Research article

Optimization studies for extraction of antioxidants from *Mucuna sanjappae* seeds: A promising natural drug for oxidative stress management

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ABSTRACT

Introduction and Aim: Cellular oxidative stress is responsible for various human diseases. Dietary antioxidant supplements from natural plants have good potential to reduce increased oxidative stress thus protecting from further medical complications. Genus *Mucuna* is well known for its promising health benefits. A common species of *Mucuna, M. pruriens* is being used for oxidative stress related diseases treatment. However, many species of this genus are not scientifically explored for their health-related beneficial properties. In the present study, we have made effort to investigate secondary metabolites composition and antioxidant potential in a *Mucuna* species namely *M. sanjappae.* In this work, we tried to understand superior system for secondary metabolites (total phenolics and flavonoids content) extraction and antioxidant activity in *M. sanjappae* seeds was demonstrated.

Methodology: Four different solvents viz., water, methanol, ethanol, and acetone in combination with four different extraction conditions (static, shaking, microwave and sonication) each with three different times have been studied. Total 48 extract samples were generated which were used for total phenolics, total flavonoids content along with antioxidant activity (DPPH, DMPD and FRAP) determination. Further major phenolics were identified using HPLC system.

Results: Water showed highest TPC and TFC level during microwave method for 120 seconds (81.627±0.728 mg GAE g^{-1} and 648.06±4.37 mg RUE g^{-1} respectively). The highest antioxidant activity was also found in *M. sanjappae* seeds extracted in water. RP-HPLC quantification of phenolics revealed tannic acid and gallic acid as predominant compounds. Correlation analysis indicated that the antioxidant of different extracted samples demonstrated a good positive relationship with TPC and TFC.

Conclusion: Present attempt indicated that, simple solvent water along with microwave method can be used for maximum antioxidants extraction from *M. sanjappae* seeds. This *Mucuna* species may be further studied to exploit as a natural antioxidant source in medicine and food *etc.*

Keywords: Antioxidant; extraction; *Mucuna*; oxidative stress; Parkinson's disease.

INTRODUCTION

Oxidation of biomolecules such as protein, DNA, electron transport chain, ubiquitin system leads to inflammation and cellular apoptosis (1). This process of oxidative damage is solely carried out by reactive oxygen and nitrogen species (ROS and RNS; 2). Non-free radical species (H_2O_2), hydroxyl radicals (OH^-), superoxide anion radicals (O^-_2), and the singlet oxygen (O_2^*) are the most reactive oxygen species. While peroxynitrite is an example of reactive nitrogen species synthesized from reaction between nitric oxide (NO) and superoxide. Peroxynitrite is a very potent, toxic, and versatile oxidant (3). Oxidative stress plays calamitous role in cell degeneration and progression of disorders. According to recent research, most of the disorders including neurodegenerative diseases, cardiovascular diseases and cancer have their progression pathways through oxidative stress caused by such hazardous oxidants (4).

At normal physiological conditions, dynamic antioxidant system of our body prevents destruction occurring because of free radicals' action (1). This system is composed of synthesized antioxidant compounds, antioxidant enzymes and dietary antioxidant molecules which contribute as a defense system against excess oxidants generated during various metabolic alterations (5). But an imbalance between oxidants and antioxidant system due to some known or unknown causes result in excessive oxidants generating oxidative stress. This ultimately leads to cell impairment and death through apoptosis.

Antioxidant potential is an important property of plants having tremendous value in the treatment of different human diseases and disorders (6). Hence, its study using *in vitro* or *in vivo* methods is an essential part of herbal medicine research. Antioxidant property of food and medicinal herbs is mainly depending on the secondary metabolites like phenolics and flavonoid compounds. Phenolics show diverse solubility in different solvents based on their

structure (7, 8). Hence, extraction of plant material using different solvents and methods is a key task to understand its exact antioxidant prospective (9) with precise conclusion.

Mucuna is one of the genera from Leguminosae family which is being used in the treatment of disorders generated due to oxidative stress especially neurodegenerative diseases (10, 11). *M. sanjappae* is one of the species from genus *Mucuna* found in Western Ghats of Maharashtra, India. The seeds of *M. sanjappae* contain higher level of L-DOPA, a promising drug for treating Parkinson's disease (10). With this background, this study was aimed to determine the superlative conditions for extraction of total phenolics and total flavonoids with superior antioxidant activity for the *M. sanjappae* seeds. For this purpose, four solvents viz., water, methanol, ethanol, and acetone in combination with four extraction methods viz., static, shaking, microwave and sonication having different incubation time was studied. Further, correlation of antioxidant activity with secondary metabolites (phenolics and flavonoids) is statistically determined.

MATERIALS AND METHODS

Chemicals and reagents

In all the experiments, we used analytical grade chemicals and solvents. Aluminium trichloride, ascorbic acid, ferric chloride, Folin-ciocalteu reagent, sodium carbonate, potassium acetate, DMPD (N, N-dimethyl-p-phenylendiamine), 2,4,6- tripyridyl-s-triazine (TPTZ), 2,2-Diphenyl-1- picrylhydrazyl (DPPH), were purchased from Sigma Chemical Co., USA. For the HPLC related studies water, methanol and acetonitrile were procured from Spectrochem Pvt. Ltd. India. Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany).

