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Design and development of herbosomes cream for the prevention and treatment of black fly bites

Chinmoy Bhuyan, Bhupen Kalita and Trishna Das

Abstract
Objective: To formulate cream containing herbosome using polyherbal extract and determine repellency effect against wild species of *Aedes albopictus* and black fly.
Methods: Herbosome was prepared using polyherbal extract in various ratios against phosphotidylcholine by rotary evaporation technique. Five formulation of herbosome were prepared. Formulations were optimized on the basis of total flavonoid content. Finally optimized herbosome was incorporated to cream.
Results: The herbosome formulation (F3) contains 92.10% total flavonoid content, so considered as optimized herbosome formulation. Similarly F1 formulation of cream was found satisfactory based on the results of viscosity, spread ability, and wash ability.
Conclusion: The herbs used in preparation of herbosomes were reported to have good repellency activity. Herbosomes of polyherbal extract incorporated into semisolid dosages form cream. The cream formulation demonstrated satisfactory properties suitable for topical application. Usefulness of the optimized cream formulation for repellant effect has to be established on wild species of black fly and *Ades albopictus* mosquito.

Keywords: Herbosome, polyherbal, phytoconstituent, cream, black fly

1. Introduction
In recent decades, several scientific works has been done towards targeted drug delivery, for development of novel drug delivery system (NDDS). Herbal drugs having a no of advantages having enhancing the solubility and bioavailability, increasing pharmacological activity, protection from physical and chemical degradation. Novel drug delivery of herbal drugs has significant scope for enhancing the activity and overcoming problems [1].
For good bioavailability natural product must have a good balance between hydrophilicity (for dissolving into the gastrointestinal fluids) and lipophilicity (to cross lipidic bio membranes). Many phytoconstituent like polyphenolics have good water solubility but are porely absorbed due to their large molecular sige, incompatibility with a process of passive diffusion or their poor miscibility with oils and other lipids [2]. Water soluble phytoconstituent mainly polyphenolics can be converted into a lipid compatible molecular complex known as Herbosome. The term itself indicate herbo means plant, while some means cell like structure.
It is a patented technology, US Patent. Herbosome are more bioavailable then simple herbal extract due to its enhanced capacity to cross the lipid biomembrance and circulation [3, 4]. A herbosome unit is a molecular level association where in primarily electrostatic forces weld the host guest aggregate (including ion dipole, dipole-dipole, and hydrogen bonding etc.) [5] Phospholipid are small lipid molecules where glycerol moiety is bonded to two fatty acid with the third hydroxyl normally one of the two primary methylenes bearing a phosphate group [6].
A black fly (sometimes called a blandford fly, buffalo gnat, turkey gnat, or white socks) is any member of the family Simuliidae. Over 1,800 species of black flies are known (of which 11 are extinct). Most species belong to the immense genus Simulium. Most black flies gain nourishment by feeding on the blood of mammals, including humans, although the males feed mainly on nectar. They are usually small, black or gray, with short legs, and antennae [7]. In India, 71 species (56 named and 15 unnamed) of black flies, all in the genus *Simulium* Laterally, have been recorded [8], of which four species are recorded from Arunachal Pradesh, the seven states in North-East India, and 16 species (eight named and eight unnamed) from Assam, a state located south-west of Arunachal Pradesh [9]. The black flies salivary extract causes unique biological effect including immunomodulation, anticoagulation and hypersensitivity reaction.

So it is of utmost important to formulate suitable dosages form to protect and heal these black fly bites. In such cases herbosomes or herbal extract novel formulation plays and immense role for prevention and protection of the diseases caused by black fly bites.

Plants were well known to be used in the tribal medicinal practices since time immemorial. More than 10,000 species of plants were patented by world health organization globally in many medicinal uses. In India (Especially north-east) represent a vast repository of diverse flora of considerable medicinal importance. Several species of plants having tremendous repellent activity against mosquito and other blood sucking insects. Among them *Flemingia strobilifera* (Makhiyoti), *Azadiracta india* (Neem), *Cuscuta reflexa* (Aakakhilota) are some of them. A special species *Polygonum hydropiper* (Bihlongoni) leaves were used by marma tribes (special tribes in Arunachal Pradesh) for repellency against black flies [10].

Materials and methods

Materials: The leaves and stem of different herb *polygonum hydropiper* (Bihlongini), *Cuscuta reflexa* (Aakakhilata), *Flemingia strobilifera* (Makhiyoti), *Azadiracta indica* (Neem) and *Cymbopogan nardus* (Citronella grass) were collected from Morigaon district of Assam. Stearic acid, Cetyl alcohol, liquid paraffin (mineral oil), triethanolamine (TEA), Glycerin, soyalecithin, Preservative (Methyl, propyl paraben), petroleum ether, ethanol and other chemical and solvents were analytical grade and procured from laboratory.

