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Original Article

DETERMINATION OF SECONDARY METABOLITES, LD_{50} VALUE AND ANTIOXIDANT ACTIVITY OF SEED EXTRACT OF *CUCURBITA PEPO* LINN.

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ABSTRACT

Objective: To quantify the secondary metabolites, acute toxicity profile and the antioxidant potential of seed extract of *Cucurbita pepo*, an edible vegetable commonly used by people of Asian countries.

Methods: The methanolic seed extract of *C.pepo* (MSCP) was analyzed by HPLC and GC to determine various phytochemicals. Free radicals scavenging activity of MSCP was measured against DPPH, NO and superoxide radicals generated *in vitro*. Male Sprague Dawley rats were administered 200, 500, 1000, 2000, 2500 and 3000 mg/kg body weight of MSCP as a single dose or in 3 equally divided doses within 8 hrs and observed for any abnormal symptoms of toxicity. Serum biochemistry and histopathological score in the ultra-sections of major organs were also determined.

Results: MSCP is found to contain alkaloids (4.09 mg), phenols (0.988 mg), flavonoids (1.223 mg) and terpenoids (2.144 mg) per gram of MSCP. The major flavonoid detected is quercetin. The MSCP is found to possess significant radical scavenging activity against DPPH (IC_{50} – 40.8 µg/ml), NO (IC_{50} – 38.70 µg/ml) and superoxide anions (IC_{50} – 38.4 µg/ml) and the effect is comparable to that of the corresponding reference compounds ascorbic acid and rutin in terms of IC_{50} value. By applying Karber's formula, 2975 mg/kg b.wt has been confirmed as the LD_{50}value of MSCP.

Conclusion: The medicinal property of *C.pepo* seeds may be attributed to the presence of flavonoids and phenolic compounds which counteract the free radicals responsible for various health complications. Based on the LD_{50}value, it could be recommended that 250 mg/kg b.wt can be administered to rats to study the therapeutic action of *C.pepo* seed extract.

Keywords: *Cucurbita pepo*, phytochemicals, free radicals, LD_{50} value, scavenging activity.

INTRODUCTION

Plants are natural source of biologically active compounds known as phytonutrients. Phytonutrients are secondary metabolites that have either defensive or disease preventive properties. Dietary intake of phytochemicals may promote health and can prolong the onset of disorders such as cancer, coronary heart disease, diabetes, and in general inflammation related disorders [1]. Majority of food ingredients such as whole grains, beans, fruits, vegetables and herbs have tremendous therapeutic potential in curing various ailments.

Harborne identified three major classes of plant chemicals terpenoids, phenolic metabolites and alkaloids [2]. Plant polyphenols are secondary metabolites that are water soluble which possess antioxidant properties. The most important dietary phenolics are the phenolic acids (including hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolysable and condensed tannins) and flavonoids. Phenols protect plants from oxidative damage. They are known to play major role in reducing the risk of coronary heart disease, diabetes, hypertension and some type of cancer [3-6].

The first line defense of antioxidants is provided by quenching free radicals which otherwise lead to propagation of lipid peroxidation and damage to critical biomolecules. Epidemiological and animal studies suggest that the regular consumption of fruits, vegetables and whole grains, reduce the risk of chronic diseases associated with oxidative damage mainly due to the antioxidant potential of the phytonutrients present in them[7, 8].

Cucurbita pepo Linn, commonly known as pumpkin or pepita is a gourd like squash of the family cucurbitacea [9]. Pumpkin seeds are smooth, flat and greenish, contain fatty acids with linoleic acid being the main component, and also contain various plant sterols and sterol glycosides, cucurbitine as well as tocopherols (Figure 1) [10-12]. Pumpkin seeds are very good source of the minerals phosphorous, magnesium, manganese, zinc, iron and copper [13]. Clinical research carried out in Thailand has shown that pumpkin seeds can reduce the risk of bladder stone disease (urolithiasis) [14]. Diets rich in pumpkin seeds were also reported to reduce the risk of gastric, breast, lung and colorectal cancer [15].

