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A complete evaluation of the antioxidant and antimicrobial potential of *Glycine max*

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ABSTRACT

Vegetable soybean is rich in phytochemicals beneficial to the human being and is therefore considered a nutraceutical or a functional food crop. Soybean has antioxidative activity and protects tissues from oxidative stress-induced injury. Although isoflavones present in soy are believed to be major components responsible for the antioxidative activity, a recent study showed that anthocyanins present in black soybean had strong antioxidative potential. The present study focuses on both the antioxidant and antimicrobial potential of *Glycine max*.

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1. Introduction

Polyphenols as antioxidant compounds are gaining a lot of importance due to their dual role in the food industry as lipid stabilizer and in prevention of oxidative stress-related disease. Antioxidants especially natural antioxidants are reported to inhibit lipid peroxidation and protect from damage due to free radical. Vegetable soybean is rich in phytochemicals beneficial to the human being and is therefore considered a nutraceutical or a functional food crop [1].

Consumption of soybeans and soy products has been associated with reducing the risks of various cancers, such as prostate and mammary and several other chronic inflammatory diseases. The health promoting activity associated with soy consumption is attributed to the presence of isoflavone [2]. The structural similarities of isoflavones to naturally occurring estrogens may protect hormone dependant cancer by modulating activity of estrogen cholesterol levels. Soybean isoflavones have reportedly increased HDL cholesterol and lowered LDL cholesterol.

Isoflavones present in soybean are in the aglycone, beta glucoside, 6-o-maloyl-beta-glucoside or 6-o-acetyl-beta-glucoside forms. The biologically active components of soy isoflavones include genistein, daidzein and biochanin A. Genistein inhibits protein tyrosine kinase activity, topoisomerases I and II, ribosomal 6S kinase and alters cell proliferation. It also has antioxidant properties and suppresses skin tumorigenesis [3].

Isoflavones belongs to the class of polyphenols. The phenolic constituents of the diet act as antioxidant by virtue of free radical scavenging properties of the constituent hydroxyl group allowing them to act as reducing agents, hydrogen-electron-donating agents or singlet oxygen scavengers.

Genistein acts as an oxidant (stimulating nitrate synthesis) and it blocks the formation of new blood vessels (antiangiogenic effects). It also act as an inhibitor of the activity of substances that regulate cell division and cell survival. Soybeans are a significant source of mammalian lignan precursor secoisolariciresinol.

The effects of fermented soybean extract on the activities of the antioxidant enzymes (AOE) such as total superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) in liver, kidney, and brain from male Sprague-Dawley rats reports that the activities of CAT, SOD, and GPX are increased in the liver. However,

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the SOD activity is decreased in the kidney. SOD and GPX activities are decreased in the brain. These results lead to the conclusion that fermented soybean extract not only has antioxidant activity but also has an effect on the activity of antioxidant enzymes in liver [5].

There is ample evidence that soybean has antioxidative activity and protect tissues from oxidative stress-induced injury. Although isoflavones present in soy are believed to be major components responsible for the antioxidative activity, a recent study showed that anthocyanins present in black soybean had strong antioxidative potential.

Several phytochemicals and micronutrients that are present in fruits and vegetables are known to exert cancer chemo preventive effects in several organs, including the colon. Among them, the soybean isoflavonoid genistein received much attention due to its potential anticarcinogenic, antiproliferative effects and its potential role in several signal transduction pathways enhancing effects may, at least in part, be related to inhibition of prostaglandin catabolic enzyme activities [6].

2. Materials and methods

2.1. Collection of sample

The sample was collected from Botanical garden, Trivandrum, Kerala and washed thoroughly to remove extraneous material and then dried at 40°C in a tray drier and packed in airtight polyethylene bags until further use.

2.2. Preparation of methanolic extract

The dried plant sample was powdered to a size of approximately 20-40 mesh size. 15 g of this was extracted with methanol in a soxhlet apparatus. The extracts were filtered through a filter paper and concentrated in a rotary evaporator at below 45°C. Each of the extract was made up to 100 ml, and stored as stock solution at 4°C in a refrigerator.

