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Research Article

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In vitro antioxidant and inhibition of monosodium urate crystals by *Sauropus androgynus* hydroalcoholic extract

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ABSTRACT

Gout is a chronic inflammatory disease characterized by hyperuricemia. Deposition of uric acid in the form of monosodium urate (MSU) crystals in the joints, may cause oxidative stress and inflammation. Current therapy for gout has many side effects, hence there is a need to develop an alternative therapy for gout management. *Sauropus androgynus* (SA), belongs to the family Euphorbiaceae. SA possesses anti-inflammatory, antihypertensive properties. SA has also been traditionally claimed to be effective in gout management, but there is no relevant scientific evidence for this claim. Hence, the present study planned to evaluate the *in vitro* effect of SA hydroalcoholic extract on antioxidant and inhibitory effect on growth of MSU crystals. The dried aerial parts of the SA were subjected to extraction by cold maceration method. Nitric oxide (NO) and ferric reducing antioxidant assay was used to evaluate antioxidant potency of the SA and ascorbic acid (standard) at different concentrations. MSU crystals were grown *in vitro*, which were characterized by FT-IR. SA hydroalcoholic extract was exposed to MSU crystals for 48 hr and observed for growth and inhibition of needle shaped MSU crystals under the microscope. FT-IR results confirm the presence of MSU crystals. SA displayed significant *in vitro* inhibition of growth of MSU crystal and antioxidant activity by nitric oxide and ferric reducing antioxidant assay. Anti-inflammatory, antioxidant and lowering effect on MSU crystals of SA helps to subside the symptoms of gout. Inhibition of growth of MSU and antioxidant activity of the plant may be due to the presence of flavonoids and vitamin C.

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INTRODUCTION

Gout is a chronic inflammatory disease characterized by high levels of uric acid and the deposition of MSU in the joints and tendons (Ragab *et al.*, 2017; Major *et al.*, 2018). Overproduction or impaired renal excretion can result in higher serum uric acid (SUA), termed as hyperuricemia which is the main predisposing factor for gout (Liu *et al.*, 2021). Accumulation of MSU in the joints leads to neutrophil activation that progressively causes synovial inflammation and joint deformity. Xanthine oxidase (XO) is the main enzyme which catalyses the formation of uric acid (Acharya *et al.*, 2015). Uric acid is the final enzymatic product of purine catabolism (Borghi *et al.*, 2015). Uric acid acts as a scavenger for oxygen, peroxyl radicals and hydroxyl radicals. It also acts as an antioxidant which helps to protect the cells from oxidative damage, thus assist to decrease the risk for many of the diseases (Sautin & Johnson, 2008). Most of the studies even show association of the hyperuricemia with the development of metabolic syndrome like cardiovascular disease, hypertension, renal disease, type 2 diabetes, etc. The pathogenesis of all these diseases are not completely clear. Although the common cause of these diseases may be increase in oxidative stress and damage to proteins and lipids. Hyperuricemia may be the cause of increase in oxidative stress as it causes vascular inflammation and endothelial dysfunction which leads to oxidative damage of lipids and proteins (Acharya *et al.*, 2015; Liu *et al.*, 2021).

Non-steroidal anti-inflammatory drugs (NSAIDs), colchicine, corticosteroids, and anti-IL-1b biologics are used in the management of gout but all these medications are associated with side effects such as gastrointestinal and hepatic toxicity (Palani *et al.*, 2018). Plants are the vital source of bioactive compounds which are used to manage various diseases (Dey *et al.*, 2021). *Sauropus androgynus* is a medicinal plant belonging to the family Euphorbiaceae (Bunawan *et al.*,

2015). It is a Southeast Asian indigenous vegetable, widely cultivated for traditional medicinal purposes (Zhang *et al.*, 2020). Traditionally, the leaves of SA are commonly used for treating genitor-urinary diseases, cardiovascular diseases, diabetes, hyperlipidemia etc. SA has also been used to reduce body weight and to improve vision according to the studies performed in Taiwan and Southeast Asia. The protective effect of SA could be due to the presence of antioxidative compounds in the plant (Yu *et al.*, 2006; Khoo *et al.*, 2015). Studies also showed that the SA contains the highest amount of flavonoids in the plant. The plant also has potential of antimicrobial or antifungal activity and it could be due to the presence of secondary metabolites like alkaloids, flavonoids, phenols, and glycosides (Hayati *et al.*, 2016). The plant has also been claimed to be beneficial for gout (Lin *et al.*, 1996). The association of antioxidant activity of SA may ameliorate the symptoms of gout. Hence, the present study is to evaluate the *in vitro* antioxidant and inhibition of growth of monosodium urate crystals by SA hydroalcoholic extract.

