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QUANTIFICATION OF URSOLIC ACID AND MINERALS FROM *HEDYOTIS HERBACEA* LINN.

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ABSTRACT: The whole plant of *Hedyotis herbacea* Linn. which was claimed to have wound healing activity, was evaluated for phytoconstituent ursolic acid determination. The literature review suggests that ursolic acid was scientifically validated for wound healing activity. So the research work aims to screen the various extract of *H. herbacea* to determine the ursolic rich fraction. So a high-performance thin-layer chromatographic method was developed for quantitative estimation of ursolic acid in *H. herbacea* family Rubiaceae. The separation was performed on silica gel 60 F254 HPTLC plates using toluene: ethyl acetate: formic acid (7:3:0.1) as mobile phase for elution of markers from the extract. The determination was carried out by using densitometric absorbance-reflection mode at 516 nm for ursolic acid, and also trace elements such as Magnesium, Calcium, Manganese, Potassium, Iron, and zinc which will favor the wound healing activity, were determined by High-performance liquid chromatography.

INTRODUCTION: Wound healing is an intricate process in which the tissue repairs by itself after an injury [1]. It is a process that involves the activation of intercellular pathways, coordination of tissue integrity, and homeostasis. The wound is actually a "disruption of normal anatomic structure and function", according to the wound healing society [2]. Wound healing is a natural biological process that is a combination of a series of independent processes where dermal cells and epidermal cells, ECM, plasma-derived proteins, growth factors, and cytokines all act together to initiate wound healing [3, 4, 5]. Depending upon the nature and depth of the injury, the wound healing can be categorized [6].

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Wound healing is categorized into four phases such as the hemostasis phase, the inflammatory phase, the proliferation phase, and the remodeling phase. For successful healing of a wound, all these four phases must take place in the correct sequence and time frame. Many factors can obstruct one or more phases of this process, thus causing improper or impaired wound healing. It was reported that Non-healing wounds affect about 3 to 6 million people in the United States, with persons 65 years and older with 85% of these events. Non-healing wounds will result in enormous health care expenditures, with the total cost estimated at more than $3 billion per year [7, 8].

The factors that influence the repair of wounds can be categorized into local and systemic factors. Local factors such as oxygenation, infection, foreign body, venous insufficiency. Systemic factors include age and gender, sex hormones, stress, ischemia, diseases: Diabetes, Keloids, Fibrosis, hereditary healing disorders, jaundice,

uremia, Obesity, Alcoholism and smoking, cancer, and Nutrition. Medications like chemotherapeutics, NSAIDs, and glucocorticoid steroids can also retard wound healing [9].

For more than 100 years, nutrition has been observed as a very crucial factor that affects wound healing. Energy, carbohydrate, protein, fat, vitamin, and mineral metabolism can affect the healing process [10]. For optimal repair, several micro-nutrients are also essential. Sequential events in wound repair require a conducive environment within the wound bed and a balanced pool of metal ions, of which at least calcium, zinc, magnesium, copper, manganese, iron, sodium, and potassium are important [11]. It was suggested that iron indirectly stimulates HDF (Human Dermal Fibro-blasts) ECM (Extracellular Matrix) deposition via modulation of macrophage behavior, with potential therapeutic application in pathologic healing [12, 13].

An analysis of wound exudates, it showed that elements such as magnesium promote tissue adhesion, migration of macrophages, keratinocytes, fibroblasts, and production of type I collagen, all of which contribute to the wound healing process [14, 15, 16]. Regulation of ion channels by KCl causes upregulation of TSP1 (Thrombospondin 1) protein, which is involved in angiogenesis and wound healing [17]. KCl-treated cells exhibited induction of TSP1 levels, which would lead to anti-angiogenic effects. It has been reported that clinical studies on the treatment of breast fissures with manganese salts with an improvement of wound healing [18], which might be due to the induction of pro-MMP-9 (Matrix metalloproteinase), stimulating keratinocyte migration *via* αvβ6. Expression of the MT gene is upregulated in the skin following topical application of zinc and in wound margins, particularly in regions of high mitotic activity. This induction of MT in the wound margin may reflect its role in promoting cell proliferation and reepithelialization [19].

