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# *In vitro* evaluation of antioxidant and antidiabetic activities of *Syzygium densiflorum* fruits

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ABSTRACT

**Objective:** To provide experimental support for the traditional knowledge of *Syzygium densiflorum* (*S. densiflorum*) fruits.
**Methods:** Powered *S. densiflorum* dried fruits were subjected to successive extraction with *n*-hexane, ethyl acetate, and ethanol using a Soxhlet extractor. Further, preliminary phytochemical screening was carried out with a series of tests. *In vitro* free radical scavenging was evaluated using total antioxidant estimation, 2,2-diphenyl-1-picrylhydrazyl radical, superoxide radical scavenging, and hydroxyl radical scavenging assays. Antidiabetic activity was estimated using α-amylase inhibition assay.
**Results:** Preliminary phytochemical estimation confirmed the presence of alkaloids, flavonoids, sterols, terpenoids, anthocyanin, phenols, carbohydrates, fixed oils, and fats in fruits of *S. densiflorum*. Ethyl acetate and *n*-hexane extracts showed less free radical scavenging and α-amylase inhibition activity than ethanol extract. $IC_{50}$ values of ethanol extracts for 2,2-diphenyl-1-picrylhydrazyl radical, superoxide radical, hydroxyl radical, lipid peroxidation, and α-amylase inhibition assays were found to be 0.01, 0.16, 0.66, 0.46, and 0.46 mg/mL respectively.
**Conclusions:** *In vitro* evaluations confirmed the antioxidant and antidiabetic potential of *S. densiflorum* fruits. Ethanol extract of *S. densiflorum* fruits showed higher activity with statistical significance *vs.* ethyl acetate and *n*-hexane extracts.

## 1. Introduction

Reactive oxygen species (ROS) and free radicals are continuously generated in pathological conditions[1]. Free radicals such as hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide, superoxide anions ($O_2^{-}$), and nitric oxide react with DNA, proteins, and lipids that eventually lead to cell death and tissue damage[2]. They play a vital role in the pathogenesis of aging, anemia, arthritis, asthma, atherosclerosis, cancer, cardiovascular diseases, diabetes, hypertension, inflammation, myocardial infarction, and neurodegenerative diseases[3]. All organisms possess defense system to protect against free radical damage through enzymatic and non-enzymatic antioxidants[1]. However, these natural antioxidants get unbalanced by exogenous and endogenous factors leading to oxidative stress[4]. The potential negative impacts of oxidants have led to increased investigations to identify potential antioxidants[5]. The harmful effects of oxidative stress can be reduced by a constant supply of natural products[6]. It is reported that herbal medicines are a safer option for prevention of diseases caused by oxidative stress[3]. The fruits of Myrtaceae family are known for their therapeutic role in the treatment of metabolic disorders[7].

*Syzygium* spp. belonging to the family Myrtaceae is widely distributed in the tropical and subtropical regions. Scientific reports show that extracts of different parts of *Syzygium* spp. possess a wide range of pharmacological properties that are antidiarrheal, antidiuretic, antihyperlipidemic, antiinflammatory, antimicrobial, antioxidant, antiscorbutic, antiulcerogenic, astringent, cardioprotective, hepatoprotective, and stomachic in nature[8-10]. The genus *Syzygium* comprises about 1 200 species, of which majority are yet to be explored by the scientific community[11]. *Syzygium densiflorum* Wall. ex Wight & Arn (Myrtaceae) (*S. densiflorum*) have been used traditionally for

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the treatment of diabetes by the tribes of the Nilgiris, Tamil Nadu, India[12]. Although, previous studies report that leaves of the *S. densiflorum* possess antioxidant[13,14], antidiabetic[12], and antimicrobial properties[15], no clear experimental evidence is available to support the traditional use of the fruits of *S. densiflorum*.

The present investigation focuses on identifying phytoconstituents in polar, mid-polar, and non-polar fractions of fruits using various qualitative assays. Polar, mid-polar, and non-polar fractions of fruits were obtained using *n*-hexane, ethyl acetate, and ethanol solvents respectively. Medicinal plants are known to consist of a mixture of chemical compounds with different functional groups, polarity, and chemical behavior, which may vary in action[16]. Hence, four different assays were performed for evaluating the antioxidant potential of polar, mid-polar, and non-polar fractions of *S. densiflorum*. In addition, the antidiabetic potential of these extracts was assessed using α-amylase inhibition assay.