MATERIALS AND METHODS

Plant Material Collection and Extraction

The fresh leaves and stems of *Sauropus androgynus* (SA) were collected, the voucher specimen of the same has been deposited in herbaria with accession number RMRC-1501. The fresh leaves and stems of SA were washed with distilled water, dried and crushed to get coarse powder. The coarse powder was soaked in 50% of ethanol for 3 days. Then the mixture was filtered using muslin cloth. The solvent was evaporated using rotary evaporator by maintaining the temperature below 50 °C. The dried extract of SA was stored in airtight container (Bouabid *et al.*, 2018, 2020).

Antioxidant Assay

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was carried out by mixing sodium nitroprusside (5 mM) in phosphate-buffered saline with 1mL of different concentrations of extract and incubated at 25 °C for 150 min. At intervals, extract (0.5 mL) of the incubation solution were removed and diluted with 0.5 mL of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance was measured at 546 nm. Ascorbic acid was used as standard. The percentage of nitrite radical scavenging activity of the extract and standard was calculated using the following formula (Boora *et al.*, 2014).

$$\%\text{Inhibition} = (1\text{-}A_I/A_0) \times 100$$

Where, A_I = absorbance of sample, A_0 = absorbance of control.

Ferric reducing antioxidant assay

A volume of 2.5 mL of 0.2M phosphate buffer (pH 6.6) and 2.5 mL of 1% w/v of potassium ferricyanide was added to the 1 mL of extract and standards. The mixture was then incubated for 20 min at a room temperature 50 °C after which 2.5 mL of trichloroacetic acid (10%) was added to the mixture and was centrifuged at 3000rpm for 10min. The supernatant 2.5 mL obtained from the centrifuge of the mixture was mixed with 0.5 mL of ferric chloride (0.1%) and 2.5 mL distilled water. Absorbance was then measured in a UV spectrometer at 700 nm against a blank sample. A high reducing power of the reaction mixture of the sample was indicated by an increased absorbance (Bhalodia *et al.*, 2013).

Monosodium Urate Crystal Study

Sodium hydroxide was solubilised in 200 mL of distilled water. Uric acid was added into the above solution and pH was adjusted to 7.2 using hydrochloric acid. The solution was heated to 120 °C for 6 hours with gentle stirring. After heating, the solution was kept in refrigerator at temperature of 4 °C overnight. The MSU crystals were filtered and dried (Palani *et al.*, 2018). Fourier transform infrared spectroscopy (FT-IR) (Shimadzu IRAffinity-1) was performed to confirm the presence of MSU crystals in the study. Hydroalcoholic extract of SA at different concentration (1000 µg/mL, 500 µg/mL, 250 µg/mL) and ascorbic acid at a concentration 1000 µg/mL were exposed to MSU crystals for 48 hours and aggregation and growth of MSU crystals were observed under the microscope.

Statistical Analysis

All *in vitro* determinations were carried out in triplicate. The results are presented in the form of Mean ± standard error mean (SEM). Calculation of IC_{50} value was carried out using GraphPad Prism 5.01 software, San Diego, CA, U.S.A. for Windows.

RESULTS

Antioxidant Assay

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was carried on hydroalcoholic extract of SA. SA at different concentrations was assessed for their nitrite free radical scavenging activity in an *in vitro* model. The NO generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize with sulphanilamide acid and couple with naphthyl ethylenediamine which forms pink colour, which was measured at 546 nm. It was observed that hydroalcoholic extract of SA at concentrations of (500 µg/mL, 250 µg/mL and 125 µg/mL) and ascorbic acid at concentrations of (500 µg/mL, 250 µg/mL and 125 µg/mL) showed antioxidant activity. Percentage inhibition of free radical scavenging was plotted against concentration of the extracts (Figure 1). The SA hydroalcoholic extract exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite radicals.

