Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6 suppl 2, 2014

Research Article

FORMULATION DEVELOPMENT, STANDARDIZATION AND ANTIMICROBIAL ACTIVITY OF AGERATUM CONYZOIDES EXTRACTS AND THEIR FORMULATION

RAJMANI PRAJAPATI[1], SUNITA ROY[1], SUDEEP MISHRA[1], S.K. RAZA[1] AND L.K. THAKUR[1]*

*[1]**Institute of Pesticide Formulation Technology (Ministry of Chemicals & Fertilizers) Sector-20, UdyogVihar, Gurgaon- 122016, Haryana (India). Email: thakurlk1964@rediffmail.com, www.ipft.gov.in**

Received: 21 Nov 2013, Revised and Accepted: 01 Feb 2014

ABSTRACT

Objective: Present study is focused on development of EC (Emulsifiable Concentrate) from extract of aerial part of *Ageratum conyzoides Linn* and evaluation of antimicrobial activity of crude extract and developed formulation. Studies of physico-chemical parameter of crude extract (methanolic) and developed formulations was carried out for standardization of crude extract as well as developed formulation. Extraction from the aerial parts of plant using different solvents were done. Development of Emulsifiable Concentrate (EC) formulations from the crude extracts using non-toxic solvents and emulsifiers and studies of their physico-chemical parameters were carried out. Comparative antimicrobial study of crude extracts and developed formulations were also done.

Methods: Extraction from the aerial parts of Ageratum conyzoides was done using different solvents like dichloromethane, methanol and hexane. Physico-chemical parameters of methanolic crude extract like loss on drying (LOD), total ash, acid soluble ash, etc. have been studied. Analysis of each crude extract (extract of methanol, dichloromethane and hexane) by HPTLC (High Performance Thin Layer Chromatography) and GC- MS (Gas Chromatography-Mass Spectrophotometry) for confirmation of bioactive compound was done. Emulsifiable concentrate (EC) was developed using bio-degradable solvents and emulsifiers. Physico-chemical parameters of these ECs were studied as per standard method. Comparative antimicrobial activity of crude extracts and developed formulations were done against *Escherichia coli* and *Staphylococcus aureus* bacteria and *Candida albicans* fungus.

Results: The GC- MS analysis result showed presence of bioactive compounds in each extract where as HPTLC showed maximum elution of components in toluene: ethyl acetate (9:1) solvent system. Developed ECs were found much more active against bacteria and fungi than crude extracts.

Conclusion: Developed formulations from aerial part of plant were found to have better antimicrobial activity than their crude extracts which can be tried in medicine formulations though lot of work is yet to be done. Physicochemical parameters and HPTLC studies were done to authenticate the adulteration for quality control of plant extracts and their formulation.

Keywords: *Ageratum conyzoides* Linn., Extraction, Physico-chemical parameters, HPTLC, GC- MS, Emulsifiable Concentrate, Antimicrobial .

INTRODUCTION

Natural plant products known as herbal medicines have long been used in control of microorganisms causing plant and human diseases [1]. Herbal medicine is readily available in our diverse vegetation which are effective, cheap and carries the potential of introducing new templates into modern medicine. Many plants synthesized substances are useful for maintenance of health in humans and other animals [2]. Micro-organisms are the causative agent of wound infections which is an important cause of morbidity in surgical patients.

Ageratum conyzoides is a small herbaceous plant which belongs to Asteraceae family. The plant is widely spread all over the world (shown in figure 1). It is very common in West Africa and some parts of Asia and South America. It is softly hairy, erect and branched annual weeds up to 80-90 cm in height. The flowers are purple to white, the fruits are achenes and easily dispersed. It has a peculiar odor that is similar to male goat in Australia and hence called 'Goat Weed' or 'Billy Goat Weeds'. It is not eaten by men due to its bad odor. The whole plant is used for medicinal purposes. The plant has insecticidal and other biological properties which may be used in agriculture [3]. The plant has long history of traditional uses in many countries in the world, especially in tropical and subtropical regions. The genus Ageratum consists of approximately 30 species but only few species have been phytochemically investigated. Extra intestinal pathogenic E. coli possesses virulence traits that allow it to invade, colonize and induce disease in bodily sites outside of the gastrointestinal tracts. This plant is a common weed easily available in field and various regions for large scale production for its commercialization. A wide range of chemical compounds including alkaloids, coumarins, chromene, flavonoids, terpines, sesquiterpenes, benzofurones, sterol and terpenoids are present in this species [4]. The leaves extract has been potentially active against bacterial infections, fungal derived skin disease and cancer of cervix, eczema, itchiness of eye and to kill lice. Efficacy of this plant has been determined against IInd and IVth star larvae of *Anapheles stephensi* [5].The ethyl acetate extract of this plant possesses antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* [6, 7]. High concentration of hexane soluble leaves extract shows larvicidal effect on *Culex quinquefasciatus* [8]. From aqueous to hexane extract of the plant, coumarin was isolated which has antifungal activity [9]. Methanolic and hexane extract of this plant shows antifungal activity against *Fusarium solani* [10].

