Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2015; 4(4): 169-174
Received: 10-09-2015
Accepted: 14-10-2015

Sreenivasa Nayaka
Department of Botany,
Karnataka University, Dharwad.
Karnataka, India.

Gireesh Babu K
BioGenics, Veena Plaza,
P.B. Road, Unkal, Hubballi
Karnataka, India

Correspondence:
Sreenivasa Nayaka
Department of Botany,
Karnataka University, Dharwad.
Karnataka, India.

Phytochemical studies on *Catharanthus roseus* grown in Eco-friendly Feather compost

Sreenivasa Nayaka, Gireesh Babu K

Abstract
The present study investigated importance of feather compost as bio-fertilizer to increase the fertility of soil and growth of *C. roseus*. The screening of primary and secondary metabolites was carried out in the effect of feather compost on the growth of a *C. roseus* plant. The presence of feather compost has significantly increased primary and secondary metabolites as compared to control one. The crude ethanol extract of *C. roseus* plant leaves analyzed over TLC, shows prominent orange brown colour band indicating the presence of vincristine to that of standard vincristine sample. The compound was identified with alkaloid extraction of Et-OH in mass spectrum. Comparing the mass spectrum obtained with database the molecular formula $C_{46}H_{57}N_4O_{10}$ and molecular ion peak at 825 was found.

Keywords: *Catharanthus roseus*, Feather compost, vincristine, ^{1}H NMR spectra, TLC, Phytochemical.

1. Introduction
Keratin, the structural component of the integument of most birds, mammals, reptiles and amphibians, occurs commonly in nature and hence arises as a waste product in variety of ways. The disposal of large quantities of keratinous wastes from leather industries, agricultural industry or from a slaughterhouse by dumping piles of unusable animal hides in natural biological zone may lead to a severe environmental impact. The growing concern for environmental production coupled with the urgent search for potentially useful materials has led to the development of a number of technologies for the bioconversion of keratinous waste into recoverable products. Keratinous waste is very important in environment, medical and agriculture and also in textile, brewery, pharmaceutical, leather and detergent industries [1, 2]. The hair, feather and sheep wool contain approximately 90% keratin and are used in composting. It is one of the more economical and environmentally safe methods of recycling the hair and feather [3]. This waste is used as an eco-friendly bio-fertilizer in agricultural fields [4]. Biodegradation of intensively formed animal waste is now viewed as an alternative avenue for creating a viable end product with visible benefits to the primary producers in environmental and economic management strategies [5]. A combination of specific waste products and process producers via biological methods would lead not only to an improved consumable product, but also to consumer confidence in waste management practices. At present, the poultry industry manages waste (carcass, feather, hair and manure) through several disposal methods. Carcass waste is generally rendered in to bone and meat meal [6]. Hair and feather waste is also rendered by either steam or chemical treatment to produce feather meal [5, 7]. The manure is stockpiled to be solid in either untreated or treated forms to consumers as fertilizer or disposed as landfill. Disposal pits and trench burial incineration are also common methods used for disposal of diseased mortality [6]. In each of these processes, however, the outcome has limitations with respect to quality, cost efficiency [5] as well as environmental management.

C. roseus now grows wild only in Madagascar, where it is endangered by the spread of agriculture and metropolitan areas. As it provides anticancer alkaloids like, vinblastine and vincristine, *C. roseus* is cultivated on plantations in tropical countries. It may even be grown under artificial conditions in bioreactors to produce large amounts of natural materials in shorter times. The present study investigated, eco-friendly bio-fertilizer feather compost prepared [8] and applied to *C. roseus* plant. On the other hand are rich in nitrogen and amino acid contents and may boost the growth and development of plant. The available literature reveals no such attempt of using feather compost in enhancing secondary metabolites in *C. roseus*. Therefore, the present study aimed at analyzing the primary and secondary metabolites in the plant grown in presence and absence of feather compost.

