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Phytochemical estimation and antioxidant activities of *Vernonia cinerea* (L) Less

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Abstract
Vernonia cinerea (L)Less is well known folk medicinal plant used in different parts of the world. It has antimicrobial, anti-inflammatory, antioxidant and anticancerous properties. In the present study phytochemical analysis of crude and partially purified extracts of root, stem and leaves of the plant was carried out. The extracts were also tested for their antioxidant potential using DPPH radical scavenging assay. The qualitative analysis has indicated the presence of different secondary metabolites. Quantitative estimation has shown considerable amount of total phenols, flavonoids and terpenoids. Thin layer chromatography results are showing the presence of triterpenoids and steroids in the extracts. There is correlation between phenolics and flavonoid content and antioxidant activity. These bioactive secondary metabolites validate the ethnomedicinal importance of this plant.
Keywords: Vernonia, Phenols, Flavonoids, Terpenoids

Introduction –
Plant secondary metabolites are important for their biological effect on other organisms. They are synthesized through different pathways and are accumulated in different places within a cell. The biologically active compounds are useful resources for development of drug in the treatment of various diseases (Balunas and Kinghorn 2005).Phytochemical investigation w.r.t secondary metabolites is a crucial step in pharmacognostic studies. It reveals the type of compounds synthesized and accumulated in different parts of the plant during different stages of its development. The analysis of primary and secondary metabolites can be done by different methods. The analysis helps in further isolation and identification of bioactive compounds. Preliminary phytochemical analysis involves qualitative identification of compounds from the extracts prepared in different solvents. These tests are based on the change in colour of extract or formation of precipitate after addition of a particular reagent (Harborne 1973). These qualitative tests help in knowing the presence or absence of particular compound of interest. The limitation of these tests is identification of a compound is tentative based on the perception of a colour. The quantity of identified compounds can be determined by spectrophometer as equivalent of standard compounds.

Thin Layer Chromatography (TLC) and paper chromatography not only involves identification of compounds but also separation of compounds based on their polarity in a standardized solvent system. Rf values or comparison with standard pure compounds are used in identification of compounds.In preparative TLC the separated compounds can be scrapped and used for assays to test their bioactivity. The modern analytical techniques such as HPLC, GC and LC hyphenated to MS are sensitive in detecting compounds present in minute quantities. Validation can be done if all the methods are used in phytochemical analysis. According to Harborne (1973) the accuracy of mass spectrometry is such to indicate the exact molecular formula of a compound so that other conventional analytical methods are not required.

Vernonia cinerea (L) Less plant is ethnomedicinally important. It is traditionally used as febrifuge, diaphoretic, diuretic, antispasmodic and anthelmintic (Khare 2007). Various parts of the plant are reported to contain sterols such as stigmasterols, sitosterols and spinasterols; triterpenoids like amyrin and lupeol; sesquiterpenes, glycosides and flavonoids (Khare 2007, Kuo 2003, Misra 1993, Chen 2006). Experimental evidences strongly suggest the anti-inflammatory and cytotoxic properties of plant extracts (Pratheeshkumar and Kuttan 2009). With this background we carried out investigation of secondary metabolites different extracts prepared from root, stem and leaves. We also estimated radical scavenging ability of these extracts by DPPH assay. The plant parts show accumulation of diverse secondary metabolites. They are rich in phenolic compounds including flavonoids and terpenoids.

Materials and Methods

Plant collection- Plants of *Vernonia cinerea* were uprooted from different regions of Pune (18^{0}32'17.6"N73^{0}48'20.4"E and 18^{0}31'35.0"N 73^{0}50'43.5"E). Plant was authenticated from BSI and Voucher specimen was submitted with ARGVEC3 code. The parts of plant, root, stem and leaves were separated and dried in shade.

Preparation and purification of extracts – Powder of dried plant parts was extracted with different organic solvents such as water, ethanol,

methanol, acetone, ethyl acetate, petroleum ether and chloroform. The filtered extracts were used for qualitative tests. Methanolic extracts were further fractionated using hexane, chloroform, ethyl acetate and methanol by column chromatography. Chloroform fractions rich in terpenoids were further purified using solvents starting with nonpolar chloroform to ethyl acetate to methanol.

Qualitative Phytochemical analysis - Following secondary metabolites are tested as per (Damodaran and Manohar 2012)

Tannins- to 1 ml plant extract 5% Ferric chloride was added. The appearance of dark blue colour indicates presence of tannins.

