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A comparative study of *in vitro* antioxidant and DNA damage protection of soxhlet vs microwave assisted extracts of *Michelia champaca* Linn. flowers

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Present study was designed to compare antioxidant and DNA damage protection of Soxhlet assisted (80% methanol) extract (SEMC) with microwave assisted (80% methanol) extract of *Michelia champaca* Linn. (MEMC). The *in vitro* antioxidant activity of SEMC and MEMC were tested for DPPH, hydroxyl radical (OH) by deoxyribose, lipid peroxidation and reducing power capacity. The extracts were also tested for protection against hydroxyl radical and tertiary butylated peroxide (t-BOOH) radical mediated DNA damage. SEMC showed highest percentage of inhibition in all the concentrations in DPPH assay. Both SEMC and MEMC showed better percentage of inhibition 93.0 and 85.0%, respectively, at 50 µg/ml concentration in hydroxyl radical assay. In lipid peroxidation inhibition assay both extracts have shown good peroxidation inhibition activity. In reducing power assay SEMC showed highest power of reduction. MEMC exhibited good protection against hydroxyl radical and t-BOOH mediated damage to DNA.

Keywords: *Michelia champaca*, Antioxidant activity, DNA damage protection.

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Introduction

Recently many alternative extraction techniques have been developed to improve the extraction method and to save the time and get maximum phytoconstituents. Conventional extraction method is used at particular temperature for several hours by refluxing. This method is time consuming and large quantity of solvent required to extract. In the extraction method developments, microwave technique is also used. Microwave assisted extraction is the most economic and time saving technique and microwave assisted extraction reduces quantity of solvent consumption. It drastically removes variety of compounds from plant material[1]. Many studies have been carried out on the microwave assisted extraction to investigate phytoconstituents from the plants.

Michelia champaca Linn. (Family-Magnoliaceae) flower commonly known as Sampige, is a tree distributed in tropical Asia and China. The plant was traditionally being used in constipation, bronchitis, antidiabetic and amenorrhea. The flowers used to cure fever, inflammation and diabetes in Indian traditional medicine[2]. The antidiabetic, antipyretic and anti-inflammatory properties of *M. champaca* flowers have been reported[3,4]. The flower was estimated for essential oil of 0.03% and the active constituents: flavonoid, quercetin, sitosterol, liriodenine, micheline A and hydrocarbons were reported from the flowers of *M. champaca*[5-10]. However, so far no comparative study of antioxidant and DNA damage protection has been reported. Thus, in the present study, we have designed the comparison of antioxidant and DNA damage protection of SEMC and MEMC by using different *in vitro* models.

Materials and Methods

Chemicals and Reagents

DPPH (2, 2'-diphenyl-1-picryl hydrazyl) were obtained from Sigma Aldrich Co, Bangalore. Potassium dihydrogen phosphate, potassium hydroxide, ferric chloride, ferrous sulphate, ethylene diamine tetraacetic acid (EDTA), ascorbic acid, potassium ferricyanide, bromophenol blue, boric acid, ethidium bromide, agarose, peptone, sodium chloride, butylated hydroxy anisole and thiobarbituric acid (TBA) were procured from Sigma (St. Louis, USA). Trichloro acetic acid (TCA) and phenol were obtained from Merck (Dermastadt, Germany). All the

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chemicals used were of analytical grade.

Collection and authentication of plant

The flowers were collected during the month of December in 2008 from Malappuram, Kerala. The plants were authenticated by Dr. Shiddamallayya N. Regional Research Institute (Ayurveda), Bangalore.

Soxhlet extraction of plant material

The flowers were shade dried and powdered separately. Powdered material (50 g) was placed in a glass Soxhlet thimble and extracted with 80% methanol for 18 h. The extract was concentrated under reduced pressure and controlled temperature (40-50°C). The nature and yields of the flower extract was brown residue and yield was 4.00 g (SEMC).

Microwave extraction of plant material

The dried flower material (50 g) was extracted at the maximum magnetron power (90 W) using 80% methanol in microwave oven for 15 min. After the extraction, the solution was left for cool and then filtered and concentrated under reduced pressure and controlled temperature by using rotary evaporator. The nature and yield of the flower extract was brown residue and yield was 2.74 g (MEMC). Both the extracts were stored in a refrigerator at 4 ºC till further use.