Study [20] revealed that calcium alginate acted as a promoter of diabetic wound healing *via* increasing the contraction of wounds, attenuating inflammatory reaction by increasing the levels of type I collagen and hydroxyproline, leading to a significant increase in the wound tensile strength. The accelerating effect of calcium alginate was due to the stimulation of collagen I expression and elevation of collagen I to III ratios to a large extent.

Herbal medicines are widely accepted because of their various advantages like lesser side effects, safety, and efficacy which ultimately increases the compatibility with humans [21]. *Oldenlandia herbacea* Roxb. (*H. herbacea*) (family- Rubiaceae) is an erect, glabrous annual shrub found in temperate and tropical regions of Africa and Asia. Its extract or decoction has been reported to be useful in the treatment of malaria. The decoction of the herb has been used for bathing rheumatic patients, and the powdered herb administered with honey for rheumatic fever and swellings. The herb is boiled in oil, and the oil is used for elephantiasis and pains in the body. The leaves have been employed as an expectorant in Asthma [22].

Ethnomedically, this plant is recognized as one of the ingredients in Jaundice treatment. A paste of leaves is considered as emollient and applied to abscesses and wound [23-24]. The plant is also reported to use in deafness.

FIG. 1: *HEDYOTIS HERBACEA*

This research work attempts to estimate the ursolic acid in various extracts of *H. herbacea* by HPTLC, which was proved to have a wound healing activity [25], and also estimate the minerals and vitamins which favors the wound healing activity. The electronic image of chromatographic fingerprint and densitogram for detecting the presence of marker compound in the sample can be provided by HPTLC [26].

H. herbacea contains an interesting triterpenoid of nature, ursolic, acid as it possesses many beneficial effects such as anti-inflammatory, hepatoprotective,

antibacterial, antiulcer, *etc*. It provoked us to identify and determine the presence of ursolic acid in the leaves also. The ursolic acid is a triterpenoid that is derived from the subgroup named ursane [27].

Moreover, trace elements like Zinc (Zn), Magnesium (Mg), Manganese (Mn), Iron (Fe), Calcium (Ca) and Potassium (K) supports wound healing property as essential trace mineral are required for cellular growth and replication. A survey of available literature showed that there was no report available on the trace element content of the leaves. So it decided to estimate the trace element content of the leaves.

MATERIALS AND METHODS:

Chemicals: Petroleum ether, Chloroform, Ethyl acetate, and Ethanol were purchased from Merck (India). Standard of ursolic acid (99% purity) were purchased from Sigma (New Delhi, India).

Plant Material: Fresh leaves of the plant *H. herbacea* were collected from the Tirunelveli district, Tamil Nadu, India. The specimen was authenticated by V. Chelladurai, Research officer-Botany (Scientist-C), Centre Council for Research in Ayurveda & Siddha, Government of India. A voucher specimen no is (CS-BOT-HH01).

Preparation of Leaves Extract: Leaves were dried in shade and ground to obtain a fine powder. The crude powder was subjected to extract with various solvent such as Petroleum ether, chloroform, ethyl acetate, and ethanol. The crude powder was kept in contact with respective solvents for 7 days at room temperature with occasional shaking by the maceration technique. Then the extract was filtered, and the filtrate was concentrated at reduced pressure to obtain the residue. Then the residue of *H. herbacea* was subjected to HPTLC estimation.

Development of HPTLC Fingerprint:

Instrument: Linomat 5 applicator (CAMAG, Switzerland), twin trough chamber (20 × 10 cm; CAMAG, Switzerland), TLC scanner IV (CAMAG, Switzerland), winCATS version 1.4.6 software (CAMAG, Switzerland), microsyringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, CAMAG, Switzerland), UV chamber (CAMAG, Switzerland), and precoated silica gel 60 F254 aluminium plates (20 × 10 cm, 100 µm thickness; Merck, Darmstadt, Germany) were used in the study.

Sample: The dried extracts of petroleum ether, Chloroform, Ethyl acetate, and Ethanol were separately dissolved in respective solvents and made up to 10mg/ml concentration.

Stationary Phase: The HPTLC plates Silica gel 254, thin-layer chromatography (20 cm × 10 cm).

Mobile Phase: The standard ursolic acid stock solution was prepared in the mobile phase toluene: ethyl acetate: formic acid (7:3:0.1) in the concentration of 100mcg/ml.

