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QUANTITATIVE PHYTOCHEMICAL PROFILE AND *IN-VITRO* ANTIOXIDANT POTENTIAL OF D5 CHOORANAM, AN ANTIDIABETIC SIDDHA FORMULATION

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Keywords:

Siddha medical system, D5 *Chooranam*, Free radicals, Phytochemicals, Diabetes mellitus

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ABSTRACT: D5 *Chooranam* is a classical *Siddha* formulation developed by Central Council for Research in *Siddha*, prescribed for the management of diabetes mellitus. The present work was carried out to assess phytochemical profile and also to explore the antioxidant potential of *Siddha* drug D5 *Chooranam*. The Phytochemical profile and antioxidant activity were evaluated in five different solvent extracts (Ethyl acetate, chloroform, ethanol, petroleum ether, and water) of D5 *Chooranam*. The phytochemical analysis of D5 *Chooranam* disclosed the existence of various phytochemicals in D5 *Chooranam*. The amount of phytochemicals present in D5 *Chooranam* also correlates with the various pharmacological activities as per its therapeutic indications. The considerable radical scavenging capacity of D5 *Chooranam*, particularly in its water extract, ethyl acetate extract, and fresh aqueous solution, is well justified by the results of all *in-vitro* antioxidant studies. These results reveal the beneficial effect of D5 *Chooranam* against oxidative stress associated disorders like diabetes mellitus and its complications. Hence it can be concluded that it may be due to the presence of vital phytochemicals in this exclusive novel blend of herbs and makes this formulation to be a virtuous choice for treatment of diabetes mellitus and its complications in *Siddha* clinical practice.

INTRODUCTION: Free radicals are the reactive oxygen species (ROS) produced during intracellular metabolic process or by exposure of human body to various exogenous agents like UV-radiation, pollutants, drugs and smoke [1]. Body encounters the deleterious effects of such ROS by eliciting antioxidant defense system or by undergoing apoptosis.

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Imbalance in body redox homeostasis results in "oxidative stress", which is responsible for etiology of various diseases like Diabetes mellitus, atherosclerosis, cardiovascular diseases, inflammatory diseases, aging, *etc*.,[2]. DM is a metabolic disorder rising to an upsetting epidemic level. This is characterized by elevated level of blood glucose as a consequence of insulin deficiency or impaired insulin action or by both.

Prevalence of DM is increasing globally. It is predicted to rise 5.4% by 2025 of the world population [3]. According to projections, 80% of the world's diabetic population will come from low- and middle-income nations by 2030. The dynamics of diabetes are changing quickly in these countries.

Uncontrolled Diabetes mellitus leads to the development of various metabolic complications which involves macro vascular and micro vascular diseases. The most common complications are Diabetic retinopathy, Diabetic nephropathy, Diabetic neuropathy, cardiovascular diseases, and stroke [4, 5]. The pathogenesis of these complications involves oxidative stress.

Although many oral hypoglycemic agents are available for the management of DM, due to limitations like high cost and undesirable adverse effects, more attention is being engrossed on identification of natural products with antidiabetic potential, strong antioxidant properties and low toxicity for discovery of ethnomedicines in the management of DM. Ethnomedicines are rich in phytochemicals like phenol, tannins, flavonoids, alkaloids, and a lot more responsible for various pharmacological activities. *Siddha* is a one of the earliest systems of medicine in India. The exclusivity of *Siddha* system is apparent by its constant service to manhood for more than 5000 years in tackling diseases and in maintaining its mental, physical, and moral health. *Siddha* system had been used by millions of people in the southern parts of India especially in Tamil Nadu and Kerala. *Siddha* system of medicine classifies diseases and disorders into 4448 types in which disease are being treated based on the three principles namely *vadham, pittam*, and *kabam* [1].

Siddha system of medicine deals with 11 metals, 64 *pashanam*, 120 *uprasams* and animal products in preparing medicines. Some *Siddha* formulations are composed of herbs with bioactive phytoconstituents, which possess wonderful antioxidant potential. D5 *Chooranam* is a one such classical polyherbal formulation composed of medicinal plants like *Avaarai (Cassia auriculata), Kontrai (Cassia fistula), Naval (Syzygium cumini), Kadazhangil (Salacia oblonga), Korai kizhangu (Cyperus rotundus), Kostam (Costus speciosus),* and *Lavanga pattai (Cinnamomum zeylanicum)* for the management of Type II Diabetes. Though all the ingredients were proved to have antidiabetic and various related pharmacological activity individually, the synergistic effect of all these medicinal plants in a single formulation may be evidenced in D5 *Chooranam* and hence it is an effective antidiabetic formulation used in the treatment of DM widely. Besides its wide usage for the treatment of DM, literatures for the elucidation of phytochemicals and antioxidant potential of D5 *Chooranam* are scanty. Hence, the current study was focussed to explore the phytochemical compositions and its antioxidant properties of D5 *Chooranam*.