Flavonoids- to the plant extract 2N NaoH is added. Yellow colour indicated the presence of flavonoids.

Alkaloids – Mayers reagent was added to plant extract. Development of white precipitate was considered the presence of alkaloids.

Saponins- Distilled water was added to the plant extract and shaken vigorously. Development and stabilization of foam is an indication of presence of saponins.

Quinones – To 1 ml of plant extract 1ml of concentrated sulphuric acid was added. The formation of red colour indicates the presence of Quinones.

Glycosides – 1 ml Plant extract was mixed with Chloroform and 10% ammonia solution. Pink colour formation indicated the presence of glycosides.

Terpenoids – to 1 ml plant extract chloroform and concentrated sulphuric acid was added. Formation of red ring at the interface indicate presence of terpenoids.

Quantitative estimation

Total phenolic content was measured from Methanolic extracts of root, stem and leaves and purified fractions using spectrophotometric analysis with Folin-Ciocalteu reagent(Sultana et al.2012). 2.5 ml Dilute Folin-Ciocalteu reagent (1:10) was added to Plant extracts followed by 2ml of 7.5% sodium carbonate. The mixture was incubated for 30 minutes. The absorbance of blue complex was measured at 760 nm. Total phenolic content was quantified with standard curve of Tannic acid. The results were expressed as mg Tannic acid equivalent(TAE)/g extract. The experiment was performed in triplicates.

Total flavonoid content was determined by the method used by (Sultana et al. 2012). To plant extracts, 0.3ml 10% $NaNO_2$was added. After 5 minutes, 0.3 ml 10% $AlCl_3$ and 2 ml 1% NaOH solution was added. Absorbance was measured at 510 nm. Flavonoid content was expressed as mg Chlorogenic acid equivalent/g extract.

Total Terpenoid content (Chang et al. 2012) - 150 µl 5% Vanillin – glacial acetic acid was added to plant extracts followed by 500 µl perchloric acid. The mixture was incubated at 70^0C for 45 minutes. The violet colour of the solution was measured at 548 nm. β sitosterol was used as standards to express Terpenoid content.

Thin Layer Chromatography (TLC)– TLC was carried out to identify Flavonoids and Terpenoids. To separate terpenoids solvent system Toluene: EA(3:1) was used and compared with Lupeol and sitosterol. The purple bands were visible after the plates spread with Vanilin-sulphuric acid and heating at 100^0C. For flavonoids Ethyl acetate: formic acid: water (82:9:9) was used and compared with Quercetin, caffeic acid, Luteolin, Chlorogenic acid. To locate bands plates were exposed to iodin vapours.

DPPH radical scavenging activity - The assay was carried out according to Blois (1958). DPPH (0.1mM) was prepared in 100% methanol. To 1 ml of DPPH solution, 200 µl of extract was added. The mixture was incubated for 30 minutes in dark. After dilution, the absorption of reduced DPPH was measured at 517nm (UV-VIS spectrophotometry). Methanolic DPPH was used as control. The Radical scavenging activity (RSA) was calculated in percentage by following formula.

RSA (%) = (Abs control - Abs sample /Abs control)* 100

Statistical analysis – Results of Total phenols, flavonoids and terpenoids are expressed as mean± std dev. ANOVA was carried out using Microsoft excel at 0.05 level of significance.

Result and Discussion

Qualitative tests results are presented in **Table 1** whichshows the presence of Terpenoids, flavonoids, Quinones, glycosides. Mild turbidity was observed after addition of Mayer's reagent in methanolic and ethanolic extracts of root, stem and leaves which indicate alkaloids might be present in these extracts.

Total phenolic content ranged from 5.32 ± 0.10 (mgTannic acid equivalent/g extract) in ethyl acetate fraction of root methanolic extract to 206.34 ± 0.75 which is highest in Leaf methanolic extract. Flavonoid content is lowest in ethyl acetate fraction of roots while highest in methanolic fraction of leaf methanolic extract. The results are shown in **Table 2**. Total phenolic content of the extracts was highest in methanolic extract followed by ethyl acetate and chloroform. There is substantial amount of terpenoids in different fractions of methanolic extracts of root,

stem and leaves. Purified elutes from chloroform fraction of leaf methanolic extract show higher amount of terpenoids compared to other fractions. The amount of terpenoids is represented in **Table 3**.

TLC plates results indicate the presence of Lupeol, sitosterol and other terpenoids in different extracts and purified fractions.