Methods

- **Extraction of selected herb species:** The extraction of selected herbs is done by simple maceration technique. First extraction is performed by using the solvent petroleum ether, as the flavonoids and terpenoids containing herbs posses fatty substances. So to remove such fatty material petroleum ether is used [11]. Fresh leaves of all the plants were collected, washed with clean water and kept the shade drying. After the leaves were dried completely, they were crushed to form coarse or moderately coarse powder. The plant material (Powder) is then kept in a closed vessel and the whole of the selected solvent (menstrum) at first petroleum ether was poured into it. The mixture is allowed to stand for seven days with occasional shaking. The liquid is strained off and the solid residue (marc) is not pressed here. After that the solid residue is dried in a news paper so that the solvent is easily evaporated [12]. After that the same residue is used for next phase of extraction using ethanol as a solvent. In case of ethanol as a solvent same procedure is followed only solid residue marc is pressed here. Strained liquid is then mixed with pressed liquid. The liquid mixture is then filtered to get clear liquid extract. The clear liquid is then subjected to rotary evaporator in order to get a solid mass to be used in the formulation. Finally extract are dried in a desiccators and hot air oven [13, 14].
- **Phytochemical Analysis:** Individual extract obtained using the solvent ethanol were subjected to the phytochemical screening of constituents by standard methods. Test for alkaloid, tannin, flavonoid, terpenoids, glycoside, volatile oil were performed in individual extract as well as combined polyherbal extract. It has been found that flavonoid, terpenoid and volatile oil were present in combined polyherbal extract.
- **Formulation of Herbosome:** The herbosome was prepared by taking ratios of herbal extract to phosphotidylcholine (PC). The formulation of herbosome is mainly prepared through rotary evaporation technique as the herbosome which are formed in this technique are stable and good in characteristics rather than other technique. In this technique at first herbal extract and soya lecithin are taken in a two different container. The herbal extract is mixed with solvent ethanol and soya lecithin is dissolved with dichloromethane. The two solution is mixed thoroughly. After that extract solution is filtered and slowly added to the previous solution and transfer to a 100 ml round bottom flask. Followed by stirring for 3hr at a temperature not exceeding 40°c.Finally a thin film of sample was obtained and solvent is evaporated [15, 16].
- **Formulation of Cream:** Cream is used as topical delivery vehicle for prepared herbosome. For preparation of topical herbosome cream it is proposed to use the following formulation composition in table-9. According to the mentioned formulation composition, all substances were weighed accurately. Then stearic acid, cetyl alcohol and liquid paraffin (oily phase) were mixed in separate beaker and melted at water bath at 60-70°c. Similarly remaining all substances except essential oil were mixed in another beaker and heated to 60-70°c. The optimized herbosome formulation was incorporated in the aqueous phase. Further molten mass of aqueous phase was added into other (oily) phase with continuous stirring and below temperature 40°c essential oil were added and then cooled down to an emulsified cream. Plant derived essential oils have been used in protection against mosquitoes and other blood-sucking insects. Many studies have documented the anti-insect potential of essential oils against a variety of insects [17]. Chattopadhyay *et al*. reported that the three essential oil cinnamon oil, eucalyptus oil and lemon grass oil were used in 2:1:1 ratio for repellent activity for mosquito and other blood-suking insects.
- **Preparation of cream containing herbosomes and essential oils:** Herbosomes was added at 2%w/w, 5%w/w and10%w/w which is incorporated in the aqueous phase of the cream. Mixture of three essential oil (Cinnamon, Lemongrass, and Eucalyptus) at 2:1:1 ratio is used in the cream. Mixture of oil is used at 1%v/v, 2%v/v and 5%v/v of cream.

Repellency Study: The efficacy of a repellent is generally evaluated under laboratory conditions in a mosquito repellent chamber. It is rectangular in shape with wooden frames fitted with glass on both sides. The top of the chamber has standard wire mesh while floor is fitted with wooden sheet. Repellency is determined against any mosquito species by releasing the required number of adult mosquitoes inside and inserting hand of subjects with the repellent applied on it. The repellency test was carried out on the best optimized formulation and triplicate study was done to check for reproducibility. Laboratory reared, three days hungry and 7 to 12 days old adult female Aedes albopictus mosquitoes were put into the repellent testing chamber (46×37×36cm) and the study was carried out for a period of 8 hours on three different days. A total of100 mosquitoes were used in each test, while the testing conditions were (24±2)°C and (65±2)%. In this

study, the left hand (without herbosome cream) was taken as control where as right hand on to which herbosome cream was applied, was used as treated. Readiness of the mosquitoes to bite was checked by exposing left hand for few seconds and the number of mosquito landing or biting (if any) was recorded. The right hand bearing herbosome cream was inserted inside the chamber to check the repellency initially after every half an hour followed by 1 hour interval. Both control and treated hand were exposed for one minute each during the trials. The results were used to calculate the percent repellency.

Repellent activity trial against black flies: Repellent trials were carried out at two locations namely Salari and Tenga in West Kameng district in Arunachal Pradesh. The black flies species recorded during the trials were Simulium himalayanse, S. indicum.

Determination of in-vivo Immunomodulatory property: Immunostimulants are the drugs those predominantly showing non-specific stimulation of immunological defense mechanisims. Most of them are not real antigens but antigenomimetics or so called mitogen causing non- specific and non-antigen dependent immunostimulation. But do not affect the formulation of a immunological memory cells [18].

Plant Materials: EXTRA IMMUNE Tablets (Charaka Pharmaceuticals) locally purchased from the medicine store. Nylon fiber purchase from local market Guwahati.Ethanolic extract of combined polyherbal extract of Polygonum hydropiper, Cuscuta reflexa, Flemingia strobilifera and Azadiracta indica used as test material.

Experimental Animals: Laboratory breed Swiss albino mice (20-25g) of either sex were housed at 25±2°C with a relative humidity of 30-70% and illumination cycle set to 12h light and 12 h dark. The animals were allowed free access to standard food pellets containing (%W/W) Protein 22.10, Oil 4.13, Fibre 3.15, Ash 5.1, Silica 1.12, and water ad libitum. Bedding material was removed and replaced with fresh paddy hask as often as necessary to keep the animal clean and dry. The animals were maintained under standard condition in animal house approved by committee for the purpose of control and supervision on experiments on animals (CPCSEA). The experimental protocol was approved by the (M.PH/PRO/06/2018) Animal Ethics Committee of Girijananda Chowdhury Institute of Pharmaceutical Science, Guwahati, Assam [19].