Plant material collection

The pods of *M. sanjappae* (MS) were collected from natural habitats at Junner, Maharashtra, India. The herbarium was maintained at Department of Botany, Shivaji University, Kolhapur. Healthy seeds were removed from the pods and fine powder was made in the mortar and pestle for further experimental work.

Experimentation

The experiment was designed using solvents viz., water, methanol, ethanol, and acetone and extraction techniques viz., static, shaking, microwave and sonication in combination with three different times (Table 1). 30mg *M. sanjappae* seed powder (MSSP) was extracted in 30 ml of respective solvent. Then centrifugation of samples was conducted at 10000 rpm for 5 min and kept in screw cap bottle until use. Detailed account about the experimental design and codes used for generated samples after extraction is shown in table 1 and 2 respectively.

Table 1: Combination of four different solvents with four different extraction techniques

	Static	**Shaking**	**Microwave**	**Sonication**
Water	3, 6 and 12hr	3, 6 and 12hr	30, 60 and 120S	5, 10 and 20min
Methanol	3, 6 and 12hr	3, 6 and 12hr	30, 60 and 120S	5, 10 and 20min
Ethanol	3, 6 and 12hr	3, 6 and 12hr	30, 60 and 120S	5, 10 and 20min
Acetone	3, 6 and 12hr	3, 6 and 12hr	30, 60 and 120S	5, 10 and 20min

Table 2: Codes used for the samples using different extraction methods

Sr. No.	**Code**	**Description**	**Sr. No.**	**Code**	**Description**
1	Wst 3	Water static 3hr	25	Etst3	Ethanol static 3hr
2	Wst6	Water static 6hr	26	Etst6	Ethanol static 6hr
3	Wst12	Water static 12hr	27	Etst12	Ethanol static 12hr
4	Wsh3	Water shaking 3hr	28	Etsh3	Ethanol shaking 3hr
5	Wsh6	Water shaking 6hr	29	Etsh6	Ethanol shaking 6hr
6	Wsh12	Water shaking 12hr	30	Etsh12	Ethanol shaking 12hr
7	Wmi30	Water microwave 30sec	31	Etmi30	Ethanol microwave 30sec
8	Wmi60	Water microwave 60sec	32	Etmi60	Ethanol microwave 60sec
9	Wmi120	Water microwave 120sec	33	Etmi120	Ethanol microwave 120sec
10	Wsn5	Water sonication 5min	34	Etsn5	Ethanol sonication 5min
11	Wsn10	Water sonication 10min	35	Etsn10	Ethanol sonication 10min
12	Wsn20	Water sonication 20min	36	Etsn20	Ethanol sonication 20min
13	Mtst3	Methanol static 3hr	37	Acst3	Acetone static 3hr
14	Mtst6	Methanol static 6hr	38	Acst6	Acetone static 6hr
15	Mtst12	Methanol static 12hr	39	Acst12	Acetone static 12hr
16	Mtsh3	Methanol shaking 3hr	40	Acsh3	Acetone shaking 3hr
17	Mtsh6	Methanol shaking 6hr	41	Acst6	Acetone shaking 6hr
18	Mtsh12	Methanol shaking 12hr	42	Acst12	Acetone shaking 12hr
19	Mmi30	Methanol microwave 30sec	43	Ami30	Acetone microwave 30sec

20	Mmi60	Methanol microwave 60sec	44	Ami60	Acetone microwave 60sec
21	Mmi120	Methanol microwave120sec	45	Ami120	Acetone microwave 120sec
22	Msn5	Methanol sonication 5min	46	Asn5	Acetone sonication 5min
23	Msn10	Methanol sonication 10min	47	Asn10	Acetone sonication 10min
24	Msn20	Methanol sonication 20min	48	Asn20	Acetone sonication 20min

Total phenolics and flavonoids content

The phenolic content of *M. sanjappae* seeds was determined spectrophotometrically according to the method reported earlier (12, 13). Results are expressed as mg of gallic acid equivalent per gram (mg GAE g^{-1}) of dry mass. The flavonoids content of extracted samples was quantified according to the standard method (14) and results are expressed as milligram of quercetin equivalents per gram (mg RUE g^{-1}) of dry weight.

Measurement of antioxidant potential

The antioxidant capacity (DPPH, DMPD, FRAP) was measured as described below using ascorbic acid as an internal standard.

a) DPPH free radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity of the MSSP extracts was determined as per the reported protocol (15) with modification (16). To prepare stock reagent, 24 mg of DPPH was added in 100 mL methanol and kept at -20^0C for future experiment work. The working solution was made by mixing of 10 mL of stored stock solution and 45 mL of methanol. This solution should show 1.1 ± 0.02 spectrophotometric absorbance value of at 515 nm. Then MSSP extract (100–500 µL) reacted with working DPPH solution after making the final reaction volume of 3 mL. The reaction mixture then incubated in dark. The decrease in colour intensity due to antioxidant mechanism was measured spectrophotometrically at 517 nm against methanol as blank. The absorbance of control DPPH solution was also determined, and the final results were depicted in µM AAE mM^{-1}.

