Extraction of Stigmasterol (saponin) and Synthesis of Their Nanoparticles From Safflower (*carthamus tinctorius* l. Var. Ssf-708) Seeds for its Anti-Acne and Anti-Dandruff Activity

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Introduction:

The most important bioactive secondary metabolites are alkaloids, saponin, flavonoids, tannins and phenolics (phytochemicals) [1]. Saponins are well-known natural surfactant, traditionally used as a detergent among these phytochemicals. They also exhibit a variety of biological activities including antimicrobial activities [2]. Based on the carbon skeletons, saponins are classified into triterpenes and steroids [3]. Saponins are group of compounds which have a number of pharmacological activities including anti-inflammatory, anti-oxidant, insecticidal and anti-microbial [4-9].

In today's basic problems, besides causing deadly diseases, microbes also cause skin infections like acne, dandruff, localized skin rashes, infections in and around nails etc. In therapeutic treatment, phytochemicals present in plant extracts have great significance with antimicrobial properties [10]. The potent antimicrobial activity of two triterpenesaponins isolated from the funicles of *Acacia auriculiformis* against various pathogenic organisms has been reported by Mandal*et al*. [11].

Safflower (*Carthamus tinctorius* L.) is well known since ancient time as a Kusum with efficient medicinal and herbal properties. This Rabi crop is mainly grown for its oil. *Carthamus* species originate from Southern Asia and is known to have been cultivated in China, India, Iran and Egypt; India being the largest producer. Plant's flowers are medicinally used for cerebrovascular, cardiovascular and gynecological problems [12]. Presence of few unwanted anti-nutritional factors (ANFs i.e. glycosides) in seeds makes it tasteless. On the other hand, these ANFs may have other biological activities [13].

Recently, nanotechnology has become increasingly important in the biomedical and pharmaceutical areas as alternative antimicrobial strategy due to re-emergence infectious diseases and the appearance of antibiotic-resistant strains especially within Gram negative microorganisms [14]. Biosynthesis of green nanoparticles using plant extracts is an interesting area in the field of nanotechnology, which has economic and eco-friendly benefits over chemical and physical methods of synthesis [15]. Reducing the particle size of metals (typically not greater than 100 nm in size, and high surface-to-volume ratio) is also an efficient and reliable tool for improving their biocompatibility, which facilitates their applications in different fields such as bioscience and medicine [16]. Plant surfactants i.e.

saponins are extensively used for synthesis of silver nanoparticles without any special reducing agent/capping agent. The green synthesis of Ag nanoparticles involves three main steps, which must be evaluated based on green chemistry perspectives, including selection of the solvent medium, selection of environment friendly reducing agents, and selection of non-toxic substances for the stability of Ag nanoparticles [17]. Sometimes the synthesis of nanoparticles using various plants and their extracts can be advantageous over other biological synthesis processes which involve the very complex procedures of maintaining microbial cultures [18]. Nanoparticles made from low toxicity immune-stimulating saponins Gg6 and Ah6, isolated from Kazakhstanian plants *G. glabra*and *A. hippocastanum* have been investigated for their abilities to stimulate humoral and cellular immunity to glycoproteins of H1N1 influenza by intranasal immunization route [19].A nano formulation for anticancer saponin with chitosan for an enhanced and sustained release has also been developed [20].

So the objective of the current research is to extract steroidal saponin (ANFs) from safflower seeds and synthesize their nanoparticles for their further use in the analysis of anti-acne activity. The current work in safflower has not been yet reported scientifically for the best of our knowledge.

2. Materials and Methods

2.1 Materials

Safflower seeds of variety SSF-708 were collected from Oilseed Research Institute, Hyderabad, India. Stigmasterol and Silver Nitrate were purchased from Sigma-Aldrich chemical company, St. Louis, USA. HPLC grade acetonitrile, water and silica 20*20 aluminum sheets were obtained from Merck, India.

2.2 Preparation of seed extract

Seeds were carefully washed/rinsed, air-dried and grounded into powder by mortar and pastel with liquid nitrogen and stored at room temperature. Successive extraction of the sterol based compounds from the seeds of Safflower were performed via solvents in a range of nonpolar - polar i.e. petroleum ether, chloroform, ethyl acetate and methanol. Selected extract was air dried to get concentrated for further analysis of different class of compounds.

2.3 Phytochemical analysis

Different solvent seed extracts were subjected to phytochemical analysis to detect the presence of biomolecules i.e. alkaloids, terpenoids, saponins, flavonoids, tannins, glycosides, steroids, proteins and carbohydrates using the standard qualitative procedures as described by Trease and Evans [21].