Ageratum conyzoides is a potential allelopathic weed. Many of the secondary metabolites of this herb are biologically active. Report on antibacterial properties of extract of this plant *on Bacillus subtilis, Staphylococcus aureus, Staphylococcus mutans, Klebsiella pneumonia* etc. has been cited [11, 12]. It shows strong larvicidal activity against *Musca domestica* third instar larvae [13].

The plant can also be used as natural herbicides to control weeds to reduce the consumption of synthetic herbicides [14]. Precocene I, precocene II and coumarin compounds are the major constituents of the plant which have been reported to be biological active compounds. Precocene I, precocene II has been used as insect regulators by inducing symptom of juvenile deficiency hormones in insects [15].

Precocene II has also been reported to completely inhibit growth of two species of fungi *Rhizoctonia solani* and *Scelerotium rolfsii* [16]. Precocene II has been found to posses hypoglycemic activity and can alter some hematological element with no toxicity to the liver, kidney and spleen tissue in Sprague dowely rats [17].

Emulsifiable concentrates (EC) is one of the most widely used formulations because it has lot of advantages such as good storage stability, easy applicability, high biological activity etc. [18, 19].

Fig. 1: Ageratum *conyzoides*

In the present work, extractions of aerial parts of *Ageratum conyzoides* using different solvents (methanol, dichloromethane (DCM) hexane) were done. The crude extract was analyzed by GC-MS and HPTLC techniques to confirm the major bioactive compounds. EC formulations were developed from these extracts using biodegradable solvents and suitable emulsifiers. Physico-chemical parameters of crude extract (methanolic) and developed Emusifiable Concentrates (ECs) were done to standardize the extract and formulations. The antimicrobial study of both crude extract and EC formulations were done against bacteria and fungi.

MATERIAL AND METHODS

The aerial parts of *Ageratum conyzoides* was collected from Etawah located in Uttar Pradesh (India) in April 2012. The plant was identified by the scientist of the Institute.

Materials

Methanol, dichloromethane (DCM) and hexane (LR grade) and surfactants like polyoxyethylene sorbitan mono oleate (tween 80), Sorbian mono oleate (Span 80) were purchased from s.d.Fine Chemical Ltd. (Mumbai, India). Triton x-100 (LR) was obtained from Thomas baker pvt. Ltd. (Mumbai). Nonyl phenol ethoxylate (Emulsol PC25A, emulsol PC 25 N) were taken from Kaiser Industries Ltd, Delhi. Methy loleate was purchased from Mohini Organic Pvt. Ltd. (Mumbai). Soya bean oil and ground nut oil were purchased from local market.

Method

Extraction

The aerial parts of *A. conyzoides* at flowering stage were collected, washed, air dried in shadow and grinded by mixer grinder. After grinding, 300 gm of plant material was extracted in 1.2 liters of different solvents (methanol, DCM, and hexane) separately three times at 40˚C to 45˚C for 6 hours. The organic solvent was filtered by whatman filter paper till clear solution was obtained. Solvent was evaporated in a rotatory evaporator (Buchi, Switzerland) under reduced pressure (vacuum) at 40˚C and the semi solid crude extract was placed in a vacuum oven at 40˚C for dryness. The crude extract was stored in air tight container at dark place [20].

Physico-chemical parameters of extract

The physicochemical parameters of methanolic *A. conyzoides* extract [21, 22] like LOD (loss on drying), total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive value, water soluble extractive value. pH and pesticide residue analysis were carried out[23].