## 2. Materials and methods

### 2.1. Extraction of plant materials

Ripened fruits were collected from the Gudalur region of Nilgiris, Tamil Nadu, India during May–June, 2013. These were authenticated by Dr. GVS Murthy, Botanical Survey of India (BSI), Southern Regional Centre, Tamil Nadu Agricultural University campus, Coimbatore, Tamil Nadu, India (Authentication No. BSI/SRC/5/23/2013-14/Tech/60) and a voucher specimen of this plant had been deposited in the herbarium of BSI, Coimbatore (Accession No. MH 174300 and MH 174301). Fruits were dried at room temperature (around 25 °C). Subsequently, the dried fruits were mechanically pulverized and extracted successively with *n*-hexane, ethyl acetate, and ethanol using a Soxhlet extractor. The obtained extracts were concentrated using a rotary evaporator under reduced pressure at 40 °C. All the dried extracts were collected in an air-tight container and refrigerated for further use.

### 2.2. Preliminary phytochemical screening

The concentrated extracts were dissolved in 100 mL of the respective solvent to obtain 1% (w/v) stock concentration and subjected to preliminary phytochemical screening according to the previously described procedures[17].

### 2.3. Estimation of free radical scavenging activity

Free radical scavenging activity of *n*-hexane, ethyl acetate, and ethanolic extracts of *S. densiflorum* fruits were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical[18], superoxide[19], $OH^{\cdot}$ scavenging assay[20], and conjugated diene method[21]. The antioxidant L-ascorbic acid was used as positive control in all the assays. Optical density (OD) of the samples was measured using a UV/vis spectrophotometer (CE 2021). The antioxidant scavenging ability was calculated using the following equation:

Antioxidant scavenging ability (%) = (OD of control - OD of test sample)/(OD of control)

$IC_{50}$ values in mg/mL were obtained by linear regression analysis to represent the concentration of the test samples that caused 50% inhibition.

### 2.4. α-Amylase inhibition assay

α-Amylase activity was determined according to previously reported method with minor modifications[22]. Briefly, 500 µL of extracts of *S. densiflorum* fruits (*n*-hexane, ethyl acetate, and ethanol) and standard drug acarbose were mixed with 500 µL of α-amylase (2 IU/mL). The mixture was pre-incubated in 20 mmol/L sodium phosphate buffer (pH 6.9) for 10 min at room temperature. Then, 1 mL of 1% (w/v) starch dissolved in the buffer was added to the reaction mixture to make a total volume of 2 mL, and the whole was incubated for 10 min at room temperature. After incubation, 1 mL of dinitrosalicylic acid color reagent was added and placed in a boiled water bath for 5 min. Further, this reaction mixture was cooled on ice to room temperature and 6 mL of deionized water was added to the reaction mixture. α-Amylase activity was determined by measuring the absorbance of the mixture at 540 nm. The results were expressed as percent of α-amylase inhibition, which was calculated according the equation:

% Inhibition = ($OD_{540}$ of control - $OD_{540}$ of sample)/($OD_{540}$ of control) × 100

### 2.5. Statistical analysis

The experimental results were represented as mean with standard deviation. Statistical differences between the means of the test and control samples were evaluated using One-way ANOVA followed by Tukey's multiple comparison test. All statistical analyses were performed using the software GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA).

## 3. Results

### 3.1. Preliminary phytochemical screening

Preliminary phytochemical estimation was carried out for *n*-hexane, ethyl acetate, and ethanol extracts and the obtained results were shown in Table 1. The presence of flavonoids, sterols, terpenoids, anthocyanin, phenols, carbohydrates, fixed oils, and fats in extracts of *S. densiflorum* fruits was observed in preliminary phytochemical screening tests. Alkaloids were found abundantly in *n*-hexane and ethyl acetate fractions, whereas alkaloids were completely absent in the ethanol extract. Overall, flavonoids, sterols, terpenoids, anthocyanin, phenols, carbohydrates, fixed oils, and fats were found to be more in the ethanol fraction when

compared to the *n*-hexane and ethyl acetate fractions.

