Indian Journal of Traditional Knowledge
Vol 21(3), July 2022, pp 505-510

Fingerprinting of *Sahaj Vati*, additive for natural lovastatin production

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Received 02 July 2020; revised 16 July 2020

The lovastatin has been used in hypercholesterolemia and heart diseases for therapeutic as well as preventative purposes and it is naturally obtained from filamentous fungi such as *Aspergillus* through biotechnological approach. The additives in fungal media which enhances the fungal growth should also increase the production of lovastatin. Finger printing of *Sahaj Vati*, a herbo-mineral formulation comprising mixture of *Plumbago zeylanica*, *Curcuma longa*, *Shilajeet*, *Commiphora mukul* and *Clerodendrum phlomidis* powder to be added in medium for fungal growth. The fungal growth activity of both batches of *Sahaj Vati* was tested for fungal isolate of *A. flavus* by contact measure through hyphal development restraint test utilizing Potato dextrose agar (PDA) and SMKY medium followed by chromatographic and different techniques for standardization. *Sahaj Vati* has lower antifungal index & increased the fungal biomass and separation of compound was higher in mobile phase Benzene: Ethyl acetate: Pyridine: 5:4.9:0.1 & Hexane: Chloroform: Pyridine: 2.7:1. *Sahaj Vati* increased the growth and biomass of *A. flauvs* which may be used for natural production of lovastatin.

Keywords: *Aspergillus flavus*, Fimger printing, Lovastatin, *Sahaj Vati*

IPC Code: Int Cl.[22]: A61K 9/00, A61K 36/00, A61K 45/06

Biotechnology can be characterized as the controlled and intentional control of organic frameworks (in the case of living cells or cell parts) for the proficient assembling or handling of valuable items for example practice of utilizing plants, creatures and small scale living beings (microbes and Growth and so forth.) for the benefit of society. It is mostly used for the production of medicine, disease diagnosis and to remove environmental waste etc.[1]. United State Food and Drug Administration (USFDA) suggested plant inferred sedate as magnificent option in contrast to engineered drug because of their faster rate of development and cheaper prices[2]. Plant inferred items are mind boggling synthetic blends, progressively being searched out as therapeutic items, nutraceuticals and makeup[3,4]. According to World Health Organization (WHO), about 80% of the total populace utilizes spices and other customary medications for their essential social insurance[5]. In addition, the herbal formulations are increasing far reaching agreeableness as restorative specialists for liver sicknesses, diabetics, arthritis, adoptogens and memory enhancers[6]. Thus, evaluation of the security, adequacy and nature of home grown medications are essential for their worldwide harmonization, which requires standardization of herbal product at point of collection, processing and after completion[7].

Metabolic syndrome is a major public health issue, characterized by dyslipidemia, hypertension & hyperglycemia[8] and lovastatin is therapeutically as well as preventatively efficacious in different disorders such as hypercholesterolemia, peripheral arterial disease, peripheral vascular disease and ischemic heart disease[9]. It has been stated that use of different additives into culture to improve production of lovastatin has been reported[10]. Thus, we tried to explore some natural product as additive to improve lovastatin production by increasing fungal biomass. Literature indicates the anti hypercholestremic and anti-obesity activity of *Plumbago zeylanica*, *Curcuma longa*, *Shilajeet*, *Commiphora mukul* and *Clerodendrum phlomidis*[11]. Thus, compound formulation (*Sahaj Vati*) prepared from aforementioned plant parts can be tested over

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filamentous fungi *A. flavus* for enhancement of natural production of lovastatin[12]. Therefore, the present investigation has been undertaken to observe the effect of poly herbal formulation "*Sahaj Vati*" (SV) on the growth of *A. flavus* biomass.

Material and Methods

Chemicals & equipments

Prepared thin layer chromatographic plate (TLC Silicagel 60 F_{254}, made in Germany, imported and marketed by Merck Pvt Ltd. Mumbai), Methanol, Benzene, Ethyl acetate, pyridine, Chloroform, TLC Chamber, UV chamber (Perfit India), hydrochloric acid, Potato dextrose agar (PDA), Plate count Agar (PCA), Violet red bile agar, Salmnella agar and Mullet Hilton Agar, SMKY (Sucrose 200 g; $MgSO_4.7H_2O$, 0.5 g; KNO3, 0.3 g and yeast extract, 7 g in 1 L medium) Autoclave, micropipette, microtip (1 mL. & 100 micro-liters) Laminar air flow chamber, Incubator, Monsant type (Make: Singhla) hardness tester, Friabulator.