2.4 Extraction of sterol rich compounds from seed sample

Ethyl acetate extract (highly rich in saponin compounds) of safflower seeds was further analyzed for extraction of sterol based compounds through two phase separation method i.e. ethyl acetate and water [22]. Aqueous phase rich in saponins was further partitioned by diethyl ether and butanol.

Butanolic phase has been obtained rich in sterol based [23-26].

2.4.1 Characterization of sterol compounds

The sterol rich butanolic extract (SB extract) was characterized by thin layer chromatography (TLC), Fourier Transform Infra-Red Spectroscopy (FTIR) followed by High Performance Liquid Chromatography (HPLC). TLC was performed by loading butanolic seed extract on aluminum coated silica sheets compare with the standard solution of stigmasterol in the solvent system of petroleum ether: chloroform (9:3) and visualized using vanillin sulphuric acid reagent. FTIR was done to identify the functional groups present in the butanolic extract [27]. FTIR spectra of solvent extract were recorded via Bruker Alpha-T spectrometer by keeping the liquid drop directly on the glass of instrument through which laser passes. Spectra were collected at room temperature, ranges from 400 to 4000cm^{-1}and the characteristic peaks with their functional groups were detected.

SB extract was subjected to HPLC for characterization under binary conditions performed by Shimadzu LC-10A (Japan) system with a UV–Vis detector set to a wavelength of 254 nm. The sample injection volume was 20 µL. Separation was carried out on a Luna C-18 Whattman ODS 5 column. The mobile phase consisted of acetonitrile and water used at a constant flow rate of 1 ml/min. Chromatographic analysis was performed Shimadzu class VP series software.

2.5 Green synthesis of sterol based silver nanoparticles

Silver nanoparticles were prepared as described by Arunachalam*et al*. [28]. Isolated SB extract was used for the bio-reduction process. Nanoparticles of SB extract were prepared via addition of 5mL of concern extract to the 95 ml of 1 mM aqueous silver nitrate in 250 ml Erlenmeyer flasks. In dark at 150 rpm the flask was incubated on a rotary shaker. Color changes in their colloidal solutions have confirmed the synthesis of silver nanoparticle.