**Table 1**

Phytochemical screening of *S. densiflorum* fruits.

| Phytochemicals | Test applied | Hexane | Ethyl acetate | Ethanol |
|---|---|---|---|---|
| Alkaloids | Mayer's test | +++ | +++ | - |
| | Wagner's test | +++ | +++ | - |
| | Hager's test | +++ | +++ | - |
| Flavonoids | NaOH test | + | + | +++ |
| | $H_2SO_4$ test | + | + | +++ |
| Sterols | Liebermann-Burchard test | + | ++ | +++ |
| Terpenoids | Liebermann-Burchard test | + | - | - |
| | Horizon test | + | ++ | +++ |
| Anthocyanin | NaOH test | + | +++ | _ |
| Proteins | Ninhydrin test (aqueous) | - | - | - |
| | Ninhydrin test (acetone) | - | - | - |
| Phenols | Ferric chloride test | - | - | +++ |
| Quinones | HCl test | + | - | +++ |
| Carbohydrates | Molisch's test for carbohydrates | +++ | +++ | +++ |
| Fixed oils and fats | Filter paper test | +++ | + | +++ |

+++: Highly present; ++: Present; +: Sparingly present; -: Completely absent.

## 3.2. Radical scavenging properties of *S. densiflorum*

### 3.2.1. DPPH radical assay

DPPH radical assay had been widely used to evaluate the antioxidant property of natural product extract in organic solvents[23]. DPPH was a free radical with an unpaired electron, which was a purple alcoholic solution that turned yellow by reduction of DPPH˙ to non-radical DPPH-H in the presence of antioxidant[24]. During reduction, unpaired electron in the DPPH˙ became paired due to hydrogen-donating ability of the antioxidant[25]. This method measured the hydrogen-donating ability of antioxidant, which was responsible for free radical scavenging activity[23]. In this study, the obtained *n*-hexane, ethyl acetate, and ethanol extracts were subjected to DPPH assay in various concentrations from 0.0125 mg/mL to 0.2 mg/mL (Figure 1). The quenching ability of the test samples was measured by color changes in OD at 517 nm. The purple color of DPPH solution provided strongest absorbance at 517 nm and decrease in the absorbance pattern represented its antioxidant activity[26]. As shown in Figure 1, dose-dependent activity was observed in the test samples and standard antioxidant showed the maximum activity in all concentrations. The ethanol extract showed the maximum inhibition ($IC_{50}$ = 0.01 mg/mL) compared to the ethyl acetate ($IC_{50}$ = 0.0155 mg/mL) and *n*-hexane ($IC_{50}$ = 0.63 mg/mL). The ethyl acetate and ethanol extract had shown maximum inhibition at 0.2 mg/mL concentration than the L-ascorbic acid. At this point of time, ethyl acetate and ethanolic extracts showed a significant increase in DPPH scavenging compared to the L-ascorbic acid ($P$ < 0.005).

### 3.2.2. Superoxide radical scavenging assay

$O_2^-$ radicals, the harmful ROS, were initial free radicals formed from mitochondrial electron transport system and numerous biological reactions[27]. Enzymes such as lipoxygenases, peroxidase, nicotinamide adenine dinucleotide phosphate oxidase, and xanthine oxidase released the reduced form of molecular oxygen ($O_2^-$) in the course of cellular reactions to protect against pathogens[28]. The release of molecular oxygen was the sole precursor of OH˙, hydrogen peroxide, and singlet oxygen. The OH˙ that induced oxidative damage in lipids, proteins and DNA also caused lipid peroxidation in foods that led to their deterioration[29]. Normal living cells had the potential to protect themselves from the toxic superoxide oxygen containing isoforms of superoxide dismutase[30]. However, the accumulation of $O_2^-$ led to oxidative stress and was also involved in the pathogenesis of many chronic diseases[31]. In this study, superoxide radicals were generated *in vitro* by the coupling reaction of nicotinamide, adenine, dinucleotide and phenazine methosulfate reducing to nitro blue tetrazolium and $O_2^-$ scavenging activity of *S. densiflorum* was estimated. The percentage of $O_2^-$ scavenging was shown in Figure 2, which illustrated the dose dependent superoxide radical scavenging activity. The test samples were not statistically significant when compared to L-ascorbic acid. As shown in Figure 2, the superoxide radical scavenging activity was high in the ethanolic extract ($IC_{50}$ = 0.16 mg/mL), followed by ethyl acetate ($IC_{50}$ = 0.22 mg/mL) and *n*-hexane ($IC_{50}$ = 0.41 mg/mL) extracts. The $IC_{50}$ for L-ascorbic acid was found to be 0.08 mg/mL. The superoxide radical scavenging assay was showed better activity in ethanol extract with significant difference compared to non-polar extract of *n*-hexane and mid-polar extract of ethyl acetate ($P$ < 0.001).

