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PHYT OCHEMICAL SCREENING, ANTIOXIDANT AND ANTIBACTRERIALEFFICACY OF MOMACARDIACHARANTIA SEED EXTRACT: AN *INVITRO* APPROACH

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ABSTRACT

The plants have been a major source of phytomedicines for human healthcare and it is prudent to analyse various phytoconstituents present in them. *Momordica charantia L.*a member of the *Cucurbitaceae* family, is widely distributed in tropical and subtropical regions of the world. It has been used in folk medicine for the treatment of diabetes mellitus, and its fruit has been used as a vegetable for thousands of years. In the present study, methanolic extract of *M. charantia* seed (MCE) were screened for their phytochemical constituents, antioxidant potential and antimicrobial activities. The antimicrobial activities of the extract was determined against gram positive bacteria and gram negative bacteria disc diffusion method. The MCE was the most active against the tested microorganisms in the study with higher inhibition zones and lower minimal bactericidal activities (MBC). Antioxidant capacity of the extract was investigated by different assays, namely, total antioxidant activity, free radical scavenging activity (DPPH assay), reducing power, hydroxy radical scavenging, activity(HRSA),metal chelating and superoxide anion scavenging activity. The total phenolic content, flavonoid, saponins and saponin were found to be 1.49 mg GAE/g, 1.45GAE/g, 0.62 mg/g respectively. MCE effectively scavenge the free radicals (DPPH, HRSA and phosphomolybdenum), chelate metal ions and possess superoxide anion radical scavenging activity. These findings indicate the potential use of seed extract as biopreservatives as they demonstrated high antimicrobial and antioxidant activities respectively.

KEYWORDS: *M. charantia*, seed, methanol extract, antioxidant potential, antimicrobial.

INTRODUCTION

Oxidative stress results from an imbalance between the generation of oxygen derived radicals and the organism's antioxidant potential which leads to chronic and degenerative diseases such as diabetes melitus, cancer, neurological dieseases etc. One of the most important routes for producing free radicals in living systems is oxidative processes. To eliminate and diminish the action of free radicals, which cause the oxidative stress the effective way is antioxidative defense mechanisms. Antioxidants are those substances which possess free radical chain reaction by inhibiting the initiation and propagation step leading to the termination of the reaction.[1]

There has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in combating with deleterious effects of oxidative stress-induced tissue injury. Among them naturally occurring antioxidants are identified as free radical scavengers; ascorbic acid, carotenoids and phenolic compounds are more effective which are known to inhibit lipid peroxidation, scavenge free radicals and active oxygen species and to chelate heavy metal ions.

*Momordica charantia*L (Bitter melon) is one of the most important species belonging to the family *Cucurbitaceae*, commonly known as bitter gourd or bitter melon in English. The origin of this crop is presumed to be India, with secondary center of diversity in China. The diverse parts of the plant are used in the treatment of ailments such as diabetes mellitus due to its hypoglycemic properties;[2] treating eye disorders and enhancing eyesight;[3] diarrheoa, pyorrhea that is bleeding from the gums;[4] respiratory problems[5] and skin infections.[6] It should also possess anti-cancerous and antileukemic activity of bitter melon against numerous cell lines including liver cancer, human leukemia melanoma andsolid sarcomas. It also posssess strong antimicrobial activity against wide range of gram positive and gram negative bacteria.[7]

Moreover phytochemicals screening of this plant indicated the presence of active components like momorcharins, momordenol, momordicilin, momordicins, momordicinin, momordin, momordolol, charantin, charine, cryptoxanthin, cucurbitins, cucurbitacins, cucurbitanes, cycloartenols, diosgenin, elaeostearicacids, erythrodiol, galacturonic acids, gentisic acid, goyaglycosides, goyasaponins and multiflorenol, which have beenisolated.[8] It is a well-

documented fact that most medicinal plants are enriched with phenolic compoundsand bioflavonoids that have excellent antioxidant property.

To the best of our knowledge, very few pharmacological studies have been reported so far on *M. charantia*. The present study was aimed to assess the phytochemical nature, antioxidant and antibacterial profiling of MCE, These useful data might help in the development of alternative controls in pharmaceutical industry.