b) DMPD radicle scavenging capacity

DMPD (N, N-dimethyl-p-phenylenediamine) antioxidant assay was done as per standard method (17). Briefly, stock of 100 mM DMPD solution was prepared by dissolving 209 mg DMPD in 10 mL D/W. Then, 1 mL of stock solution was mixed in 100 mL of 0.1 M acetate buffer (pH 5.2). To produce $DMPD^{\bullet+}$ radicals, 0.2 mL of a 0.05 M ferric chloride was added to the above solution. Respective volumes (10–50 µL) of MSSP extract was mixed in 2.0 mL of $DMPD^{\bullet+}$ solution. Further volume was made to 3 ml by D/W and reaction mixture was incubated for 10 min. Spectrophotometric reading were recorded at 505 nm. The DPPH radical scavenging capacity is expressed in µM AAE mM^{-1}.

c) FRAP (ferric reducing/antioxidant power) assay

FRAP assay was carried out as per method described (18). Respective volumes of MSSP extract were added in 3.0 mL FRAP reagent. D/W was added to the mixture to make up volume up to 4 ml and incubated in dark for 30 min at room temperature. The spectrophotometric absorbance of ferrous tripyridyl triazine complex was determined at 593 nm. The FRAP activity was expressed in µM AAE mM^{-1}.

Statistical analysis

The experimental assays were performed in triplicates and final outcomes are presented as Mean ± SEM using Microsoft excel program for Windows XP. Statistical correlation between secondary metabolites (Phenolics and flavonoids) and antioxidant potential was performed using GraphPad Prism 5 with one-way analysis of variance followed by Dunnett's multiple comparison tests.

RESULTS

Total phenolics content (TPC)

Effect of extraction solvent on TPC

Table 3 depicts phenolics content from the differentially extracted samples. Polyphenol contents determined using water extract was ranged from 67.34±0.56 to 81.62±0.72 mg GAE g^{-1}. TPC for methanol extract ranged from 25.71±0.39 to 77.82±0.35 mg GAE g^{-1}. The highest TPC content for ethanol and acetone extract was 7.92±0.52 and 2.95±0.01 mg GAE g^{-1}, which indicates that, these two solvents do not possess potential of TPC extraction from *M. sanjappae* seeds. The order of TPC extraction capacity for the various solvents under study was water > methanol > ethanol > acetone.

Table 3: Total phenolics content (mg GAE g^{-1}) and flavonoids content (mg RUE g^{-1}) of differentially extracted *M. sanjappae* seed samples.

Sample	Total phenolics (mg GAE g-1)	Total Flavonoids (mg RUE g-1)	Sample	Total phenolics (mg GAE g-1)	Total Flavonoids (mg RUE g-1)
Wst 3	67.34±0.56	438.06±8.125	Etst3	1.98±0.13	7.11±1.12
Wst6	75.38±0.70	501.81±6.25	Etst6	4.67±0.56	20.92±1.18

Wst12	79.55±1.07	497.12±4.68	Etst12	5.43±0.46	15.42±0.93
Wsh3	68.39±0.44	463.37±5.93	Etsh3	3.98±0.06	26.55±1.1
Wsh6	69.98±0.38	487.12±11.56	Etsh6	3.19±0.09	18.8±0.43
Wsh12	74.88±0.64	435.56±0.62	Etsh12	4.79±0.24	33.48±0.75
Wmi30	76.82±0.78	531.5±9.68	Etmi30	4.54±0.12	40.77±0.41
Wmi60	76.91±0.47	592.12±11.56	Etmi60	5.82±0.20	32.86±1.41
Wmi120	81.62±0.72	648.06±4.37	Etmi120	7.92±0.52	39.48±0.88
Wsn5	66.62±0.05	455.25±2.18	Etsn5	3.47±0.05	21.05±0.44
Wsn10	71.66±0.60	505.87±7.18	Etsn10	5.91±0.53	4.11±0.94
Wsn20	73.98±0.48	509±9.06	Etsn20	5.64±0.2	21.73±2.47
Mtst3	37.44±0.90	164.62±0.93	Acst3	1.93±0.32	1.28±0.04
Mtst6	44.29±0.63	230.87±4.68	Acst6	2.95±0.01	2.86±0.01
Mtst12	50.66±1.04	252.75±0.93	Acst12	0.07±0.00	2.51±0.12
Mtsh3	45.55±1.04	250.87±12.81	Acsh3	0.03±0.00	0.24±0.006
Mtsh6	54.29±0.90	273.37±0.9	Acst6	0.29±0.00	0.85±0.03
Mtsh12	59.43±0.45	319.93±5	Acst12	0.41±0.01	0.96±0.02
Mmi30	40.81±0.73	213.37±18.43	Ami30	0.13±0.02	0.41±0.02
Mmi60	71.29±0.22	245.25±2.81	Ami60	0.17±0.06	0.6±0.03
Mmi120	77.82±0.35	357.12±10.31	Ami120	0.26±0.00	0.92±0.05
Msn5	25.71±0.39	89.62±2.81	Asn5	0.19±0.00	0.60±0.01
Msn10	35.67±0.11	164.93±2.5	Asn10	0.49±0.01	1.56±0.05
Msn20	37.74±0.05	192.75±1.56	Asn20	1.67±0.04	1.78±0.03

Note: All the samples for reaction were taken 1% w/v except Acst3, Ami30 and Ami60 which were taken as 2%w/v).

Effect of extraction method on TPC

As like extraction solvent, extraction method and time have influenced greatly on the TPC of MS bean. In the present study, we had used four different extraction methods each with three different extraction time (Table 1). Water and methanol showed highest TPC extraction during microwave method for 120 seconds (81.627±0.728 and 77.827±0.355 mg GAE g^{-1} respectively). Though ethanol and acetone showed negligible TPC extraction, but among all the methods microwave 120 sec was found to be better method of extraction. Present study indicated, microwave and sonication assisted extraction effectively reduces time required for maximum amount of phenolics.