Ferric reducing antioxidant assay

Ferric reducing antioxidant assay was carried out on SA. It was observed that SA hydroalcoholic extract at concentrations of

(500 µg/mL, 250 µg/mL and 125 µg/mL) and ascorbic acid at concentrations of (500 µg/mL, 250 µg/mL and 125 µg/mL) showed antioxidant activity. The reducing power of standard and hydroalcoholic extracts of SA increases with the increase in concentrations of extract and standard (Figure 2). The results of SA and ascorbic acid shows reducing power at all concentration. IC_{50} value for SA was found to be 1518 µg/mL and for ascorbic acid 203.6 µg/mL.

Effect of Hydroalcoholic Extract of SA on Monosodium Urate Crystals

MSU crystals were grown *in vitro*, which were characterized by FTIR (Figure 3). The characterization study confirmed the presence of MSU crystals in the experiments. The FTIR spectrum has shown the presence of functional groups and bonds in MSU crystals like ketone group (C = O), carbon-nitrogen bond (C-N), O-H stretching, N-H stretching and rocking and also observed vibrational frequencies for different functional groups (Table 1).

Hydroalcoholic extract of SA at the concentrations (1000 µg/mL, 500 µg/mL and 250 µg/mL) and standard drug ascorbic acid at a concentration of 1000 µg/mL were used for the growth inhibition study of MSU crystals. After exposure of MSU crystals to hydroalcoholic extract of SA for 48 hr the microscopic and FTIR studies were performed. Microscopic study reveals the presence of needle shaped MSU crystals. The control group showed more aggregation and rate of growth of MSU crystals. Ascorbic acid at a concentration of 1000 µg/mL showed less aggregation and suppressed the rate of growth of MSU as shown in Figure 4. As compared to the control, SA at all different concentrations, significantly decreases the aggregation and rate of growth of MSU crystals in a dose dependent manner (Figure 5). These results suggest an inhibitory effect of ascorbic acid and SA on MSU crystals.

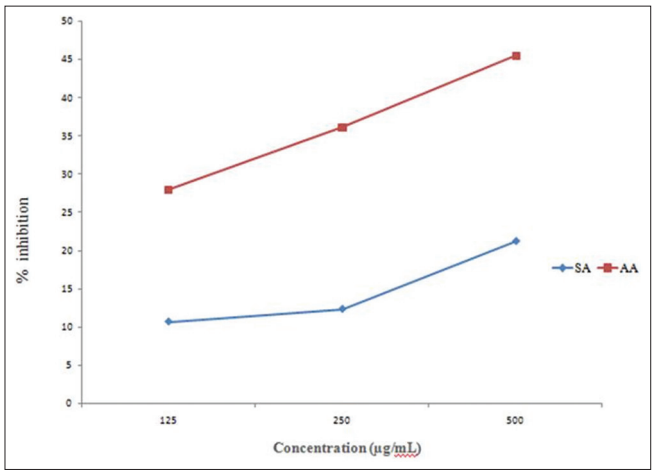

Figure 1: Effect of SA hydroalcoholic extract on Nitric oxide assay

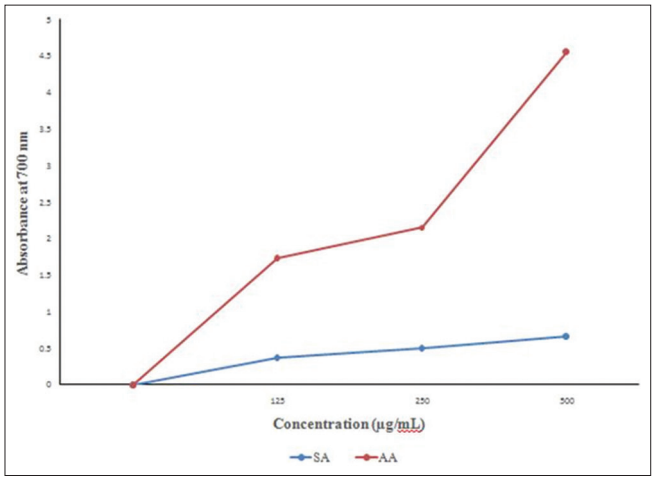

Figure 2: Effect of SA hydroalcoholic extract on reducing power assay

DISCUSSION

Gout is a metabolic disorder characterized by hyperuricemia (Pillinger & Mandell, 2020). Hyperuricemia is a condition which may lead to an increase in oxidative stress, inflammation and endothelial dysfunction. These are the pathological condition associated with multiple comorbidities including hypertension, cardiovascular disease, chronic kidney disease (CKD), stroke and metabolic syndrome (Xiong *et al.*, 2019). Traditionally, plants are used as medicines which provide therapeutically significant secondary metabolites which are essential to treat various diseases (Naive *et al.*, 2021). There are claims suggesting the use of SA traditionally in gout management but the effect of SA on the growth of MSU crystals is unclear. SA is a medicinal plant, rich in antioxidant properties which may have beneficials effect in the management of gout. In this context the current study will help to understand the *in vitro* antioxidant and inhibition of monosodium urate crystals by hydroalcoholic extract of SA.