Formulation of test compound

Selection of Dose: It is found that polyherbal extract (Polygonum hydropiper, Cuscuta reflexa, Flemingia strobilifera, Azadiracta indica) were safe at limit dose of 2000mg/kg with no mortality in studied subjects respectively, 1/20th, 1/4th and 1/2 of this dose i.e 100mg/kg, 500mg/kg and 1000mg/kg for polyherbal extract was used in the subsequent study. Oral suspensions of the polyherbal extract were prepared by suspending them separetly in 1% solution of Sodium Carboxy methyl cellulose to obtain suitable dosages form [20, 21, 22, 23].

Carbon ink suspension: Carbon ink suspension, n was prepared from camel fountain pen ink. Ink suspension was diluted eight times with normal saline solution. Then a dose of 10µl/gm body weight of mice was recommended for Carbon Clearance Test [24].

Experimental Protocol: The drug solutions were prepared in distilled water for oral administration. Immunomodulatory activity was checked in both cellular and humoral levels. Cellular immunity was evaluated by Carbon clearance test where as humoral immunity was analyzed by Delayed type hypersensitivity test. All the experimental model had 5 (Five) common groups consisting of 2 (Two) animal each. Group 1 was served as vehicle control and received (Vehicle i.e, Normal saline 1ml/100g/day p.o). Group 2 was treated with extraimmune Tablet powdered and suspended in distilled water (100mg/kg/day p.o). Group 3, Group 4 and Group 5 administered low (100mg/kg, Oral) Medium dose. (500mg/kg, Oral) and high dose (1000mg/kg, oral) of ethanolic extract of combined polyherbal extract respectively.

Evaluation of immunomodulatory activity

Carbon Clearance test: Vehicle, Combined polyherbal extract were administered orally to the respective group of Swiss albino mice for 10 days as per experimental protocol. Forty eight hours after the last dose of the drugs animals were received intravenous injection of (10µl/gm) Indian colloidal carbon ink (Camel fountain pen ink) suspension via the tail vein. Blood samples were withdrawn from each mouse by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. A 50µl of the blood sample is mixed with 4ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660nm taking 0.1% Sodium carbonate solution as blank. The phagocytic index K was calculated using the following formula- K= (Log e OD1- Log e OD2)/15

Where, OD1 and OD2 are the optical densities at 0 min and 15 min, respectively**.**

Results and discussion

Determination of total flavonoid content: Total flavonoid content (TFC) is use to determine for the selection of optimized herbosome formulation before and after herbosome formulation. The total flavonoid content was estimated by aluminium chloride calorimetric method. The principal involved in the aluminium chloride calorimetric method ($Alcl_3$) is that $Alcl_3$ form acid stable complexs with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition it also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids. Chang.*et al* reported that quercetin to be suitable reference for determination of total flavonoid content in plant sample extract. Therefore, quercetin solutions of various concentrations were used to make the standard calibration curve.

Procedure: The method was based on the formation of a flavonoid–aluminum complex having the absorption maxima at 415 nm. 100 µL of plant extracts in methanol (10 mg/mL) was mixed with 100 µL of aluminum trichloride (20%) in methanol. A drop of acetic acid was added and the mixture was diluted with methanol up to 5 mL. After 40 min the absorbance was measured spectrophotometric ally at 415 nm. Blank sample was prepared using 100 µL of methanol in place of plant extract. The absorbance of standard quercetin solution (0.5 mg/mL) in methanol was also measured under the same conditions and the total flavonoid content (mg quercetin equivalent/mg plant extract) was calculated using the following equation:

Total flavonoid content = (A × mo)/(Ao × m)

Where, 'A' is the absorbance of plant extract solution, 'Ao' is the absorbance of standard quercetin solution, 'm' is the weight of plant extract, and 'mo' is the weight of quercetin in the solution [25].

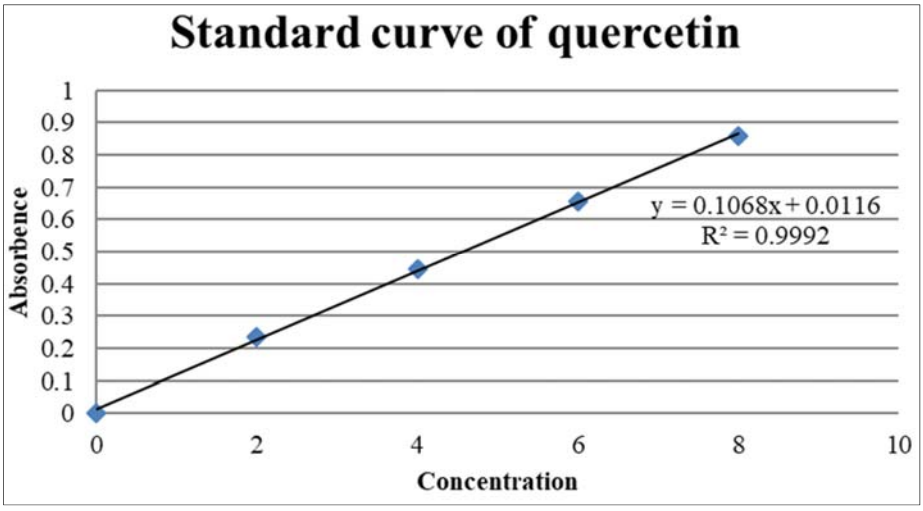

Fig 1: Standard curve of quercetin

Table 1: Determination of TFC before and after herbosome formulation

Determination of flavonoid	Total flavnoid content
Before herbosome formulation (Combined herbal extract)	95.39%
F1 (After formulation)	72.37%
F2	77.30%
F3	92.10%
F4	83.88%
F5	78.94%

From the table-1 it can be easily conclude that the total flavonoid content (TFC) of herbosome formulation F3 (1:1), contains 1gm of herbal extract and soya lecithin is more than that of other formulation. So it is the optimized herbosome formulation for this plan of work.