Total flavonoids content (TFC)

Effect of extraction solvent on TFC

The concentration of TFC was investigated in various solvent extracts of MSSP as is revealed in Table 3. The order of TFC extraction capacity of four different solvents was water > methanol > ethanol > acetone. The highest TFC (648.06±4.37 mg RUE g-1) was obtained in seed extracted by the water using microwave treatment for the 120 seconds. Methanol showed maximum TFC extraction (357.12±10.31 mg RUE g-1) for the same treatment. Ethanol and acetone showed very poor capacity of TFC extraction. The maximum TFC extraction using ethanol and acetone was 40.775±0.41 and 2.862±1 mg RUE g-1 respectively.

Effect of extraction method on TFC

The extraction method has strong effect on the TFC content of MSSP. The results revealed that among all the extraction methods, microwave treatment for 120 sec is the superior method for the maximum extraction of TFC. Water, methanol, and ethanol indicated maximum TFC content for the microwave treatment for 120 sec. (648.06±4.37, 357.12±10.31 and 40.77±0.41 mg RUE g-1 respectively) whereas acetone showed maximum TFC during the static condition for 6 hrs (2.862±1 mg RUE g-1).

From the TPC and TFC results, it was found that, water and methanol showed increase in content as time of extraction increases. But ethanol and acetone does not follow that ascending trend. For both the phytoconstituents (TPC and TFC) extraction from MS beans, water is proved to be best solvent.

Antioxidant activity studies

DPPH Radical Scavenging Assay (DPPH RSA)

For DPPH assay, 1mg/ml conc. of ascorbic acid was considered as a standard compound. The concentration of water and methanol extract for the reaction was taken 0.1mg/1ml while ethanol and acetone were 1mg/ml. It means that, water and methanol samples were of 10 times diluted suggesting the higher antioxidant capacity of sample extracted using these two solvents. The scavenging activity was found to be high for the water extract (707.66±1.38 um AAE mM^{-1}). The order for activity was water > methanol > ethanol > acetone (Fig. 1).

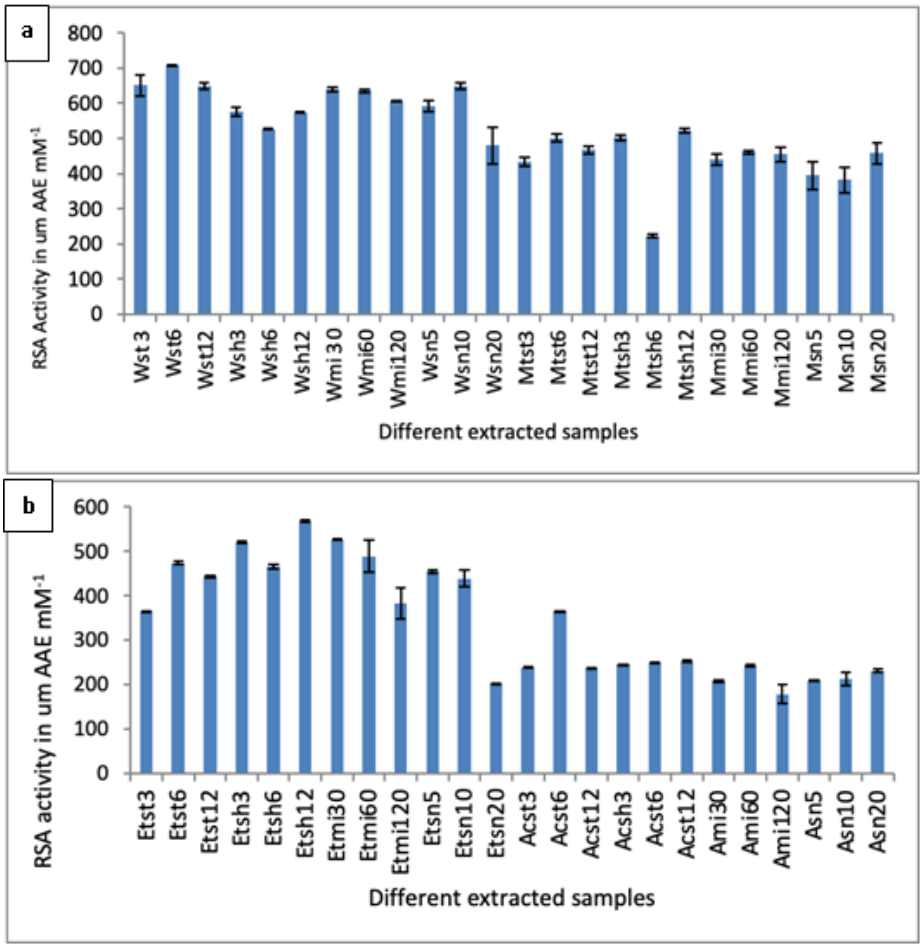
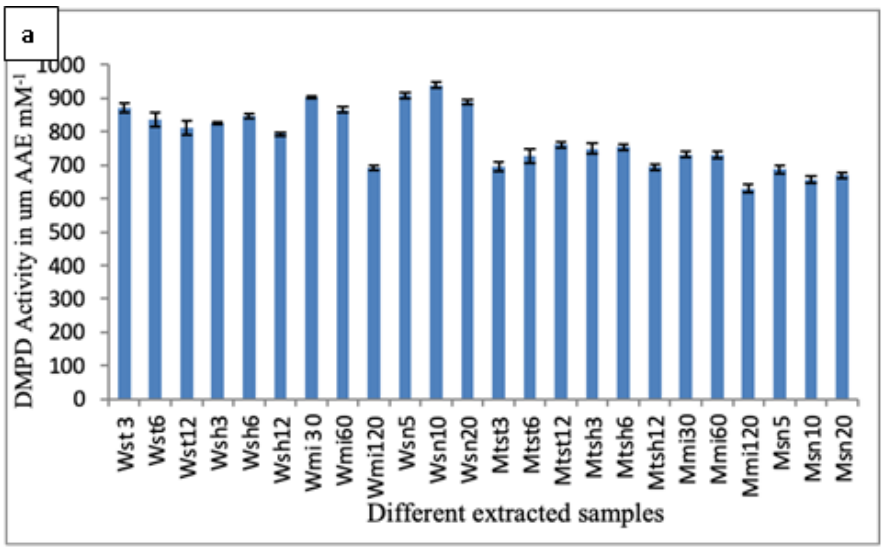