Preparation of *Sahaj Vati*

SV I & II were set up according to standard operative procedure by utilizing Suddha Shilajeet, Suddha Guggul, Haridra and Chitrak as fixings and seven bhavana (levigation) of Agnimantha kwatha (decoction) with slight alteration in pharmaceutical innovation[13]. *SV*- I contents *Shilajeet*, *Guggul*, *Haridra* and *Chitrak* in equivalent extent (25% each by weight) while in *S V-II Shilajeet* (44.7%), *Guggul* (44.7%), *Haridra* (4.8%) and *Chitrak* (5.8%) by weight[14].

Hardness

The hardness test was performed by utilizing hardness analyzer. The instrument quantifies the power required to break the tablet by anvils of the instrument. The tablet was set between two anvils; power applied to the anvils, and the devastating quality that worthy motivations the tablet to break was recorded. The devastating quality test was performed on 20 tablets from each formulation.

Friability

The Friabilator (Roche type) comprises of a plastic chamber partitioned into two sections and spins at 25 rpm. Twenty tablets are gauged, set in the tumbling chamber and turned for four minutes of 100 insurgencies. During every insurgency the tablets tumble from a separation of six crawls to experience stun. After 100 unrests the tablets are again gauged. The misfortune in weight shows the friability. The satisfactory furthest reaches of weight reduction ought not be over 0.8%. The friability (f) is given by: f = 100 (1-wo/w), where, wo = beginning load of the example before friability and w = weight of the examples after friability test for each formulation.

$$\text{Friability} = \frac{\textit{Tablet weight before friability} - \textit{Tablet weight after friability}}{\textit{Tablet weight after friability}} X\ 100$$

Ash value

3 g of accurately weight samples of *Shilajeet*, *Guggul*, *Haridra*, *Chitrak* and two batches of *SV* was taken in to silica crucible placed over electric muffle furnace at 450°C for 90 min. After self-cooling silica crucible was removed and colour as well as weight of the Ash was observed.

Acid insoluble ash

The ash acquired was overflowed with 25 mL of hydrochloric corrosive (~70 g/L) for 5 min and afterward sifted with ash less filter paper to isolate insoluble issue. Gathered insoluble issue was washed with high temp water and touched off at about 450°C till consistent weight. The substance of corrosive insoluble debris in mg per g of air-dried material was determined.

Weight variation

Weight variation test was carried out by weighing 20 tablets individually using electronic. The average weight of the tablets was then calculated. The percentage of weight is determined with the assistance of following equation.

$$\% \text{ of Weight variation} = \frac{\text{Individual weight} - \text{Average weight}}{\text{Average weight}} X\ 100$$

Thin layer Chromatography

For preparation of methanolic extract samples of both *SV*, were dissolved in methanol and TLC study was carried out by using mobile phase I and II Benzene: Ethyl acetate: Pyridine: 5:4.9:0.1 & Hexane: Chloroform: Pyridine: 2.7:1 respectively.

Microbial load

Microbial load was calculated following "Manual of methods of analysis of Foods" Food Safety and Standards authority of India, Ministry of Health and Family Welfare Government of India, New Delhi 2012[15].

Sample preparation

For sample preparation, 1 g of both two *SV* was transferred to a sterile glass taste tubes filled with 9 mL. of saline distilled water and mixed thoroughly. The suspension acted as stock. Further, samples were diluted by serial dilution method.

Preparation of Potato Dextrose Agar (PDA)

The medium comprises 300 g potatoes, 20 g dextrose and 15 g agar for every liter, and the pH was acclimated to 5.6±0.2.

Platelet Count Agar (PCA) medium

The PCA medium was used for the total vival count, which was made out of 5 g casein catalyst hydrolyses, 2.5 g yeast separate, 1 g dextrose and 9 g agar for each liter and the pH was changed in accordance with 7.0±0.2.