High Performance Thin Layer chromatography Technique (HPTLC)

The technique was used to separate chemical compounds present in extract. Samples of methanol, dichloromethane and hexane extract with Linamate IV applicator, bandwidth 5mm, gap 10mm among each extracts were loaded on merk aluminum plate pre coated with silica gel 60 F_{254} of 0.2mm thicknesses. The plates were developed in twin trough chamber using mobile phase toluene: ethyl acetate (9:1). The observation was taken under 254 nm, 366 nm and white light after derivatization with anisaldehyde sulphuric acid [24].

GC-MS analysis

The chromatographic procedure was performed using Shimadzu GC-MS system, GC.2010, MSQP2010 (Shimadzu, Kyoto, Japan) with auto sampler. 1000 ppm solution in methanol, dichloromethane and hexane were prepared from three extracts (methanol, dichloromethane, and hexane) and 1 µL of each extract was injected using DB -5MS column (30 meter × 0.25 mm, film thickness 0.25µm). Helium gas was used at flow rate1ml/min. as a carrier gas. The analysis was carried out using oven programming of initial temperature 50˚C for 2 minutes followed by ramp rate of 20˚C/minute up to 130˚C followed by ramp of 12˚C/min. to a temperature of 180˚C, finally raised temperature to 280˚C at 3˚C per minute and hold for 15 minutes. The ion source temperature was set at 250˚C. The injection port temperature was set as a 250˚C and the total run time was 58.5 minute. The instrument was operated in electron impact (EI) mode with electron energy 70 ev. Confirmation of analytes was done by SIM (selective ion mode) mode [25].

Development of Emulsifiable Concentrate (EC) formulation

EC formulations from different *A. conyzoides* extract were developed using methyl oleate (biodegradable solvent) to avoid toxicity of formulation. Screening of emulsifier was done carefully. Suitable emulsifiers and their mixtures (blended emulsifiers) and biodegradable solvent were mixed in the crude extract to get homogenous dark brown solution of EC. The developed ECs were dispersed in hard water (342 ppm) to check blooming. The EC formulation which showed best characteristic was selected for further studies. The detail of developed EC formulation is given in table 1. The physicochemical parameters and antimicrobial study were carried out for crude plant extract and their developed EC formulations.

Table 1: Extraction, yield and its Emulsifiable Concentrates (EC) formulation

S. No.	Extraction			Formulation				
	Extract Code	Solvent	Yield (%)	Name of Emulsifier	Emulsifier (%)	Solvent (q.s.)	Crude extract (%)	Formulation code
1	ACM	Methanol	9.33	Emulsol PC-25 A Tween-80	10 4	Methyl oleate	5	FM
2	ACD	Dichloromethane	3.47	Emulsol PC-25 A Tween-80	10 4	Methyloleate	5	FD
3	ACH	Hexane	2.40	Emulsol PC-25 A Tween-80	10 4	Methyloleate	5	FH

Physicochemical studies of EC formulation

Physicochemical parameters of developed Emulsifiable Concentrates (EC) like accelerated temperature stability (ATS), blooming, emulsion stability, cold stability, pH values and flash point were done as per Bureau Indian standard [26].The viscosity data of these formulations was measured at 100 RPM at room temperature by Rotational Viscometer (Make- Fungilab, Spain).

Evaluations of antimicrobial studies

Antimicrobial studies of different extracts (methanol, dichloromethane and hexane) of *A. conyzoides* and their EC formulations were done against two bacteria species *Escherichia coli* (Gram - ve), *Staphylococcus aureus* (Gram + ve) and one fungus species *Candida albicans*. The study was carried out by Broth Dilution method to determine minimum inhibitory concentration

(MIC). The antimicrobial study was done from Micropharm Diagnostic Center, Gandhinagar, Gujarat, India.

RESULTS AND DISCUSSION

The extract of Ariel part of *A. conyzoides* was dark brown semisolid with oily nature. The extraction and formulation details are given in table -1, Maximum yield from plant (9.33 %) was obtained in methanol. Physico-chemical parameters of methanolic extract of *A. conyzoides* were studied and are tabulated in table-2. Deterioration time depends upon the amount of water present in crude extract. If water content is high, the crude extract can be easily deteriorated due to fungus. The total ash value of plant material indicates the amount of mineral and earthly material attached to the plant material while the amount of acid insoluble ash shows siliceous matter of plant.