It is generally known that many of the diseases are a result of oxidative stress that leads to an imbalance between the formation of reactive oxygen species (ROS) and their neutralization (Boora *et al.*, 2014). Oxygen radicals are hydrogen peroxide molecules, alkoxy radicals, peroxide hydroxyl radicals, hydroxyl radicals and superoxide anion radicals which are commonly referred to as ROS (Liu *et al.*, 2021). When endogenous antioxidant mechanisms are inadequate to remove the free radicals then synthetic antioxidants are known to scavenge the free radicals, but due to their adverse side effects, natural antioxidants are safer alternatives (Boora *et al.*, 2014; Kumar *et al.*, 2020).

A huge number of naturally occurring substances have been documented to have antioxidant potential and various *in vitro* methods have been used to assess their free radical scavenging and antioxidant activity. Therefore, in the present study SA

Table 1: Assignments of observed absorptions in FTIR spectrum of MSU

Assignments	Observed vibrational frequencies (cm^{-1})
C=O	1707.08
C=C	1540.23
C-N	1280.79
N-H stretching	2913.60
N-H rocking	720.44
O-H stretching	3032.23-2625.23

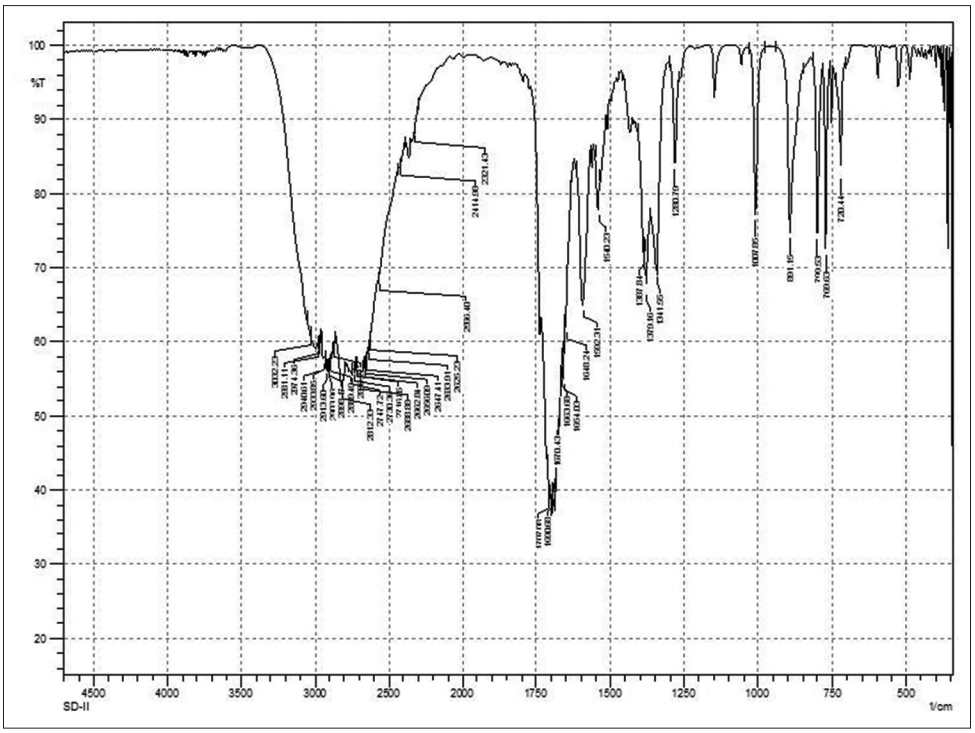

Figure 3: FT-IR spectrum of MSU crystals. FTIR spectrum showed the presence of functional groups and bonds in MSU crystals like keton group (C = O), carbon-nitrogen bond (C-N), O-H stretching, N-H stretching and rocking. This shows presence of MSU crystals

