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A Study on Phytochemical Compounds of Verbesina Encelioides Leaves

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ABSTRACT
Verbesina encelioides (Asteraceae family) is a significant medicinal plant, known as golden crown beard and has aggressive and dominant growth characteristics. The present study has been carried out to qualitatively and quantitatively analysis of primary and secondary metabolites present in the V. encelioides leaf. The results indicated the presence of ample number of phyto-constituents like 4389 µg/gm total soluble sugar, 5521 µg/gm starch, 201 mg/gm proteins, 7.8 gm lipid, 126 µg/gm phenols, 56.88 µg/gm tannins and 20% saponins. By using thin layer chromatography, kaempferol and terpeniol were characterized. This study proves that V. encelioides leaves have a significant amount of nutritional values and secondary metabolites which can be beneficial to human health. But still more research using chemical approaches is required to identify numerous applications of V. encelioides biomolecules.

Keywords:Verbesina encelioides, Asteraceae, Phyto-constituents, Thin layer chromatography.

I. INTRODUCTION

Nature has provided a plethora of remedies to cure all of humanity's ailments. Herbal medicines have a history as old as human civilization. Today, there is a wealth of information available about the therapeutic properties of various plants. Plants continue to be a major source of drugs in both modern and traditional medical systems **(Rupasinghe et al, 2003; Savithramma et al, 2011)**. The preliminary step in the standardization of crude drugs is pharmacognostical research. The detailed pharmacognostical evaluation provides useful information about the morphology, microscopical, and physical properties of the crude drugs. Many important drugs have undergone pharmacognostical studies, and the findings have been incorporated into various pharmacopoeias **(Sharma et al., 2004).** A number of crude drugs have yet to be scientifically identified as having a plant source. As a result, pharmacognostical research provides scientific data on the quantitative and qualitative value of plant drugs **(Dhanabal et al., 2005)**. Secondary metabolites in plants are non-nutritive compounds that have protective or disease preventive properties. Plants produce these compounds to protect themselves, as well as also helpful to protect humans from disease. There are numerous phytochemicals isolated from herbs with a variety of health benefits, including antioxidant, anti-microbial, anti-inflammatory, and cancer-prevention properties **(Rupasinghe et al, 2003; Savithramma et al, 2011)**.

The Asteraceae family is one of the largest flowering plant families, with over 23,600 species and approximately 1620 genera **(Funk et al., 2009)**. The genus Verbesina contains about 300 species that range from Canada to Argentina, with the greatest diversity in Mexico's highlands and the north and central Andes **(Panero et al., 1997)**. Verbesina encelioides, also known as wild sunflower or golden crown beard, is an exotic invasive weed that is thought to have originated in the United States and Mexico. Golden crown beard is an erect, annual, wild herb with a wide range of tolerance to climatic conditions and a competitive growth habit **(Singh et al., 2017)**. It is a broad-leaved, 30-60 cm tall herb with yellow flowers 2-5 cm wide on long peduncles that resemble sunflowers. As a result, it is also known as wild sunflower **(Kaur et al., 2018)**. Terpenoids, flavonoids, aromatic compounds, and other phytoconstituents are the most abundant in the various parts **(Bohlmann et al., 1980)**. In addition, Galegine and ferulic acid have been reported as key phytochemicals present in this plant. Galegine, an alkaloid, has both antimicrobial and anti-tumor properties. Ferulic acid has been claimed to reduce the side effects of chemo- and radiotherapy of carcinomas by increasing natural immune defenses. V. encelioides used to treat spider bite symptoms **(Ramakrishnan et al., 2017)** as well as stomach diseases known as heamorrhoids **(Bhattacharjee et al., 2001)**. The leaves are used as a poultice to treat

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rheumatism and the juice is used as a laxative **(Parrota et al., 2001).** Therefore, the aim of the present study was to identify the phytochemical compounds qualitatively by using thin layer chromatography and quantitatively from the leaf part of V. encelioides.

II. MATERIAL AND METHOD

Plant Materials

Healthy plants of Verbesina encelioides were collected in the month of April, 2013 from the Mansarovar area, Jaipur. The authenticity of the plant was confirmed by renowned taxonomist Prof. S. Kshetrapal, Department of Botany University of Rajasthan, Jaipur, India.The freshly collected V. encelioides leaves were washed thoroughly with tap water and then air-dried under shade for about 2-3 weeks. The dried samples were ground into powder form by using a homogenizer. The fine powder form was used as a samplefor further analysis.