Qualitative phytochemical analysis

The Soxhlet and microwave extracts of *M. champaca* flower were evaluated for chemical tests for the identification of various active constituents[11].

***In vitro* antioxidant activity**

The SEMC and MEMC were tested for *in vitro* antioxidant activity using several standard methods. The final concentration of the extract was 150, 100 and 50 µg/ml. The absorbance was measured spectrophotometrically against the corresponding blank solutions.

DPPH$^{\bullet}$ free radical scavenging activity

DPPH$^{\bullet}$ radical scavenging activity was assessed with minor modifications[12]. Different concentrations of the extracts/standard antioxidants ranging from 50, 100 and 150 µg/ml were mixed with 1 ml of freshly prepared 0.5 µM DPPH in methanolic solution and 2 ml of pure methanol. The resulting solutions were then kept for 30 min at 37°C. The absorbance was measured spectrophotometrically at 517 nm. Blank test or control are without any inhibitor or test sample. The % inhibition was calculated.

Hydroxyl radical ($^{\bullet}$OH) scavenging activity

OH radical scavenging was determined by the method of Aruoma and Halliwell (1987)[13]. OH radical were generated by incubating the potassium dihydrogen phosphate (10 mM), potassium hydroxide buffer (pH 7.4) at 37ºC for 60 min. Hydrogen peroxide (1.4 mM), ferric chloride (20 mM) and deoxy ribose (2.8 mM), ethylene diamine tetra acetic acid (100 µM) and ascorbic acid (100 µM) were added to the solutions with extracts/without extracts. Ascorbic acid was added in the end to start the reaction. Degradation of deoxyribose sugar induced by $^{\bullet}$OH was determined by addition of 1 ml TBA (1%, w/v) and 1 ml TCA (5.0% w/v) and heating at 100ºC for 20 min. The pink chromogen formed was determined by measuring its absorbance at 535 nm.

Lipid peroxidation inhibitory assay

A spectrophotometric assay for evaluating antioxidant activity was based on the inhibition of peroxidation in human erythrocytes ghosts. An assessment of oxidation was achieved by measurement of thiobarbituric acid reactive substances. The human erythrocyte ghost was isolated according to the method of Dodge *et al* (2002)[14]. The ghost suspension (100 m1 containing 300 mg membrane protein equivalent) was subjected to peroxidation by ferrous sulphate and ascorbic acid (10:100 mM) in final volume of 1 ml of tris buffered saline (20 mM, pH 7.4, sodium chloride 150 mM). The reaction mixture was treated with or without extract (50, 100 and 150 µg/ml). Butylated hydroxy anisole (BHA) and curcumin were used as positive control and the concentration was 400 mM. The contents were incubated for 1 h at 37ºC. The reaction was terminated by the addition of phenol (5%, 10 ml) and TCA (1%, 1 ml). To each system TBA (1%, 1 ml) was added and the mixture were kept in a boiling water bath for 15 min, cooled and centrifuged at 6000 rpm for 10 min. The absorbance of supernatants was measured colorimetrically at 535 nm. The negative control without any test sample was considered as 100% peroxidation[15].

Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986)[16] with minor modification. Potassium ferricyanide solution (100 µl)

was mixed with phosphate buffer (200 µM, 200 µl, pH 6.5) in the presence or absence of extracts or BHA at 50, 100 and 150 µg/ml concentrations. The contents were incubated at 50°C for 20 min and TCA (10%, 200 µl) was added to the reaction mixture and centrifuged at 5000 rpm. The resulting supernatant was taken and mixed with 100 µl of ferric chloride solution and final volume was made up to 1 ml with water and then incubated at 37°C for 10 min. The absorbance was measured at 700 nm. The absorbance increases, reducing power of the extracts increases.

Hydroxyl radical mediated DNA damage assay by electrophoresis

The ability of Soxhlet and microwave extracts of *M. champaca* to protect Calf thymus DNA from devastating effects of hydroxyl radicals. The assay was described by Lee *et al* (2002)[17] with minor modifications. The reaction mixture contained 10 µl of 30 µM H_2O_2 followed by addition of extracts and the standard curcumin and the final volume of the mixture was 30 µl. The mixture was then incubated for 30 min at 37ºC and the DNA was analyzed on 1% agarose gel (prepared by dissolving 1.0 g of agarose in 100 ml of TBE buffer) followed by ethidium bromide staining. In this curcumin was used as positive control and without extract was negative control.