Fig.1: *Cucurbita pepo* Linn

The traditionally used plant products, if recommended scientifically for therapeutic purpose, they need to be validated in terms of their phytochemical components and the acute toxicity profile. Also the minimum lethal dosage need to be determined to evaluate the biological efficacy of the test compound to be recommended.

Hence the aim of the present study is to determine the acute toxicity profile, LD_{50} value and to investigate the antioxidant activity of MSCP by using *in vitro* models of free radicals.

MATERIALS AND METHODS

Collection and identification of seeds

The seeds were collected from fresh pumpkins and authenticated by Dr.P. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai (Voucher No. PARC/2013/2103). The pumpkin seeds were powdered using domestic grinder. This fine powder was sieved and used for preparing extract.

Preparation of MSCP

100 gm of *C. pepo* seed powder was soaked in 150ml of methanol (w/v) for 3-5 days with intermediate shaking. This was filtered through a fine cheese cloth and the filtrate was pooled after 3 repeated extractions. The filtrate obtained was evaporated to dryness using rotary evaporator. The concentrate was lyophilized and used for the study.

HPLC-UV analysis

Solid phase extraction (SPE) using C18 column and fractionation of total phenolics

MSCP was subjected to solid phase extraction using C18 Hypersil Gold column (5µm, 150 x 4.6 mm) and peptides, small molecules were removed. Fractionation of neutral and acidic phenolic acids was also carried out simultaneously. The resulting fraction was then subjected to reverse phase high performance liquid chromatography (RP-HPLC). The total phenolics in MSCP was detected using the stationary phase octadecylsilyl silica and mobile phases [A – phosphoric acid: water (0.5:99.5 v/v), B – acetonitrile]. The UV detector was set at 220nm with the flow rate adjusted to 1.0 ml/min. The major peaks were identified and the retention times were compared with those of the standards.

Fractionation of total flavonoids

HPLC chromatography (System name: LACHROM L-7000 MERCK, Proc.method-HITACHI) was applied to estimate the amount of total flavonoids. The total flavonoids in the extract was determined by using the stationary phase as octadecylsilyl silica gel and mobile phase as acetonitrile, sodium dihydrogen phosphate with dilute orthophosphoric acid. The detector used for analysis was a UV detector, set at 350nm with a flow rate of 0.5ml/min. The major peaks in MSCP were determined according to the retention times obtained from standards run at identical conditions.

Gas chromatography (GC) analysis of terpenoids in MSCP

The terpenoids level was measured using GC using capillary column coated with macrogol 20000 R and nitrogen as carrier gas. The flame ionization detector was set at the flow rate of 0.4 ml/min and anethole was used as standard.

Free radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging assay is a commonly recommended method for the assessment of antioxidant potential of plant extracts. The assay is based on the ability of DPPH, a free radical, to get decolorized in the presence of antioxidants. To 300 µl of methanolic solution of DPPH (100 µM) various concentrations of MSCP (20-100 µg/ml) in water were added and incubated at 37°C for 30 min in dark and the absorbance was measured at 490nm. Ascorbic acid was used as the reference standard. The percentage scavenging of DPPH free radicals was calculated and compared with that of the standard ascorbic acid.The IC_{50} value was also determined.

Superoxide anion scavenging activity

The method of Nishkimi *et al* [16] was applied for the measurement the superoxide anion scavenging activity of MSCP. Briefly, 156 µM NBT in 100 mM phosphate buffer, pH 7.4, 1 ml of nictionamide adenine dinucleotide (NADH) in 100mM phosphate buffer, pH 7.4 were added to an aliquot of MSCP (20-100 µg). The reaction was started by adding 100 µl of phenazine methosulphate (60 µM in 100mM phosphate buffer, pH 7.4) and the reaction mixture was incubated at 25°C for 5 min. The decrease in absorbance was measured at 560nm against water blank. Rutin was used as the positive control.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of the aqueous extract was measured by taking various concentrations of MSCP and standard rutin (20-100 µg) dissolved in phosphate buffer (0.025 M, pH 7.4) and incubated with sodium nitroprusside (5 µM) in standard phosphate buffer at 25°C for 5 hrs. After incubation, 0.5 ml of the reaction mixture was added with 0.5 ml of Griess reagent (equal volumes of 1% sulphanilamide in 2% phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride in water). The absorbance of the chromophore formed was read at 540nm. The activity was compared with that of rutin [17].