Table 2: Determination of percentage yield of extracted plant

Herbal Extract	Percentage yield (%)
Polygonum hydropiper	5.53%
Cuscuta reflexa	4.65%
Flemingia strobilifera	5.89%
Azadirachta indica	9.8%
Cymbopogan nardus	6.7%

Table 3: Determination of loss on drying

Name of the herb (powder sample)	%Loss in weight (w/w)
Polygonum hydropiper	0.164
Cuscuta reflexa	0.177
Flemingia strobilifera	0.163
Azadirachta indica	0.156
Cymbopogan nardus	0.161

Table 4: Determination of ash value

Name of the sample (powder)	Total ash (%w/w)
Polygonum hydropiper	9.33
Cuscuta reflexa	9.67
Flemingia strobilifera	9.55
Azadirachta indica	10.27
Cymbopogan nardus	9.89

The herbosome was prepared by taking ratios of herbal extract to PC of 0.5:1, 0.75:1, 1:1, 1:0.75 and 1:0.5 as mentioned below table.

Table 5: Formulation of different phospholipid complex

Formulation	Ratio of herbal extract to phosphotidylcholine	Solvent
F1	0.5:1	Dichloromethane + ethanol
F2	0.75:1	Dichloromethane + ethanol
F3	1:1	Dichloromethane + ethanol
F4	1:0.75	Dichloromethane + ethanol
F5	1:0.5	Dichloromethane + ethanol

Evaluation of optimized herbosome

Determination of particle size: The prepared herbosome samples were dispersed in Isopropyl alcohol by stirring on a magnetic stirrer for 10 minutes. The dispersion was analyzed in size analyzer (Malvern, Nanoseries, S90 Zetasizer).

Table 6: Determination of particle size

Formulation	Average particle size (nm) ±SD (n=3)
Herbosome (0.5:1) (F1)	328±1.44
Herbosome (0.75:1) (F2)	383±1.89
Herbosome (1:1) (F3)	547±1.74
Herbosome (1:0.75) (F4)	682±2.14
Herbosome (1:0.5) (F5)	482±2.11

From the above table it can be concluded that the average particle size varied between 328nm to 628nm for different combination of herbal extract and soyalecithin. The particle size of herbal extract was found to increase as the herbal extract fraction was increases in the complex. Except formulation (F5). The reason may be attributed to the availability of number of herbal extract molecule as compared to phospholipid molecule in contact during complex formation.

Differential scanning calorimetry (DSC study): DSC studies for herbal extract, phosphatidylcholine (PC), Physical mixture of herbal extract and phosphatidylcholine and herbosome (1:1) were performed on a Perkin Elmer differential scanning calorimeter by heating samples over a temperature range of 40-400°C in closed metal pans at the rate of10°C per minute under the environment of nitrogen gas.