Fig. 1: DPPH radical scavenging activity of various solvent extracted MSSP samples in um AAE mM^{-1}. a) Water and methanol extract b) Ethanol and acetone extract (Note: Water and methanol extract samples were taken as 1mg/10ml whereas ethanol and acetone were taken as 1mg/ml).

DMPD radical scavenging activity (DMPD RSA)

In the present DMPD activity determination, MSSP water extract showed superlative activity for the scavenging of free $DMPD^{+}$ radicals as compared to different extracts under study. The order of the activity was water > methanol > ethanol > acetone (Fig. 2). The highest DMPD RSA was observed for the water extraction using sonication for 10 min (938.42±8.5 um AAE mM^{-1}). Methanol showed maximum DMPD scavenging activity of 760.57±9.28 um AAE mM^{-1} for extraction at static condition for 12 hrs. The concentration of water and methanol extract for DMPD reaction was 0.1mg/1ml while ethanol and acetone samples were taken as 1mg/ml. The study indicates that, ethanol and acetone have poor DMPD scavenging activity.



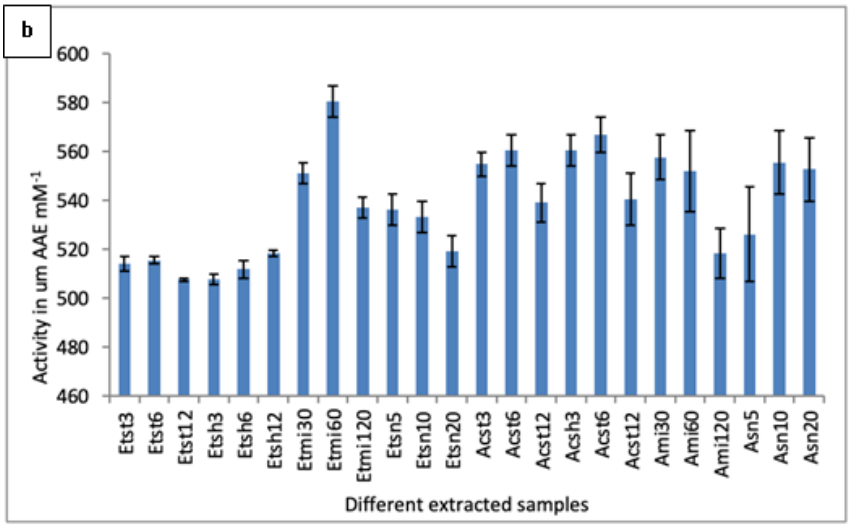


Fig. 2: DMPD radical scavenging activity of different solvent extracted MSSP samples in um AAE mM^{-1}. a) Water and methanol extract b) Ethanol and acetone extract (Note: Water and methanol extract samples were taken as 1mg/10ml whereas ethanol and acetone were taken as 1mg/ml).

FRAP assay

Standard used for FRAP assay was ascorbic acid. According to the present study, water is the best solvent for extracting phytocomponents with FRAP radical scavenging capacity. The order of activity was same as that of TPC, TFC, and other antioxidant assays that is water > methanol > ethanol > acetone (Fig. 3). The highest FRAP activity was found for the *M. sanjappae* seeds extracted using water at static condition for 6 hrs (803.4±11.5 uM AAE mM^{-1}). Methanol extract showed maximum FRAP activity of 358.9±18 uM AAE mM^{-1} for extraction at shaking for 3 hrs. As like DMPD assay, the concentration of water and methanol extract for DMPD reaction was 1 mg/10 ml while ethanol and methanol samples were taken as 1mg/ml. The study indicates that, ethanol and acetone have poor FRAP activity.