Biochemical Analysis

Analysis of Primary metabolies

Proteins

Protein content was evaluated by **Lowry et al. (1951)** method in which 0.05gm V. encelioides leaves powder was homogenized individually with 10ml TCA (10%). The homogenized samples were centrifuged at the speed of 10000 rpm for 10 minutes tentatively. The supernatant was used to determine protein concentration. 0.5ml of supernatant was diluted with distilled water up to 1ml. Then 5 ml of a freshly prepared alkaline solution that prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5 % $CuSO_4.5H_2O$ in 1% Sodium potassium tartrate was added and incubated at 37°C for 10 minutes. After incubation 0.5ml of Folin-Ciocalteau (FC) reagent was added and vortexed properly. The absorbance was read at 750nm by spectrophotometer after 30 minutes of incubation. Bovine serum albumin (1mg/ml) was used to prepare the standard curve. Blank has all reagents except samples.

Total Soluble Sugars (TSS)

TSS was estimated by using the phenol-sulphuric acid method of **Dubois et al. (1951)**. The 0.05gm powder sample was homogenized with 20 ml of 80% ethanol with the help of mortar-pestle and then centrifuged at the speed of 12,000 rpm for 15 minutes tentatively and the supernatants were collected separately for TSS estimation. 0.5ml of supernatant was diluted up to 1ml with distilled water. 1 ml of 5% phenol was added to the sample and mixed completely. 5ml conc.H_2SO_4 was added gently in the tube and kept on ice for cooling. Then tubes were allowed to stand for 20 minutes in a water bath at 26-30°C. Glucose (100µg/ml) was used as standard. The intensity of the yellow colour was measured at 490nm against blank.

Starch

The 0.05gm powder sample was homogenized with 20 ml of 80% ethanol by using mortar-pestle. Each sample was centrifuged at the speed of 12,000 rpm for 15 minutes tentatively and the pellets were separated individually to perform a starch test. Pellet was suspended in 5 ml of 52% perchloric acid, followed by 6.5 ml of water and the mixture was shaken vigorously for 5 minutes. Later, above mentioned **Dubois et al. (1951)** were followed with 1ml of sample solution to calculate starch concentration.

Lipids

Lipid contents were measured by using **Jayaram, 1981** method. The 0.1 gm sample was homogenized with 10 ml distilled water, thereafter 30 ml of chloroform and methanol (2:1) was added. The mixture was thoroughly mixed and left overnight at room temperature in the dark for complete extraction. Later, 20 ml of chloroform mixed with 2 ml of water were added and centrifuged. Two layers were separated,the lower layer of chloroform, which contained all the lipids, was carefully collected in the pre-weighed glass vials. The chloroform layers dried in vacuo and weighed. Each treatment was repeated thrice and their mean values were calculated.

Phenols

Total phenol content in the sample was estimated by the spectrophotometer method of **Bray and Thorpe (1954)**. The 0.2gm leaf sample was macerated with 10 ml of 80% ethanol. The mixtures were centrifuged at the speed of 15000 rpm for 15 minutes and 0.5 ml supernatant collected separately was made up to 1 ml with 80% ethanol. 1ml of Folin-Ciocalteau (FC) reagent and 2 ml of 20% sodium carbonate solution was added and the mixture was vortexed properly. The sample was placed in boiling water for 1 min and cooled under running water. These reaction mixtures were diluted to 25 ml by adding distilled water and optical density was read at 750 nm against a blank. Gallic acid (100µg/ml) was used to plot the standard curve.

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Analysis of Secondary Metabolites

Tannin

One gram of sample was added to 100 ml of distilled water and boiled gently for 1hour on an electric hot plate and filtered using Whatman 42 filter paper (125 mm). Addition of 5.0 ml of Folin-Denis reagent **(LOB Chemie Pvt. Ltd.)** and 10 ml of saturated Na_2CO_3 solution into 50 ml of distilled water and 10 ml of diluent extract was mixed for colour development. Dissolution of 0.20 g of tannic acid in distilled water and dilution to 200ml were used to obtain a tannic curve. Varying concentrations (0.2-1.0 mg/ml) of the standard tannic acid solution were pipetted into five different test tubes to which Folin-Denis reagent (5 ml) and saturated Na_2CO_3 (10 ml) solution were added and made up to the 100 ml with distilled water. Then the solution was left to stand for 30 minutes in a water bath at 25ºC. Optical density was ascertained at 700 nm with the aid of a spectrophotometer **(Polshettiwar et al., 2007)**.