METHODS AND MATERIALS:

Study Drug: The study drug, D5 *Chooranam* was purchased from Department of Pharmacy, *Siddha* Central Research Institute, Central Council for Research in *Siddha*, Arumbakkam, Chennai (Batch No: D505042021).

Chemicals: Fine chemicals like Quercetin, Curcumin, Nicotinamide Adenine Dinucleotide Hydrogen (NADH) used in this study were obtained from HiMedia Laboratories Private Limited (Mumbai, India). Chemicals such as N-(1-naphthyl) ethylenediamine dihydrochloride), Ascorbic acid, BHT (Butylated Hydroxy Toluene) and Phenazonium Methosulphate (PMS) were procured from LOBA chemie private limited (Mumbai, India). NBT (Nitro Blue Tetrazolium) and Gallic acid were of Sisco Research Laboratories Private Limited (Maharashtra, India) make. All other chemicals and reagents used in this study were of analytical grade.

Preparation of Extracts: Five different solvent extracts of *Siddha* drug, D5 *Chooranam* was prepared with increasing polarity namely Petroleum ether, Chloroform, Ethyl acetate, Ethanol and Water by cold percolation method [6]. 100g of D5 *Chooranam* was soaked in 250ml of each solvent and kept aside in room temperature for 72 hours and mixed with glass rod on daily basis. Then the mixture was filtered using whatman No.1 filter paper. The filtrate was evaporated to dryness and stored at 4°C. The yield of drug extract was calculated and expressed by following the formula,

Yield of drug extract = Final amount of drug obtained / Initial amount of drug taken for extraction × 100

To prepare a 2 mg/ml of stock solution, 100mg of Chloroform, Petroleum Ether, Ethyl acetate, Ethanol and Water extract was weighed and dissolved individually in 50ml of Dimethyl Sulfoxide (DMSO) and stored in refrigerator for further analysis. In addition, an aqueous extract

was also freshly prepared by dissolving 100mg of D5 *Chooranam* (drug) directly in 50ml of distilled water.

Phytochemical Analysis:

Qualitative Phytochemical Analysis: The standard procedures of Sofowara (1993), Trease and Evans (1989) and Harborne (1973) [7, 8, 12]. were used for the analysis of D5 *Chooranam* extracts to confirm the presence of various phytochemicals like Alkaloids, Coumarins, Tannins, Saponins, Flavonoids, Quinones, Phenols, Carbohydrate, Proteins, Cardiac Glycosides, Gum, and Starch.

Quantitative Phytochemical Analysis:

Total Phenol Estimation: The phenol content in various D5 *Chooranam* extracts were determined by using the procedure of McDonald *et al.,* (2001) [9]. 200µl of different concentration of extracts were mixed with 1ml of Folin-Ciocalteau reagent (1:2 diluted) and 3ml of 20% sodium carbonate after incubation at room temperature in dark for 90 minutes, the absorbance of the reaction mixture was measured at 765nm using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). The resultant values were expressed in mg of Gallic acid equivalents (GAE)/g of drug.

Total Flavonoid Estimation: The flavonoid content was evaluated by Chang *et al*., (2002) [10]. 0.25ml of different concentration of extracts were taken and the volume was made up to 1.25ml using distilled water. Into the mixture 75µl of sodium nitrate (5%) and 0.15ml of aluminum chloride (10%) solution was added and gently mixed. Finally, 0.5ml of 0.1M Sodium hydroxide was added, and the volume was made up to 2.5ml using distilled water. After the resulting solution shaken well, absorbance was measured at 510nm using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). Total flavonoid in D5 *Chooranam* was expressed in mg of Quercetin Equivalents (QE)/g of the drug.

Total Tannin Estimation: Peri and Pompei (1971) method as used for determination of tannin content in D5 *Chooranam* extracts. 1ml of various concentrations of drug extracts was taken and the volume was made up to 2 ml with distilled water. Then 0.5ml of Folin's phenol reagent (1:2 diluted) and 5ml of 35% Sodium carbonate solution was mixed. The resulting mixture was incubated for 5mins at room temperature for the development of blue color. The color intensity was read at 640nm using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). The total tannin content was expressed as mg of Gallic acid Equivalents (GAE)/g of drug.

Alkaloid Estimation: The alkaloid content of D5 *Chooranam* was estimated by Harborne, (1973) [12]. To 5g of D5 *Chooranam*, 200ml of 20% acetic acid was added and kept undisturbed for 4 hrs in a beaker covered with parafilm. Then the resulting mixture was filtered and heated in boiling water bath to concentrate it into one fourth of the volume. The alkaloids were precipitated by adding concentrated ammonium hydroxide solution in drop wise manner. After allowing the solution to settle down, precipitate was collected and weighed.