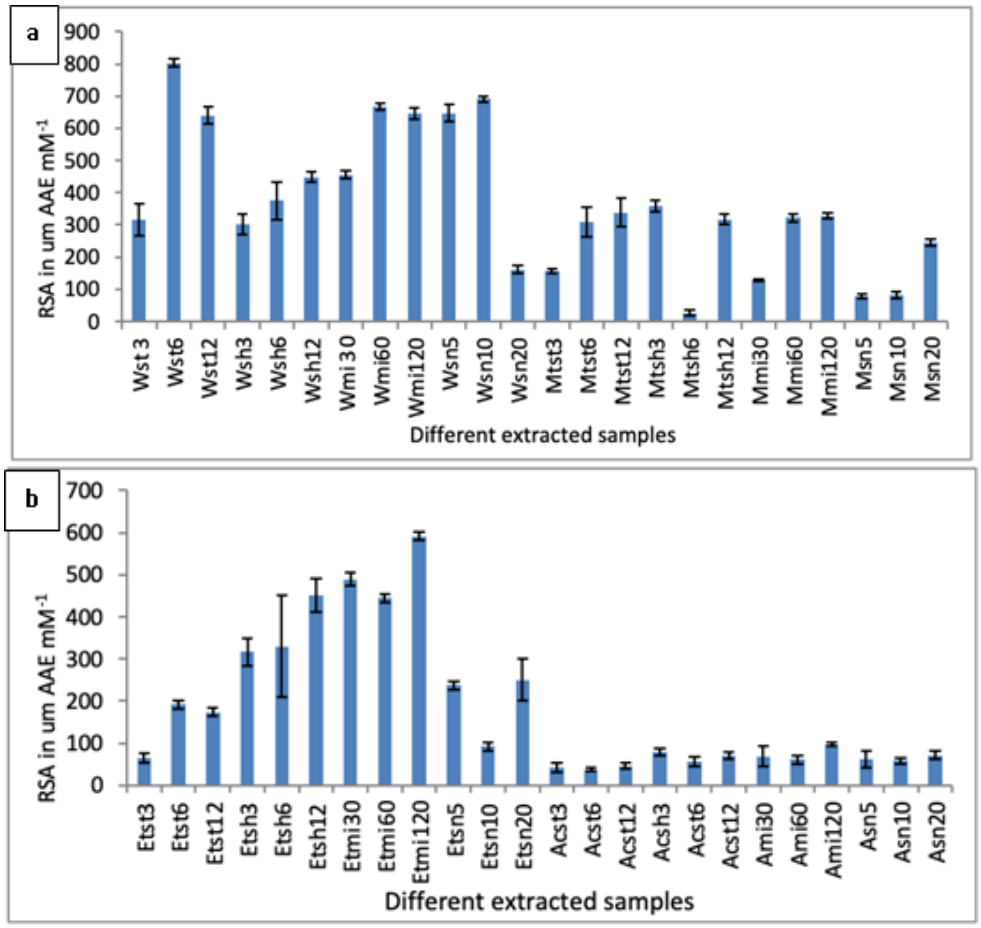


Fig. 3: FRAP activity of different various solvent extracted MSSP samples in uM AAE mM^{-1}. a) Water and methanol extract b) Ethanol and acetone extract (Note: Water and methanol extract samples were taken as 1mg/10ml whereas ethanol and acetone were taken as 1mg/ml).

Correlation of antioxidant activity with TPC and TFC

In the present study we carried out statistical analysis using GraphPad Prism 5 for correlation using three different antioxidant assays under study.

Fig. 4 presents the correlation between TPC with antioxidant activity in *M. sanjappae* bean extract which was determined using GraphPad Prism 5. The data showed a good positive correlation between TPC with DPPH (y = 3.3609x + 324.09; R^2 = 0.5092), DMPD (y = 3.9213x + 533.02; R^2 = 0.8266) however less correlation with FRAP (y = 4.1176x + 144.1; R^2 = 0.375) activity. The good correlation between TFC was found with DPPH (y = 0.53x + 320.54; R^2 = 0.5535) and DMPD (y = 0.5952x + 544.95; R^2 = 0.8324) and poor correlation with FRAP (y = 0.6869x + 144.88; R^2 = 0.4561) activity respectively (Fig 4).