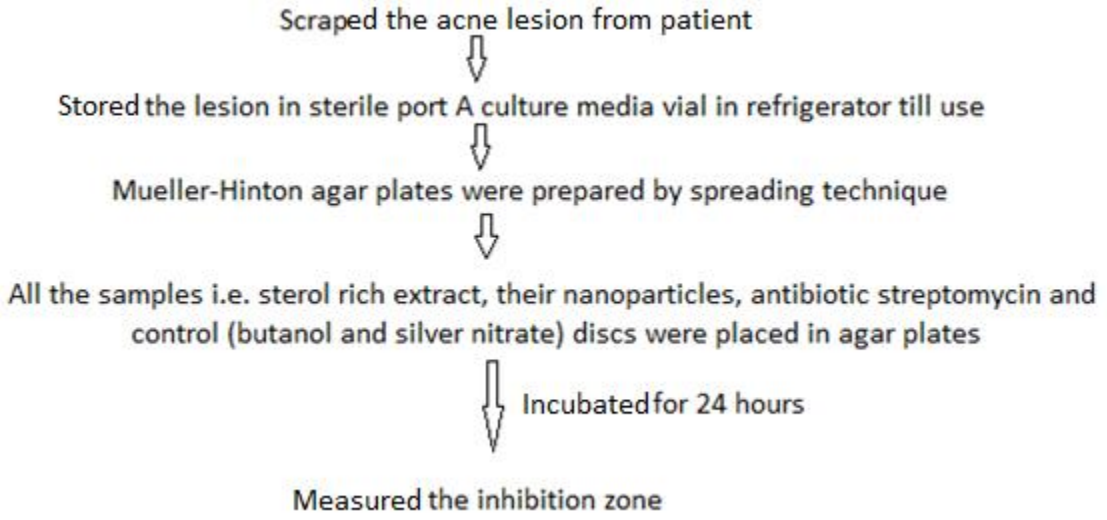
2.5.1 Characterization

Nanoparticles bio-reduction by silver nitrate was monitored using a double beam UV spectrophotometer, FTIR Spectroscopy, Zeta sizer and potential and their particle size was analyzed by SEM. The samples were subsequently measured in UV-Vis spectrum at regular time intervals at room temperature between 200-800 nm using Perkin Elmer Lambda 750 UV-Vis –NIR spectrophotometer using Milli Q water as reference [29,30]. Further FTIR was analyzed for the group reduction in comparison to the SB extracts [27]. FTIR spectra of AgNPs were recorded using Bruker Alpha-T spectrometer. Spectra were collected in the range of 400 to 4000cm^{-1}at room temperature [31].Zeta Potential and Zeta sizer were used for accurate information (stability of sterol based nanoparticle). The experiment was carried out in particle size analyzer (Malvern Zetasizer-90 Nanoseries) with DTS0012 cuvette for particle size analysis and DTS1070 cuvette was for the analysis of AgNPs potential. Scanning electron microscopy (SEM) (TESCAN)

analysis was done with fine powder of the extracted sterol Nano-fractions coated with the palladium. In the SEM analysis, an electron beam was focused into the affine probe and subsequently raster scanned over a small rectangular area. The energy of the electron beam was continuously adjusted to suit the examination in progress.

2.6 Comparative anti-acne activity of sterol based seed compounds and their nanoparticle

Anti-acne activity was performed by the method of Maatalah*et al*. [32] and Collins and Lyne [33] as shown in above graphical pathway. The minimum inhibitory concentration (MIC) of the SB extract and their nanoparticles (in triplet) were determined for the acne causing microorganisms. 0.1 ml of varying diluted (2,4,6 and 8 times) concentrations of this SB extract and their nanoparticles were taken in test tubes, loaded with 2 ml of nutrient broth followed by loopful of acne lesion. The procedure was repeated for the acne lesion using the standard antibiotic (streptomycin) without plant extracts (standard). A tube with nutrient broth only for the acne lesion served as control.

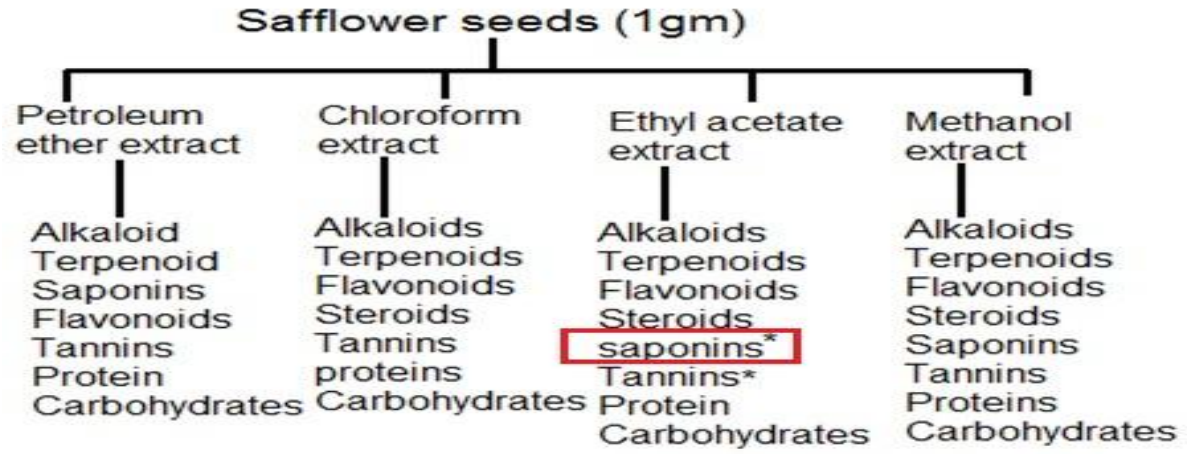


Briefly, the Mueller-Hinton agar (HiMedia, Mumbai, India) plates were inoculated by spread plating. Sterile filter paper discs (Whatman No 3 mm in diameter) were soaked with isolated SB fraction, their nanoparticle and control (butanol and $AgNO_3$) and after five minutes drying they were placed at the agar plates with standard antibiotic (streptomycin) followed by incubation at 37°C for 24 hours under the vertical laminar air flow (Labnicsequipments, L60B) After incubation, microbial growth inhibition was estimated in millimeter by measuring inhibitory zones around the discs. The anti-acne activity was done in the triplicates.

3. Results And Discussion

3.1 Phytochemical analysis

The phytochemical analysis of ethyl acetate seed extract showed the maximum presence of saponins in safflower seeds than methanol, petroleum ether respectively while in chloroform extract it was absent. The presence of saponins was confirmed by the foam test.