The water soluble extractive value indicates the presence of sugar, acid, inorganic compounds and water soluble matter while the alcohol soluble extractive value shows the presence of polar constituents like phenol, alkaloids, steroids, glycosides, terpenes, flavanoids and other organic matter.

Table 2: Pysicochemical parameters of *A. conyzoides* extract

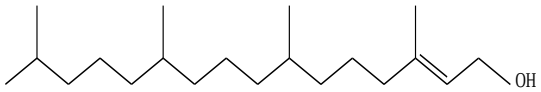
S. No.	Parameters	Results
1	Description	Dark brown semisolid with oily
2	Extraction medium	Methanol
3	Loss on drying at 105˚C	8.82 %
4	Total ash	16.95 %
5	Acid insoluble ash	3.87 %
6	pH	4.12
7	Water soluble extractive value	56.43 %
8	Alcohol soluble extractive value	34.26 %
9	Water soluble ash	96.9 %
10	Pesticide residue	Nil

The extracts of Ariel part of *A. conyzoides* (methanol, dichloromethane and hexane) were analyzed by GC-MS. The presences of components were confirmed by comparing mass spectra of analyzed components with standard mass spectra of NIST and Willey library.

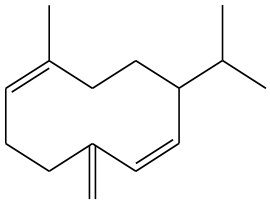
Details of analyzed components and mass fragment (m/z) ratio are given in table-3-5. Maximum number of constituents (nine) was confirmed in hexane extract of the plant while minimum number of constituents (seven) was confirmed in DCM extracts. In all three extracts precocene II was found as a major constituent as compared to other constituents. In quantitative analysis, maximum quantity of preccocene II (48.34 %) was found in DCM extract while minimum quantity in methanol extracts 31.35 %. In hexane extracts precocene II was found as 34.05 %.

The maximum unknown chemical components were found in methanol extract i.e 52.07% while minimum unknown chemical components were found in DCM extracts i.e 34.23 %. In hexane extract the unknown chemical constituents was found to be 46.33 %.

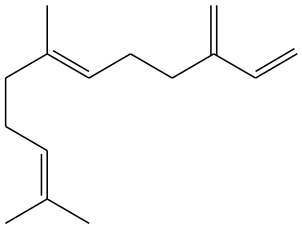
The following components of aerial parts of *A. conyzoides* extracts were :1,6 cyclodecadiene 1 methyl 5 methylene (germacrene D),4,11,11 trimethyl-8-methylene bicycle [7,2,0] undec-4-ene (trans β carryophlene),1,6,10 cyclodecacatrene 7, 11 dimethyl -3-methylene (β fernesene),7 methoxy 2, 2 dimethyl- 3-chromene(precocene I) ,5-oxatricyclo[8,2,0,0,(4,6)] dodecane 4, 12, 12 trimethyl-9- methylene (β carryophlene epoxide),6, 7 dimethoxy 2,2 dimethyl-3-chromene (precoceneII),Hexadecanoic acid, 2 hexadecan-1-ol 3, 7, 11, 15tetra methyl (phytol isomer),(6E, 10E, 14E, 18E) 2, 6, 10, 15, 19, 23 Hexamethyltetracosa 2, 6, 10, 14, 18, 22 hexaene (transsqualene). The structures of analyzed components are given below.



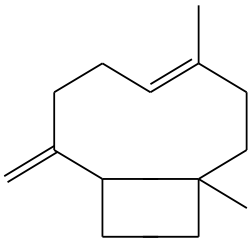
Phytol Isomer



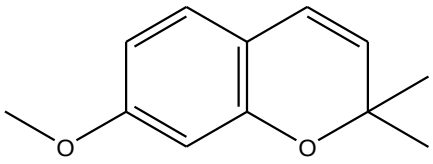
Germacrene (D)