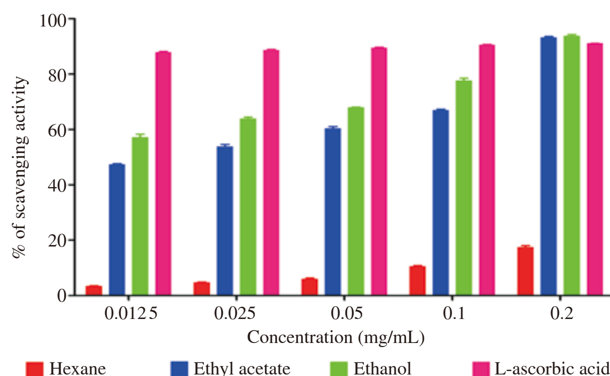


**Figure 1.** Antioxidant efficacy of *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits on DPPH radical.

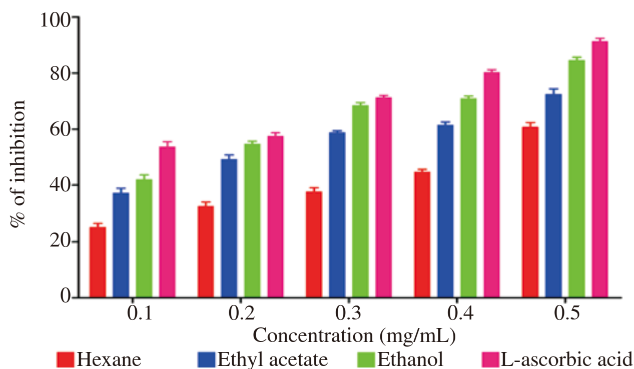


**Figure 2.** Antioxidant efficacy of *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits on Superoxide radical.

#### 3.2.3. $OH^{\cdot}$ scavenging assay

The $OH^{\cdot}$ was a neutral form of hydroxide ion, which was generated from hydrogen peroxide and $Fe^{2+}$ through Fenton reaction[32]. It was reported as an extreme non-radical reactive species that could easily cross cell membranes, initiating the lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids. Subsequently, it attacked the biological macromolecules and, as a consequence, led to cell death[33]. It had a very short life of about $10^{-9}$ s because of its high reactivity[34]. In this study, the deoxyribose assay was used to measure the $OH^{\cdot}$ scavenging of extracts of *S. densiflorum* fruits in the presence of $Fe^{3+}$-EDTA and $H_2O_2$. The percentage of $OH^{\cdot}$ scavenging was illustrated in the bar diagram (Figure 3). The dose-dependent activity of test samples was also observed. As shown in the Figure 3, the L-ascorbic acid showed better radical activity with $IC_{50}$ of 0.019 mg/mL concentration, followed by ethanol ($IC_{50}$ = 0.66 mg/mL), ethyl acetate ($IC_{50}$ = 1.53 mg/mL), and *n*-hexane extracts ($IC_{50}$ = 2.63 mg/mL). Among the test samples, ethanol extract showed significant increase in the $OH^{\cdot}$ scavenging activity compared to the *n*-hexane and ethyl acetate extracts ($P < 0.001$).