2. Materials and methods

2.1. Preparation of feather compost

Chicken feather was washed with tap water and detergent followed by defatting (Chloroform: methanol) [9]. After making them fat free, the solvent was evaporated, washed three to four times with distilled water and air-dried. In the preparation of feather compost the slight changes, wheat husk was used and mixed uniformly with small pieces of feather in 1:1 ratio (wheat husk: feather) [4, 8] then, it was placed in a plastic bin. This mixture was autoclaved at 110ºC for 15 min. The preparation was then inoculated with 50 ml aqueous spore suspension (7 X 10^7 spore/ml) of *Streptomyces albus* prepared from 7 days old culture grown on SCA medium. Feather compost preparations were kept for degradation for 14 days, respectively. The pots were covered with polythene bag to avoid contamination and were regularly moistened with sterilized water to maintain the moisture content of about 60%. The degradation of feather was examined at regular intervals. After complete degradation of feather compost powder taken and applied to soil. The assessment of the impact of feather compost in pot experiments growing *C. roseus* plant were carried out in triplicate [8].

2.2. Phytochemical Studies in *C. roseus*

The feather compost treated powdered plant material was subjected for successive extraction starting from non polar solvents such as, petroleum ether (PE), chloroform, ethanol and distilled water (Aq) using soxhlet extracts. The extracts were concentrated to dryness. These extracts were used for preliminary screening of secondary metabolites.

2.2.1. Test for phenolics

(a) Phenol test

To the extract, 0.5 ml of ferric chloride solution was added and observed for the formation of intense colour for the presence of phenolics.

(b) Ellagic acid test

The extract was treated with a few drops of 5% (v/v) glacial acetic acid and 5% (w/v) sodium nitrate solution. Observe the formation of muddy yellow, olive brown, Niger brown and deep chocolate colours for the presence of phenolics.

(c) Hot water test

The slice of leaves was partially dipped in a hot water and observed the formation of intense colour at the junction for the presence of phenolics.

2.2.2. Test for tannins

(a) Tannin test

Gelatine (1% w/v) prepared in sodium chloride (10% w/v) treated with extracts and observed the appearance of white precipitate for the presence of tannins.

(b) Sodium chloride test

Few drops of sodium chloride solution were treated with extracts and observed the formation of precipitate for the presence of tannins.

2.2.3. Test for flavonoids

(a) Flavonoid test

Few magnesium turnings were added to the extract and conc. sulphuric acid dropped through the sides of the test tube. Observe the formation of magenta colour for flavonols, scarlet colour for flavones and deep cherry colour for the presence of flavonoids.

(b) Ferric chloride Test

The extract was treated with neutral ferric chloride solution and observed the formation of blackish green colour for the presence of flavonoids.

(c) Lead acetate test

The extracts were treated with lead acetate solution (10% v/v) and observed the formation of yellow precipitate for the presence of flavonoids.

2.2.4. Test for Alkaloids

(a) Mayer's reagent test

2 ml of Mayer's reagent and 1 ml of dilute hydrochloric acid were added to the extract and observed the formation of yellow precipitation for the presence of alkaloids.

(b) Wagner's reagent test

2 ml of Wagner's reagent and 1ml of dilute hydrochloric acid were added to the extract and observed the formation of white precipitation for the presence of alkaloids.

(c) Dragendorff's reagent test

2 ml of Dragendorff's reagent and 1ml of dilute hydrochloric acid were added to the extract and observed the formation of orange precipitation for the presence of alkaloids.

2.2.5. Test for steroids

(a) Salkowski test

The observed the formation of wine red colour, when concentrated sulphuric acid was added to the extracts for the presence of steroids.

(b) Liebermann-Burchard's test

To the extract, a few drops of acetic anhydride was added and mixed well and 1ml of conc. sulphuric acid was dropped from the sides of the test tube. Observe the formation of red ring at the junction of two layers for the presence of steroids.

2.2.6. Test for glycosides

(a) Keller-Killani test

The extract was mixed with a few drops of glacial acetic acid for a minute and cooled. To this, 2 drops of ferric chloride solution were added. The contents were transferred to another test tube containing conc. sulphuric acid and observed the formation of reddish ring at the junction of two layers for the presence of glycosides.

(b) Molischs' test: To the extract, 1 ml of Molisch's reagent was added and 1ml of conc. sulphuric acid dropped through the sides of the test tube and observed the formation of reddish violet ring at the junction of two layers for the presence of glycosides.

2.2.7. Test for triterpenes

(a) Salkowski test

To the extract, a few drops of concentrated sulphuric acid were added and observed the appearance of golden yellow colour to the lower layer for the presence of triterpenes.