Tertiary butylated hydroperoxide (t-BOOH) mediated DNA damage assay by electrophoresis

To prepare agarose solution (1.0%, 100 ml) 0.7 g of agarose was added to 100 ml of TBE. Microwave or on a hot plate stirred until agarose dissolved. The solution was cooled to 55ºC before pouring (Ethidium bromide can be added at this point to a concentration 0.5 µg/µl). Gel tray was prepared by sealing ends with tape or other custom made dam. The comb was placed in gel tray about 1 inch from one end of the tray. Gel solution (50ºC) was poured into tray to a depth of 5 mm and gel was allowed to solidify for 20 minutes at room temperature. The comb was removed from the tray and covered the electrophoresis with buffer (the same buffer used to prepare the agarose). To prepare sample for electrophoresis, add 1 µl of 6 x gel loading dye for every 5 µl of DNA solution. Mix well, lode 5-12 µl of DNA solution per well. Electrophoresis at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized. If the gel was not stained with ethidium during the run stain the gel in 0.5 µg/ml ethidium bromide until the DNA has taken up the dye and is visible under short wave UV light[18].

Results and Discussion

Qualitative phytochemical analysis

The preliminary phytochemical analysis results of SEMC and MEMC is given in the Table 1. The SEMC yield was more when compared to MEMC. Both extracts contains carbohydrates, alkaloids, terpenoids, flavonoids and steroids. Proteins and amino acids are absent in both the extracts.

Scavenging activity of extracts against DPPH radical

Free radicals are major contributors for ageing and for degenerative diseases, such as cancer, cardio vascular disease, cataracts and declined immunity system[19], therefore, scavenging of free radical is having importance. DPPH gives a stable free radical in methanol solution receiving proton from any hydrogen donor, mainly from flavonoids or phenolics, it reduces its chromophore and will become pink to yellow. In order to measure the antioxidant activity of both the extracts (SEMC and MEMC), through free radical scavenging the results are shown in the

Table 1— *In vitro* antioxidant properties of *M. champaca* flower extracts

*Average of three determinations

Extract/Standard (concentration in µg/ml)	% inhibition ± SEM*		
M. champaca soxhlet assisted extract (SEMC)	DPPH assay	Hydroxyl radical by deoxyribose	Lipid peroxidation
150	70.0 ± 3.03	97.8 ± 0.70	94.0 ± 2.6
100	51.6 ± 0.28	90.8 ±1.41	91.0 ± 0.40
50	10.5 ± 0.30	93.0 ± 4.9	82.0 ± 0.70
M. champaca microwave assisted extract (MEMC)			
150	48.5 ± 1.76	97.4 ± 8.48	92.0 ± 2.12
100	31.6 ± 3.53	88.8 ± 6.36	91.0 ± 2.82
50	7.06 ± 2.82	85.0 ± 2.82	89.0 ± 1.4
Curcumin (10 µg/ml)	29.0	90.0	92.0
BHA (10 µg/ml)	-	95.0	98.0

Table 1. According to values provided in Table 1, SEMC exhibited highest percentage of scavenging than the MEMC in all tested concentrations. A dose dependent response was observed in DPPH radical scavenging activity and the concentration increased as the activity increased for both the extracts. The scavenging SEMC in all the three concentration is more than the BHA or curcumin.

Scavenging activity of extracts on OH radical

OH radical, singlet oxygen, hydrogen peroxide and organic peroxide produce strong bioactivity. In normal amount of free radicals shows positive activity on division and growth of cells, as well as in detoxification. But excess of free radical produce toxic effect on human bodies, such as damage to macromolecules and change in cellular activity results series of harmful biochemical reactions. OH is the most active one which can react with any biomolecules in high speed. Therefore, $^{\bullet}OH$ may be the most harmful ROS to the organism[20,21]. Table 1 depicts scavenging activities of both extracts against OH radicals. The scavenging activity of SEMC and MEMC on OH radicals were comparable with the standards used (curcumin and BHA). The scavenging activity was increased as the concentration of the extracts increased, with a maximum scavenging activity >97.8% at 150 µg/ml. At all the concentrations selected except 50 µg/ml, the order of scavenging activity of tested extracts was SEMC>MEMC>BHA>curcumin, indicating scavenging activities of the extracts were stronger than the standard antioxidants.