Note- * represented the higher presence

The ethyl acetate extract has also shown the presence of alkaloids, terpenoids, flavonoids, tannins, steroids, proteins and carbohydrates besides saponin (Table 1). The present work was concerned with the presence of ANFs in the form of saponins and tannins in safflower seed and proceded with sterol based compounds as a present demand of the work.Similarly, Zhou *et al*. [34] also found the compounds like phenylethanoid glycosides, steroids, flavonoids, coumarins, polysaccharides and fatty acids in extracts of safflower.

Table 1 List of Phytochemicals analyzed in ethyl acetate seed extract of Safflower

S. No.	Tests	Methods	Seeds material
1.	Alkaloids	Mayer's test	+
		Wagner's test	+
		Dragendorff's test	+
2.	Terpenoids	Libermann-Buchard test	++
3.	Saponins	Foam test	+++
4.	Flavonoids	NaOH test	+
		Lead acetate test	+
5.	Tannins	Braymer's test	+
		$FeCl_3$ test	++
		Lead acetate test	++

6.	Steroids	$CHCl_3$ test	+
7.	Proteins	Ninhydrin test	++
		Nitric acid test	++
		NaOH test	++
8.	Carbohydrates	Benedict's test	++
		Mollisch test	++
		Fehling's test	++

3.2 Characterization of sterol compounds

Normal and reversed-phases thin layer chromatography provides excellent qualitative information for estimation of saponins in plant material [35]. The qualitative analysis i.e. TLC played an important role in the identification of saponins.Thin layer chromatography of sterol compounds presents in the extracted seed samples and with stigmasterol as a standard showed the presence of violet and yellow brown colored spots by vanillin reagent. The Rf values for these spots were calculated as 0.685 for the standard and 0.69 for safflower seed extract (Fig. 1).

Fig. 1. TLC fingerprinting profile of SB extracts of *Carthamustinctorius*(CB) and stigmasterol standard (S).

FTIR spectroscopy is sensitive and reliable for detection of bio-molecular composition [36]. The SB extract was passed into the FTIR and the functional groups of components were separated based on their peak ratio. The FTIR analysis showed characteristic peaks at 3325.73, 2930.01, 2869.95, 1460.39, 1377.01, 953.64, 899.42, 733.92 corresponding to alcohols, alkanes, alkanes, alkanes, alkanes, alkenes, alkenes, alkenes, alkenes respectively (Fig. 2). Similar work was also reported in aqueous methanol extract of sea grasses in infrared spectra from the mid-infrared region (4000-400 cm^{-1}) [37]. Verma*et al*. [38] has also detected steroidal saponin isolated from the leaves extract of *Asparagus racemosus*through FTIR spectra which showed the presence of C-H

stretch, C=O carbonyl stretch, C-O Stretch of carbonyl etc.