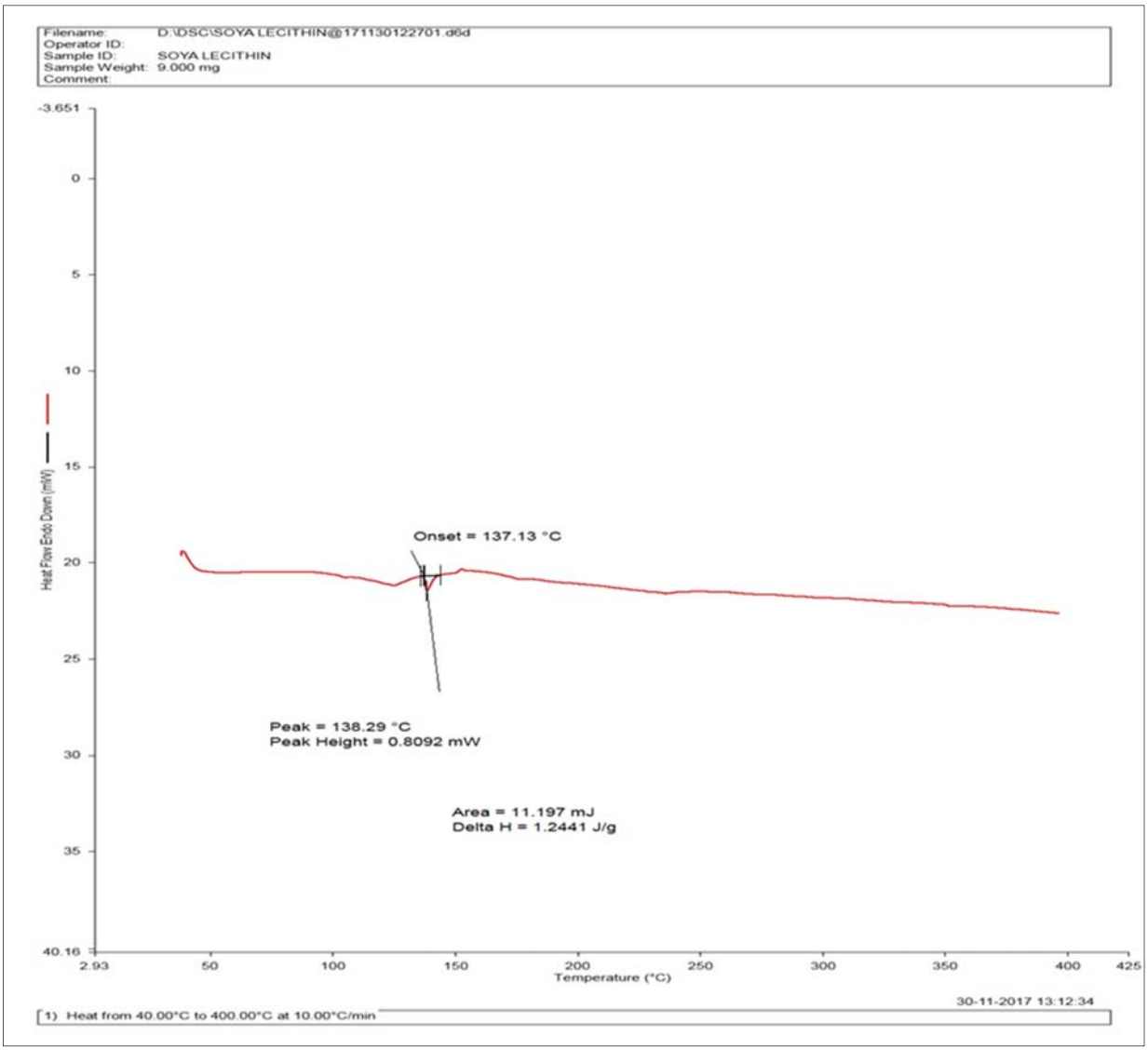

Fig 2: DSC thermogram of soya lecithin

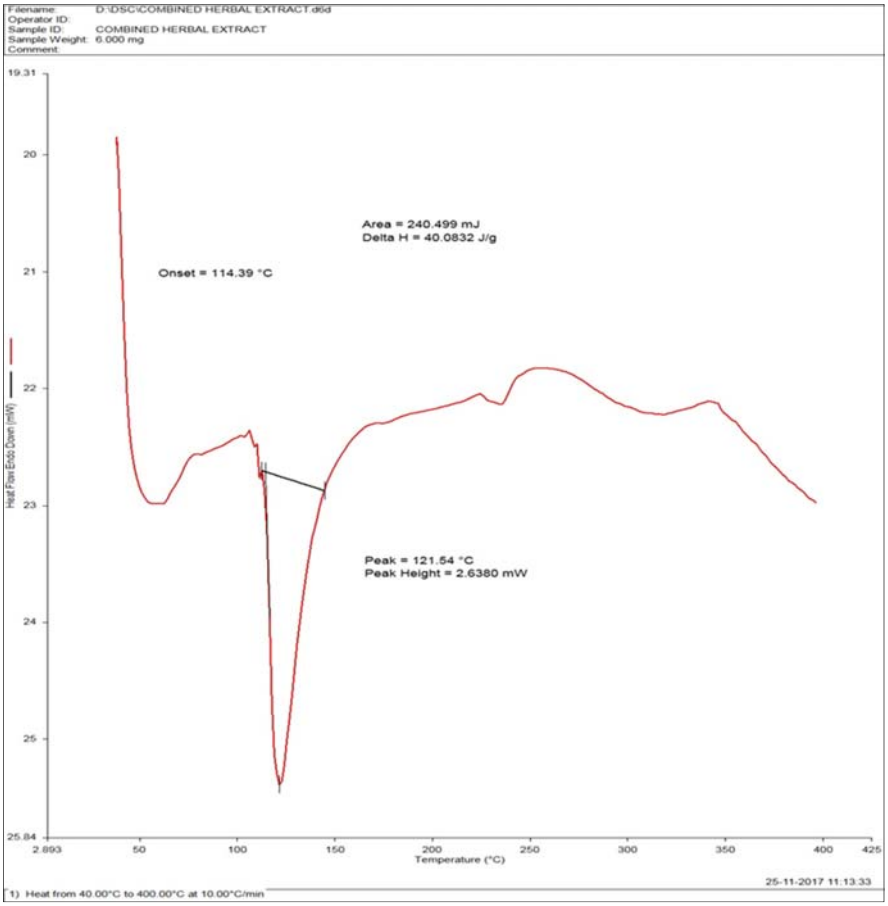

Fig 3: DSC thermogram of combined herbal extract

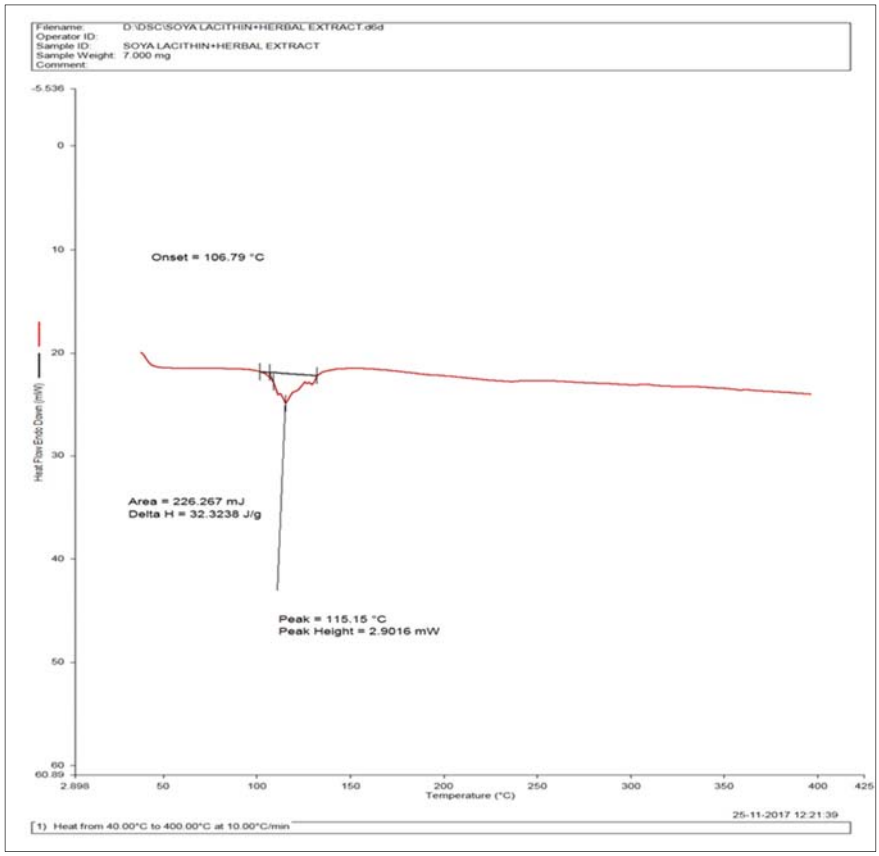

Fig 4: DSC thermogram of physical mixture (Soyalecithin+ herbal extract)