DPPH radical scavenging activity is significantly ($P< 0.05$) highest in methanolic and ethyl acetate fractions compared to nonpolar chloroform extracts. We could observe correlation between amount of phenolics and flavonoid content and DPPH radical scavenging activity.

There is considerable amount of total phenols and flavonoids in different extracts. Phenolicsincluding flavonoidsare accumulated in more amount in leafcompared to stem and root. Our earlier study has shown that methanolic extract of leafhas high antioxidant activity (Goggi and Malpathak 2017). This activity is attributed to presence of different types of phenolic compounds including flavonoids. High amount of phenols and flavonoids in methanolic fractions compared to ethyl acetate fractions indicate that during partitioning most of the phenolic compounds are partitioned in methanol and very less amount was detected in Ethyl acetate fraction of leaf and root extracts.

Phenolic compounds are water soluble possessing aromatic ring with one or more hydroxyl group. They are accumulated in vacuoles. Flavonoids are largest group of phenolic compounds. Nonflavonoids include monocyclic phenols, phenylpropanoids , phenolic quinones and polyphenols such as tannins and lignins. Phenolic compounds are known for their diverse biological activities. They have antioxidant (Rice-Evans et al. 1997), anti-inflammatory(Kazlowaska et al. 2010), anticancer(Sawadogo et al. 2012) and antidiabetic(Kusirisin et al. 2009) property.

Table1: Qualitative phytochemical analysis of the extracts of *Vernonia cinerea* plant parts

	Aq			Eth			Meth			Ace			EA			PE			Chl		
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
Alkaloids	-	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Quinones	-	+	+	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-

+ = detected, - = not detected; Aq= Aqueous, Eth = Ethanol, Meth= Methanol, Ace= Acetone, EA= Ethyl acetate, PE= Petroleum ether, Chl- Chloroform; L= leaf, S = Stem, R = Root.

Table 2- Total phenol and Total flavonoid content of plant extracts expressed as mean ± std dev

Extracts	Total phenolics (mgTannic acid equivalent/g extract)	Total Flavonoids (mg Chlorogenic acid equivalent/g extract)
VR Me	192.72 ± 0.16	79.07 ± 0.91
VS Me	138.96 ± 0.24	67.38 ± 1.18
VL Me	206.34 ± 0.75	104.95 ± 5.37
VLA- Me	137.52 ± 6.83	118.13 ± 0.29
VSA-Me	135.73 ± 0.43	69.87 ± 1.21
VRA-Me	48.99 ± 1.18	49.22 ± 0.08
VRA-EA	5.32 ± 0.10	9.99 ± 0.31
VL A-EA	15.17 ± 2.01	58.18 ± 1.57

VR Me, VS Me, VL Me= Methanolic extracts of Root, Stem and Leaf; VLA- Me, VSA- Me, VRA-Me = Methanolic fractions of VR Me, VS Me, VL Me; VRA-EA, VL A-EA = Ethyl acetate fractions of VR Me, VS Me, VL Me

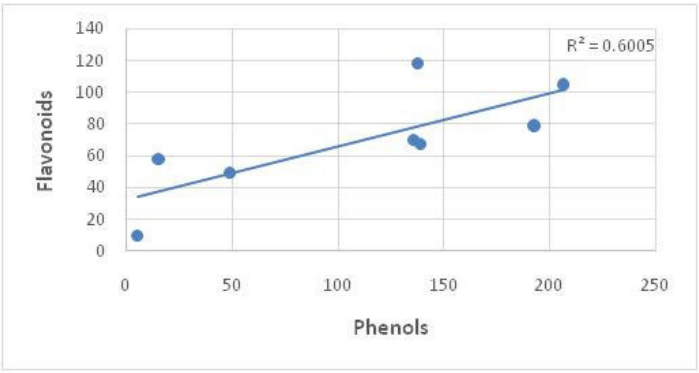
Table 3- Total terpenoid content of plant extracts expressed as mean ± std dev

Extracts	Total Terpenoids (µg β sitosterol equivalent/g extract)
VR Me	264. ± 5.55
VRA-EA	229.47 ± 3.52
VRA-Hex	271.85 ± 1.01
VS Me	189 ± 1.88
VS-Chl-a	134.71± 1.88
VS-Chl-b	122.33 ± 1.48
VS-Chl-c	-
VS-Chl-d	192.33 ± 1.48
VS-Chl-e	451.38 ± 6.63
VS-Chl-f	102.80 ± 1.48
VS-Chl-g	318.28 ± 3.53
VL Me	309.47 ± 3.59