2.3. Determination of dry weight

One ml of methanol extract of sample (MES) was taken in a previously weighed petriplate to constant weight. The extract was dried at 105°C till constant weight. Dry weight was calculated by the following formula

$$\text{Dry weight (1ml)} = A - B.$$

Where, 'A' is the weight of petriplate with dry sample. 'B' is the weight of petriplate.

2.4. Proximate composition

Proximate composition ie., amount of moisture, total fatty matter, carbohydrate, ash content and protein of the Glycine max were analysed according to the AOAC procedures.

2.5. Evaluation of Antioxidant activity

The antioxidant property was assessed in terms of total phenolic content, total reducing power, DPPH radical scavenging activity, metal chelating activity, ABTS radical cation scavenging assay, hydroxyl radical (OH⁻) scavenging activity and antioxidant activity in linoleic acid emulsion system.

2.6. Thin-layer chromatography

Three thin-layer chromatography (TLC) plates, coated with silica gel G (Fluka Chemie, Switzerland) to 0.25 mm thickness, were spotted with methanolic extract of soybean. All plates were then developed in a solvent system of ethyl acetate/methanol/water (10:2:1; v/v/v).

2.7. Evaluation of antibacterial activity

The antibacterial activity of soybean methanolic extract was studied against gram positive and gram negative bacteria using disc diffusion method.

3. Results and discussion

Soybeans contain isoflavones that have several known biological activities. Hence the present study was carried out to investigate the antioxidant, antimicrobial properties of Glycine max.

Glycine max powder was taken for the present study and was extracted by refluxing with methanol and subjected to proximate analysis for the determination of moisture, ash, crude fiber, fat and carbohydrate content. The total phenolic content was also studied and antioxidant properties were investigated [8].

3.1. Proximate composition

The results from the proximate analysis of Glycine max showed that moisture content was higher with 63.06±1.06 followed by total carbohydrate content 20.05±1.14%. The crude protein content was 1.04±0.09%, crude fiber content was 3.47±0.50% whereas the total fat and ash content were 5.31±0.46 and 7.34±0.54% respectively (Table 1).

The results of proximate analysis showed a high content of moisture and carbohydrate (Table 1).

Table 1. Proximate composition of vegetable soybean

Compositional Analysis	Values In %wet Weight Basis
MOISTURE	63.06 ± 1.06
ASH	7.34 ± 0.54
FAT	5.31 ± 0.46
PROTEIN	1.04 ± 0.09
CARBOHYDRATE	20.05 ± 1.14
FIBRE	3.47 ± 0.50

3.2. Dry weight of extract

The dry weight of the sample was calculated to be 24.6 mg/ml which corresponds to a yield of 16.4% of the dry sample.

3.3. Antioxidant activities

Antioxidant activities of soybean methanolic extract against DPPH radical, superoxide radical, hydroxyl radical, ABTS radical and metal chelation were evaluated using standard assay methods [9]. In addition, total phenolic content (TPC) and total reducing power (TRP) were also evaluated. The antioxidant activity of soybean extract in linolenic acid emulsion system was also evaluated. All the experiments were carried out in triplicates.

3.3.1. Total phenolic content (TPC)

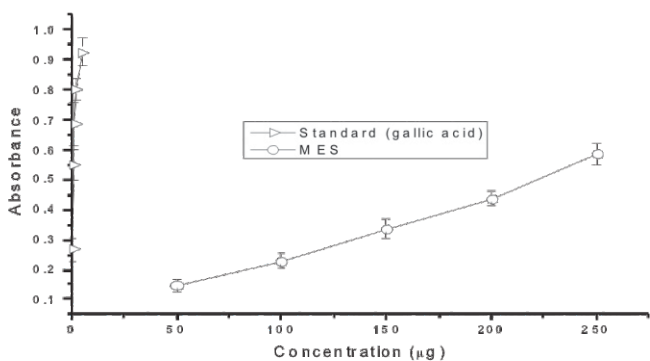
Total polyphenol present in the methanolic extract was determined by using Folin ciocalteau method and expressed as mg of gallic acid equivalents (GAE) per g of extract [10].