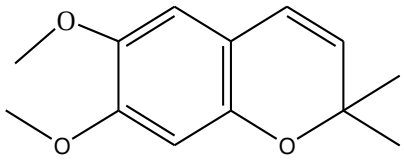
Cisβ -Farnesene



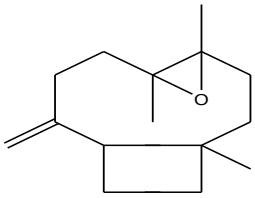
Trans β caryophyllene



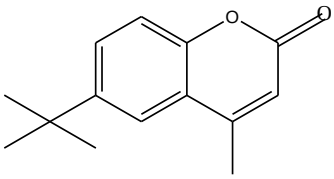
Precocene I



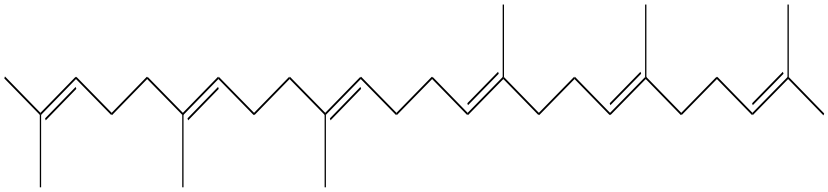
Precocene II



Trans β caryophyllene epoxcide



Tertiary-butyl cumarin



Trans Squalene

The HPTLC fingerprint observed under UV light range at 254 nm, 366 nm and white light are shown in figure 2. At 254 nm, dichloromethane extract showed 4 bands with R_f values 0.32, 0.45, 0.54 and 0.59 and methanolic extract showed 2 bands with R_f values 0.34 and 0.62 and hexane extract showed 5 bands with R_f values 0.22, 0.34, 0.47, 0.54 and 0.60. At 366 nm and dichloromethane extract showed 6 bands with R_f values 0.07, 0.12, 0.29, 0.33, 0.48, and 0.57. The methanolic extract showed 2 bands with R_f values 0.07, 0.29, 0.33, 0.48 and 0.57and hexane extract showed 3 bands with R_f values 0.08, 0.12, 0.22, 0.33, 0.47, 0.57 and 0.71.Under white light (after derivatization with anisaldehyde sulphuric acid) the dichloromethane extract showed 4 bands with R_f values 0.25, 0.47, 0.56 and 0.61and methanol extract showed 5 bands with R_f values 0.20 and 0.65 and hexane extract showed 7 bands with R_f values 0.28, 0.35, and 0.63. The HPTLC Fingerprint showed that maximum no. of bands observed at 366 nm and minimum no. of bands observed at 254nm. In present study, toxic organic solvents and emulsifiers were replaced with biodegradable oil and emulsifiers.

Emulsifiable Concentrates were developed using methyl oleate from the plant extracts. EC developed formed stable emulsion when dispersed in hard water (342 ppm). Good blooming was observed and light yellow greenish emulsion was formed. No creamy layer was formed in the emulsion even after 2 hours. The different parameters like Emulsion stability, pH value, accelerated temperature stability, cold stability, flash point etc. of developed EC passed standard tests as given in table 6. The developed ECs were stable at accelerated temperature (54˚C±1˚C) for 14 days and cold condition (i.e.10˚C for 1 hour). No phase separation or sedimentation was found in developed EC. The pH value range of crude extracts and developed EC formulations were 6.78 to 5.12 and 4.48 to 4.83 respectively. The pH values of formulated Emulsifiable Concentrates were slightly lower than pure crude extract. All developed EC did not catch fire till 85˚C. These formulations compiled flash point test as they were above (24.5˚C). The viscosity data of these ECs were found in the range of (16.8 cps to 18.4cps) at 100 rpm and room temperature.

Table 3: DCM extracts chromatogram and Mass fragmentation pattern in SIM[a] mode

Compound name	Molecular weight	Retention time	% Area	Molecular ion (m/z)	Base ion (m/z)	Other fragments ion(m/z)
Trans -β-caryophyllene	204	10.242	2.91	204	93	189, 161,147, 133, 79, 53
Cis- β- farnesene	204	10.385	.85	204	69	206, 161, 147, 133, 93, 55
Precocene I	190	10.582	1.12	190	175	144, 132, 115, 81, 77, 51
Trans -β-caryophyllene epoxide	220	12.086	1.13	220	79	177, 149, 123, 109, 69
Precocene II	220	12.878	48.34	220	205	207, 189, 173, 162, 161, 144, 119, 110, 102, 91, 85, 77, 69, 51
Phytol isomer	296	21.228	1.46	296	71	123, 95, 55
Trans squalene	410	38.103	9.96	410	69	203, 175, 149 121, 95, 68
Unknown chemical constituents			34.23			

a: Selective Ion Mode(SIM)