Scavenging activity of extracts in lipid peroxidation inhibitory assay

Lipid peroxidation leads to the formation of several products such as diene conjugates malondialdehyde and hydroperoxides that can act as oxidation indicates measuring the ability of a compound to decrease these oxidation indicators[18]. The results are shown in the Table 1. According to the results, this is dose dependent activity and comparable with the standards curcumin (10 µg/ml) and BHA (10 µg/ml). All effects increased with increasing concentration. The scavenging activity of lipid peroxidation was decreasing, SEMC>MEMC>curcumin.

Reducing power assay

Measurement of reducing power gives antioxidant activity in the extracts. In this method reduction of the

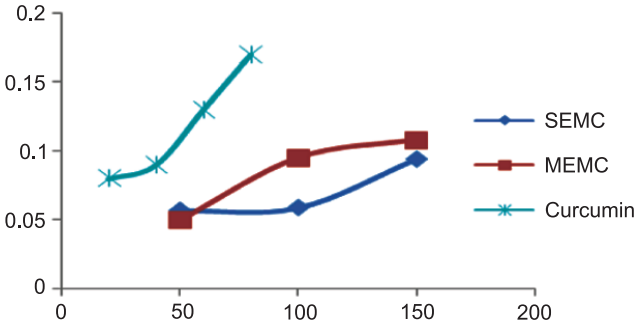

Fig. 1— Reducing power of SEMC and MEMC

Fe^{3+} (ferric)/ferricyanide complex to Fe^{2+} (ferrous) ion in presence of reductants (antioxidants) present in the extracts. Formation of ferrous complex of blue color is measured at 700 nm[22]. Higher absorbance indicates higher reducing power. Reducing capacity of extracts has been shown in Figure 1. The concentrations of extracts measured 150, 100 and 50 µg/ml with the absorbance of MEMC at 700 nm were 0.108±0.07, 0.095±0.05 and 0.050±0.07, respectively, while those of SEMC were 0.094±0.08, 0.059±0.02 and 0.057± 0.02, respectively. MEMC exhibited stronger reducing power than the SEMC.

Hydroxyl radical mediated DNA damage assay by electrophoresis

The antioxidant activity of SEMC and MEMC in hydroxyl radical mediated DNA damage was checked by electrophoresis. The results were in confirmation with the hydroxyl radical scavenging ability of SEMC and MEMC in DNA degradation method. It had been found that when the calf thymus DNA was dissolved in Fenton's reaction mixture, the DNA molecules were broken into small sections[23] due to hydroxyl radicals generated in reaction mixture. However, addition of extracts reduced the hydroxyl radical mediated strand breaking markedly. Significant protection of DNA from damage caused by hydroxyl radicals was also observed. However, the curcumin treated (positive control) exhibited more significant protective effect. MEMC exhibited more significant effect than the standard curcumin.

t-BOOH radical mediated DNA damage assay by electrophoresis

The protection of DNA damage induced by t-BOOH radical of SEMC and MEMC, were measured by electrophoresis. The results confirmed radical scavenging activity of SEMC and MEMC in broken DNA. Significant protection of DNA from damage caused by hydroxyl radicals was observed.

However, the curcumin treated (positive control) exhibited more significant protective effect. MEMC showed highest significant effect then the standard curcumin. In both the extracts MEMC is showing more protection.

Conclusion

The present study demonstrated that SEMC exhibited stronger antioxidant activity against DPPH, OH radical and in lipid peroxidation. The microwave assisted extract of *M. champaca* effectively inhibited hydroxyl radical and t-BOOH radical-mediated DNA damage under *in vitro* conditions and showed stronger reducing capacity. This suggests that the phytoconstituents which are present in the extracts may be good indicators for potential antioxidant activity of the extracts. This may be due to the type of phenolics extracted or some unidentified antioxidants. These observations suggest that the nature of the biologically active constituents of the SEMC may be different from those present in the MEMC. The investigation results revealed that both SEMC and MEMC can be used as the easily accessible source of natural antioxidants.

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