Antioxidant Assays: The antioxidant nature of D5 *Chooranam* was studied in terms of free radical scavenging activity. Various concentrations (10-1500µg) of D5 *Chooranam* extracts were analyzed for antioxidant activity in a dose specific manner.

Nitric Oxide Radical Scavenging Activity of D5 *Chooranam:* Nitric oxide radical scavenging potential of D5 *Chooranam* extracts were analyzed by Green *et al*., (1982) and Jagetia *et al*., (2004) [13] method. Nitric oxide produced in the reaction involving sodium nitroprusside and oxygen was measured by Griess reaction. Sulfanilamide (1%) and naphthyl ethylene diamine dihydrochloride (0.1%) in ortho-phosphoric acid (2.5%) were mixed thoroughly to prepare Griess reagent right before use.

The reaction mixture of 0.5ml of sodium nitroprusside in phosphate buffered saline and 1 ml of different concentration of D5 *Chooranam* extracts (20, 40, 60, 80,100µg) were incubated for 150 minutes at 25ºC. After incubation 0.5ml of reaction mixture was mix up with equal volume of fresh Griess reagent. The absorbance of the solution at 546nm was measured in Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). Standard Ascorbic acid was also treated similarly. Percentage of inhibition was determined using the following formula:

Percentage (%) of nitric oxide radical scavenging assay = $[(A_0-A_1)/A_0] \times 100$

Superoxide Anion Radical Scavenging Activity of D5 *Chooranam*: Superoxide radical scavenging assay of D5 *Chooranam* extract were performed as mentioned by Nishi Miki *et al*., (1972) [14] method. Reaction solution was prepared by mixing 1ml of 156µM NBT solution (pH-7.4), 468µM NADH (pH-7.4) solution and 0.1ml of various concentrations of D5 *Chooranam* extracts ranging from 20-1500µg.

The reaction was then initiated by adding 100µl of PMS (pH-7.4) and incubated for 5 minutes at 25ºC. The absorbance of the sample mixture was read at 560nm against control using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). Quercetin was used as the standard, and it was also treated the same way. Percentage of inhibition was calculated according to formula as under:

Percentage (%) of superoxide scavenging = $(A_0 - A_1)/A_0 \times 100$

Hydroxyl Radical Scavenging Activity of D5 *Chooranam*: To assess the hydroxyl radical scavenging activity of five different solvent extracts of *Siddha* formulation D5 *Chooranam*, To 1ml of iron-EDTA solution, 0.5ml of EDTA (0.018%) and 1ml of DMSO were added to prepare the reaction mixture. The reaction was initiated by the addition of 0.5ml of 0.22% ascorbic acid solution. The experimental mixture was placed in water bath for 15 minutes at 80ºC-90ºC. To terminate the ongoing reaction 1ml ice-cold TCA was added to the final mixture containing 3ml of Nash reagent. After incubating at room temperature for 15 minutes, the developed yellow color was read spectrophotometrically at 412nm using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India) [15]. The activity of standard (BHT) was assayed similarly. The % of inhibition the test extract was calculated using the below cited formula:

Percentage (%) hydroxyl radical scavenging activity = $[A_0 - (A_1 - A_2] \times 100/A_0$

Total Reducing Power Activity of D5 *Chooranam*: D5 *Chooranam* extracts were investigated for its total reducing power activity according to Oyaizu, (1986) [5] method with slight modification. The various concentrations (20, 40, 50, 80, 100, 150, 200, 250µg) of D5 *Chooranam* extracts were added with 2.5ml of phosphate buffer (pH-6.6) and 2.5ml of potassium ferricyanide (1%). After incubating at 50ºC for 20 minutes, 2.5ml of 10% TCA was added and the tubes were then centrifuged at 2500rpm for 10 minutes.

Followed by centrifugation, the resultant Supernatant was mixed with 2.5ml of distilled water and 0.5ml of 0.1% $FeCl_3$. The absorbance of the experimental mixture was read at 700nm using Microprocessor based Double beam UV-Visible Spectrophotometer (Igene Labserve, India). BHT was treated as standard, and it was also treated similarly.

Statistical Analysis: All the values were expressed as Mean ± Standard Deviation (SD). All Statistical analysis including IC_{50} value determinations were performed using GraphPad Prism software (9.3.1). Results of the study was statistically analyzed using ANOVA (One-Way) followed by Least Significant Difference (LSD) test and the P value was calculated to ascertain the statistical significance of the observed changes. $P<0.05$ was considered as significant. All the assays were done in triplicates.