Table 4: Hexane extracts chromatogram and Mass fragmentation pattern in SIM[a] mode

Compound name	Molecular weight	Retention time	% Aera	Molecular ion (m/z)	Base ion (m/z)	Other fragments ion(m/z)
Germacrene (D)	204	9.878	0.35	204	161	133, 105, 91
Trans -β-caryophyllene	204	10.242	2.33	204	93	189, 161,147, 133, 79, 53
Cis- β- farnesene	204	10.378	0.78	204	69	206, 161, 147, 133, 93, 55
Precocene I	190	10.570	0.92	190	175	144, 132, 115, 81, 77, 51
Trans -β-caryophyllene epoxide	220	12.078	1.23	220	79	177, 149, 123, 109, 69
Precocene II	220	12.878	34.05	220	205	207, 189, 173, 162, 161, 144, 119, 110, 102, 91, 85, 77, 69, 51
Hexadecanoic acid	256	17.103	2.21	256	74	87, 41
Phytol isomer	296	21.228	1.84	296	71	123, 95, 55
Trans squalene	410	38.103	9.96	410	69	203, 175, 149, 121, 95, 68
Rest chemical constituents %			46.33			

a: Selective Ion Mode(SIM)

Table 5: Methanol extracts chromatogram and Mass fragmentation pattern in SIM[a] mode:

Compound name	Molecular weight	Retention time	% Area	Moleculr ion (m/z)	Base ion (m/z)	Other fragments ion(m/z)
Trans -β-caryophyllene	204	10.237	1.52	204	93	189, 161,147, 133, 79, 53
Cis- β- farnesene	204	10.378	0.39	204	69	206, 161, 147, 133, 93, 55
Precocene I	190	10.570	0.51	190	175	144, 132, 115, 81, 77, 51
Trans -β-caryophyllene epoxide	220	12.078	0.46	220	79	177, 149, 123, 109, 69
Precocene II	220	12.878	31.35	220	205	207, 189, 173, 162, 161, 144, 119, 110, 102, 91, 85, 77, 69, 51
Hexadecanoic acid	256	17.103	6.14	256	74	87, 41
Phytol isomer	296	21.228	1.53	296	71	123, 95, 55
Trans squalene	410	38.103	6.03	410	69	203, 175, 149, 121, 95, 68
Rest chemical constituents %			52.07			

a: Selective Ion Mode(SIM)

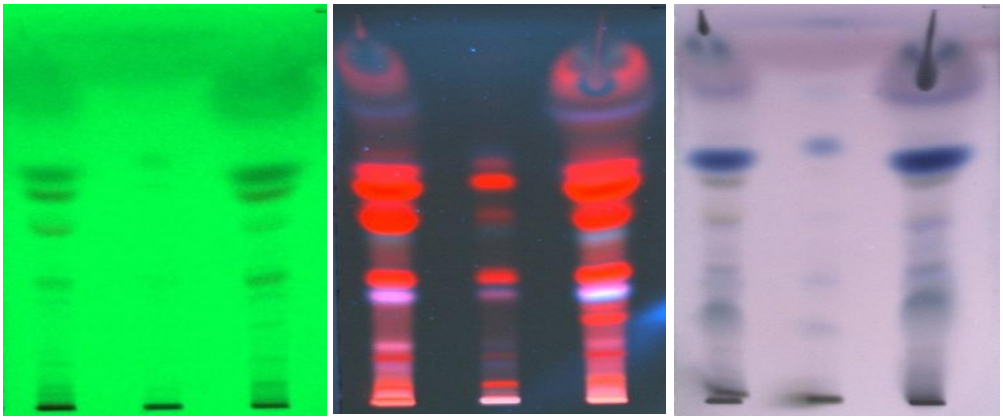


Fig. 2: HPTLC Fingerprint of extracts in mobile phase toluene : ethylacetate (9:1)254nm 366nm White light