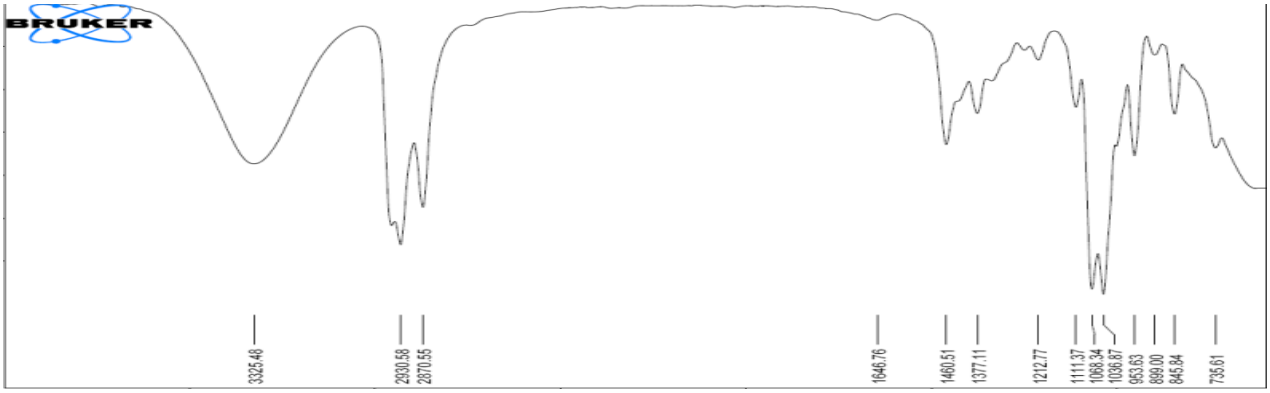


Fig. 2. FTIR peaks of SB extract

HPLC technique has been used efficiently for the characterization of various saponins and other glycosides present in crude plant extract [39]. Separation of different saponins can be affected by HPLC using variety of stationary and mobile phases [40].The chromatographic behaviors of steroidal saponins of *Asparagus officinalis, Convallariamajalis and Digitalis purpurea* have been effectively studied by HPLC-MS on C_{18} reverse-phase column and aqueous methanol or aqueous acetonitrile as mobile phase in gradient manner with or without the addition of 1% acetic acid [41].HPLC quantification of Stigmasterol as a standard, showed retention time at 6.775 min (fig. 3) while SB extract showed retention time at 6.790 min (fig. 4) indicated the presence of different sterol rich compounds like Stigmasterol and other glycosides derivatives as their chromatographic profile being quite similar, differing only in the relative abundance and presence or absence of some other phytosterols. Earlier studies have shown saikosaponin a, c, and d compounds in *Bupleurumfalcatum*throughreversed-phase (ODS C18) column [39]