MATERIALS AND METHODS

Plant Material The mature green *M. charantia* were collected from local market, Mayiladuthurai. The seeds were separated from the mature fruit.

Preparation of Momocardia Alcoholic Extract (MCE)

Alcoholic extract of the seed was prepared according to the method developed by Shibib et al.[16] Unripe fruit (1Kg) bought from the local market was thoroughly washed, and the seeds were removed. The seed was blended in1500 mL of 95% alcohol and left at room temperature with occasional shaking for 48 h. The suspension was filtered through cheesecloth, and the filtrate was evaporated in a Rotovac (BuchiLabortechnik AG, Switzerland) at 40°C to remove the alcohol. The final residue was stored at −20◦Cuntil further use. The residual extract was suspended in1% (w/v) carboxymethyl cellulose (CMC), and used in the investigation.

Preliminary phytochemical screening

The seed extract of *M. charantia* was subjected to different chemical tests for the detection of phytoconstituents such as carbohydrates, glycosides, alkaloids, amino acids, phenolics, flavonoids, triterpenoids, steroids, etc. They were identified by characteristics colour change using standard procedures. 24-26.[9]

Quantification of phytochemical compounds

The phytochemical compounds were quantified using the following methods.

Total Phenolic content (TPC)

Total phenolic content (TPC) of MCE were estimated by Folin Ciocalteau's method using Folin-Ciocalteau reagent.[10] Gallic acid was used as a standard with varied concentration (10-50µg). Both samples and standards (10-50µg) were mixed with Folin- Ciocalteu reagent (1:1), 7.5% (w/v) sodium carbonate and diluted with water. The blue colour formed was mat

760 nm. Each measurement was repeated fivet imes and the TPC were expressed as gallicacid equivalents (GAE).

Total Flavonoid Content (TFC)

Aluminium chloride method was employed to quantify the total flavonoid content in the MCE using Quercetin as the standards with concentration varied from10-50µg. 5% sodium nitrite, 10% aluminium chloride and 1M sodium hydroxide were added to MCE and standard. The colour formed was measured at 510 nm using UV- Vis spectrophotometer. Each measurement was repeated five times and TFC was expressed as mg Quercetin equivalent per gram extract weight (mg QE/g extract weight).[11]

Total Alkaloid Content (TAC)

The total alkaloid content of the MCE was determined by Sutharsingh[12] method in which. 5 g of the sample was filtered and concentrated to one-quarter of the original volume on a water bath after treatment with 200ml of 10% acetic acid in ethanol. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide, filtered and weighed.

Total Saponin Content

Powdered sample (10g) was treated with 100ml of 20% aqueous ethanol, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re-extracted. The combined extracts were reduced to 40ml over a water bath at about 90°C and the concentrate was transferred into a separating funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60ml of n-butanol was added to the combined extracts and washed twice with 10ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath, dried in an oven to a constant weight and the saponin content was calculated as a percentage.[12]

***In vitro* radical scavenging activity of MCE**

DPPH radical scavenging assay

The antioxidant activity of the MCE was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH, according to Chang et al.[13] Sample extract at various concentrations (10-50µg) was taken and the volume was adjusted to 100µL with

methanol. 5 ml of 0.1 mm methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20min at 27ºC. The absorbance of the sample was measured at 517nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% DPPH radical scavenging activity = (Control OD – Sample OD /ControlOD) × 100

Phosphomolybdenum assay

The antioxidant activity of MCE was evaluated by the formation of green phosphomolybdenum complex according to the method of Prieto et al.[14] An aliquot of 100µL of sample solution was combined with 1ml of phosphomolybdenum reagent solution (0.6M sulphuric acid, 28mm sodium phosphate and 4mm ammonium molybdate) in a 4ml vial. The vials were capped and incubatedin a water bath at 95ºC for 90 min. Samples were cooled to room temperature, the absorbance of the mixture was measured at 695 nm againsta blank. Gallic acid (10mg/mL in DMSO) was used as standard. The results were reported as % phosphomolybdenum reduction potential.