Table 6: Physicochemical stability data of Emulsifiable Concentrate (EC) formulation

S. No.	Formulation code	Emulsion stability	Viscosity (in cps)	ATS[b] stability at 54±1˚C for 14 days	Cold stability at 10˚C for 1hr	pH	Flash point (˚C)
1	FM	Stable	18.4 ± 0.3	Pass	Pass	4.48 ± 0.1	Above 85
2	FD	Stable	17.2 ± 0.4	Pass	Pass	4.83 ± 0.1	Above 85
3	FH	Stable	16.8 ± 0.3	Pass	Pass	4.75 ± 0.1	Above 85

b: Accelerated Temperature Stability

All the extract and developed formulations showed antibacterial as well as antifungal activity. Table -7 shows that methanolic extract showed antimicrobial activity against *E.coli*, *S. Aureus* and *C. Albicans* with MIC 100 µg/ml, 200 µg/ml and 100 µg/ml respectively while developed EC (5 % w/w) gave MIC 5 µg/ml, 5 µg/ml and1.25 µg/ml against these bacteria and fungus.

Hexane extract showed MIC 200 µg/ml, 200 µg/ml and 100 µg/ml respectively while developed EC (5 % w/w) gave MIC 1.25 µg/ml, 2.5 µg/ml and 2.5 µg/ml. DCM extract showed MIC 25 µg/ml, 25 µg/mland 200µg/ml respectively while developed EC (5 % W/W) gave MIC 5 µg/ml, 10 andµg/ml 5 µg/ml against them.

The all developed EC formulations showed marked lower MIC than crude extracts against all selected bacterial and fungus strains.

Table 7: Antimicrobial data of extracts and EC formulation

S. No.	Sample code	MIC[c] (µg/ml)		
		Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
1	ACM	100	200	100
2	FM	5	5	1.25
3	ACH	200	200	100
4	FH	1.25	2.5	2.5
5	ACD	25	25	200
6	FD	5	10	5

c: Minimum Inhibitory Concentration

CONCLUSION

The plant has several chemical constituents like precocene I, precocene II, coumarin, etc.as confirmed by GC-MS. Extraction from the plant using methanol as solvent gave good yield. EC Formulation of these extracts was best possible with the help of Methyl Oleate as a solvent and mixture of non-ionic emulsifiers. These extracts as well as their formulation possess antimicrobial activity that can be used for agriculture purposes. Physicochemical parameters and HPTLC studies were done to authenticate the adulteration for quality control of plant extracts and their formulation From the above study, we can conclude that the plant extract from *Ageratum conyzoides* can be tried in medicine formulations though lot of work is yet to be done.

ACKNOWLEDGEMENT

Authors are thankful to Department of Chemicals and Petrochemicals, Ministry of Chemicals and Fertilizers, Government of India for their financial support.