Activity against *A. flavus*

The fungal activity of both batches of *SV* was verified against *A. flavus* isolate by contact measure through hyphal development restraint[16] using PDA solution. 10, 50 and 100 mg both batches of *SV* were dissolved in 10 mL of distilled water and 0.5 mL of this solution was added to 9.5 mL liquid PDA in various petri plates to accomplish last focuses (0.20 to 1.0 mg/mL). Furthermore, PDA plates rewarded with Wettasul-80 (1 mg/mL) were utilized as positive control. Control plate contained just PDA. A 5 mm plate of *A. flavus* (obtained from seven days old culture) was placed upside down on focal point of the plate and was hatched in the dark at 28±2°C for 7 days. Antifungal index was determined as the following-

$$\text{Antifungal index \%} = \left(1 - \frac{Dt}{Dc}\right) \times 100$$

Where, Dt: the diameter of growth zone in the test plate; Dc: the diameter of growth zone in the control plate.

Fungal Biomass

Requisite amounts of batches of SV were added to 25 mL SMKY medium to accomplish the various focuses from 1000 to 10000 µg/mL. The medium was immunized independently with 20 µL spore suspension (10^6 spores/mL) of *A. flavus* confined from *Pistachia vera* and brooded for ten days at (27±2°C). The substance of each flask was separated and dried in oven at 120°C before biomass measurement.

Result

S V II has higher hardness and passed the friability test but *S V I* has not passed friability test because of lower hardness. The variation in weight was more in *S V* II (Table 1). In chromatographic study showed the similar separation of compounds in mobile phase II & III (Table 2) and both SV was free from mocrobes (Table 3) Both *S V* has lower antifungal index (Table 4) and increased the fungal biomass at1000 µg/mL (Table 5).

Discussion

Friability of tablet is defined in term of tolerance of shock during packaging & transportation whereas hardness indicates the probability of loss on handling and transport. From Table 1, it is clear that *SV*-I do

Table 1 — Ash value, acid insoluble ash of weight variation, Hardness & Friability *SV*

Formulation	Weight variation (Mean ±SD)	Ash value	Acid Insoluble Ash	Hardness (Kg/cm-1)	Friability Test
SV-I	1.30±.034	0.44±0.13	0.036±00	1.2±0.16	Fail
SV -II	4.48±0.22	0.26=15	0.033±.00	14.66±2.05	Pass

Table 2 — R_f values of *SV* in different mobile phase

Name of Sample	R_f value in Mobil phase-1 (Benzene: Ethyl acetate: 2: 3)	R_f value in Mobil phase -2 (Benzene: Ethyl acetate: Pyridine: 5: 4.9: 0.1)	R_f value in Mobil phase -3 (Hexane: Chloroform: Pyridine: 2.7: 1)
SV-I	0.47, 0.52, 0.73, 0.79, 0.91	0.59, 0.73, 0.91, 0.82, 0.86	0.84, 0.93, 0.66, 0.76, 0.84, 0.91
SV-II	0.47, 0.52, 0.57, 0.82, 0.91, 0.76	0.53, 0.59, 0.62, 0.73, 0.78, 0.82, 0.69, 0.80, 0.86, 0.93	0.53, 0.66, 0.71, 0.84, 0.91, 0.76, 0.84, 0.91, 0.98

R_f Retention factor

Table 3 — Microbial load in *SV*

Name of Sample	Bacterial Count	Fungal Count	*E. Coli*	*Salmonella* sp.	*Staphylococcus aureus*	*Pseudomonas aurigenosa*
SV-I	No	No	No	No	No	No
SV-II	No	No	No	No	No	No

Table 4 — Antifungal Index of *SV I* and *II* on *A. flavus*

Sample name	Concentration (mg/mL)	Diameter (cm) (Mean ± SD)	Antifungal Index (%)
Control	------	5.87±0.12	
SV-I	1	6.70±7 1.54	15
	5	6.67±1.04	13
	10	6.47±0.50	10
SV- II	1	6.27±0.65	6.8
	5	6.50±0.50	10
	10	6.47±0.50	10

Note: Values are Mean ±SD

Table 5 — Effect of *SV* over *A.* flavus biomass

Formulation	Concentration(µg/mL)	Fungal Biomass (g) (Mean ± SD)	% Growth Inhibition
Control	-	0.309±0.01	-
SV-I	1000	0.290±0.02	-6.14
	5000	0.387±0.07	25.24
	10000	0.436±0.02	41.10
SV- II	1000	0.320±0.03	3.5
	5000	0.483±0.02	56.31
	10000	0.513±0.02	66.01