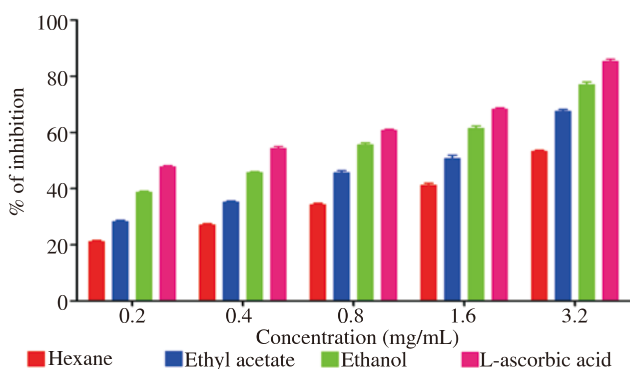


**Figure 3.** Antioxidant efficacy of *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits on $OH^{\cdot}$.

#### 3.2.4. Total antioxidant estimation by conjugated diene method

Lipid peroxidation was a widely reported biologically relevant free radical chain reaction for human diseases[35]. The $OH^{\cdot}$ was the most powerful oxidant that attacked polyunsaturated fatty acids to initiate lipid peroxidation. An increase in lipid peroxidation caused cell damages[36]. Natural antioxidants scavenged/neutralized the free radical and minimized the detrimental lipid peroxidation[37]. The conjugated diene method had been widely used to measure the oxidation of linoleic acid in order to determine the antioxidant potential of natural products[38]. In this study, the potential of fruit extracts of *S. densiflorum* to inhibit the peroxidation of linoleic acid was estimated using the conjugated diene method (Figure 4). The oxidation of linoleic acid due to conjugated diene formation was measured at various concentrations (0.1–10 mg/mL) from the extracts of *S. densiflorum* fruits at 234 nm[39]. The bar diagram represents the dose dependent total antioxidant capacity in tested samples. It was observed that the ethanolic extract showed a significant increase in the total antioxidant with $IC_{50}$ of 0.46 mg/mL ($P < 0.001$ *vs.* ethyl acetate and *n*-hexane), followed by ethyl acetate extract ($IC_{50}$ = 1.24 mg/mL) which showed better activity than the *n*-hexane ($IC_{50}$ = 5.64 mg/mL). However, the antioxidant activity of the test samples was found to be less than the L-ascorbic acid ($IC_{50}$ = 0.25 mg/mL).

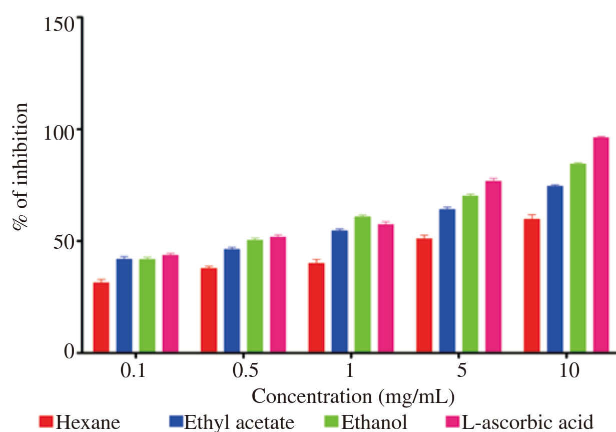


**Figure 4.** Total antioxidant effect of *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits.

### 3.3. α-Amylase inhibition assay

Antidiabetic property of extracts of *S. densiflorum* fruits was estimated by α-amylase inhibition assay. The standard drug acarbose was used as control along with crude extracts of *S. densiflorum* fruits (Figure 5). Figure 5 shows the percentage inhibition of α-amylase of *S. densiflorum* and standard drug acarbose. As shown in Figure 5, the maximum inhibition effect was observed in standard drug acarbose, followed by ethanol, ethyl acetate, and *n*-hexane extracts. The $IC_{50}$ of *n*-hexane, ethyl acetate, and ethanolic extracts were found to be 2.87 mg/mL, 1.23 mg/mL, and 0.46 mg/mL respectively. The ethanolic extract showed maximum inhibition among the test samples with significance at concentration 0.5 mg/mL ($P < 0.001$ compared to ethyl acetate and *n*-hexane).