Superoxide anion radical scavenging activity (SOD)

The superoxide anion radical scavenging ability was assessed using the method of Nishikimi.[15] 1ml of NBT solution, 1ml of NADH solution, 0.1ml of varying concentrations of MCE (10-50µg) and 0.1ml of PMS solution were added together and incubated at 25°C for 5min. After 5min, the absorbance was read at 560 nm. The results were reported as %SOD.

Hydroxyl radical scavenging activity (HRSA)

The scavenging activity of the methanol extracts on hydroxyl radical was measured according to the method of Klein.[16] Various concentrations (10-50µg) of MCE were added with 1.0 mL of iron-EDTA solution, 0.5ml of EDTA solution, and 1.0ml of Dimethyl Sulphoxide (DMSO). The reaction was initiated by adding 0.5 mL of ascorbic acid and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0 mL of ice-cold TCA. 3ml of Nash reagent was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412nm against reagent blank.

The % hydroxyl radical scavenging activity (HRSA) is calculated by the following formula:

$$\% \text{ HRSA} = [(Abs_{control} - Ab_{ssample})/Abscontrol] \times 100.$$

Where, $Abs_{control}$ is the absorbance of the control; $Ab_{ssample}$ is the absorbance of the extract/standard.

Metal chelating activity

The chelating effect of ferrous ions by the MCE was estimated by the method of Dinis.[17] 100µL of the extract was added to 0.05ml of 2mm of ferric chloride. The reaction was initiated by the addition of 0.2ml of ferrozine (5mm) and the mixture was shaken vigorously and left at room temperature for 10min. Absorbance of the solution was then measured spectrophotometrically at 562nm. The chelating activity of the MCE was evaluated using EDTA as standard. The results were expressed as % metal chelating activity.

The ratio of inhibition of ferrozine -Fe^{2+} complex was calculated as follows:

% inhibition= (Control OD – Sample OD /Control OD) × 100.

Antimicrobial activity

Bacterial Strains and Growth Conditions

E.coli O157: H7 (ATCC 43890), *Staphylococcus aureus* (ATCC65384 Bacillus subtilis (ATCC 14028) were employed in the experiments. These strains were maintained in tryptone soy broth (TSB) containing glycerol (20%) at −40°C until use.

Media preparation

Mueller Hilton Agar and Nutrient broth wereprepared according to manufacturer' sspecifications.

Standardization of Inoculum

Using sterile inoculation wire loop, 3-4colonies from an overnight culture of the test organism was transferred into a tube of saline until the turbidity of the suspension matched the turbidity of the 0.5 McFarland Standard as described by the National Committee for Clinical Laboratory Standard(NCCLS, 2008).[18]

Antimicrobial Susceptibility Test

The agar well diffusion method was used for the antimicrobial susceptibility test. Mueller Hilton agar was prepared according to manufacturer's specification. The media were autoclaved and dispensed into sterile petri-dishes and allowed to gel. Standardized inocula of each bacterial isolates were streaked on the agar plate. Four wells of 6mm each was made in each plate with a central well for control using a sterile cork borer. The wells were filled with

0.1ml of different concentrations (400µ/ml, 200µ/ml, 100µ/ml and 50µ/ml) of the extract with the aid of sterile pipettes per well. Likewise, 400µ/ml, 200µ/ml, 100µ/ml and 50µ/ml of the standard antibiotic (amoxicillin) were used in separate plates to serve as positive control. While sterile distilled water was used as negative control on separate plates. The plates were allowed to stand for 15 minutes on a table to allow free diffusion of the extracts. Diameters of zones of inhibition were measured using transparent plasticmeter rule after 24 hours of incubation at 37^{o}C.[19]

Minimum Inhibitory concentration

MIC was determined by preparing various concentrations of the MCE by serial doubling dilution and incorporated into test tubes containing 2ml nutrient broth. Standardized inocula of 0.1ml of the isolates were inoculated and the tubes were incubated at 37°C for 24 h (NCCLS, 2008).