Saponin

A 20 g leaf sample was added in a conical flask containing 100 ml of 20 % aqueous ethanol. The solution was heated for 4 h with constant stirring at 55ºC. Solution was filtered and residue was extracted with 200 ml 20 % ethanol. After that both extracts were mixed and solvent was evaporated till 40 ml volume of extract. The concentrate was extracted with 20 ml of diethyl ether in a separating funnel. The aqueous layer was recovered while the ether layer was discarded. The aqueous extracts were purified by adding 60 ml n-butanol. Then it was washed twice with 10 ml of 5 % aqueous NaCl. The solution was dried and the saponin content was calculated as percentage **(Eleazu et al., 2012).**

Xanthoprotein

1ml of methanol extract was treated separately with few drops of conc. HNO_3 and NH_3 solution. Formation of reddish orange precipitate indicates the presence of xanthoproteins **(Beena et al., 2016)**.

Thin Layer Chromatography to Detection of Flavonoid and Terpenoid

a) Detection of Flavonoid

Powder leaves sample (10 g) was extracted with 100 ml of 80% methanol in a water bath **(Subramanian and Nagarajan, 1969)** for 24 hr. The methanol soluble fractions were filtered, concentrated in vacuo and aqueous fractions were fractionated by sequential extraction with petroleum ether (FrI), diethyl ether (FrII), and ethyl acetate (FrIII) separately. Each step was repeated thrice for complete extraction, fraction I was discarded in each case because it contained fatty substances, whereas fractions II and fraction III were concentrated and used for flavonoids.Fraction III was further hydrolysed by refluxing with 7% sulphuric acid (10 ml/g plant material for 2 h), filtered and the filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings, and concentrated in vacuo. Both fractions II and III were taken up in a small volume of ethanol (2-5ml) before the chromatographic examination.

b) Detection of Terpenoid

The powder sample was extracted using a maceration method by dissolving powder into chloroform or methanol 96% which had been placed in the Erlenmeyer glass. Then, closed it with plastic and gave Erlenmeyer rubber. After that, shake the solution with a magnetic stirrer and allow it to stand for 48 h. Subsequently, the solution was filtered using filter paper and then, filtrate was evaporated in a water bath until it dried.

c)Thin layer Chromatography

Thin glass plates (20x20 cm^2) were coated with Silica gel G (250 µm thick). The freshly prepared plates were air-dried at room temperature; thereafter these were kept at 100°C in an oven for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis.

d) Running and staining

Each extract was spotted on silica gel plates, followed by plates were developed in an air-tight chromatographic chamber saturated with a solvent mixture Benzene: Acetic Acid: Water (125:72:3) for flavonoid **(Wong and Francis, 1968)** and toluene: ethyl acetate (93:7) for terpenoid **(Kristanti and Tunjung, 2015)**. The developed plates were air-dried and visualized under UV light at 254 nm and 365 nm then sprayed with I_2 vapour for flavonoid and sulfate vanillin as a colouring reagent that produced purple colour for terpenoid. Reference compounds used quercetin for flavonoid and terpeniol for terpenoid. The coloured spots thus developed were noted and the retention factor (R_f) value of each spot was calculated. R_f value was calculated by using following formula:

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$$R_f = \frac{\text{Distance traveled by the solute (cm)}}{\text{Distance traveled by the solvent (cm)}}$$

III. RESULT AND DISCUSSION

Phytochemical analysis is used to identify new sources of therapeutically and industrially valuable compounds derived from medicinal plants that have been chemically evaluated **(Ambasta et al., 1986)**. The primary and secondary metabolites in Verbesina encelioides leaves were studied qualitatively and quantitatively in this study. The quantitative estimation of primary metabolites revealed the presence of various chemical constituents in the experimental plant with variable concentration as shown in Table-1.

Analysis of Primary Metabolites

Protein

Proteins dictates the occurrence of disorders and stimulate the biosynthesis of natural hormones such as IAA, cytokinin, and gibberellins, cell division, plant pigments, enzymes, organic foods, and plant metabolism **(Oretili et al., 1987)**. In the present study the total estimated protein in the leaf was 201 mg/gm dry weight. According to **Velasco et al., 2021** the proximal chemistry evaluations performed on V. sphaerocephala displayed 17.7% proteins. **Kaszás et al., 2020**determined 33.3% average total protein level on a dry mass of Jerusalem artichoke leaves. Soluble proteins play an important role in the growth of the plants and are a very important component of numerous plant enzymes that reflect the overall plant metabolism **(Berry and Downton, 1982)**.