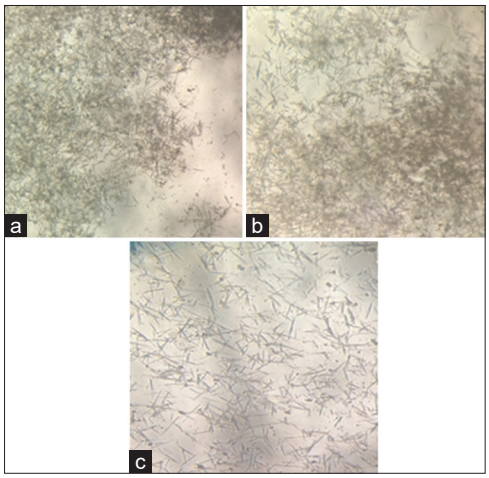

Figure 4: Microscopic structure of MSU crystals, a) Blank: Blank showed more aggregation and growth of needle shaped MSU crystals, b) Control: Control group showed more aggregation and growth of needle shaped MSU crystals and c) Ascorbic acid (1000 µg/mL): Ascorbic acid showed less aggregation and growth of MSU crystals

at different concentrations were evaluated for their reducing power activities and nitrite free radical scavenging activity in an *in vitro* model. Nitric oxide radical scavenging activity is based on the principle that sodium nitroprusside generates NO which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. In the body NO is generated from L-arginine amino acid by phagocytes and vascular endothelial cells. NO is a free radical because of its unpaired electron and possesses important reactivity with particular types of proteins and other free radicals. The NO becomes more toxic when it reacts with superoxide radicals, forming a highly reactive compound (Boora *et al.*, 2014). The hydroalcoholic extract of SA and standard at all concentrations showed inhibition of nitrite radicals.

Ferric reducing antioxidant assay is based on the principle that substances, which have reduction ability, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which further reacts with ferric chloride to form ferric–ferrous complex that has maximum absorption at 700 nm (Bhalodia *et al.*, 2013). The reducing power of the hydroalcoholic extracts of SA and standard increases with the increase in the concentration of extract and standard.

Oxidant-producing enzymes are upregulated in many of the diseases as it is the major source of ROS. In the human body, xanthine oxidoreductases (XORs) are important enzymes for uric acid production, which includes xanthine oxidase (XO) and xanthine dehydrogenase (XDH) (Liu *et al.*, 2021). XO is an enzyme which catalyses the oxidation of hypoxanthine to xanthine as a results in the formation of uric acid which on deposition in the joints causes painful inflammation and has a key role in the development of gout (Deng *et al.*, 2019; Bakar *et al.*, 2020). XO is also involved in the generation of ROS which is the main cause of many of the diseases (Sautin & Johnson, 2008). Overproduction of uric acid increases the simultaneous production of superoxide anion which leads to oxidative stress. Superoxide radicals further form hydroxyl radicals which promote oxidative damage and elevate oxidative stress (Acharya *et al.*, 2015). Previous clinical studies reported that oxidative stress was significantly higher in asymptomatic