Minimum Bactericidal Concentration (MBC)

Nutrient agar plates were inoculated with sample from each of the tubes that show no turbidity and the plates were incubated at 37°C for 24 h to determine the MBC. MBC was determined by inoculating samples from the MIC tubes that showed no bacterial growth on Mueller Hilton agar plates separately and then incubated at 37oC for 24hours. After the incubation the plates were observed for presence or absence of growth. The least concentration of the extract that showed no bacterial growth was considered as the MBC (NCCLS, 2008).

RESULTS

Phytochemical screening

The MCE was used for preliminary screening of phytochemical such as alkaloids, tannin, phenolic compound, saponin, glycoside, flavonoid, protein, reducing sugar and phytosterol by using standard procedures described by Khan et al.[20] Table 1 describes about the presence of alkaloid, phenol, saponin, protein, glycoside, tannin and reducing sugar and the absence of protein.

Total phenolic, flavonoid and saponin content

Polyphenolic compounds much gained importance due to their free-radical scavenging ability and antioxidant capacity. The total phenolic flavonoid and saponin contents of MCE was presented in Table2.

Antioxidant activity of MCE

It is well known that the antioxidant activity of plant extract is due to the presence of polyphenolic components which is having the capacity as donors of hydrogen atoms or electrons and to capture the free radicals. The following results were obtained in our study.

The free radical- scavenging activity of the MCE was tested using DPPH scavenging system. The essence of DPPH method is that the antioxidants react with the stable free radical i.e., α, α diphenyl β--picrylhydrazyl (deep violet colour) and convert it to α, α -diphenyl-β-picrylhydrazine with discoloration. The degree of discoloration indicates the scavenging potential of the antioxidant sample. In the present study MCE was able to decolorize DPPH and it appears that MCE possesses hydrogen- donating capabilities to act as antioxidant.

Inhibition of $DPPH^{\bullet}$ radical formation was proportional to increasing concentrations of the MCE (Fig. 1). The inhibition percentage of MCE was 47 % at 10µg GAE and 77 % at 50µg GAE while GA exhibited the 34 % at 20µg and 67 % at 50µg. MCE exhibited strong inhibition against all the free radicals and the effect was more pronounced than GA.

The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of plant extracts. In the presence of the MCE, Mo (VI) is reduced to Mo (V) and forms a green-coloured phosphomolybdenum V complex, which shows maximum absorbance at 700nm. From the results obtained, it can be seen that MCE possesses significant antioxidant activity which increased in a concentration-dependent manner (Fig.2).

MCE was accounted for differential degree of metal ion chelation ability which is indicated by reduction in formation of red coloured complex. Maximum % inhibition observed with MCE was about 65% while GA exhibited 65% inhibition. Percent inhibition of colour production as a function of activity of MCE has been graphically represented in Fig. 3.

The percentage superoxide anion scavenging activity of MCE was proportional to increasing concentrations of the extract added and was comparable with that of GA (Fig. 3). The scavenging capacity of MCE was 53% at 10 µg GAE and around 72% at 40 µg GAE.

The percentage hydroxyl radical-scavenging activity of MCE and GA at various concentrations are presented in Fig.4. Both MCE and GA showed significant inhibitory

activity in a concentration-dependent manner. However, MCE showed higher scavenging potential than GA. For MCE, the maximum inhibition was 72% at 40 µg GAE.

The results obtained in the present study clearly shows that MCE can effectively scavenge free radical species like superoxide radical, hydroxyl radical, and $DPPH^{\bullet}$ and metal chelating ability.

Antibacterial Activity

To the best of our knowledge, no previous publications havebeen reported the antibacterial activity of ourplant extract. Therefore, the antibacterial activity of MCE against the tested bacteria strains was assessed by the presence and absence of inhibition zones using the disk diffusion method.