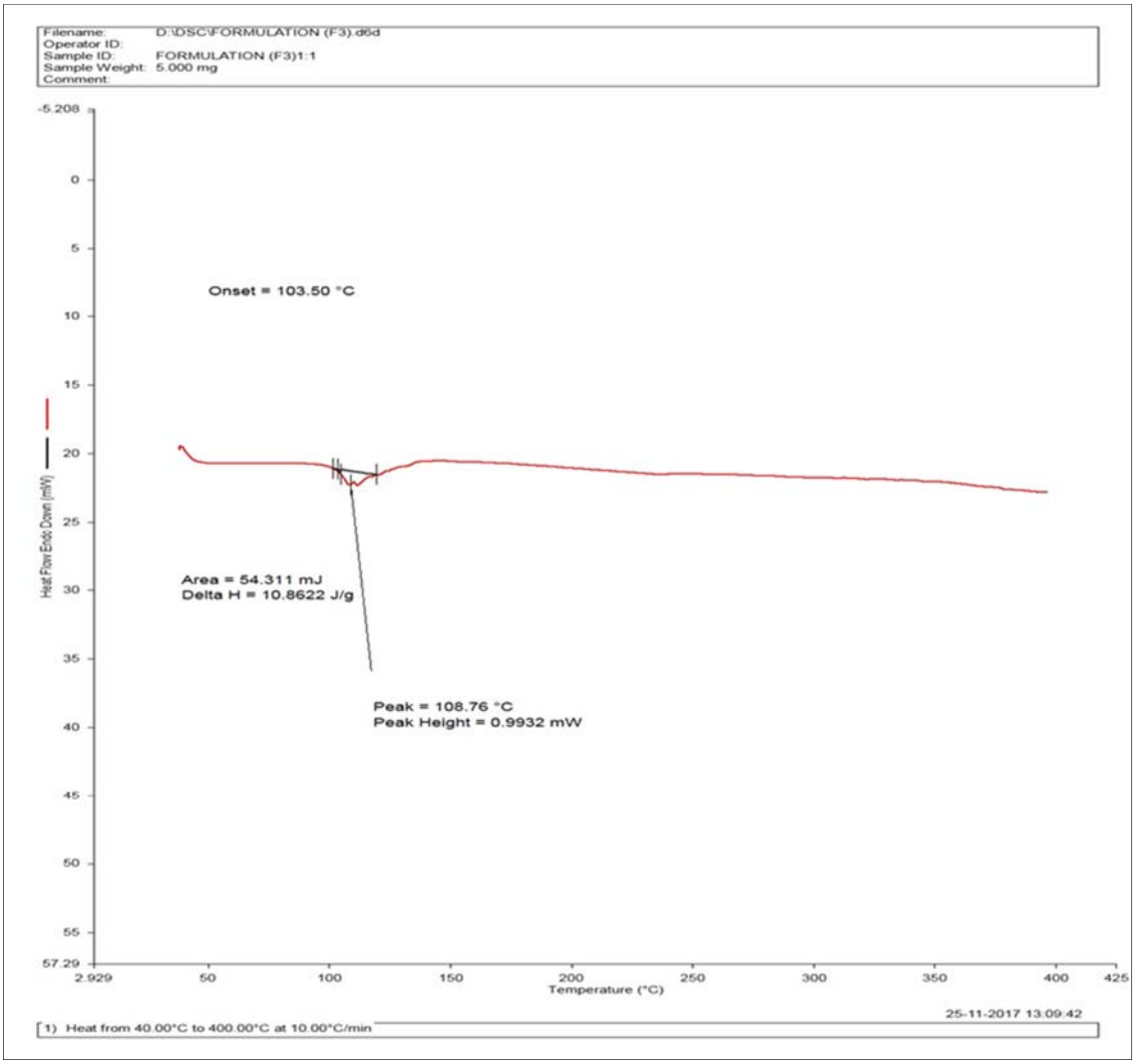

Fig 5: DSC thermogram of optimized formulation (1:1)

From the above DSC thermogram it can be concluded that phosphotidylcholine shows peaks at 138.29°C. Combined herbal extract shows broad endothermic peak at 121.54°C. The physical mixture of herbal extract and soyalecithin shows peak at 115.15°C. Which are lower than that of individual single compound. The DSC thermogram of herbosome gives a peak at 108.76°C, which was at still lower temperature than that of physical mixture. For the complexes the phase transition temperature was lower than the phase transition temperature of PC. The thermogram suggests some kind of interaction between herbal extract and phosphatidylcholine. Such interaction according to Xu *et al.,* 2009 results from combination of hydrogen bonds or van der Waals forces, but the interaction does not lead to the formation of new compound, and in other DSC curve there will be no such interaction and no such other peaks are seen.

Table 7: Formulation composition of topical herbosome cream

Ingrediants (%w/w)	F1	F2	F3
Stearic acid	6	8	4
Cetyl alcohol	2	1	3
Liquid paraffin (Mineral oil)	20	20	20
Triethanolamine (TEA)	2	1	3
Glycerin	10	10	10
Methyl paraben	0.5	0.5	0.5
Phytosome (ratio 1:1)	0.8	2	4
Essential oil	0.4	0.8	2

Evaluation of herbosome cream

Apperance: By visual inspection colour and homogeneity of the formulation were evaluated which are summarised in table.