Reducing power

Aliquots of MSCP with concentration ranging from 20-100 µg were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 20 min. 1.5 ml of 10% trichloroacetic acid was then added to the reaction mixture and the contents were centrifuged at 3000 rpm for 10 min. 0.5 ml of the supernatant was collected and mixed with 1 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Control was processed similarly with distilled water. Ascorbic acid was used as the standard.

Acute toxicity study

Acute toxicity test was performed according to the World Health Organization (WHO) guideline (WHO 2000) and the organization of Economic Co-operation and Development (OECD) guideline 420 for testing of chemicals (OECD 2001).

Animals and husbandry

Male Sprague Dawley rats (175-200g) were housed four per polycarbonate cage under controlled conditions (20 – 25°C/ RH 40-45) in a 12 :12 h L:D cycle and fed standard pellet diet containing required amount of minerals and vitamins obtained from M/S : Provimi Animal Nutrition India Pvt Ltd., Bangalore, during 7 days acclimatization period. Diet and water were provided *ad libitum.*

LD_{50} value determination

Different group of rats were administered 200/ 500/ 1000/ 2000/ 2500 /3000 mg/kg of b.wt of MSCP in three equally divided doses during a period of 8 hrs. The drug was dissolved in 2 to 3 ml of distilled water (freshly prepared) and administered orally by gastric intubation. The animals were observed for behavioral changes like alertness, grooming, aggressiveness, touch response, tremor, sleep, convulsion, muscle spasm, analgesia, lacrimation, diarrhea, salivation and numbers of deaths (mortality) for 72 hrs. The animals were sacrificed at the end of 72 hrs and immediately after the sacrifice, blood samples collected were used for serum separation. Enzyme assays were performed within 2 hrs of sample collection. The whole blood collected was subjected to hemoglobin estimation and cell counting. Blood levels of transaminases, alkaline phosphatase, glucose, urea, creatinine and protein were determined by standard kits using semi auto analyzer.

RESULTS AND DISCUSSION

Different phytochemicals present in the daily food have various therapeutic effects which are essential to prevent diseases and to maintain a state of wellbeing. Recent studies have been focused on finding the natural substances present in foods or medicinal plants that decrease the risk of cardiovascular and degenerative diseases by reducing oxidative stress and counteracting macromolecular oxidation [18].

Phytochemical analysis

Table 1 shows that MSCP contains rich amount of bioactive compounds which exhibit antioxidant property. The quantitative analysis revealed that MSCP contains a rich amount of phenolic compounds that include flavonoids, alkaloids and terpenoids. It is well known that plant flavonoids and phenols in general are highly effective in scavenging free radicals and providing antioxidant defense to living cells. Polyphenols and flavonoids are isolated from plants used for the prevention and cure of various diseases which are mainly associated with free radicals. MSCP was found to be rich in quercetin which is the aglycone or sugarless form of rutin, the major bioflavonoid in the human diet.

Table 1: Quantitative phytochemical analysis

Phytochemicals	Quantity (mg/g of MSCP)
Alkaloids	4.09
Total phenols	0.988
Terpenoids	2.144
Flavonoids	1.223
Rutin	0.031
Galangin	0.041
Quercetin	0.907

Values are mean of three individual samples collected from different parts of Chennai.