The result of the antibacterial activity of MCE is shown in Table 3). Highest zone of inhibition was observed in MCE with 13.0mm for E.coli, 1mm for *B.subtilis*and 10.0mm for *S.aureus*. It is pertinent however, to statethat, lowest concentration of 50 and 100µ/ml showed little or no activity on the test bacteria. While the hierarchy ofthesusceptibility pattern of the testedbacteria to the MCE is: *E.coli*>*B.subtilis*>*S.aureus.*

In similar research study Odunbaku *et al., (2012)* reported that the two trees extracts *(Mangiferaindica and Citrus aurantifolia)* have considerable inhibitory effects on *Staphylococcus albus, Pseudomonas aeruginosa, Aspergillus terreus, Aspergillus niger and Penicillium oxalicum.* Shanjidaet al., (2015) reported that, different concentrations of the methanolic extracts of the leaves of *Mangifera indica* exhibited antimicrobial activities against all the isolates of bacteria *(Bacillus cereus, Bacillus subtilis, Escherichia coli and Salmonella typhi).*

The MIC and MBC result result is shown in (Table 4). From the result MCE indicated an MIC range of 12.5µg/ml -50µg/ml and MBC range of 50µg/ml - 100µg/ml, While the standard antibiotic (amoxicillin) had MIC and MBC ranges of (6.25µg/m-25µg/m).However, from the finding of this study it is enough to state that MCE had the lowest MIC values.

The inhibition zone produced by the MCE on different bacterial strains was between 4mm and 20mm. The antimicrobial studies revealed that the MCE showed inhibitory effects on *B. subtilis, S. aureus and E. coli* as shown in Table 4. The MIC value was lowest for the MCE is (0.25 mg mL−1) against *B. subtilis.*

Tables

Table 1: Phytochemical composition of MCE.

S.No	Phytochemical	Presence/Absence
1	Alkaloid	++
2	Phenol	++
3.	Tannin	+
4	Saponin	++
5	Glycoside	+
6	Protein	+
7	Phytosterol	-
8	Reducing sugar	+

Where ++: moderate, +; indicartes normal, -; indicates absence

Table 2: Total phenolic,flavonoids and saponin content.

S.No	Chemical constituents	Standard equivalent in MCE (mg/g)
1.	Total phenolic content	1.49 ± 0.17
2.	Total Flavonoids	1.17 ± 0.04
3	Saponin content	0.62 ± 0.02

Table 3: Antibacterial Activity of MCE.

Bacteria	MCE(µg/ml)				AMOX(µg/ml)			
Concentration of Zone inhibition	50	100	200	400	50	100	200	400
E.coli	0	0	9	10	12	12	15	18
S.aureus	0	7	9	0	0	9	0	11
B.subtilis	0	0	0	11	9	11	14	16

Table 4: MIC and MBC of MCE.

Bacteria	MCE		AMOX	
	MIC (µg/ml)	MBC(µg/ml)	MIC (µg/ml)	MBC(µg/ml)
E.coli	**50**	**100**	**50**	**22.5**
S.aureus	**12.5**	**400**	**6.25**	**25**
B.subtilis	**12.5**	**25**	**6.25**	**12.5**

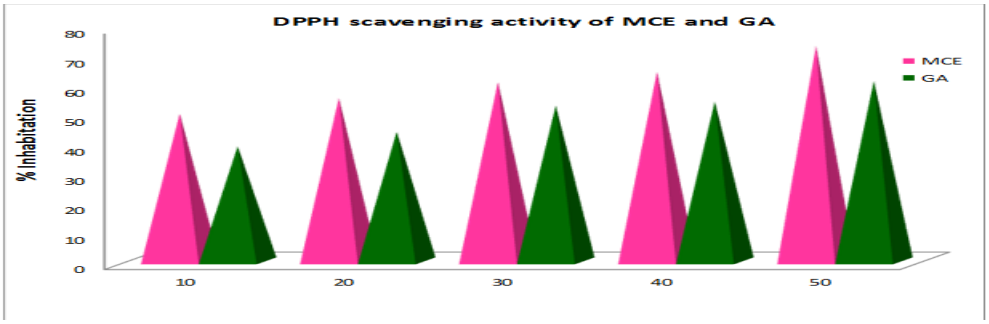

Fig 1: DPPH radical scavenging activity of MCE and GA.