(b) Liebermann-Burchard's test

To the extract few drops of acetic anhydride were added and mixed well. 1ml of conc. sulphuric acid was dropped from the sides of the test tube and observed the formation of red ring at the junction of two layers for the presence of triterpenes.

2.3. Isolation of Vincristine by Chemical Method

A 5 g powdered sample was mixed with 100 ml of 0.1% sulphuric acid for 15 min. Filtered and washed 20 ml of 0.1N sulphuric acid. Basified with concentrated ammonia (pH 9.0) while shaking and partitioned twice with diethyl ether (50 ml). Diethyl ether was evaporated and 5 ml of methanol was added. This sample was used for TLC profiling of the drug.

Preparation of TLC Plates

A 30 g of silica gel and 60 ml of distilled water were stirred and coated on glass plate to a thickness of 0.2 mm the plates were allowed to dry at room temperature, the plate was activated by heating for 1 h at 110ºC just prior to spotting.

Standard solution

N-Butanol: Acetic acid: Water (5: 1: 1).

Procedure

A sample of 20 µl test solution and 5 µl standard solution were applied on precoated silica gel 60 F254 of uniform thickness of 0.2 mm. The gel was developed in the solvent system to distance of ¾ of the height of the gel. The plate was dried and sprayed with Dragendorff's reagent.

2.4. Identification and characterization of Crude Alkaloid by LCMS/^{1}H NMR Spectra

A sample of 5 g of powdered plant material was suspended in 200 ml of surfactant solution in a glass beaker and sonicated for 2.5 h in an ultrasonic bath at a constant temperature of 25ºC. The extract was separated by simple filtration and residual material washed with 20 ml of pure water. The solution of combined filtrates was acidified with sulfuric acid solution to pH 3-4 and the alkaloids were precipitated with 15 ml of Mayer's reagent. The precipitate was dissolved in an alkaline solution of sodium carbonate (5%) and extracted with Et-OH. The organic layer washed with water to neutral pH, dried with Na_2SO_4 and concentrated to dryness under reduced pressure to obtain alkaloids [10].

3.0. Results

3.1. Preliminary Screening of Secondary Metabolites

The effort was made to screen the presence of secondary metabolites such as, alkaloids, phenolics, tannins, flavonoids, steroids, glycosides and triterpenoides in leaves of *C. roseus* grown in feather compost extracted in petroleum ether (PE), chloroform ($CHCl_3$), ethanol (Et-OH) and distilled water (Aq). In Phenolics the ethanolic extract of plant material displayed positive response to all three tests (Phenol test, Ellagic test and Hot water test) pointing out the presence of phenolics. The tannin and sodium chloride tests and showed the presence of tannin. Chloroform, ethanolic and aqueous leaf extracts responded positively to flavonoid test, ferric chloride and lead acetate test. The alkaloids test, chloroform, ethanol and aqueous leaf extracts reacted positively to Mayer's reagent, Wagner's reagent and Dragendorff's reagent tests showing the presence of alkaloids. Steroid's positive response to Salkowski and Leibermann-Burchard's test imparting the presence of steroids. Glycosides test shows positive response to Kellar-Kiliani and Molishsch's tests pointing out the presence of glycosides and Triterpenes test shows all the four leaf extracts have reacted positively to Salkowski, Liebermann-Burchard's tests importing the presence of triterpenes as shown in table 1.

Table 1: Preliminary screening of Secondary metabolites in the leaves of C. roseus

Sl.No	Tests	PE extract	$CHCl_3$	Et-OH	Aq Extract
1	Test for phenolics				
	a. Phenol test		-	+	-
	b. Ellagic acid test		-	+	-
	c. Hot water Test		-	+	-
2	Test for Tannins				
	a. Tannin test	-	-	+	+
	b. Sodium chloride test	-	-	+	+
3	Test for Flavonodies				
	a. Flavonoid test	-	+	+	+
	b. Ferric chloride test			+	+
	c. Lead acetate test.	-		+	+
4	Test for Alkaloids				
	a. Mayer's reagent test	--	+	+	-
	b. Wagner's reagent test	-	+	+	-
	c. Dragendorff's reagent test	-	+	+	-
5	Test for Steroids test				
	a. Salkowski test	-	+	+	-
	b. Libermann-Burchardt's test	-	+	+	-
6	Test for Glycosides				
	a. Keller-Kiliani's test	-	+	+	+
	b. Molisch's test	-	+	+	+
7	Test for Triterpenoids				
	Salkowski	+	+	+	+
	Libermann-Burchardt's test	+	+	+	+