Detection Wavelength: Different amounts of (200, 400, 600, 800, and 1000ng) ursolic acid and various residues of extracts (200ng) were loaded onto a TLC plate and scanned at 516nm to get the calibration curve in the ascending mode.

RESULTS AND DISCUSSION: Various proportions of toluene and ethyl acetate were tried as the mobile phase on silica gel HPTLC plates, and a ratio of (7:3:0.1) provides a good elution. The calibration curve of ursolic acid was prepared by plotting the concentration of ursolic acid versus the area of the peak was depicted in **Fig. 5**.

Petroleum ether, chloroform, ethyl acetate, and ethanolic extract of *H. herbacea* were analyzed by the optimized chromatographic condition of HPTLC analysis which showed 4mg/g of ursolic acid in chloroform extract as mentioned in **Fig. 2** and **4**, **Table 1** and other extracts doesn't show the presence of it. The screened chloroform extract was subjected to trace element, and vitamin content analysis by HPLC as listed in **Tables 2** and **3**.

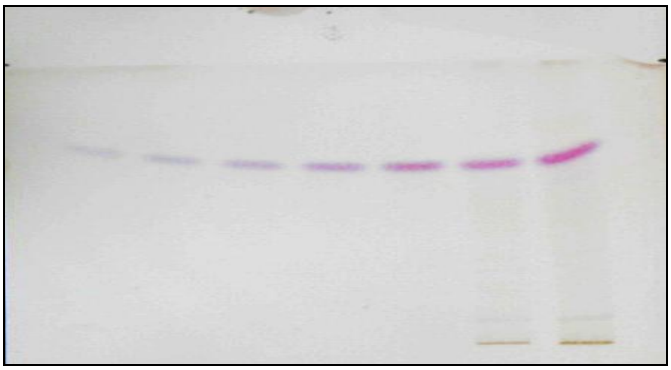

FIG. 2: QUANTITATIVE ESTIMATION OF URSOLIC ACID AT 516 nm (AT WHITE LIGHT)

TABLE 1: ESTIMATION OF URSOLIC ACID IN *H. HERBACEA*

Track ID	Stock	Injection volume	Injection amount	R_f value	Area
Ursolic acid	0.1mg/ml	10µl	1 µg	0.64	8545.2
Chloroform extract	10mg/ml	2µl	20 µg	0.66	8386.8

TABLE 2: LIST OF MINERALS PRESENT IN *H. HERBACEA*

S. no.	Trace elements	Amount (mg/g)
1	Calcium	20.67
2	Potassium	13.59
3	Manganese	0.984
4	Iron	1.136
5	Zinc	14.08
6	Magnesium	17.835