Carbohydrate

Carbohydrates are the basic compounds needed to produce phenolic compounds via the shikimic acid pathway, which converts extra carbohydrates from glycolysis and the pentose phosphate pathway into aromatic amino acids. The accumulation of carbohydrates in different parts of the plant followed a descending order of leaf > stem > root**(Reddy et al., 1996)**. In the present study the total soluble sugar estimated in V. encelioides leaf was 4389 µg/gm dry weight and the level of starch was 5521 µg/gm. In **2021, Velasco et al**. displayed 45.2% carbohydrates in V. sphaerocephala which is a large amount of carbohydrates, thus explaining the high capacity to absorb water and as per **Munné-Bosch and Alegre, 2002** the critical roles of adequate water supply for various physiological processes such as photosynthesis, respiration, transpiration, translocation, enzyme reaction, and cell turgidity occur concurrently. The increase in carbohydrates content in sunflower leaves may be due to the role of antioxidants in protecting chloroplast membranes from photooxidation and help to provide an optimal environment for the photosynthetic machinery.

Lipid

Plant lipids, include fatty acid derivatives such as triacylglycerols (TAGs) and glycerophospholipids (GPLs), aromatic and hydrocarbon-like compounds such as sterols, carotenoids, and terpenes, and waxes serving many important functions, including storage of metabolic energy, protection against dehydration **(Tao et al., 2007).** In the present study the lipid was calculated to be 7.8 gm from the leaves sample. In the study of **Kaszáset al., 2019** the linolenic acid in Helianthus tuberosus represented 39-43% of total lipid content; moreover, the ratio between ω-6 and ω-3 essential fatty acids was ~0.6: 1.

Phenol

Phenols constitute the largest group of plant metabolites, varying in size from a simple structure with an aromatic ring to complex ones. The higher amount of phenol is important in regulation of plant growth, development and disease resistance**(Weber et al., 2010)**. In current study the phenol content was estimated to be 126 µg/gm. In study of **Rodríguez et al., 2021** the total phenolic contents of the V. sphaerocephala extracts ranged from 6.55 ± 0.71 to 10.50 ± 0.76 mg whereas in estimation of **Ramakrishnan et al., 2017** the maximum phenol content was 3.6 mg/g.

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Table 1: Primary metabolites content in leaves of Verbesina encelioides

Primary metabolites	Concentration
Total Soluble Sugar	4389 µg/gm
Starch	5521µg/gm
Lipids	7.8 gm
Protein	201 mg/gm
Phenol	126µg/gm

Analysis of Secondary Metabolites

Tannin

Tannins are the most abundant secondary metabolite produced by plants, accounting for 5% to 10% of the dry weight of tree leaves. Tannins can protect leaves from insect herbivores through deterrence and/or toxicity **(Barbehenn, et al., 2011)**. In the present study the tannin was estimated to be 56.88µg/gm from V. encelioides leaves. **Salazar et al., 2018** identified the tannins content in aqueous extract of V. encelioides by using $FeCl_3$ test and revealed that it is as supposedly responsible for diuretic activity in rats along with other phytochemicals in V. crocata. **Ramakrishna et al., 2018** estimatedtannin content of 4.72% in plant parts of V. encelioides.

Saponins

Saponins are glycosidic compounds found in abundance in plants. Certain saponins can hemolyzed blood cells and form insoluble complexes with cholesterol **(Shin et al., 2015)**. Current study showed 20% of saponin in the tested plant leaf. **Salazar et al., 2018** conducted a frothing test for saponins and found significant amount of saponins in V. crocata. **Lim et al., 2020** identified total saponin concentration in quinoa seeds, leaves, and root with 1.26 g/100 g, 0.97 g/100 g and 13.39 g/100 g, respectively. **Roonjho et al., 2022**quantified highest 53063 mg/l saponin content in Porterandia anisophylla thereafter 42829 and 42510 mg/l in Antidesma cuspidatum and Clidemia hirta, respectively by using HPLC. **Ojiako (2014)** reported higher percentage of saponin in M. Olieifera leaves than alkaloid and phenols.