REFERENCES

1. Pattnaik MM, Kar M, Sahu RK. Bioefficacy of some plant extracts on growth parameters and control of diseases in *Lycopersicum esculentum*. Asian J. of Pant Sci. and Res. 2012; 2(2):129-142.
2. Agu GC, Thomas BT. Antibacterial activity of ethanol and aqueous ext. of five Nigerian Medicinal plants on some wound pathogens. Nature and Science 2012; 10(2): 78-84.
3. Kamboj A, Saluja AK. Isolation and stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae).Int Jof PharmPharm Sci. (2011) ;2(1):94-96.
4. Okunade A L. *Ageratum conyzoides* (Asteraceae). Fitoterapia. (2002) 73; 1-16.
5. Arya N S, Chaurasia A, Shakya M, Bharti, Sahai N. Efficacy of *Ageratum conyzoides* against the control of mosquitoes. IJPSR (2011); 2(12):3235-3237.
6. Nuralih P. Chemical components and biological activities of volatile oil of *Ageratum conyzoides* L. Proceedings of the Int. Seminar of Chem. (2008); 586-588.
7. Chukwuka KS, IKheloa JO, Moody JO, Mankinde TA. The antimicrobial activity of some medicinal plants on Escherichia coli as an agent of livestock. Adv. Appl.Sci. Res. (2011); 2(4):37-48.
8. Saxena RC, Jayashree S, Padma S, Dixit OP. High concentration of hexane soluble leaves extract shows larvicidal effect on *Culex quinquefasciatus* J Environ Biol.(1994); 15(1):67-74.
9. Widodo GP, Sukandar EY, Sukrasno, Adnyana IK. A coumarin from *Ageratum* leaves (*Ageratum conyzoides* L.). Int. J.Pharmacol. (2008); 4(1):56-59.
10. Javed S, Bashir U. Antifungal activity of different extracts of *Ageratum conyzoides* for the management of *Fusarium sola.* Afr.J.Biotechnol. (2012); 11(49):11022-11029.
11. Okwori AEJ, Dina CO, Junaid S, OkekeI, Adetunji OJA, Olabode AO. Antibacterial activity of *Ageratum conyzoides* Extracts on selected bacterial pathogens, The Internet J. Microbiol. (2007);4(1).
12. UdayaPrakash NK, Bhuvaneswari S, Jahnavi B, Abhinaya K, Gulbsy Rajalin A, Prathap M, Sundraraman G, Elumalai K, Devipriya S, KannanV, Sriraman V, Kathiravan G. A study on antibacterial activity of Common Weeds in Northern Districts of Tamil Nadu, India. Res. J. Med. Plant. (2012) ;6: 341-345.
13. Calle J, Rivera A, Luis JG, Aguiar ZE, Niemeyer HM, Nathan PJ.Insecticidal activity of the petroleum Ether Extract of *Ageratum conyzoides* L. RevistaColombiana De Quimica. (1990);19(1): 91-96.
14. Tran DX, Shinkich TI, Hong NH, Khanh TD, Min CI. Assessment of phytotoxic action of *Ageratum conyzoides*L. (billy goat weed)on weeds. Crop Prot. (2004); 23:915-922.
15. Schrankel KR, Grossman SJ. Hsia MT. Precocene II nephrotoxicity in the rats. Toxicol. Lett. (1982) ; 12: 95-100.
16. Iqbal MCM, Jayasasinghe ULB, Herath HMTB, Wiljesekara KB, Fujimoto Y. A fungistatic chromrne from *Ageratum conyzoides*. Phytoparasitica (2004);32: 119-126
17. Adebayo AH, Zeng GZ, Zhang YM, Ji CJ, Akindahunsi AA, Tan NH. Toxicological evaluation of precocene II isolated from *Ageratum conyzoides* L. (Asteraceae) in Sprague Dawley rats. Afr.J. Biotechnol. (2010) ; 9(20):2938-2944.
18. KozuksY, Ohtsubo TA. Predictive Solubility Tool for Pesticide Emulsifiable Concentrate Formulations. ASTM International mem. ofCrossRef. (2009).
19. Vanitha S. Developing new formulation using plant oils and testing their physical stability and antifungal activity against *alternaria chlamydospora* causing leaf blight in *solanum nigrum.* RJAS. (2010); 1(4): 385-390.
20. Ruedeekorn W, Attawadee SY, Jiraporn P, Chitchamai O, Arunporn I. Development and evaluation of granule and emulsifiable concentrate formulations containing derris elliptica extract for crop pest control. JAFC. (2009); 57:11234-11241.
21. World Health organization, Quality control methods for medicinal plant materials. Published by WHO. Geneva(1998).
22. CCRAS Laboratory guide for analysis of Ayurveda and Siddha Formulations. New Delhi.(2010).
23. Kamboj A, Saluja A K. Microscopical and preliminary phytochemical studies on aerial parts (Leaves and stem) of *Ageratum conyzoides* Linn. Journal of Pharmacy Research. (2010); 3(3):432-434.
24. Singh A, Meena AK, Sudeep, Meena, Pant P, Padhi MM. Studies on standardization of Andrographispaniculatanees and indentification by HPTLC using Andrographolide as a marker compound. Int. J Pharm Pharm Sci.(2012) ; 4(2): 1-4.
25. Abirami P, Rajendran A.GC-MS analysis of methanol extracts of *Vernoniacinerea*.Euro. J. Exp. Bio. (2012); 2(1): 9-12.
26. BIS, specification, Indian Standard Methods of Test for Pesticides and Their Formulations, IS: 6940-1982, Reaffirmed.(1997) ;21-26.