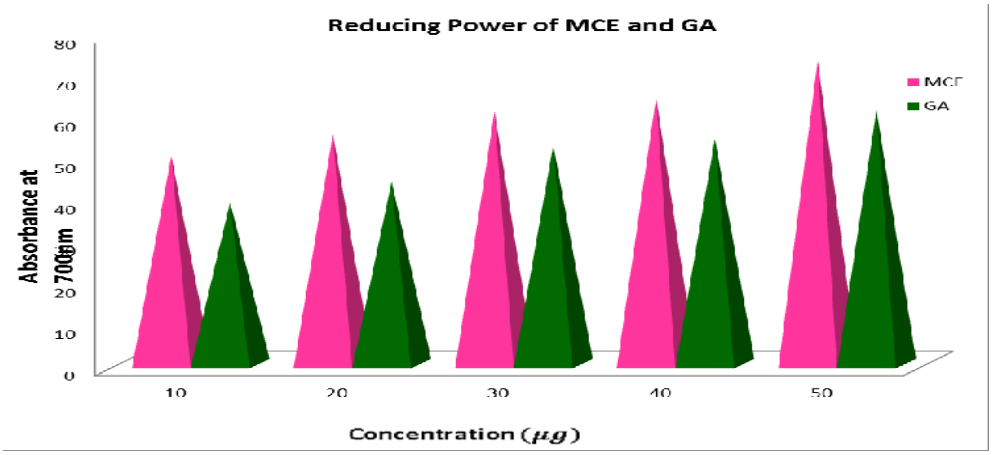

Fig 2: Phosphomolebdenum assay of MCE and GA.

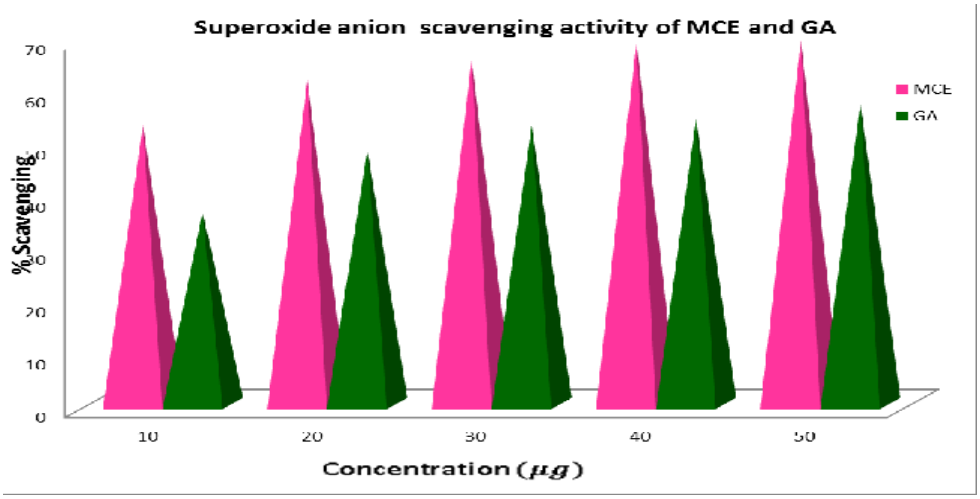

Fig 3: Superoxide anion scavenging activity of MCE and GA.