Table 8: Determination of physical appearance of herbosome cream

Batch no	Colour	Homogeneity
F1	Creemish semisolid cream	Good
F2	Creemish semisolid cream	Good
F3	Creemish semisolid cream	Good

Determination of viscosity: The viscosity of the formulated batches was determined using a Brookfield Viscometer with spindle 25 and 64. The formulation whose viscosity was to be determined was added to the beaker and was allowed to settle down for 30 min. at room temperature before the measurement was taken. Spindle was lowered into the centre of formulation, taking care that spindle does not touch bottom of the cylindrical vessel and rotated at a speed of 10, 20, 50, 100 rpm. The viscosity reading was noted down and the averages of three readings were taken.

Table 9: Determination of viscosity

Spindle no	Formulation	RPM	Viscosity (cps)
S-25	F1	10	1710
		20	1240
		50	982
		100	875
	F2	10	1840
		20	1430
		50	1068
		100	986
	F3	10	1885
		20	1556
		50	1132
		100	1040
S-64	F1	10	1842
		20	1654
		50	1248
		100	1047
	F2	10	1886
		20	1746
		50	1332
		100	1123
S-64	F3	10	1945
		20	1789
		50	1465
		100	1145

Determination of Spread ability: The parallel plate method is the most widely used method for determining and quantifying the spreadability of semisolid preparations. The advantages of the method are simplicity and relative lack of expense. Also, the assemblies can be designed and fabricated according to individual requirements to type of data required. On other hand, the method is less precise and sensitive, and the data it generates must be manually interpreted and presented. Later, Vennat *et al*. validated the spreading diameter measurements of creams on the basis of cellulose derivatives and established the linearity of spreading diameter measurements. The linear relationship between viscosity and spreading diameter was independent of the derivative. The spreading capacity of the cream formulations was measured 48 h after preparation by measuring the spreading diameter of 1 g of the cream between two 20X20 cm glass plates after 1 min. the mass of the upper plate was standardized at 125 g. The following equation is used for the purpose-

$$[S = M.L / T]$$

Where, M = weight tied to upper slide; L = length of glass slides/distance of travel; and T = time taken to travel a fixed distance.

Table 10: Determination of spread ability of cream

Batch no	Load apply (gm)	Distance in cm	Spread ability (gm.cm/sec)
F1	15	0.720	9.16
F2	15	0.675	9.14
F3	15	0.760	9.11

Determination of wash ability: A little amount of cream was applied on hand and washed under running tap water for 10 minutes.

Table 11: Determination of wash ability of cream

Batch number	Washable
F1	Washable
F2	Washable
F3	Washable

Estimation of mosquito repellent activity (*Aedes albopictus)*

Table 12: Mosquito landing data in control and test (F1)

S. no	Application Time (min)	Landing of mosquito on control (F1)	Landing on mosquito on test (F1)
1	0	15	No Landing
2	30	14	1
3	60	12	3
4	105	10	4

Table 13: Mosquito landing data in control and test (F2)

S. no	Application Time (min)	Landing of mosquito On control (F2)	Landing of mosquito on test (F2)
1	0	10	No Landing
2	30	8	2
3	60	9	3
4	120	7	3
5	165	8	4

Table 14: Mosquito landing data in control and test (F3)

S. no	Application Time (min)	Landing of mosquito on control (F3)	Landing of mosquito on test (F3)
1	0	9	No Landing
2	30	10	No Landing
3	60	7	2
4	120	9	3
5	180	8	4
6	240	7	4
7	285	9	5

Table 15: Determination of percentage repellency in three formulations (F1,F2, F3)

S no	Formulation	Mosquito Species	Percentage Repellency (%)							
			15 min	30 min	60 min	120 min	180 Min	240 min	300 min	360 min
1	F1	*Aedes albopictus*	93.33	86.67	66.67	-	-	-	-	-
2	F2	*Aedes albopictus*	100	100	83.33	66.67	-	-	-	-
3	F3	*Aedes albopictus*	100	100	87.5	66.66	50	36.36	25	-

Table 16: Determination of Repellency index in three formulations (F1, F2, F3)

S no	Formulation	Mosquito Species	Repellency Index (RI)							
			15 min	30 min	60 min	120 min	180 min	240 min	300 min	360 min
1	F1	*Aedes albopictus*	87.5	76.47	50	-	-	-	-	-
2	F2	*Aedes albopictus*	100	100	71.43	50	-	-	-	-
3	F3	*Aedes albopictus*	100	100	87.5	80	66.67	53.33	40	-

Estimation of repellent activity against Black Fly

Table 17: Determination of percent repellency against simuliids

S. No	Formula Tion	Simulid. Spp	Percent Repellency (%) At Tenga (Arunachal Pradesh)										
			0 min	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 Min	270 min	300 min
1	F1 (2%)	*S namurivagum*	100	90.9	75	50	42.85	-	-	-	-	-	-
2	F2 (5%)	*S namurivagum*	100	100	81.81	80	62.5	66.66	42.8	33.33	-	-	-
3	F3 (10%)	*S namurivagum*	100	100	90	80	88.88	70	66.6	72.7	55.5	50	55.5

Table 18: Repellency (Average protection time in minutes±SEM) of herbosome cream against simuliid species at two different location Arunachal Pradesh

Formulation	Conc.	Average Protection time (minutes ± SEM)	
		Salari (*S indicum*)	Tenga (*S nemorivagum*)
Herbosome Cream	2%	115±6.1	120±7.0
	5%	190±7.9	210±11.0
	10%	270±8.5	300±10.2

Determination of *in vivo* immunostimulant property
Determination of carbon clearance test

Table 19: Determination of Phagocytic Index of different treatment groups (All values are expressed as Mean±SE, n=6

Treatment Group	Phagocytic Index
Group-1 (Control)	0.0262±0.0060
Group-2 (Extra immune Tablet) Standard	0.05105±0.0031
Group-3 (Polyherbal Extract 100)	0.03319±0.0024
Group-4 (Polyherbal Extract 500)	0.03687±0.0029
Group-5 (Polyherbal Extract 1000)	0.03872±0.0038

Discussion
Evaluation of prepared herbosome
Determination of total flavonoid Content
The total flavonoid Content (TFC) is calculated by aluminium chloride calorimetric method. It has been found that formulation containing (1:1) 1gm of herbal extract and 1 gm of soyalecithin shows more amount TFC. So it is selected for optimized phytosome formulation for the study.