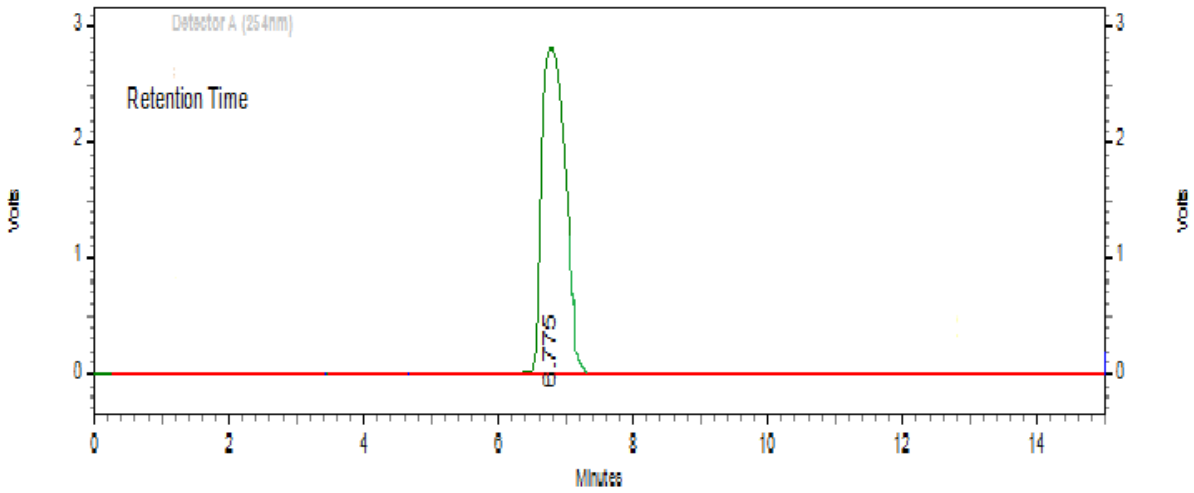


Fig. 3. HPLC chromatogram of standard Stigmasterol

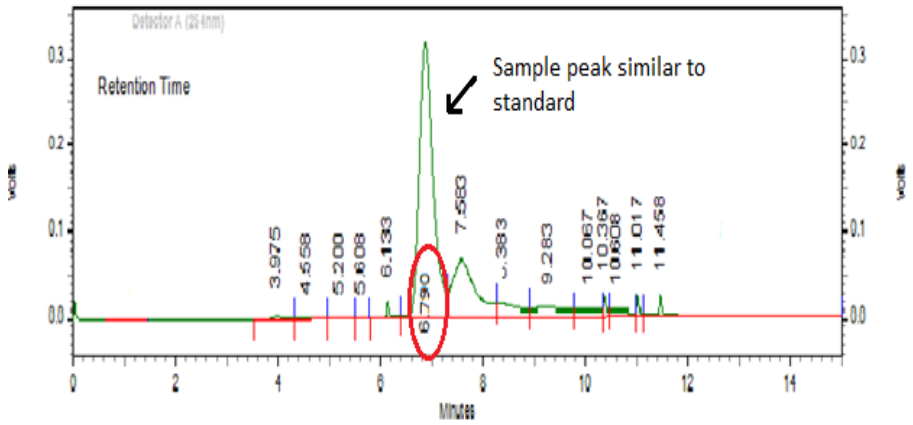


Fig. 4. HPLC chromatogram of SB extract

3.3 Green synthesis of sterol based silver nanoparticles

Green silver nanoparticles can be advantageous over other biological synthesis processes to maintain microbial cultures [42].Nanoparticles made from low toxicity immune-stimulating saponins Gg6 and Ah 6, from *G. glabra*and *A. hippocastanum*were used for their abilities to stimulate humoral and cellular immunity to glycoproteins of H1N1 influenza by intranasal immunization route [43].A nanoformulation for anticancer saponin with chitosan for an enhanced and sustained release has also been developed [44].Green synthesis of silver nanoparticles of the isolated SB extract (might be rich in saponin) was performed as by Arunachalam*et al*. [28]. Fig. 5 showed the reduction of silver ions into silver nanoparticles during exposure to plant extracts as a result of

change in color. The color change was due to the Surface Plasmon Resonance phenomenon.

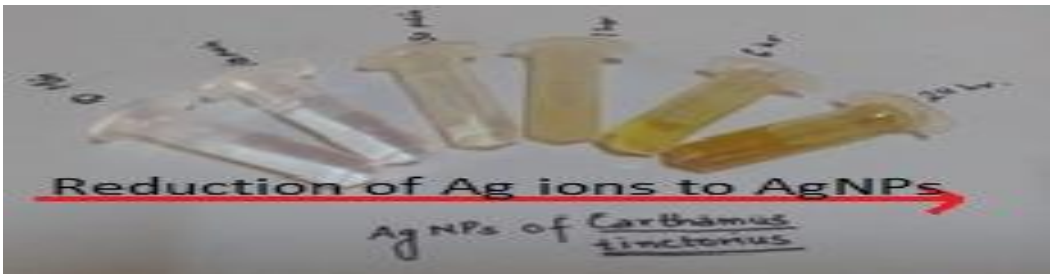


Fig. 5.Synthesis of silver nanoparticle in the SB extract

3.3.1 Characterization of sterol based nanoparticles

The UV-Vis analysis of the silver nanoparticles showed the maximum absorbance at 421nm, depicting that SB extract has the maximum ability to reduce Ag+ ions at this wavelength after 24 hours (fig. 6).

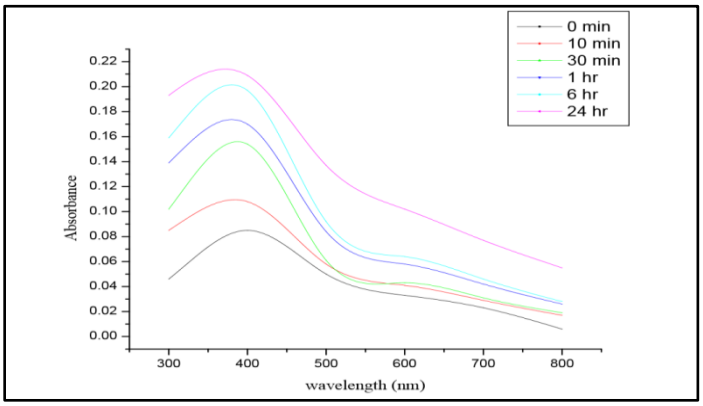


Fig. 6. UV-Vis spectrum of silver nanoparticles of SB extract

Synthesized metal nanoparticles were used for capping and efficient stabilization via FTIR. The FTIR analysis of nanoparticles showed characteristic peaks at 3744.37, 3678.67, 3649.64, 3621.10, 2855.26, 1741.85, 1694.28, 1477.99, 1463.23, 1396.44 and 760.90 (Fig. 7). The functional groups detected in nanoparticle sample included alcohol (stretch), alkane (stretch), carbonyl (stretch), aromatic (stretch), alkane (bending) and alkene (bending) at different wave numbers. These groups depicted the capping of the nanoparticles by the identified saponin, i.e. sterol compounds and some other glycosides.