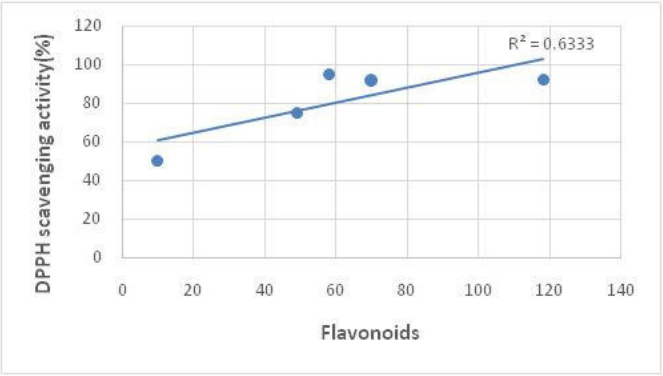
Extracts	Total Terpenoids (µg β sitosterol equivalent/g extract)
VL-A Hex	121.14 ± 6.56
VL-Chl-AA	287.57± 8.92
VL-Chl-AB	272.33 ± 2.88
VL-Chl-AC	759.47 ± 3.29
VL-Chl-AD	552.80 ± 2.70
VL-Chl-AE	433.28 ± 5.05
VL-Chl-AJ	448.52 ± 7.63
VL-Chl-AI	340.90 ± 4.36

VRA-Hex = Hexane fraction of VR Me; VS-Chl-a - VS-Chl-g = subfractions of chloroform fraction of VS Me; VL-Chl-AA - VL-Chl-AI = subfractions of chloroform fraction of VL Me

A)



B)



C)

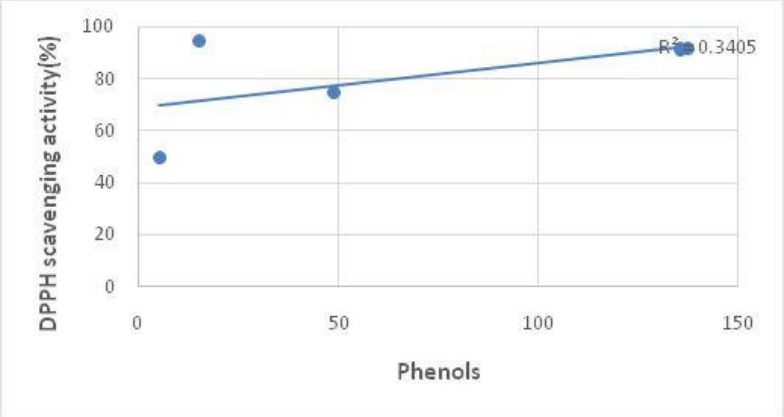


Figure 1- A) Correlation between amount of total phenols and flavonoids B) Correlation between % radical scavenging and flavonoids C) Correlation between % radical scavenging and phenols

According to (Wojdylo et al. 2007)Phenolic acids and flavonoids are major contributors to the antioxidant activity of phenolic compounds. The study has reported the presence of more amount of flavonoids in the Asteraceae species compared to species belonging to other families. It is proposed that the hydroxyl group of flavonoids is responsible for antioxidation. 3', 4' - orthodihydroxy configuration in ring B and 4-carbonyl group in ring Cis essential for radical scavenging.In addition 3-OH group or 3- and 5-OH groups in ring C adds to the antioxidant activity of flavonoids. The presence of the C2–C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B and it increases the radical-scavenging activity. In the absence of the o-dihydroxy structure in ring B, a catechol structure in ring A can compensate for flavonoid antioxidant activity.

Terpenoids are known for their diverse biological effects(Wang et al.2005). Large amount of terpenoids in the extracts and fractions indicates the dominance of terpenoid pathway in V.cinerea. Our earlier studies have shown the presence of different classes of terpenoids including steroids in methanolic extracts of root, stem and leaves. DPPH radical scavenging activity was significantly

less in terpenoid rich fractions when we compare high activity in flavonoid and phenol rich fractions which indicate phenolic compounds including flavonoids are contributing towards antioxidation.

Conclusion–Our study clearly shows that all the plant parts under investigation are rich in phenolic compounds and terpenoids which possess therapeutic importance. It is possible to correlate with the ethnomedicinal importance of this plant where whole plant is used in the treatment of several diseases.

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