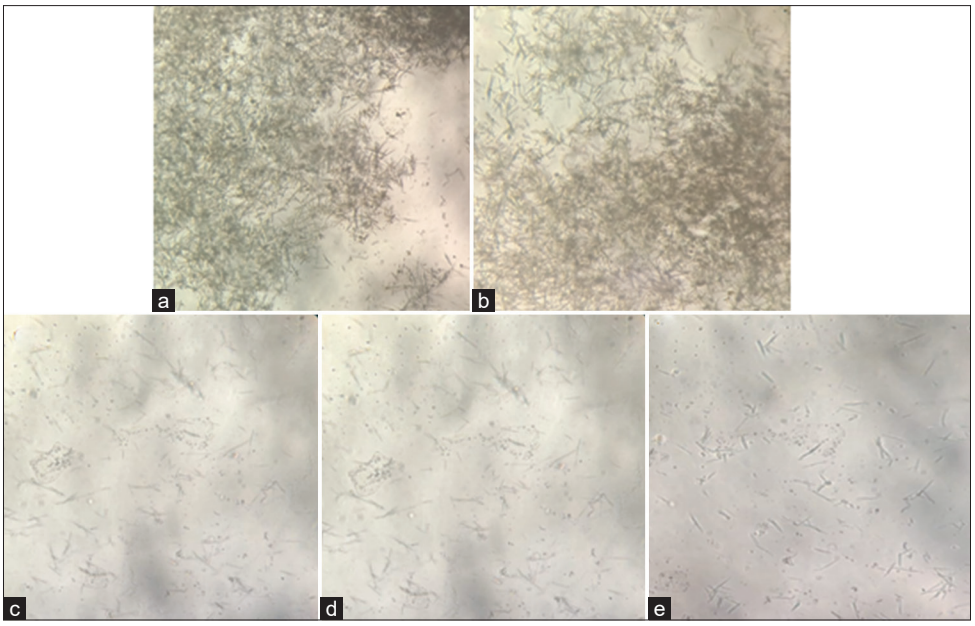

Figure 5: Microscopic structure of MSU crystals, a) Blank: Blank showed more aggregation and growth of needle shaped MSU crystals, b) Control: Control group showed more aggregation and growth of needle shaped MSU crystals, c, d and e) SA (1000 µg/mL-250 µg/mL): SA showed less aggregation and growth of MSU crystals in a dose dependent manner

young patients with primary hyperuricemia than the healthy individuals (Liu *et al.*, 2021). As hyperuricemia causes oxidative stress which leads to oxidative damage of lipids and proteins, it is the principal cause in pathology of hypertension, visceral obesity, type 2 diabetes, etc (Sautin & Johnson, 2008). Some studies supported the increase in cardiovascular mortality rate in those patients with higher serum uric acid levels than the patients with normal serum uric acid levels. This suggests that hyperuricemia itself causes oxidative stress and has a role in the pathology in gout (Acharya *et al.*, 2015).

The *in vitro* MSU inhibition study provides the direct observation of the growth of crystals. The growth of MSU crystals along with plant extract gives crucial information on crystallization promotion or inhibition by analyzing the changes. The changes include size, shape, aggregation number etc. This *in vitro* model helps in characterizing the grown crystals and assists in formulating a strategy for the suppression or dissolution of MSU crystals (Ahmed *et al.*, 2016). In the current study, hydroalcohoilc extract of SA at different concentrations was assessed for its MSU suppression activity by forming *in vitro* MSU crystals. The MSU crystals mainly form due to the reaction between the Na^+ ion and the urate ion of uric acid (Parekh *et al.*, 2009). MSU crystals were grown in vitro, microscopic study confirms the presence of needle shaped MSU crystals. MSU crystals were characterized by FTIR. The FTIR spectroscopic study has proved the formation of MSU crystals in the experiments as it contains all functional groups and bonds. SA at all different concentrations showed significant inhibition of aggregation and growth of MSU crystals. Several literatures reported to have relationship between serum vitamin C and uric acid. Vitamin C supplementation may have a positive effect on purine metabolism which reduces the risk of formation of uric acid (Huang *et al.*, 2005; Brzezinska *et al.*, 2021).

In certain conditions uric acid also contributes to the formation of free radicals (Glantzounis *et al.*, 2005). Chronic exposure to free radicals contributes to the etiology of many diseases. Naturally occurring phytochemicals are the most potential agents to manage chronic diseases. As they possess antioxidant, anti-inflammatory, anticancer, antidiabetic activity. In this context SA which is known for its potential antioxidant and anti-inflammatory activity was selected. The phytochemical studies of SA are reported to contain flavonoids, alkaloids, carotenoids, phenolics, polyphenols, vitamin C etc. These constituents are individually known for their antioxidant potential (Bunawan *et al.*, 2015; Zhang *et al.*, 2015). Polyphenols, vitamin C, flavonoids, etc. acts as antioxidants and are reported to scavenge free radicals (Huang *et al.*, 2005). The presence of these phytoconstituents in SA could be contributing to its nitric oxide radical scavenging potential. Also, the hydroalcoholic extract of SA demonstrated ferric reducing ability pertaining to its antioxidant phytoconstituents. The present study exhibits antioxidant activity based on two assays carried out. The presence of flavonoids and phenolics reported to have XO inhibitory activity (Bakar *et al.*, 2020). Thus, the potential anti-inflammatory and antioxidant activities of flavonoids may be beneficial to overcome hyperuricemia and its complications.

CONCLUSION

The hydroalcoholic extract of SA showed *in vitro* antioxidant activity and suppression of the growth of MSU crystals. The *in vitro* inhibition of the growth of MSU crystal and antioxidant activity of the SA hydroalcoholic extract may be due to the presence of vitamin C and flavonoids. These results were found to be encouraging for the *in vivo* studies, which may be helpful to design therapies for gout management.

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