The total phenolic content in methanolic extract of Glycine max were 8.8% of the dry extract which corresponded to 1.4% of the dry sample. Overall, Glycine max revealed better antioxidant properties than *T.portentosum*, which was in agreement with the higher content of phenols found in the first species.

3.3.2. Total reducing power

The Fe³⁺- Fe²⁺ transformation in the presence of MES (sample) and gallic acid were investigated to measure reductive ability according to Oyaizu [11]. The presences of reducers (antioxidants) cause the reduction of Ferricyanide complex to the ferrous form. The absorbance was measured at 700 nm. Evaluation of total reducing power showed that gallic acid had reducing activity greater than that of soybean sample. A linear relation of reducing activity was observed between sample and standard (Fig.1). The reducing power of soybean extract might be due to the di and monohydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities [12].

Fig.1. Total reducing power of MES and standard



3.3.3. DPPH radical scavenging activity

Scavenging the stable DPPH radical model is another widely used method to evaluate antioxidant activity.

Fig.2. showed the scavenging activity of MES and standard on DPPH radicals at various concentrations. The scavenging activity on DPPH radicals increased with increasing concentrations (5g - 100g). A linear relation was obtained between radical scavenging activity and sample concentration.

Fig.2. DPPH scavenging activity of MES and standard

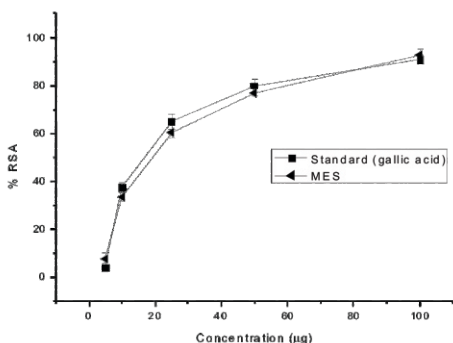


Table 2. gives the percentage DPPH scavenging activity of the extract and standard (gallic acid). There was an inverse relationship between IC₅₀ and antioxidant activity. The IC₅₀ value of sample was 16.48µg/ml and was high as compared to the standard (26.98µg/ml). This indicated antioxidant activity of sample was higher when compared to standard, indicating MES to be a potential antioxidant.

Table 2. Percentage DPPH radical scavenging activity and IC₅₀ values of MES & standard

Standard			Sample		
Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)	Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
5	3.83		5	7.66	
10	33.28		10	33.33	
25	47.13	26.98	25	60.48	16.48
50	79.9		50	76.99	
100	90.86		100	92.68	

3.3.4. Metal chelating activity

The chelation of ferrous ions by the extracts and standards was estimated by the method of Dinis et al [13]. As shown in Fig.3. the formation of the ferrozine-Fe²⁺ complex is not complete in the presence of the extracts indicating that MES can chelate ions. The absorbance of the complex decreased linearly in a dose dependant manner for the extracts (Table 3).

The IC₅₀ values for metal chelating activity of MES were found to be 116.83 g/ml. However the chelating ability of the extract was much lower than that of EDTA (IC₅₀ of 7.27 g/ml), which acted as a standard.

Fig.3. Metal chelating efficacy of MES and standard at varying concentration

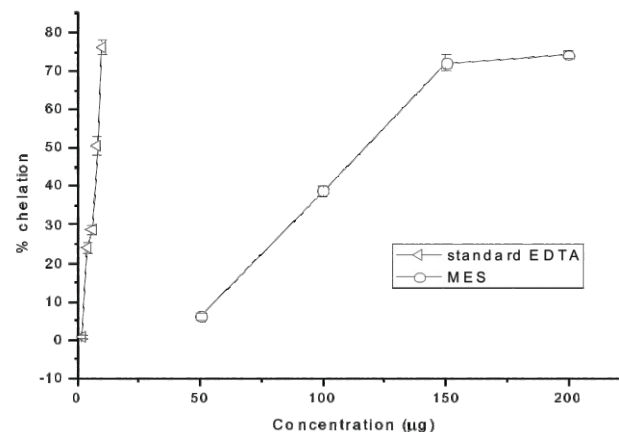


Table 3. % Metal chelating capacity of standard and sample at varying concentrations