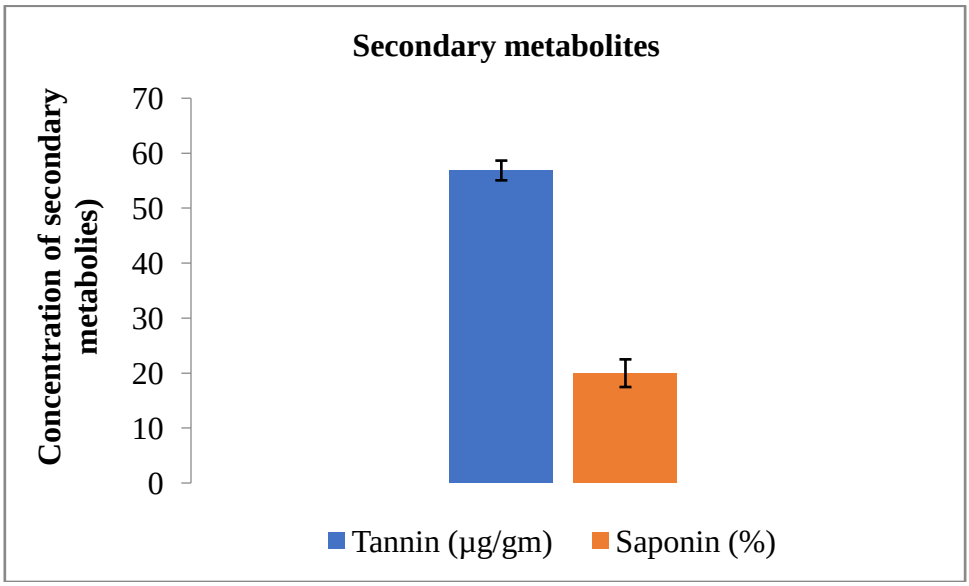

Figure 1: Secondary metabolites content in in leaves of Verbesina encelioides `

Xanthoprotein

In the current study a very high amount (+++) of xanthophyll was estimated in leaves of Verbesina encelioides. According to **Kumar et al., 2013**, xanthoproteins are highly present in all solvent extracts of Holoptelea integrifolia,

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including distilled water, methanol, chloroform, acetone, and petroleum ether, whereas Celestrus emarginata showed extremely low (+) presence in distilled water, chloroform, and very low in petroleum ether. In the results of qualitative phytochemical analysis of Acalypha fruticosa leaf extracts conducted by **Devi et al., 2021** the concentration of xanthoprotein was low (+).

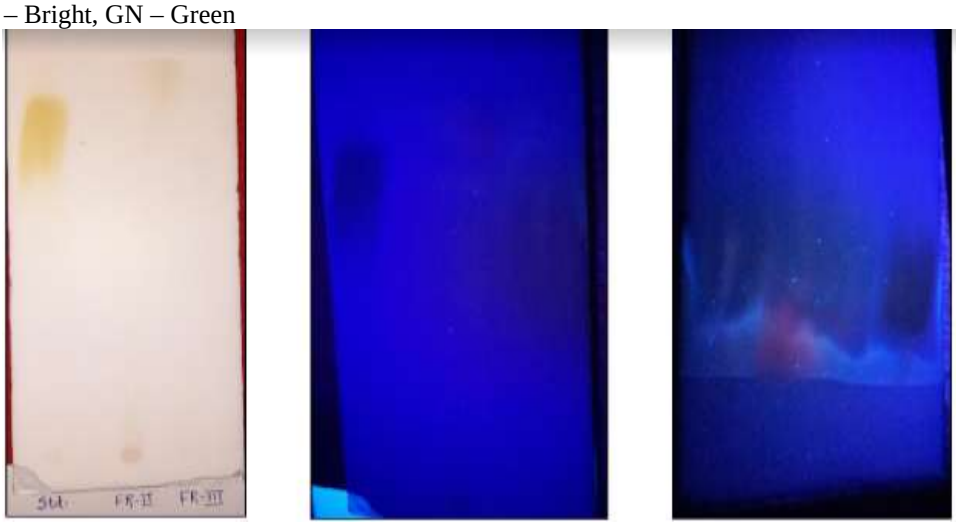
Detection of Flavonoid

Flavonoids contain hydroxyl functional groups, identified as a class of antioxidant compounds in plants that act as free radical terminators **(Shahidi et al., 1992; Das et al., 1990; Younes et al., 1981)**. In the current study thin layer chromatography was performed to identify the flavonoid compounds present in the tested plant sample. As shown in Figure 2, spots of flavonoid compound on the TLC plate were observed at 0.77, 0.88 and 0.86 R_f values, respectively with standard (Quercetin), FR-II and FR-III. In UV treatment all spots were shown in black colour. According to Table 2, both FR-II and FR-III have Kaempferol as a flavonoid compound. As per the study of **Kristanti and Tunjung, 2015** the qualitative estimation of Artocarpus communis leaf revealed Kaempferol with 0.89 R_f value. The flavonoid rutin was identified by **Rodríguez-Valdovinoset al., 2021** in methanolic extracts ofV. sphaerocephala flower and leaves by using high-performance thin-layer chromatography (HPTLC).