TABLE 3: LIST OF VITAMINS PRESENT IN *H. HERBACEA*

S. no.	Vitamins	Amount (µg/g)
1	Vitamin A	0.245
2	Vitamin c	5.805
3	Vitamin E	0.0091

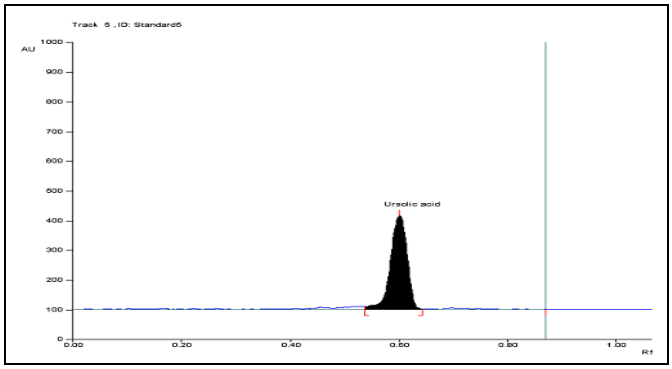

FIG. 3: HPTLC CHROMATOGRAM OF STANDARD URSOLIC ACID

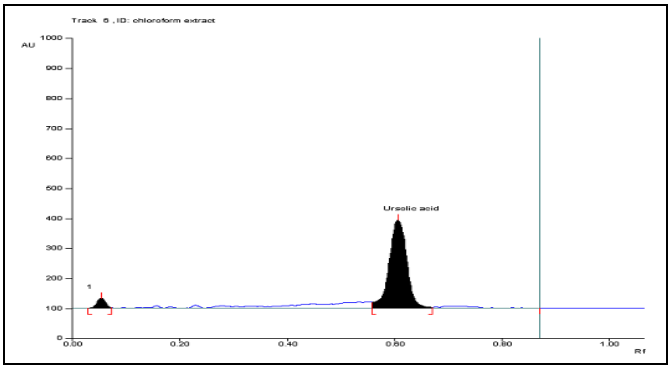

FIG. 4: HPTLC CHROMATOGRAM OF SAMPLE URSOLIC ACID

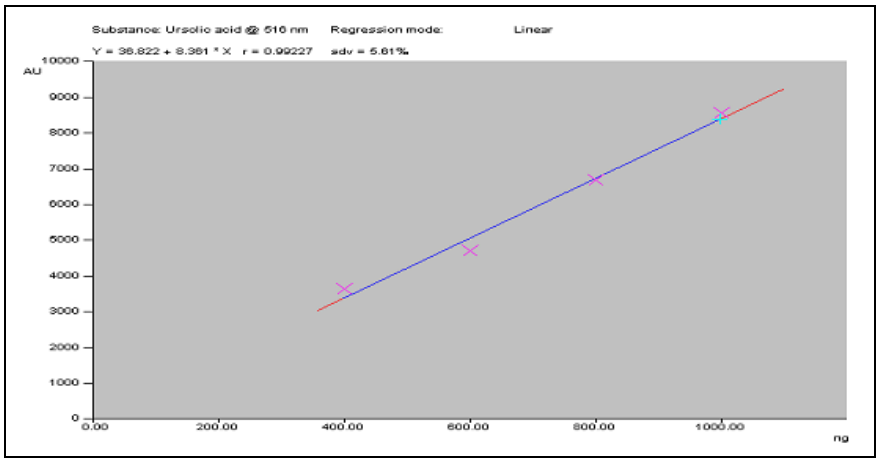

FIG. 5: STANDARD CALIBRATION FOR URSOLIC ACID IN ALL TRACK (CONCENTRATION *VS*. AREA)

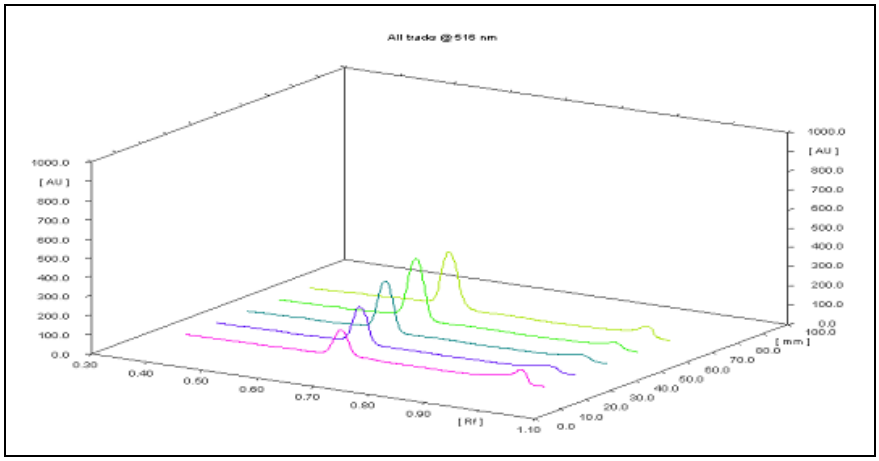

FIG. 6: HPTLC ESTIMATION OF URSOLIC ACID (3D DISPLAY)

CONCLUSION: The proposed HPTLC method was used for the quantitative estimation of ursolic acid. Ursolic acid is one of the factors that supports wound healing, and also the nutritional factor which promotes the wound healing activity was also estimated by HPLC. The HPTLC estimation of ursolic acid confirms its presence in chloroform extract of *H. herbacea* , which is having a significant effect on wound healing. The extract also indicated the presence of various minerals such as zinc, calcium, magnesium, manganese, potassium and iron at different concentrations which thereby provides an added advantage along with ursolic acid in wound healing activity, since all the minerals are reported for their significance in wound healing activity. Hence based on this report, chloroform extract of *H. herbacea* will be utilized to evaluate the *in-vitro* and *in-vivo* wound healing activity for further confirmation of ursolic acid-enriched extract to prove its efficiency in wound healing.

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