PE: Petroleum ether; CHCl3: Chloroform; Et-OH: Ethanol; Aq: Aqueous; L: Leaf; R: Root; +: Present, -: Absent

3.2. Quantitative Estimation of Primary and Secondary Metabolites

The leaves of *C. roseus* plant grown in feather compost and control plant were extracted in Et-OH and analysed for the presence of primary and secondary metabolites and presented the results in table 2. The quantitative estimation has shown that (11.04±0.32 mg/g) proteins were present. Likewise, flavonoids was found to be present in an amount of 4.27±0.26 mg/g, phenols 2.38±0.79 mg/g, amino acids 1.23±0.76 mg/g, reducing sugars (1.02±0.6 mg/g), carbohydrates (12.5±0.12 mg/g) and tannins 3.14±0.66 mg/g and alkaloids (0.82±0.54 mg/g). In control plant protein (7.2±0.2 mg/g), reducing sugar (0.88±0.52 mg/g), carbohydrates (9.80±0.16 mg/g), flavonoids, (2.88±0.3 mg/g), phenols (1.60±0.8 mg/g), amino acid (0.96±0.26 mg/g), tannins (2.16±0.12 mg/g) and alkaloids (0.59±0.45 mg/g). In the treated feather compost the increase was observed when compared to control one.

Table-2: Effect of feather compost and control on the quantitative estimation of primary and secondary metabolites in *C. roseus* leaves.

Plant material	Feather compost (%)	Control (%)
Primary metabolites		
Amino acids	1.23 ± 0.76	0.82 ± 0.26
Proteins	11.04 ± 0.32	7.2 ± 0.20
Reducing sugar	1.02 ± 0.60	0.88 ± 0.52
Carbohydrates	12.5 ± 0.12	9.8 ± 0.16
Secondary metabolites		
Phenols	2.38 ± 0.79	1.60 ± 0.80
Flavonoids	4.27 ± 0.26	2.88 ± 0.30
Tanins	3.14 ± 0.66	2.16 ± 0.12
Alkaloids	0.82 ± 0.54	0.59 ± 0.45

3.3. Qualitative Separation of Alkaloid by Thin Layer Chromatography (TLC) Method: Among the various chromatographic technique TLC is extensively employed for drug analysis and also major active constituents of drug. The Rf values of 0.12 and 0.24 with orange brown colour were recorded feather compost growing in *C. roseus* plant (Fig 1) and compared with standard vincristine sample.