Determination of Particle Size
The particle size of herbosome is calculated through size analyzer (Malvern, nanoseries, S90 zetasizer). It has been found that the average particle size varied between 328nm to 628nm for different combination of herbal extract and soyalecithin. The particle size of herbal extract was found to increase as the herbal extract fraction was increase in the complex. Except formulation (F5). The reason may be attributed to the availability of herbal extract molecule as compare to phospholipid molecule in contact during complex formulation.

Differential Scanning calorimetry
DSC studies for herbal extract, Phosphatidylcholine (PC), Physical mixture of herbal extract and phosphatidylcholine and Phytosome (1:1) were shown in the figure. DSC thermogram of phosphatidycholine shows peaks at 138.29°C. Combined Herbal extract shows broad endothermic peak at 121.54°C. The physical mixture of herbal extract and soyalecithin shows peaks at 115.15°C. Which are lower than that of combined extract. The DSC thermogram of phytosome gives a peak at 108.76°C, which was at still lower temperature than physical mixture. For the complexes phase transition temperature was lower than that of phase transition temperature of PC. The thermogram suggest some kind of interaction between herbal extract and phosphotidylcholine. Such interaction according to Xu et.al,2009 result from combination of hydrogen bond or vander wall forces, but the interaction does not lead to the formation of new compound, and in other DSC Curves there will be no such interaction and no such other peaks are seen.

Evaluation Of herbosome cream

Physical Apperance: It was seen that all the formulations were creemish semisolid in nature and good characteristics and good homogeneity.

Viscosity: The viscosity of the formulated batch was determined using a brook field viscometer with spindle no 25 and 64. It has been found that viscosity of the prepared herbosome cream was found to decrease in general with increase in concentration of herbosome. It is seen that there is a shear thinning effect that is if we decrease the rate of shear it increases the viscosity of cream. Viscosity of cream is inversely proportional to the rate of shear (RPM)

Spreadability: The spreadability indicates the ease with which cream is spreadable by the amount of shear. The spreading coefficient for the prepared herbosome cream is found in the asending order F1>F2>F3

Estimation of repellent activity against *Aedes albopictus* spp

It has been found that with increasing in time both percent repellency and repellency index gradually decreases for three formulations. The complete protection time of F3 formulation is more almost 5hr, Compare to F2 (2hr), and F3 (1hr).Many studies have been carried out to evaluate the repellent activity of herbal based insecticides against vector mosquitoes. Which suggest their effectiveness in repelling the mosquitoes and other biting insects. The plant based repellent either singly or combination has been proved to be useful in providing considerable protection against the hematophagous insect in the field. Pant derived repellent has been much accepted because these have no or comparatively lesser harmful effect to the user and environment.

Estimation Of repellent activity against Black Fly (Simuliids)

The average protection time (APT) Achieved with 10% concentration of herbosome cream is significantly higher than the other two concentrations (2%,5%) tested in two different locations. There was no high significant difference in the protection time produced by the similar concentration of herbosome cream among the two testing locations. No bite was observed up to two hours with 2% and up to five hour with 10% topical application of herbosome cream. However percent repellency gradually decreases after 2hr in 5% and 10% formulations. In the present study the combined polyherbal extract of herbosome cream at 10% concentration repelled the black flies for >4hr during their peak biting hours. Which indicates that topical application of these herbosome cream could provide effective repellency for 3-4hr during the active biting period of black flies.

Determination of *in vivo* Immunomodulatory properties

The phagocytic activity of raticulo endothelial systems is generally measured by the rate of removal of carbon particles from the blood stream. This indicates the stimulation of raticulo endothelial systems. The effect of combined polyherbal extracts (100mg/kg, 500mg/kg and 1000mg/kg) within different dosages form were evaluated with respect to the phagocytic activity of carbon clearance. The phagocytic index of treatment group -5 (1000mg/Kg) shows much more phagocytic index as compare to treatment group -4 and treatment group -3. So it can be concluded that combined polyherbal extract having immunostimulant property.

Conclusion

The present study aimed at successful development of herbosome cream against black flies and other blood sucking insects. Indigenous herbs Bihlongoni, Makhiyoti, Aakakhilata and Neem are selected for this research work as they reported to have antimosquito property. Extraction of crude extract of selected plants are done using solvent ethanol. The equal proportion of crude extract was used for formulation of herbosome. Herbosome was prepared by rotary evaporation technique. Based upon total flavonoid content (TFC) herbosome was optimized and incorporated The optimized herbosome (1:1) was incorporated into cream and perform repellency study against Aedes albopicta and Simuliids. It has been found that formulation F3 (10%) herbosome cream showed excellent percent repellency (%PR) and repellency Index (RI). Black flies repellency study was performed in Arunachal Pradesh. The results obtained indicates that average protection time (APT) achieved with 10% concentration of herbosome cream is significantly higher than other two concentrations (2%,5%) tested in two different locations. *In vivo* study (Determination of Immunostimulant) property is performed in Swiss albino mice, using carbon clearance test. Result suggests that as the number of dosage increases there is an increase in phagocytic index. Which indicates that the plant species which are selected for repellency study having immunostimulant property.

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Conflict of interests

Author's have no conflict of Interest.

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