti-oxidant activity, apoptosis	25, 26, 27
3'-hydroxy-5, 6, 7, 4'-tetramethoxy flavone	Anti-oxidant, Anti-cancer (colon cancer, breast cancer)	27, 28, 29
Benzoic acid	Antibacterial, antifungal, anti-oxidant properties	30, 31
Gallic acid	Anti-inflammatory, anti-oxidant, Anti-metastasis effects, wound healing activity	32, 33, 34, 35

CONCLUSION: Unavailability of pharmacopoeial standard for herbal based medicinal product is a foremost problem. High-Performance liquid chromatography (HPLC) is one of the major chromatographic technique for identification of biomarker. Using HPLC helps to improve the quality of product and helps to sustain the shelf life of the product for a longer period.

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