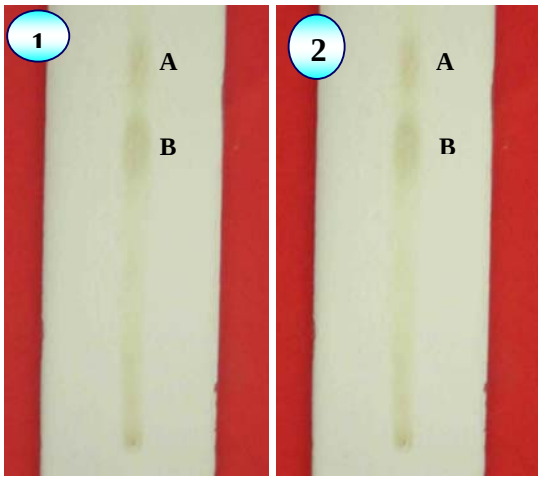
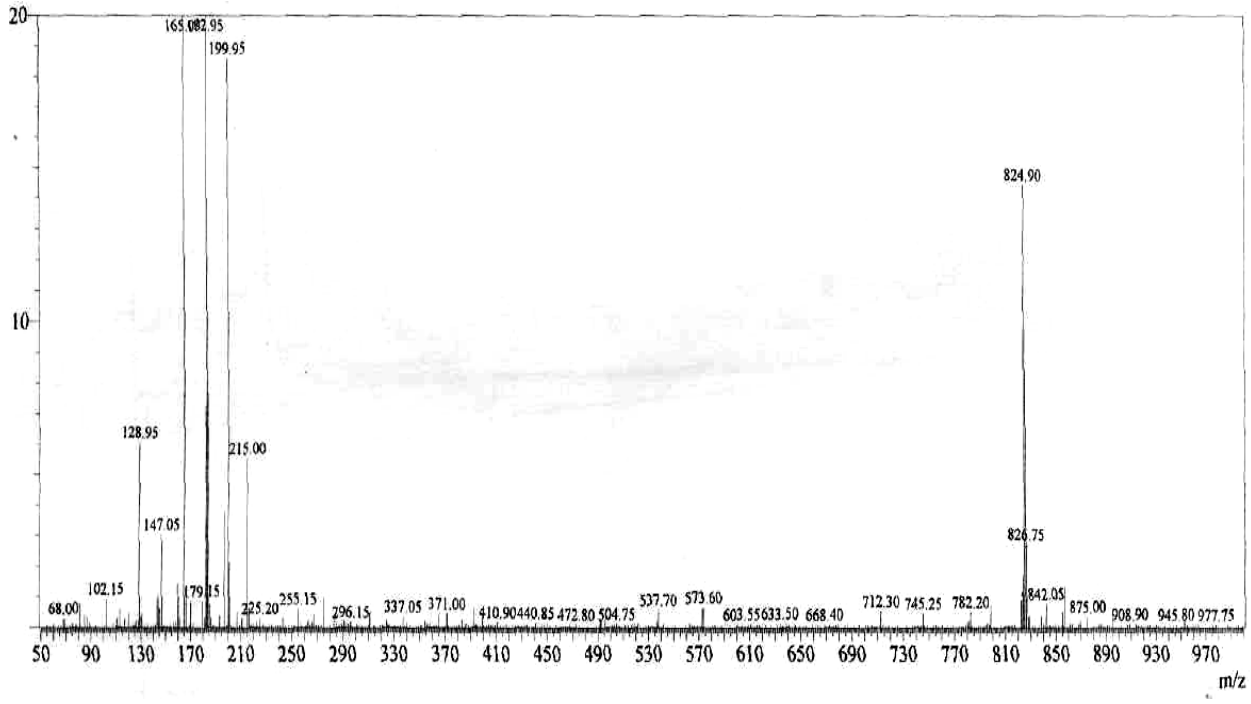


Fig 1: TLC-Profile of test solution of *C. roseus* leaf extract growing in feather compost with standard Vincristine sample. (i) Ethanolic extract and (ii) Standard (Vincristine)

3.4. LCMS/^{1}HNMR Analysis of Crude Extract: The compound was identified with alkaloid extraction of Et-OH in mass spectrum. Comparing the mass spectrum obtained with this database for the molecular formula $C_{46}H_{57}N_4O_{10}$ (Fig 2), Molecular ion peak at 825 was found. ^{1}H NMR (CDCl3 400 MHZ) spectrum (Fig 2) showed a multiplet accounting for aromatic proteins at δ 0.8 to 0.95 and δ 1.20 to 1.45, a single at δ 8.1 for aldehydic protons, a singlet at δ 9.5 for NH protons along with these peaks 1.60 to 1.76 (2H, m), 2.0 to 2.3 (2H, m), 2 to 5 (3H, s), 2-60 (1H, m), 2.80 (3H, m), 2.85 to 2.90 (2H, m), 3.25 (1H, s), 3.0 to 3.55 (2H, m), 3.70 (3H, s), 3.75 (3H, s), 4.61 (1H, s), 5.92 (1H, m), 7.0 (1H, s), 7.2 to 7.3 (3H, m) were also found. On the basis of above spectroscopic studies the structure was identified as vincristine.



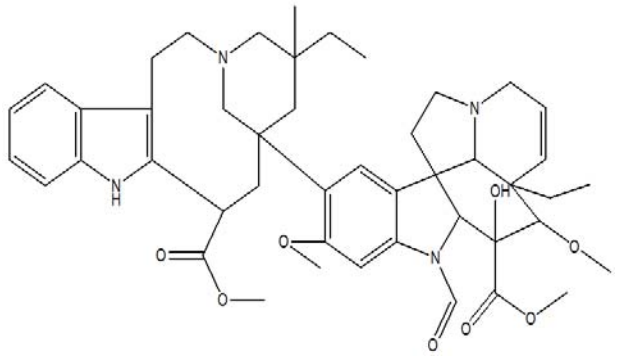


Fig 2: Elucidated structure of vincristine based on ^{1}H NMR spectra.