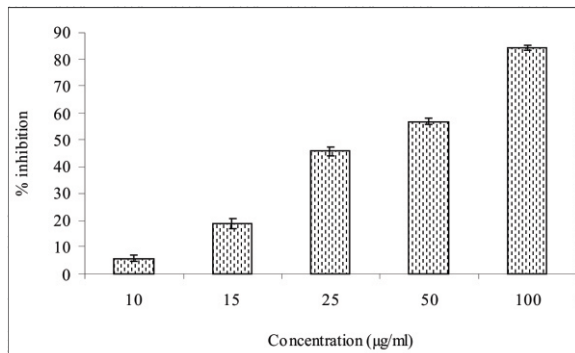
Standard			MSE		
Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)	Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
2	0.75		50	6.1	
4	23.93		100	38.8	
6	28.63	7.27	150	72.06	116.83
8	50.60		200	74.27	
10	76.06				

3.3.5. ABTS radical cation scavenging activity

Scavenging the stable ABTS radical model was widely used method to evaluate antioxidant activity [13].

Fig.4. depicted a steady increase in the ABTS radical scavenging capacity of the extract with increase in concentration. The TEAC value for the extract at the maximum concentration studied (100 µg/ml) was found to be 47.96 which means that 47.96 µg of trolox will give the same scavenging capacity as that of 100 µg of MES.

Fig.4. ABTS radical scavenging capacity of methanolic extract of soybean



3.3.6. Hydroxyl Radical Scavenging Activity

Hydroxyl radical generated through Fenton reagent in a buffered system can be used to evaluate the scavenging activity of antioxidant [14]. Fig.5. showed the dose dependant curve for the radical scavenging activity of extract and standard. The IC 50 values for standard catechin and MES were found to be 452.2 & 1153.3 µg/ml respectively. MSE exhibited lower hydroxyl radical scavenging activity as compared to standard, but still possess scavenging capacity (Table 4).

Fig.5. Hydroxyl radical scavenging efficacy of MES and standard at varying concentrations.

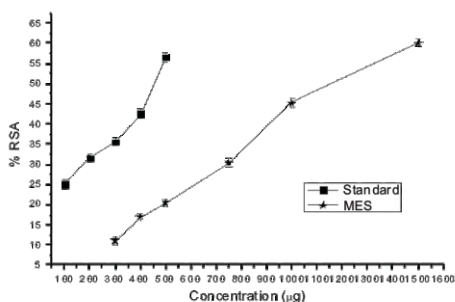


Table 4. % Hydroxy radical scavenging activity of standard and sample at varying concentrations

Standard			MSE		
Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)	Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
100	25		300	11.8	
200	31.5		400	17.1	
300	35.5		500	20.4	
400	42.53	452.2	750	30.32	1153.3
500	56.57		1000	45.23	
			1500	60.1	

3.3.7. Superoxide radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals [15].

The investigations in the present study on the superoxide radical scavenging capacities, showed that the MES inhibited superoxide radicals in a dose dependent manner and the extract exhibited super oxide scavenging activity at all the concentrations studied (100-300) µg/ml. IC50 value of standard was found to be 52.96 µg/ml and the same for MES was 284.1 µg/ml (Table 5).