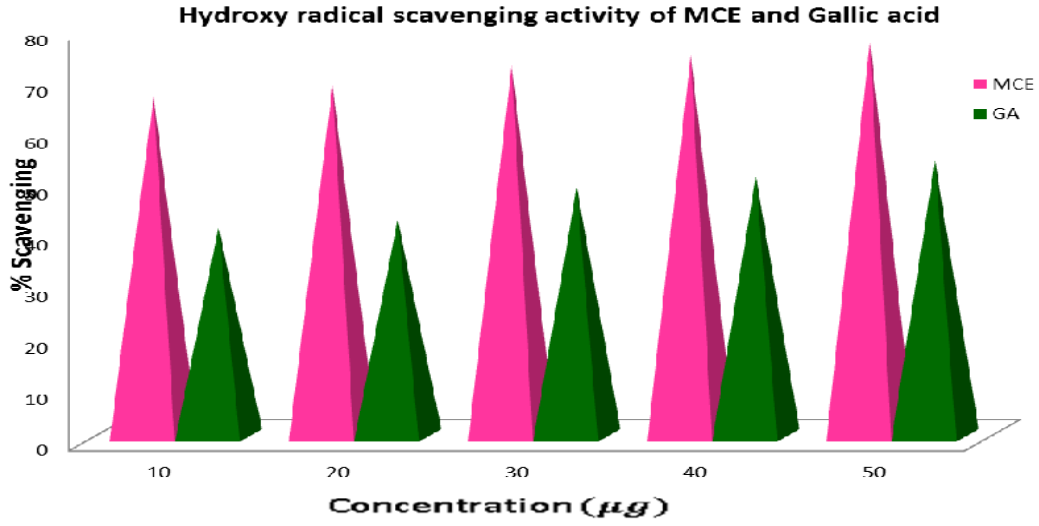

Fig 4: Metal chelating activity of MCE and GA.

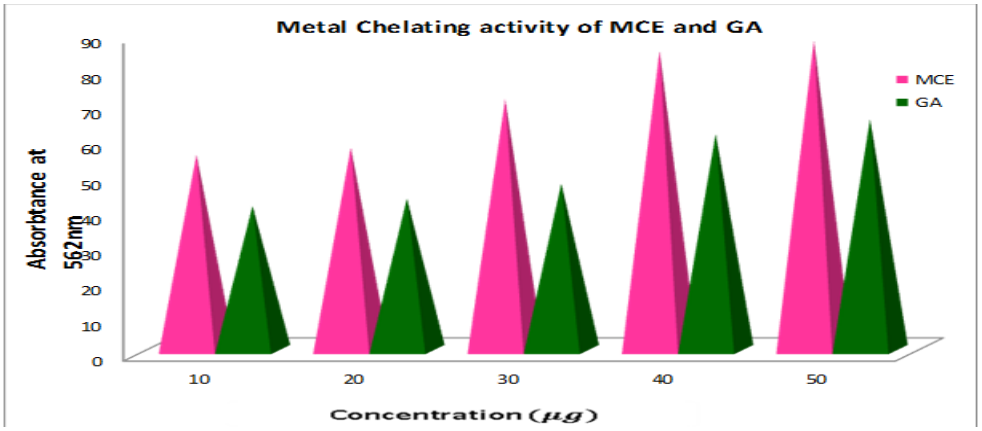

FIG 5:

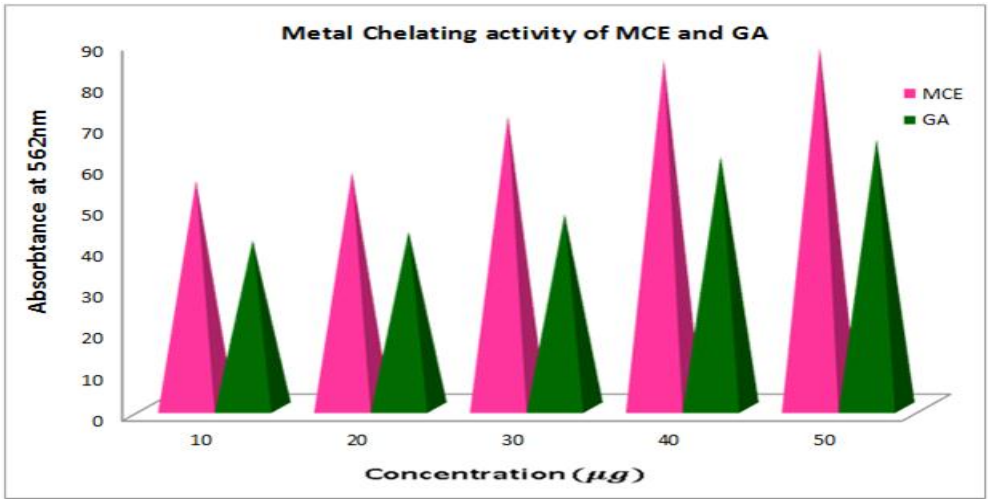

FIG 6:

DISCUSSION

Phytochemical analysis reveals that MCE are rich in Phenolics, alkaloids and saponins. Tanins and terpenoids showed distributory effect. Our results were correlate with Rashid et al (2017).[21] studies in which they have shown that aqueous extract of *M. charantia* contain notable amount of alkaloid, phenolic and saponin compounds. In addition, they also found the presence of tannin, glycoside, protein, reducing sugaretc. in the aqueous extract of *M. charantia.* Similar work has been carried by Adegbola et al (2016)[23], in their studies they have shown the presence of these secondary metabolites in water and ethanolic extract . Due to the presences of these secondary metabolites that are responsible for the antioxidant and antimicrobial activities of *M. charantia.* Okwu and Okwu, 2004 in their study they proven that the due to the presence of saponnis and alkaloids *M. Charantia* can be used for cure of intestinal problems and as a antimalarial drug.[24] To strengthen our result, Prarthna et al., 2014 also confirmed the presence of flavanoids, saponins, terpenoids, coumarins, emodins,

alkaloids, proteins, cardiac glycosides, anthraquinones, anthocyanins, steroids etc in the *M. charantia.*

The DPPH method is used to estimate the radical scavenging ability of antioxidant compounds.

The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The results obtained in this study were corroborated with the studies by [Oriji et al 2018].[25]

The scavenging of H_2O_2, by extracts may attribute to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells because it may give rise to hydroxylradicals in the cells. Addition of H_2O_2, to cells in culture can lead to transition metal ion dependent OH radica lmediated oxidative DNA damage Thus, removing hydrogen peroxideas well as superoxide anion is very important for protection of pharmaceuticals and food products.[26]

Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as via non- enzymatic reaction such as autoxidation by catecholamines. In the present study, superoxide radical reduces NBT to a blue coloured formazan that is measured at 560nm. Superoxide anions indirectly initiated lipidperoxidation as a result of superoxide and hydrogenperoxide serving as precursors of single O_2 and hydroxylradicals.

The super oxide radical scavenging activity of fresh and dried bitter gourd types is presented in Fig 4. The results of above table revealed that dark green big possessed lowest superoxide anion radical scavenging activity with an IC_{50}value of 57.23µg/ml. According to Hamissou et al. (2013).[27] an average of 1.55units of super oxide dismutase activity per µg total proteins was recorded for bitter gourd fruits. A study conducted by Tsai et al. (2014) on antioxidant, cell protective and antimelanogenic activities of leaf extracts from wild bitter melon cultivars reported an activity of 9.12mg/ml in leaf extracts.

Many research studies revealed that plants play an important role in the development of new therapeutic sources. In accordance with the review of literature, due to the presence of the phytochemicals like saponins, tannins, flavonoids, coumarin, phenol, and glycosides has a promising activity against pathogens and make the plant to possess antibacterial activity.[28] Waterhouse, 2003 stated the ability of plant extracts against bacteria depends on the solubility of the bioactive constituents.[29] Our results clarified that the MCE proved its efficiency to be used as the source for antibacterial compounds compared with some standard antibiotics due to its inhibitory effects on *B. subtilis*, *S. aureus*, and *E. coli* with lowest MIC value (0.25mg mL−1) against *B. subtilis*.

CONCLUSION

The study was able to provide insights as to the solubility of antioxidant compounds present in *Momocardia charantia* seeds in ethanol. The bioactivity and the radical scavenging efficacy of MCE in vitro is a worthy exploration and could be considered as the next step following this screening process. Whether the identified antioxidant activities could be initiated in actual physiological conditions has always been the challenge since the chemical assays have not been essentially able to capture the true potential of antioxidants and plant-based food products containing antioxidant compounds. The possible synergistic effects between different antioxidant compounds also need to be explored. Such studies may help elucidate the mechanisms explaining the effect of consuming different types of seeds or their byproducts on oxidative stress status and their resulting or associated disease conditions in humans.

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