HPLC analysis of MSCP

HPLC analysis reveals that the extract was found to be rich in alkaloids (4.09mg/g), terpenoids (2.144mg/g) and phenolics (0.988mg/g). MSCP also contains flavonoids such as rutin: 0.031mg, quercetin: 0.907 mg and galangin: 0.041mg per gram of seed extract [Figure 2 (A) & (B)]. Many reports conclude that antioxidant principles present in medicinal plants are responsible for their therapeutic potential [19]. The phenolic compounds have been recognized as antioxidant agents, which act as free radical terminators [20]. It has been reported that compounds such as the flavonoids which contain hydroxyl groups are responsible for the radical scavenging effect of medicinal plants. The mechanism of action of the flavonoids is proved with respect to their free radicals scavenging and or metal chelating effects [21, 22].Flavonoids are known to reduce body lipid levels significantly. The result shows that MSCP contains rich amount of these bioactive compounds which could be accounted for the antioxidant and hypolipidemic effects.

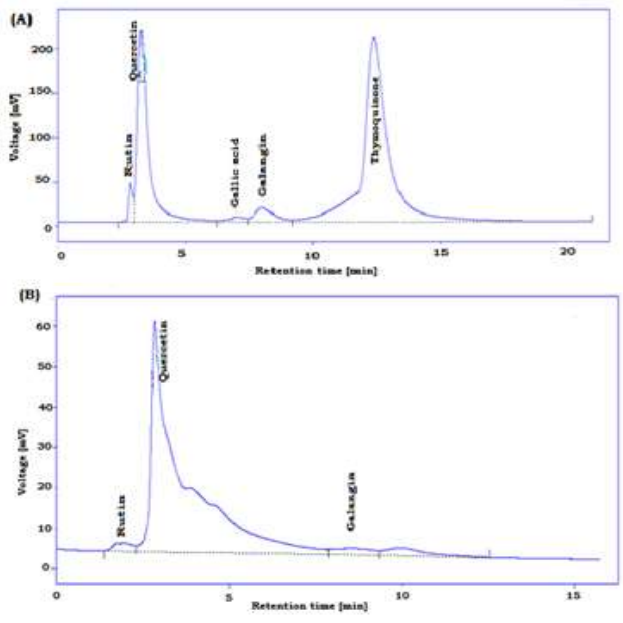

Fig.2: (A) HPLC fingerprint of standard flavonoids (B) HPLC fingerprint of flavonoids present in MSCP

Free radical scavenging activity

Free radicals and other reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide are an entire class of highly reactive species derived from the normal metabolism of major nutrients present in food[23]. In recent years there is an increasing interest in finding antioxidant phytochemicals because they can inhibit the propagation of free radical reactions and protect the human body from diseases. Hence MSCP was subjected to screening for its antioxidant potential.

DPPH scavenging activity

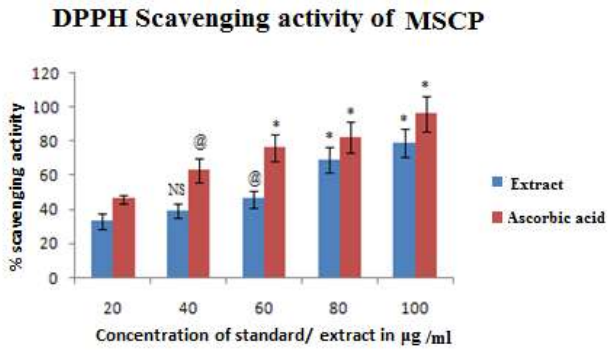

Fig. 3: DPPH scavenging activity of MSCP and standard ascorbic acid. Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

Antioxidants neutralize the free radical nature of DPPH by transferring electrons or donating hydrogen atoms and causing the change in color of the reaction mixture from purple to yellow [24, 25]. The degree of the discoloration indicates the scavenging potential of the antioxidants. The results obtained in this investigation show that MSCP possess a potent scavenging activity against DPPH radicals (Fig. 3). The scavenging activity was comparable to that of standard ascorbic acid. The IC_{50} value of MSCP (40.8 µg /ml) was found to be nearer to that of standard ascorbic acid (38.7 µg /ml).