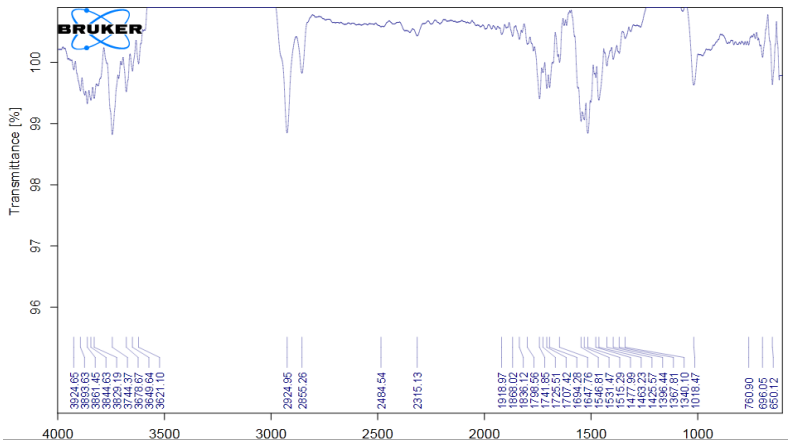


Fig. 7. FTIR spectrum of SBE nanoparticles

The Zeta potential and zeta sizer analysis of the silver nanoparticles of the seed samples was carried out in computer controlled particle size analyzer (Malvern Zetasizer-90 Nanoseries) to find out the particles size distribution and electric potential of AgNPs. The size and potential of nanoparticles synthesized from butanolic extract was 81 nm and -31.2 mv respectively (fig. 8, 9). The size of the prepared silver nanoparticles of the seed extract was within the range of standard nanoparticle i.e. between 1-100 nm.

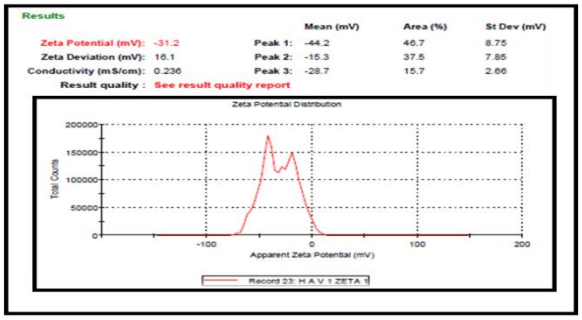


Fig. 8. Zeta potential of SBE nanoparticle

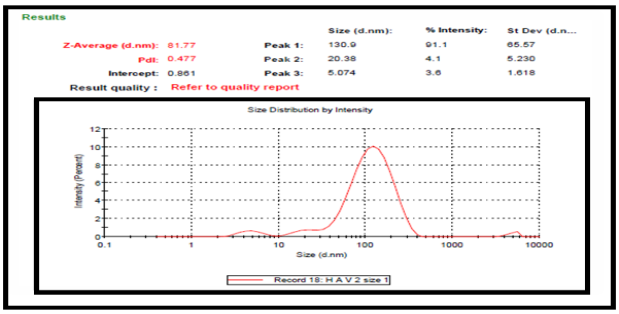


Fig. 9. Zeta sizer of SBE nanoparticles

The microstructure and size of the biosynthesized silver nanoparticles were studied using SEM (TESCAN) analysis. The micrograph of nanoparticles synthesized from SB extract showed formation of the round shape particles. The size of the nanoparticles is ranging between 59-61 nm (fig. 10).

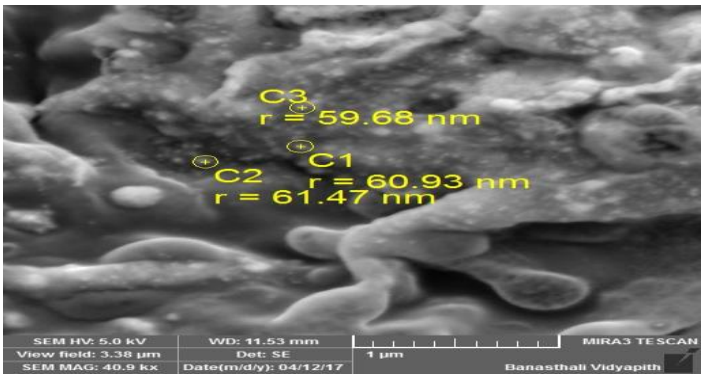


Fig. 10. SEM image of the nanoparticles of SB extract

Similarly, the results cited for Eclipta prostrate aqueous extract's SEM analyses of the synthesized AgNPs were clearly distinguishable measured 35–60 nm in size. The SEM analyses of *Euphorbia hirta* leaves showed relatively spherical shape nanoparticles formed with diameter range 40-50 nm [45]. Dimitrijević*et al*. [46] also synthesized nanoparticles from organometallic solution precursor and characterized by SEM. They found that particles were mutually different in size varying between 30 to 140 nm with preferred bimodal (40 and 70 nm) distribution for Nano-film which was grown from the solution with concentration of 15 % w/w Ag+.