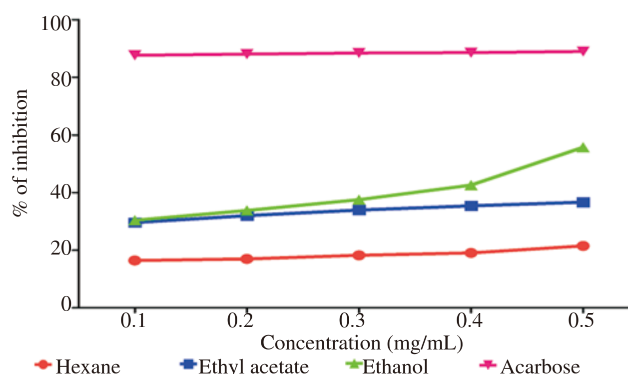


**Figure 5.** α-Amylase inhibition potential of *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits.

## 4. Discussion

The secondary metabolites of crude drug play a diverse role in the treatment and management of chronic disorders. Phytoconstituents are qualitatively and quantitatively various among plants. Qualitative estimation with natural crude extract provides preliminary information about the chemical composition of plant products[40]. The phytoconstituents of the crude extract possess

a wide range of pharmacological activities due to the presence of phenols, alkaloids, terpenoids, flavonoids, saponins, tannins, and carotenoids[41]. Thus, preliminary phytochemical estimation helps to understand the medicinal value of the extracts of *S. densiflorum* fruits. In the present study, the presence of flavonoids, sterols, terpenoids, anthocyanin, phenols, carbohydrates, fixed oils, and fats in extracts of *S. densiflorum* fruits was observed in preliminary phytochemical screening tests. Phenolic compounds contribute more to the antioxidant potential of the crude extract and also possess antiinflammatory, antiatherosclerotic and anticarcinogenic activities[42]. It is reported that plants with glycosides, terpenoids, alkaloids, flavonoids and phenolics have antidiabetic potential[43]. Preliminary phytochemical screening test confirms that the antidiabetic and antioxidant property of *S. densiflorum* fruits may be due to the presence of phytoconstituents. However, further detailed investigations are necessary to explore the pharmaceutical property of *S. densiflorum* fruits.

In addition, the antioxidant property of *S. densiflorum* fruits was investigated. The ROS are natural byproducts of oxygen metabolism produced in aerobic organisms. ROS stimulates signaling pathways in response to changes in intra and extracellular environmental conditions of living cells[44]. However, augmented levels of ROS lead to oxidative stress and involve in the pathogenesis of many chronic diseases[31]. Thus, estimating the antioxidants property of natural product in *in vitro* is an initial process to determine their therapeutic potential. The standard radical systems are available to evaluate the antioxidant of the natural products. However, it has been suggested that selected plant samples should be evaluated in two or more radical systems for estimating their radical scavenging capacity. In this investigation, the free radical scavenging of *S. densiflorum* fruits extracts were evaluated using DPPH radical, $O_2^-$, $OH^·$, and lipid peroxidation systems. It has been observed that ethanol extract of *S. densiflorum* fruits showed better activity than other extracts due to presence of phytoconstituents in the extract. Besides, α-amylase inhibition assay was performed to estimate the antidiabetic property of extracts of *S. densiflorum* fruits. The α-amylase enzyme is one of the major secretory products of the pancreas and salivary glands. It plays a vital role in starch digestion by hydrolyzing the internal alpha 1,4-glucosidic linkages in starch[45]. Starch digestion is the principal source of glucose in the diet and inhibition of this enzyme significantly reduces the postprandial increase of blood glucose level. Therefore, it has been recognized as the therapeutic target for modulating the earliest metabolic abnormality of diabetes mellitus[46]. Natural products control the glucose level by inhibiting starch hydrolysis[47]. In this study, ethanol extract of *S. densiflorum* fruits showed better activity than the other two extracts and it confirmed its therapeutic role in diabetes treatment.

Preliminary phytochemical estimation with *n*-hexane, ethyl acetate, and ethanol extracts of *S. densiflorum* fruits shows that alkaloids are rich in *n*-hexane and ethyl acetate extracts. On the other hand, flavonoids, sterols, terpenoids, anthocyanin, phenols, carbohydrates, fixed oils, and fats are present in ethanol extracts. *In vitro* evaluation of free radical scavenging and α-amylase inhibition potential confirmed the pharmacological potential of *S. densiflorum* fruits. The ethanol extracts of *S. densiflorum* fruits possess more free radical scavenging and α-amylase inhibition potential compared to *n*-hexane and ethyl acetate extracts due to its various phytoconstituents. Overall, this investigation provides scientific evidence of the traditional knowledge and therapeutic potential of *S. densiflorum* fruits.

## Conflict of interest statement

We declare that we have no conflict of interest.

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