Superoxide anion scavenging activity

MSCP was found to possess comparable free radical scavenging activity against superoxide anions when compared to that of standard rutin (Fig. 4). The IC_{50} value was found to be 38.4 µg/ml and 40.57 µg/ml for MSCP and rutin respectively. Superoxide anions are toxic intermediates formed during lipid peroxidation and found to enhance the risk of major diseases in humans.

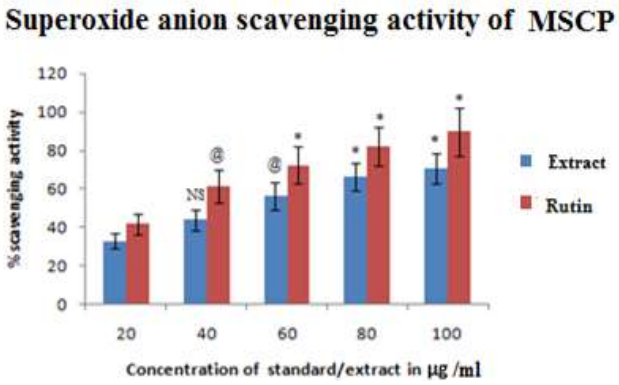

Fig.4: Superoxide anion scavenging activity of MSCP and standard rutin. Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non - significant when compared to the initial concentration.

Superoxide anion is an initial free radical that plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen in living organisms. Hence the superoxide scavenging activity is essential for medicinal plants to exhibit therapeutic activity as explained by Korycka [26].

Nitric oxide scavenging activity

Nitric oxide (NO) is an essential bio-regulatory molecule required for several physiological processes like neural signal transmission, immune response, vasodilation and control of blood pressure [27, 28]. But, NO formation in excess is toxic to living organisms. In the present investigation, it was found that MSCP significantly scavenges the nitric oxide and the effect was comparable to that of standard rutin at similar concentrations with the IC_{50} value of 38.70µg/ml and 45.84µg/ml respectively (Fig.5).

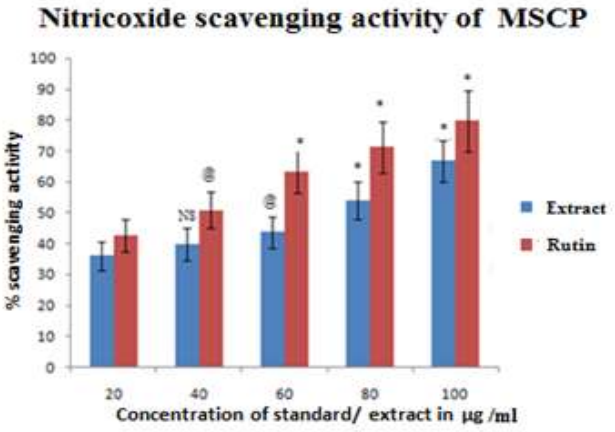

Fig. 5: Nitric oxide scavenging activity of MSCP and standard rutin. Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai. Statistically significant values are expressed as @p<0.05, *p<0.001, NS- non significant when compared to the initial concentration.

Reducing power

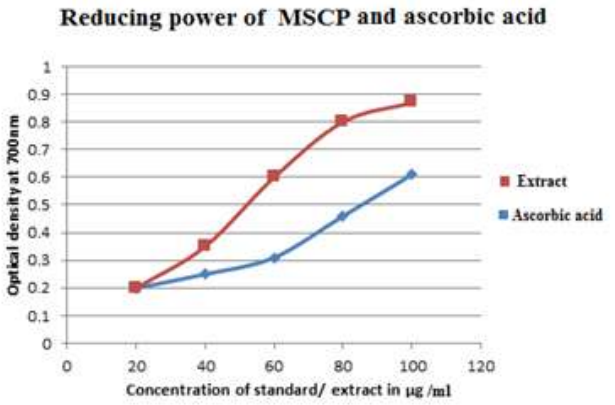

Fig.6: Reducing power of MSCP and standard ascorbic acid. Data are mean ± S.D obtained from 3 samples collected from different parts of Chennai.