4. Discussion

In the preliminary tests, the primary and secondary metabolites were found to be present. Phenols displayed positive response in two tests *viz.,* hot water and Ellagic test pointed out the presence of phenolics. In hot water test, dipped leaf showed prominent brownish demarcation at the junction of dipped and undipped part. The ethanolic and aqueous extracts reacted positively to tannin and sodium chloride test importing the presence of tannins and showed white precipitation. The chloroform, ethanolic and aqueous extracts of *C. roseus* plant respond positively to flavonoid, ferric chloride, lead acetate and flavanoid tests indicating the presence of flavonoids. The alkaloid test chloroform and ethyl alcohol extracts showed positive result. Mayer's, Wagner's and Dragendroff's reagent showed the presence of alkaloid [11]. While other extracts did not show any positive response to indicate the occurrence of alkaloids. The chloroform and ethyal alcohol leaf extract showed distinct red ring in Liebermann-Burchard's test imparting the presence of steroids. Among the various extracts screened for glycosides, chloroform, ethyl alcohol and aqueous extracts have shown positive response to Kellar-Kiliani and Molisch's test pointing out the presence of glycosidies [12]. All the extracts responded positively to Salkowaski and Liebermann- Burchard's tests imparting the presence of triterpenes. Quantitative estimation of protein, flavonol, phenol, amino acid, alkaloids were done. Proteins showed more as compared to other secondary metabolites. Among the various chromatographic techniques till the date, thin-layer chromatography (TLC) is extensively employed for the rapid drug analysis and subsequent drug preparations. It is very useful for monitoring the identity and purity of drugs and for detecting adulterations and substitutions. TLC also provides semi-quantitative information on the major active constituents of a drug. In the present study, the sample displayed two distinct bands imparting orange brown colour with R_f values 6.5 and 7.2. This shows the presence of vincristine [11]. Crude plant extracts often need to be concentrated and fractioned in order to remove lipids, proteins, pigments and other plant cell compounds. Once the samples have been purified, the separation and quantification is performed. Thin-layer chromatography (TLC) and colorimetric have been used to isolate and quantify indole alkaloids of *C. roseus* [13, 14].

In the present study, ethyl alcohol extract of *C. roseus* led to the isolation of vincristine compounds. The high-resolution 400 MZ X LCMS and NMR spectral data obtained with the database for the molecular formula $C_{46}H_{57}N_4O_{10}$ and molecular ion peak at 825 was found. Recently, HPLC method was proposed to quantify these compounds in a crude extract of *C. roseus* [15]. High-performance liquid chromatography (HPLC) systems equipped with an autosampler provide a powerful tool to analyze numerous samples. The separation of indole alkaloids is based on reversed-phase chromatography using C_{18} as a stationary phase. Mobile phases usually consist of a mixture of buffer solution such as, n-heptane sulfonic acid [16, 13], diammonium phosphate [17] or ammonium acetate supplemented with triethylamine [18] and an organic phase such as, methanol or acetonitrile. Detection is performed using UV detector at fixed wavelength [16, 18] or a fluorescence detector [17]. All these methods allowed quantification of most of the indole alkaloids of *C. roseu*s. However, to study the overall secondary metabolites of *C. roseu*s, the iridoids precursors such as, secologanine and the indole precursors such as, tryptophan and tryptamine need to be quantified. Recently, a novel HPLC method was proposed to quantify these compounds in a crude extract of *C. roseus* [15]. ^{1}H NMR has both advantages and limitations as technique for metabolomics. Sample preparation is usually simple and rapid, measurement times are short and readily automated and advanced data analysis methods are available. The structure of known compounds from database may provide sufficient information for the structures of unknown compounds to be deduced. ^{1}H NMR spectrum of plant extracts are inevitably crowded because of a large number of contributing compounds with multiply signals. Each chemically distinct hydrogen atom, or group of hydrogen's, has its own chemical shift, which gives rise to the dispersion of signals across the spectrum [19]. A different technological approach has been taken for high screening of plant extracts and building of natural product libraries [20]. It employs a sequence of fractionation (fractions contain up to 5 compounds), biological activity screening to identify active fractions, further chromatography and screening to identity active compounds and characterization compounds by MS and ^{1}H NMR.