Fig.6. Superoxide radical scavenging efficacy of MES and standard at varying concentration

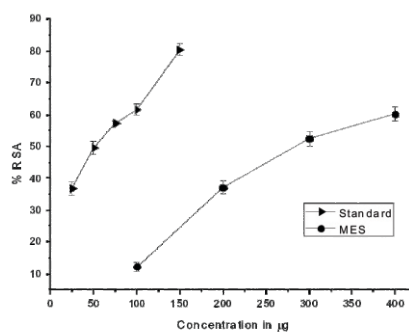


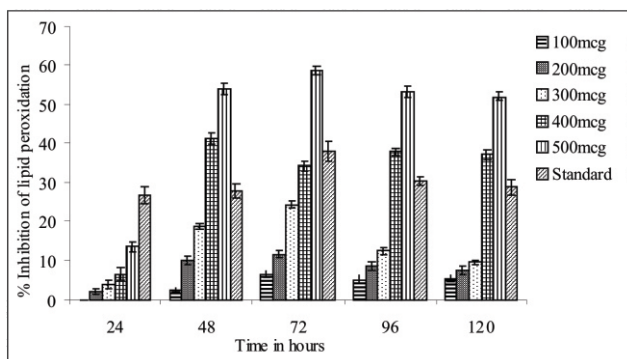
Table 5. % Superoxide scavenging activity of standard and sample at varying concentrations

Standard (Ascorbic acid)			MSE		
Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)	Concentration (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
25	36.7		100	12.46	
50	49.5	52.96	200	37.12	284.1
75	57.3		300	52.30	
100	61.4		400	60.13	
150	80.4				

3.3.8.Total antioxidant capacity determination in linoleic acid emulsion system

The sample exhibited effective and powerful antioxidant activity at all the concentrations tested. The effect of various concentrations of MES (100–500 µg ml⁻¹) on peroxidation of linoleic acid emulsions are represented in Fig.7. However, the maximum inhibition of catechin, at concentration of 500 µg ml⁻¹ was found to be only 37.02% after 72 hours of incubation. As can be seen, sample at concentrations of 400 and 500 µg ml⁻¹ was found to have better inhibition on linoelic acid peroxidation than the standard at the maximum concentration studied (500 µg ml⁻¹).

Fig 7. Antioxidant activity of sample at different concentrations and catechin (500 µg ml⁻¹) in the linoleic acid emulsion system using the thiocyanate method. Results are expressed as means ± SD of three parallel measurements.



3.3.9.Thin-layer chromatography

The chemical components, especially the phenolic compounds in the sub fractions, of the methanol TLC detected extracts with the use of UV absorption and specific spraying reagents [16]. A solvent system, ethyl acetate: methanol: water (EMW) (10:2:1, v/v/v) was used to separate the chemical components in MES. With the EMW solvent system, each fraction was separated into three to six UV-distinct spots (Fig.8). The results of further testing of these UV-positive TLC spots for their antioxidation activity (DPPH radical-scavenging) and phenolic compound identification (spray tests) are shown in Table 6. MES had two TLC spots that showed antioxidant activity in DPPH test. The presence of phenolic compounds were confirmed by the spray 1 test (Bartons test) where 5 distinct spots of phenolic compounds were separated. Spray 2 test, which indicated the presence of trihydroxy/dihydroxy phenolic compounds or indicated the presence of both trihydroxy (green colour with Rf 0.6 cm) and dihydroxy phenolic compounds (brown colour with Rf 1.5 and 3.1 cm) in the MES.

Table 6: Rf values of the chemical compounds of the methanolic extracts of vegetable soybean which showed positive results to the tests of antioxidant activity and presence of phenolics in TLC under a mobile phase of ethyl acetate:methanol:water

Antioxidant Test	MSE				
	R _{t1}	R _{t2}	R _{t3}	R _{t4}	R _{t5}
DPPH test	1.1	2.5			
Spray 1	0.6	1.2	6	6.3	6.7
Spray 2	0.6 (green)	1.5 (blue)	3.1 (blue)		

Spray 1. solution: 1% solution of iron(III) chloride in water mixed immediately before use with an equal volume of a 1% solution of potassium hexacyanoferrate (iii) in water (Bartons reagent).

Spray 2. solution: 2%iron(III) chloride in ethanol.