Note: Values are Mean ±SD

not passed friability test with lower hardness whereas *SV*-II passed friability test with higher hardness. Higher hardness of *SV*-II is due to higher concentration (44.7%) of *Guggul* and *Shilajeet* having high binding capacity[17,18]. Thus by adding suitable binding agent in *SV*-I and *SV*-II either will be broken before administration or dissolving in water at time of administration for better pharmaceutical and therapeutic efficacy. Total ash represents inorganic contents in any formulation whereas acid-insoluble ash indicates acid insoluble inorganic content. Higher inorganic content indicates adulteration of raw ingredients by substances such as silica etc. Inorganic content of both *SV* is was very less indicating the presence of adulterant like silica in very small amount.

Thin layer chromatography is a semi quantitative analytical technique based on the principle of separation and used in the field of phytochemicals, biochemistry etc. to identify the components. Separation of compound during thin layer chromatography depends on relative affinity of compound towards stationary and mobile phase i.e., it depend on either solubility of compound in mobile phase or adherence to stationary phase and separated compounds are visualized as spots on the plate. Number of spot on TLC plate was more in mobile phase III in comparison to mobile phase I & II in both *SV*. Furthermore, higher number of spot was present in *SV*-II as compare to *SV*-I in same mobile phase, suggesting the presence of extra compounds in *SV*- II as compare to *SV*-I. This was further confirmed through Infra-red and UV-visible spectroscopy. Furthermore, if the compound is soluble in mobile phase, it will travel up on TLC plate (higher R_f value) whereas if the compound likes the fixed stage, it will adhere to it that will cause it not to move far (lower R_f value). The compound with the larger R_f value is less polar due to sticking to the stationary phase[19]. Thus we can assume that compounds of *S V* are polar in nature because of higher R_f value in all mobile phase (Table 2).

Lovastatin is well established anti hypercholesterolemic drug[20], naturally produced by filamentous fungi like *A. flavus*[21]. The microbial creation of Lovastatins has given a great helpful operator to hypercholesterolemia and lovastatin obtained by *A. flavus* UICC 360 increases HDL along with reducing cholesterol, triglycerides etc.[22] and also economical in comparison to chemically synthesized lovastatin[10] dragging the attention of researcher to natural production of efficacious hypocholesteromic drug. The most commonly agreed postulate suggested that secondary metabolites are formed when large amounts of primary metabolic precursors, such as acetate, malonate, pyruvate, and amino acids, accumulate[23]. The fungus gets rid of these precursors by diverting them into secondary metabolites[24,25] and this may be the reason of lovastatin production as secondary metabolism. Further, it is also well

established that aflatoxin, a chemical secreted by *A flavus* is toxic and carcinogenic. It has been observed that both SV increased the fungal biomass suggesting that its help in fungal growth (Table 5) and it has been previously reported that Aflatoxin, which is carcinogen, is also produced by fungus[26-29]. So we have assessed the antiaflatoxic potential of both *SV* and found that both *SV* inhibited 96% of Aflatoxin B_1 production[14]. This indicates that *SV* significantly reduces aflatoxin B_1 whereas it increases anti-fungal index as well as fungal biomass (Table 5). It has been stated that numerous added substances have been put into culture to improve creation of lovastatin[10], thus we assume that *SV* may be one of the additive used for enhancing the growth of *A. flavus* as along with higher percentage of lovastatin. Quantitative estimation of lovastatin should be done from the *A. flavus* by using *SV* as additive in growth media for further research.

Conclusion

Binding agent added in *SV-I* during preparation and *SV-II* should be broken or dissolve in water at time of administration have better pharmaceutical & therapeutic effect. Both *SV*-I and *SV*-II differ in their composition and may be used as additive during culturing *A flavus* for enhancing growth without producing aflatoxin B_1 and may enhance natural production of lovastatin.

Acknowledgement

The authors are thankful to the Head, Department of Rasa Shastra & Bhaishjya Kalpana, Faculty of Ayurveda, Institute of Medical Sciences and Head Department of Botany, Institute of Science, Banaras Hindu University for providing opportunity to carry out experiment.

Conflict of Interest

Authors declare they do not have any conflict of interest.

Authors' Contributions

The concepts and design of article by KDY and AKD, written by KDY and critically reviewed by NKD, AKC and VKY. All authors read and approved the final manuscript.

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