Table 2: Chromatographic and Physico-chemical characteristics of isolated Flavonoids from Leaf of Verbesina encelioides

Isolated compounds	R_f value	Physical appearance		Colour of spots
	S_1	Daylight	I_2 Vapour (R_1)	UV
Kaempferol	0.84	GN-YW	YW-BN	BK
Quercetin	0.79	GN-YW	YW-BN	BK

Abbreviations: S_1 – Benzene: acetic acid: water (125: 72: 3), R_1 – I_2 Vapour, YW – Yellow, BK – Black, BN – Brown, BT – Bright, GN – Green



(a) (b) (c)

Figure 2: Detection of flavonoid compounds with light treatment (a) Spray with I_2 vapour (b) UV 254nm (c) 365nm

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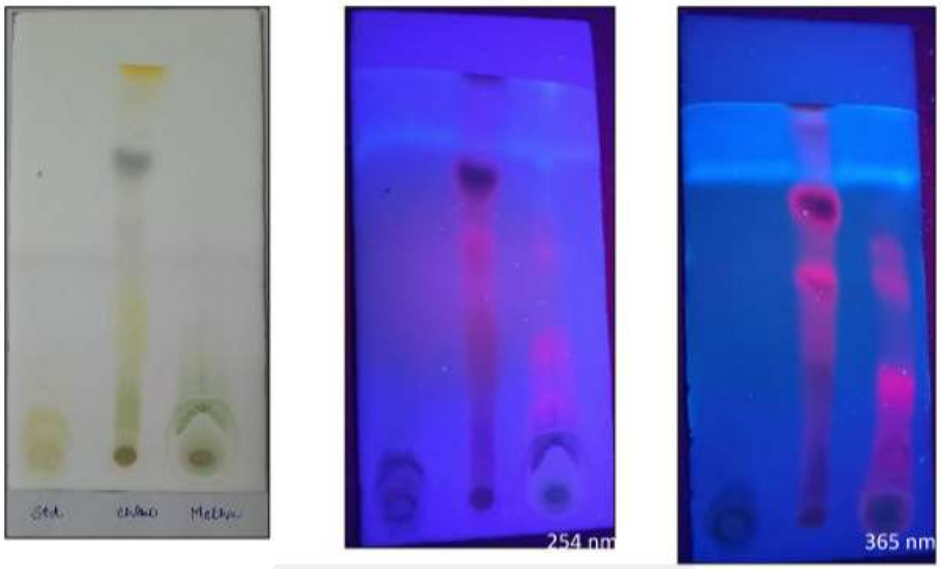
Terpenoid

Terpenoids are secondary metabolites that have an isoprene framework. Terpenoids have antiseptic, antimicrobial, antibiotic, and plant growth regulator properties, as well as the ability to treat skin disorders and diabetes **(Lenny, 2006)**. In the current study terpenoids were detected in the methanol and chloroform extract of Verbesina encelioides leaves with R_f values being 0.315 and 0.310, respectively with purple colour that resemble to R_f value of terpeniol shown in figure 3. As per the study of **Kristanti and Tunjung, 2015** the qualitative estimation of pulp of Artocarpus communis revealed the 0.31 R_f value for both methanol and chloroform extract respectively that indicated the presence of terpeniol. **Yamunadevi et al., 2011** studied the methanolic extract of stem, leaves, root, flower and seeds of Aerva lanata showed the presence of 27 different types of terpenoids with 27 different R_f values in the range of 0.06 to 0.97.



(a) (b) (c)

Figure 3: Detection of terpenoid with various treatment (a) Spray with sulfate vanillin (b) UV 254nm (c) 365nm

IV. CONCLUSION

The phytochemical analysis of V. encelioides leaf yielded significant results in various tests, confirming the presence of many primary and secondary metabolic compounds. Such evaluation of different natural biomolecules and identification of natural organic compounds is crucial for successful pharmaceutical research and predictions.

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