Fig.6 shows the reducing power of phytochemicals in MSCP. The reducing power was found to be concentration dependent and found to possess similar effect as that of ascorbic acid, the reference standard. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of MSCP was comparable to that of the reference standard, ascorbic acid at similar concentrations.

Acute toxicity study

The acute toxicity test revealed that all the rats received different concentration of MSCP as a single dose, did not show any elevation in the activities of marker enzymes when compared to normal rats. LD_{50} value was also determined which was found to 2975 mg/kg b.wt (Table 2).

Table 2: Determination of LD_{50} value of MSCP by arithmetic method of Karber

Group	Dose (mg/Kg b.wt)	No of animals dead
1	0	0
2	200	0
3	500	0
4	1000	0
5	2000	0
6	2500	0
7	3000	1

Group	Dose difference(a)	Mean mortality(b)	Probit(axb)
8	(3000-2500) 500	0.5	250

Sum of the product = 250

LD_{50} = Least lethal dose in a group – Σ (axb)/N

LD_{50} = 3000-250/10

= 3000-25

= 2975 mg/ kg body weight

Biochemical analysis

The hematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status in both animals and humans [29].Biochemical analysis also shows that MSCP administration did not cause any pathological alteration in the level of blood cell count & also in the blood level of metabolites such as glucose, protein, urea and creatinine (Table 3).The results also showed that there were no significant changes in serum levels of ALP, ALT and AST verifying the nontoxic nature of MSCP. This finding also supports the use of these seeds in traditional medicine. Apart from that, histological analysis was done to rule out the pathological alteration in the organs.

Table 3: Concentration of serum biochemical and the blood cells in rats administered with MSCP (2500 mg/kg b.wt)

Parameters	Control	Test
Aspartate transaminase(IU/L)	43.6 ± 5.2	45 ± 5.6 NS
Alanine transaminase (IU/L)	42.1 ± 5.6	39.0 ± 3.6 NS
Alkaline phosphatase (IU/L)	100 ± 11.6	95.0 ± 10.1 NS
RBC count(million/cu.mm)	6.3 ± 0.70	6.1 ± 0.72 NS
WBC count(cells/cu.mm)	10,000 ± 800	9700 ± 900 NS
Lymphocyte (%)	90 ±10.1	88.0± 9.1 NS
Eosinophil (%)	2.2 ±0.26	2.0 ±0.19 NS
Platelet count (lakhs/cumm)	4.9 ± 0.31	5.2± 0.41 NS
Hemoglobin (gm %)	15.6 ± 1.6	15.9 ± 1.8 NS
Glucose (mg/100 ml)	73 ± 8.1	70± 7.9 NS
Protein (g/100 ml)	7.9± 0.80	7.5± 0.68 NS
Urea (mg/100 ml)	40± 4.5	36 ± 4.2 NS
Creatinine (mg/100 ml)	0.81 ± 0.09	0.76 ± 0.08 NS

The histological examination is highly essential for evaluating toxicity related pathological changes in tissues and organs [30]. Histopathological examinations did not reveal any abnormal changes after the administration of MSCP, evidenced by 'zero' histopathological score.

Values are expressed as mean ± S.D for n=6. Statistically significant values are expressed as NS- non significant when compared to that of control rats.

CONCLUSION

The results of the present study showed that MSCP contains phytonutrients such as quercetin which could prevent the formation of harmful free radicals. The acute toxicity profile of MSCP revealed that up to 2500 mg/kg b.wt of MSCP, there is no toxic symptom observed in experimental rats and hence up to 250 mg/kg b.wt of the test material can be safely used for the determination of therapeutic activity. The free radical scavenging activity of *C.pepo* seeds revealed that they can be used for the prevention or treatment of human diseases such as cardiovascular diseases, type II diabetes mellitus and obesity which are associated with oxidative stress.

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