5. Conclusion

These results led to the conclusion that addition feather compost was significant resulted in to a high valued product acceptable in agricultural, as suitable organic nitrogen source obtained from feather. Applications of feather compost improve soil fertility along with other soil properties and could be an attractive natural fertilizer for plants. This method could be an alternative to farm composting to produce an eco-friendly compost.

6. References

1. Shih JCH. Recent development in poultry waste digestion and feather utilization a review. Poultry Science. 1993; 72:1617-1620.
2. Matsumoto T. Fungal diseases in dermatology. In principal and practice of clinical mycology (Eds.), Kibbler CC, Mackenzie DW, Odds FCJ, New York, J Wiley and Sons Ltd. 1996; 103-129.
3. Ichida JM, Krizova L, Lefevre CA, Keener HM, Elwell DL, Burtt EH. Bacterial inoculum enhances keratin degradation and biofilm formation in poultry compost, J Microbiol Methods. 2001; 47(2):199-208.

4. Lyndall M, Pettett, Ipek Kurtboke D. Development of an environmentally friendly biofertilizer with keratin degrading and antibiotic producing actinomycetes. Actinomycetologia 2004; 18:34-42.
5. Kim WK, Patterson PH. Nutritional value of enzyme or sodium hydroxide-treated feathers from dead hens. Poultry Sci. 2000; 79:528-534.
6. Environmental protection agency Review on farm disposed treatment risks and the potential for recycling of wastes produced form commercial chicken farms and process. Consolation draft. Produced for the environment protection. Agency by PPK. Environment and Infra structure pvt. Ltd., 1999.
7. Valtcho D, Zheljazkov. Assessment of wool waste and hair waste as soil amendment and nutrient source, J Environ Qual. 2005; 34:2310-2317.
8. Sreenivasa Nayaka, Vidyasagar GM. Development of an ecofriendly biofertilizer with keratin degrdeaing *S. albus,* International journal of Annals of plant sciences. 2013; 2(07):238-244.
9. Sangeeta Lal, Ram C, Rahak, Suresh K, Hasiju. *In vitro* degradation of keratin by 2 sps of *Bacillus,* J Appl Microbiol. 1999; 48:283-287.
10. Abdelouaheb Djilani, Belgacem Legseir, Rachid Soulimani, Amadou Dicko, Chaffique Younos. New extraction technique for alkaloids, J Braz Chem Soc. 2006; 17(3):518-520
11. Gupta AK, Tandon N, Sharma M. Quality standards of Indian Medicinal Plants. Indian council of medicinal research New Delhi. 2005; 3:307.
12. Sanjay K, Iwan J, Jesper L, Jacobsen, Anthoni J, Guttamann. Role of conformational entropy in force induced biopolymer unfolding. 2007; 19:103-111.
13. Morris P, Scragg AH, Smart NJ, Stafford A. Secondary production formation by cell suspension cultures. In: Dixon RA (Eds.), 1985;127
14. Fransworth NR, Blomster RN, Damratoski D, Meer WA, Camarato LV. Studies on *Catharanthus* alkaloids, VI. Evaluation by means of thin layer chromatography and cerric ammonium sulphate spray reagent, J. of. Nat. Prod. 1964; 27:302-314.
15. Dagnino D, Schripsema J, Verpoorte R. Analysis of several iridoid and indole precursors of terpenoid indole alkaloids with a single HPLC run. Planta Med. 1996; 62:278.
16. Bhadra R, Vani J, Shanks V. Production of indole alkaloids by selected by hairy root lines of *Catharanthus roseus*. Biotechnol Bioeng 1993; 41:581.
17. Renaudin. Analysis of *C. roseus* alaklid by HPLC. Steve Hisiger and Marrio Jolicoeur. Springer. 1985; 6:207-234.
18. Toivonen L, Ojala M, Kauppinen V. studies on the optimization of growth and indole alkaloid production by hairy root culture of *Catharanthus roseus.* Biotechnology Boeing 1991; 37:378-673.
19. Ian JC. Use of NMR for metabolic profiling in plant systems, J Pestic Sci. 2007; 32(3):200-212.
20. Eldridge GR, Vervoort HC, Lee CM, Cremin PA, Williams CT, Hart SM *et al*. 2002; 74:3963-3971.