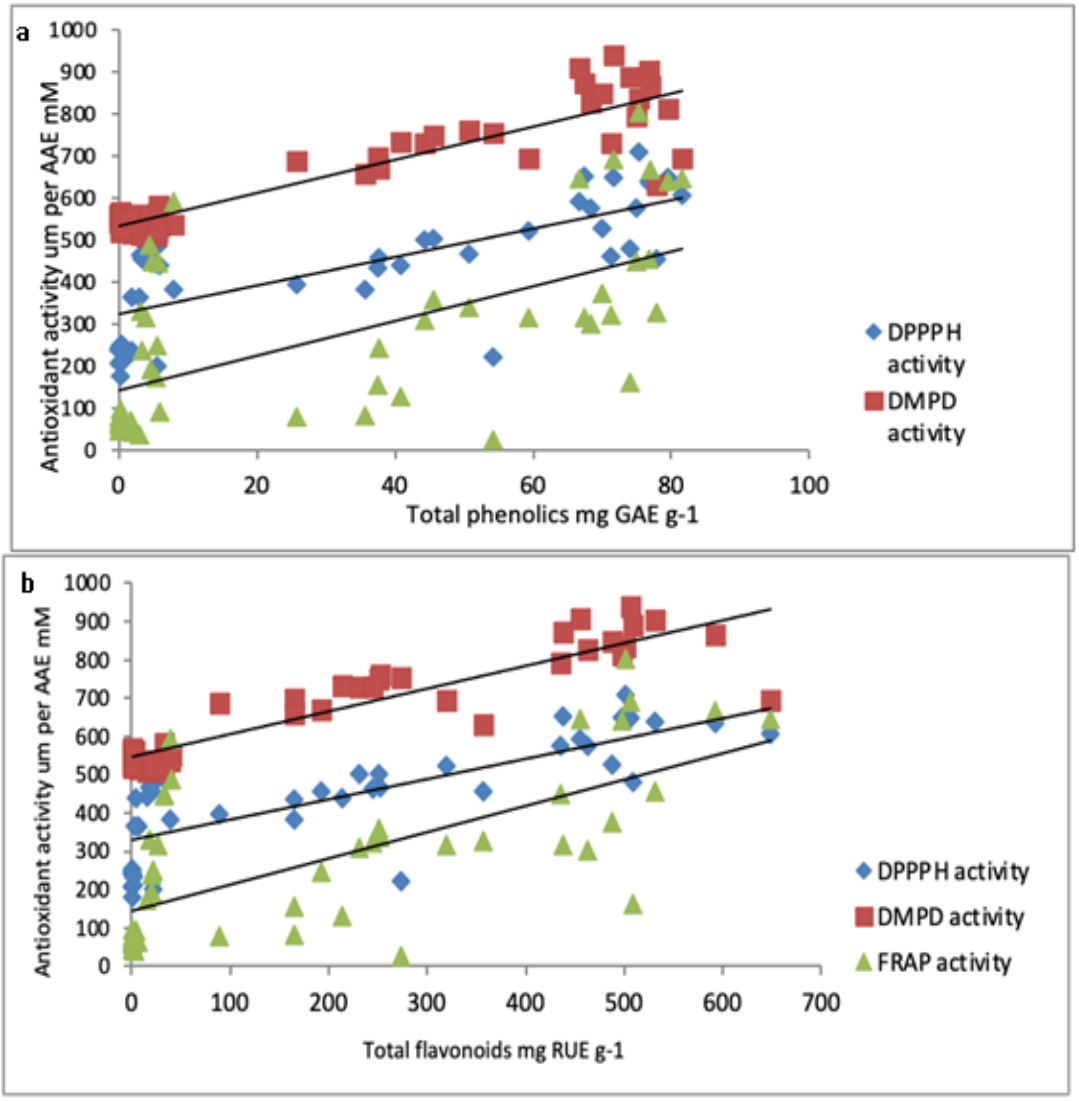


Fig. 4: Correlation of antioxidant activity (DPPH, DMPD and FRAP) with (a) total phenolic content and (b) total flavonoids content carried out using GraphPad Prism 5.

RP-HPLC quantification of gallic acid (GA) and tannic acid (TA)

RP-HPLC analysis has shown presence of gallic acid and tannic acid in *M. sanjappae* seed samples extracted using different solvents (Table 4). The higher level of GA and TA was found in water whereas, methanol showed moderate, and ethanol showed least content. Acetone extract does not show any presence of phenolic compounds. The present results of GA and TA level in different extracts were in accordance with TPC and antioxidant activity of respective extracts. The higher level of GA was achieved in water extract at microwave for 120sec (16.53 mg/gm) and sonication for 20min (16.37 mg/gm). However, maximum amount of TA was achieved in water extract at static for 12 hrs (32.47 mg/gm), sonication for 20min (32.11 mg/gm) and microwave for 120sec (31.30 mg/gm) treatment.

Table 4: Gallic acid and tannic acid quantification from MS seeds using RP-HPLC

Sample code	Gallic acid content (mg/gm)	Tannic acid content (mg/gm)	Sample code	Gallic acid content (mg/gm)	Tannic acid content (mg/gm)
Wst 3	1.57	15.73	Etst3	0.021	1.32
Wst6	8.27	29.65	Etst6	0.037	1.46
Wst12	9.71	32.47	Etst12	0.161	2.15
Wsh3	4.91	16.47	Etsh3	0.022	2.15
Wsh6	5.47	14.84	Etsh6	0.029	2.38
Wsh12	6.29	17.94	Etsh12	0.029	2.73
Wmi30	13.32	24.96	Etmi30	0.029	2.60
Wmi60	15.65	27.72	Etmi60	0.033	2.83
Wmi120	16.53	31.30	Etmi120	0.037	2.92
Wsn5	5.41	22.05	Etsn5	0.006	2.14
Wsn10	7.27	29.48	Etsn10	0.025	1.57
Wsn20	16.37	32.11	Etsn20	0.347	2.50
Mtst3	0.73	15.24	Acst3	ND	ND
Mtst6	3.20	15.45	Acst6	ND	ND
Mtst12	3.66	20.60	Acst12	ND	ND
Mtsh3	0.35	2.15	Acsh3	ND	ND
Mtsh6	1.68	2.38	Acst6	ND	ND
Mtsh12	5.97	2.73	Acst12	ND	ND
Mmi30	3.58	16.06	Ami30	ND	ND
Mmi60	4.93	19.13	Ami60	ND	ND
Mmi120	5.02	19.21	Ami120	ND	ND
Msn5	0.80	12.66	Asn5	ND	ND
Msn10	0.96	15.00	Asn10	ND	ND
Msn20	2.05	19.81	Asn20	ND	ND

ND: Not Detected

DISCUSSION

Investigation of chemical composition of food and medicinal plants is an essential task to ensure their beneficial activities for human health. Evaluation of important secondary metabolites and their medicinal properties is the prime research aspect for exploring novel plant species for sustainable human benefits. To this point, present efforts were made to investigate optimum conditions for quantification of phenolic and flavonoids level, antioxidant activity and their statistical correlation in *M. sanjappae* seeds.