3.4 Anti-acne assay

The plant based antimicrobial compounds are good source for medicine due to their enormous therapeutic potential. Silver in medical and industrial process, has long been used as having inhibitory effect on microbes i.e. topical ointments to prevent infection against burn and open wounds [47, 48].

MIC values of the micro-organisms present in acne have been showed in table 2. The concentration of active anti-acne compounds was decreased along with increasing the dilution. The highest MIC value was observed in 2 times diluted SB extract as well as in their nanoparticles. The lowest MIC value was found in 8 times diluted SB extract and their nanoparticles. In comparison, the sterol based nanoparticles showed less MIC value than SB extract.

Table 2 MIC values of the diluted SB extract and their nanoparticles with standard

Diluted concentrations	MIC (mg/L) of SB extract	MIC (mg/L) of SBE nanoparticles
Standard	0.630	0.625
2 times	0.448	0.412
4 times	0.398	0.362
6 times	0.293	0.251
8 times	0.268	0.217

In the initial study of anti-acne activity, the zone of inhibition of 26mm of isolated sterol rich compounds against butanol control (4mm) showed that isolated sterol rich compounds have good potential against acne bacteria (fig. 11). Further study has been conducted with the isolated sterol rich compounds, their nanoparticles, antibiotic streptomycin and control (butanol and silver nitrate). In this assay, isolated sterol rich compounds showed highest inhibition zone i.e. 32 mm while their nanoparticle showed 28 mm inhibition zone which is less than the sterol rich compounds. The minimum inhibition zone was observed in the case of control i.e. butanol (7 mm) and silver nitrate (22 mm) while antibiotic streptomycin showed highest inhibition zone i.e. 50mm. This assay confirmed that sterol rich compounds have higher potential against acne than sterol nanoparticle (fig. 12). Further, it has been observed that minimum zone of inhibition was still at 8 times dilution of the sterol rich fraction as well as in their nanoparticles but comparatively less than 2 times dilutions.

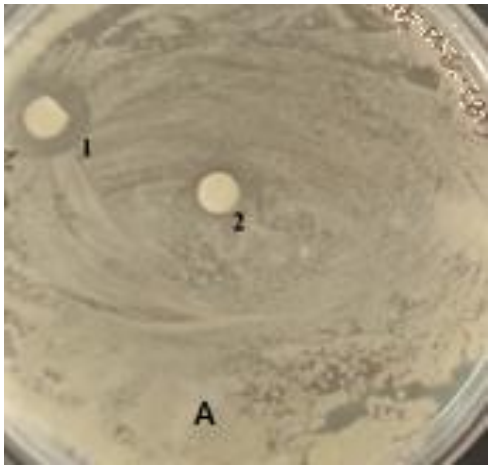


Fig. 11. Anti-acne activity of butanolic seed extract (1) and butanol solvent as a control (2).

Fig. 12. Comparative anti-acne activities of solvent butanol control (DS), butanolic seed extract (D1), AgNO3control (D2) and silver nanoparticles of butanolic seed extract (D3) with antibiotic streptomycin (S) in center

The same thing has been evaluated in the petroleum ether extract of *Tridaxprocumbens* (Asteraceae) against dandruff [49].The effect of germinating seeds of fenugreek (Fabeaceae) on the hair dandruff has been found to act as an anti-dandruff component.Saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell have antimicrobial property [50,51]. In a new study, flavonoid silver nanoparticles synthesized from *Coriandrumsativum*leaf extract also exhibit anti-acne, anti-dandruff and anti-breast cancer activity which havegreat potential in biomedical applications [52].

4. Conclusion

In present study, first time sterol based ANFs present in different form in safflower seeds have been reported as their anti-acne activity. However, many herbal drugs possessed poor solubility, low absorption, lower bioavailability and slow pharmacological action. To overcome these disadvantages, nanocoating herbal drugs produced by various methods have improved pharmacokinetics and bio-distribution of therapeutic agent. With the recent progress and ongoing efforts, it is hopeful that the implementation of our approach by using safflower may contribute with high pharmaceutical properties. It is equally observed that stigmasterol i.e. saponins has a wide range of spectrum against the acne causing micro-organisms that can be used in the formulation of anti-acne cream/face wash in future and for other herbal formulations.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgement

Authors are thankful to Prof. AdityaShastri, Hon. Vice Chancellor, Banasthali University, Banasthali (Rajasthan), India for providing facilities for experimental work. Also we are thankful to Directorate of Oilseed Research (ICAR), Rajendranagar, Hyderabad (A.P.) India, for safflower seed facilities being an important part of our study.

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