3.4.Evaluation of antibacterial activity

It is well known that phenolic antioxidants act as inhibitors for radical chain reactions on autoxidation of organic substrates, and the sulfuric antioxidants act as decomposers for hydroperoxides [17]. Moreover, some phenolic compounds were known to show antimicrobial activities in addition to their antioxidant effects.

The antibacterial activity of methanolic extract of soybean was tested against Gram positive and Gram negative bacteria using disc diffusion method. The organisms used were *Bacillus subtilis* and *Pseudomonas aeruginosa*. The antibacterial activity was tested using Kirby Bauer method. The paper discs were treated with known concentration of plant sample (24.6mg/ml) and then placed in agar plates inoculated with the respective organism. Methanol was kept as the control. The methanolic extract of soybean showed more inhibition against *Pseudomonas* (Plate III) and growth inhibition was less in the case of *Bacillus subtilis* (Plate II). The results of the antibacterial activity are shown in Table 7. As can be seen there is a clear zone of inhibition in both the cases. The inhibition was higher for G-ve *Pseudomonas aeruginosa*.

As the present study was very preliminary in nature, the antioxidant activity can be systematically studied using different concentration of the extracts against different strains of microbial organisms including pathogenic bacteria and fungi. Such a study will establish the actual antimicrobial potential of the extract and its use as a potential anti-microbial agent. However, the current study revealed that the MES possess antimicrobial activity.

Table 7. Zone of inhibition of MES in mm against the bacterial strains.

Bacterial strain	Zone of inhibition in mm
<i>Bacillus subtilis</i>	0.2
<i>Pseudomonas aeruginosa</i>	0.5

4. Discussion

The main objective of the present study was to investigate the antioxidant and antimicrobial potential of vegetable soybean using various in-vitro studies. The total phenolic content (TPC) and total reducing power (TRP) were evaluated for the methanolic extract of the dried sample. DPPH radical scavenging activity, metal chelating activity, ABTS radical cation scavenging assay, hydroxyl radical (OH-)

scavenging activity, superoxide radical scavenging activity and antioxidant activity in linoleic acid emulsion system were carried out for the methanolic extract of the sample (MES) to assess the in-vitro antioxidant activities.

The methanolic extract of the dried sample (MES) had good TRP as compared to the standard. The IC50 for DPPH radical scavenging activity of the standard gallic acid is 26.98 µg/ml and that of sample is 16.48 µg/ml indicating that MES possess promising antioxidant potential. MES exhibited very effective metal chelating activity (IC50 of 116.83µg/ml) though the chelating ability was less as compared to the standard EDTA (IC50 of 7.27 µg/ml). The TEAC value for the extract at the maximum concentration studied (100 µg/ml) was found to be 47.96 which means that 47.96 µg of trolox will give the same scavenging capacity as that of 100µg of MES.

Also, MES at concentrations of 400 and 500 µg ml⁻¹ is found to have better inhibition on linoelic acid peroxidation than the standard at the maximum concentration studied (500 µg ml⁻¹).

The presence of phenolic compounds was confirmed by TLC, by using three different spray tests as reported in the literature. Bartons test gave 5 distinct spots of phenolic compounds where as Spray 2 (solution: 2% iron(III) chloride in ethanol) test, indicated the presence of both trihydroxy (green colour with Rf 0.6 cm) and dihydroxy phenolic compounds (brown colour with Rf 1.5 and 3.1 cm) in the MES. A detailed study on chemical characterization is required to identify the active compounds in the MES.

Further studies were carried out to evaluate the antioxidant potential of MES as some phenolic compounds are known to show antimicrobial activities in addition to their antioxidant effects. As discussed in previous chapter, MES exhibited zone of inhibition against both Gram positive and Gram negative bacteria. The inhibition was higher for Gram negative *Pseudomonas aeruginosa*. As the current study is very preliminary in nature, the antimicrobial activity can be systematically studied using different concentration of the extracts against different strains of microbial organisms including bacteria and fungi. Such a study will establish the actual antimicrobial potential of the extract and its use as a potential anti-microbial agent.

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