Our study clearly showed that, extraction solvent significantly affects the TPC content of MS seed. This result agrees with earlier findings (19) which showed the recovery of phenolic compounds from Dalmatian Wild Sage (*Salvia officinalis* L.) is affected by the nature of solvent used, its polarity and the solubility of phenolic compounds. As like extraction solvent, extraction method and time have influenced greatly on the TPC of MS bean. Among the four methods, microwave and sonication were superior methods in phenolics compounds isolation. As found in phenolics, total flavonoids content was similarly affected by the type of solvent used for the extraction. Among the solvents, water proved to be the best solvent for the maximum extraction of TFC followed by methanol. Our study showed that, ethanol and acetone is the poor extractor of TPC and TFC from the *M. sanjappae* seeds and should not be consider for the phenolics or flavonoids extraction from it. Furthermore, method of extraction also showed considerable effects on flavonoids extraction. Microwave treatment for 120 sec. proved to be best for flavonoid isolation.

Due to the diverse properties of phytochemicals, total antioxidant of plant sample could not be understood appropriately using either single antioxidant assay method or single extraction system (20). Hence, present research effort was taken with three different antioxidant assays. In the present research attempt, measurement of antioxidant activity was carried out using simple and low-cost antioxidant assays. Scavenging of synthetic free radicals like $DPPH^{\bullet+}$, $DMPD^{\bullet+}$ using antioxidant compounds present in sample gives defined conclusion about antioxidant potential and its possible therapeutic use. The relative DPPH, DMPD and FRAP activity of the four different solvents extract using four different extraction methods with different time was investigated (Fig. 1).

Hydrogen donating antioxidant compounds present in sample causes reduction of DPPH radicals in the solution which is the principal of DPPH assay (21). There are several reports on successful application of DPPH assay for the antioxidant study of plant extracts (22). Water extract showed high antioxidant activity. Role of polyphenols in the DPPH radical scavenging activity is well described in earlier published scientific reports throughout world (7). In DMPD experiment, the reduction of colored unstable

$DMPD^{+}$ radicals by donating hydrogen atom resulting in colorless solution is the principal of assay. This assay is popularly used for determining the antioxidant capacity of diverse categories of food products (23). DMPD scavenging potential of water extract was superior followed by methanol and other solvent extracts. In addition, FRAP assay is a regular assay used in the antioxidant activity measurement. It has several advantages like simple, inexpensive, and rapid test. Fe^{3+} to Fe^{2+} reduction by electron present in the antioxidant compound of test drug/sample is the basis of FRAP assay. The formed complex of Fe^{+2} is monitored spectrophotometrically at 700nm (24). There is direct proportion of increased absorbance to the reduction capacity of the sample. This assay is successfully employed to investigate antioxidant properties of various plant species (25). We found common trend of antioxidant potential in various solvent extracted samples as: water > methanol > ethanol > acetone.

Statistical correlation of phenolics and flavonoids with antioxidant activity was determined. Phenolics and flavonoids are the compounds highly responsible for antioxidant potential of herbal medicine. Number of studies proved positive correlation of antioxidant activity either with TPC or TFC (24). In the present study, we also found that, there is direct correlation of these secondary metabolites concentration on antioxidant activity suggesting their role in the antioxidant properties of plants. Combinatorial effects of different biomolecules, principle and mechanism of antioxidant reactions and experimental conditions used for study have strong effect on the correlation of antioxidant activity with major secondary metabolites like TPC and TFC (11). Bioactive herbs containing higher level of phenolic and flavonoid compounds shows promising antioxidant and anti-inflammatory properties. It indicates herbal food and medicines possessing higher level of such compounds will exerts good health benefits against oxidative stress related disorders. Phenolic compounds have ability to donate electron to unstable free radicals. By terminating free radical chain reactions, these compounds can also improve quality and stability of food. Gallic acid and tannic acid were prime compounds quantified using HPLC. Gallic acid shows strong antioxidant and anti-Parkinson's activity (26). Different studies have proved antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, antiallergic, stopping bleeding, antimicrobial activity of tannic acid (27-29). Oxidative stress is one of the prominent causes in the inflammation, rheumatoid arthritis, cancer, aging, cardiovascular illnesses, and neurodegenerative ailments such as Parkinson's and Alzheimer's disorders progression (30). The data obtained from present study revealed potential of *M. sanjappae* seeds in reducing oxidative stress which may be further verified by *in vivo* and *in vitro* models.

CONCLUSION

Secondary metabolites isolation and antioxidant potential is strongly depending on the solvent and condition used for the extraction process. In our present study, we have reported four solvents in combination with four extraction methods. We found that, among various solvents, water has superior ability of extracting phenolic compounds and antioxidant activity from *M. sanjappae* beans. Among various extraction methods, microwave followed by sonication were superior methods. A positive correlation was observed between total phenolics or flavonoids content and antioxidant activity. HPLC analysis successfully quantified gallic acid and tannic acid as a major phenolics compounds. Overall, universal, and easily available solvent water in combination with microwave or sonication technique with minimum time proved to be best antioxidant extraction system for *M. sanjappae* seeds. Future studies can be focused on molecular level investigation of antioxidant mechanism of *M. sanjappae* and its further implementation in oxidative stress related disorders using *in vivo* and *in vitro* techniques.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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