RESULTS:

Yield of D5 *Chooranam* Extracts: Five different solvent extracts of D5 *Chooranam* were prepared using solvents with increasing polarity like Water, Ethyl acetate, Chloroform, Petroleum ether, and Ethanol by cold percolation method. The yield of the extract was calculated and depicted graphically in **Fig. 1.**

The order of extract yield was found to be Water > Ethanol > Ethyl acetate > Chloroform > Petroleum Ether. It showed that the yield of water extract was comparatively higher than that of other extracts. This result infers the nature of phytochemicals present in D5 *Chooranam* as hydrophilic. As most of the physiological conditions are hydrophilic in nature, the bioavailability of the D5 *Chooranam* would be greater when consumed with its adjuvant, warm water and thereby it elicits its biological activity effectively.

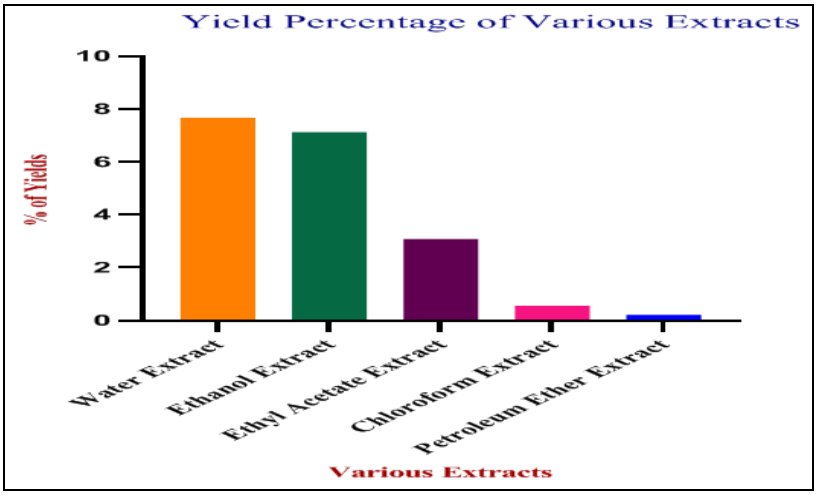


FIG. 1: YIELD OF D5 *CHOORANAM EXTRACTS* IN PERCENTAGE

Qualitative Phytochemical Analysis: The results of preliminary phytochemical analysis were delineated in **Table 1**. The presence and absence of phytochemicals were illustrated as "+" (positive) and "-" (negative) respectively. Grades like 1+, 2+, 3+ were given to represent the degree of colour intensity compared with the other extracts. Qualitative phytochemical analysis of D5 *Chooranam* extracts of five different solvents has exhibited the presence of phytochemicals like Flavonoids, Quinones, Phenols, Alkaloids, Tannins, Coumarins, Saponins, and Cardiac Glycosides. The analysis of fresh aqueous solution also revealed the presence of Coumarins, Tannins, Saponins, Flavonoids, Quinones, Phenols and Cardiac Glycosides whereas, alkaloid, was found to be absent.

The existence of many phytochemicals in both water extract and fresh aqueous solution of D5 *Chooranam* correlates with the increased yield of water extract. Due to the presence of many phytochemicals like phenol, flavonoids *etc*, an increased quantity of water extract was resulted. Hence, it can be arrived that water extract is the best extract of D5 *Chooranam* drug. Presence of significant phytochemicals like Tannins, Flavonoids, Alkaloids, Phenols, Saponins and Cardiac glycoside were also confirmed in chloroform, ethyl acetate and ethanol extracts of D5 *Chooranam*. Whereas petroleum ether extract was found to contain tannins, phenols, flavonoids, quinones only.

TABLE 1: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF D5 *CHOORANAM*

S. no.	Name of the Test	Petroleum Ether Extract	Chloroform Extract	Ethanol Extract	Ethyl Acetate Extract	Water Extract	Aqueous Drug Solution
1	Alkaloids	-	+	-	-	+	-
2	Coumarins	-	-	-	-	+	+
3	Tannins	+	+	+	+	+	+
4	Saponins	-	-	2+	-	+	+
5	Flavonoids	+	2+	+	+	2+	+
6	Quinones	3+	3+	-	-	3+	2+
7	Phenols	+	+	3+	2+	3+	2+
8	Carbohydrates	-	-	-	-	-	-
9	Proteins	-	-	-	-	-	-
10	Cardiac Glycosides	+	2+	2+	2+	3+	2+
11	Gum	-	-	-	-	-	-
12	Starch	-	-	-	-	-	-

Quantitative Phytochemical Analysis: The amount of Phenols, Flavonoids, Tannins and Alkaloids in various solvent extracts were shown in **Fig. 2A-C.**

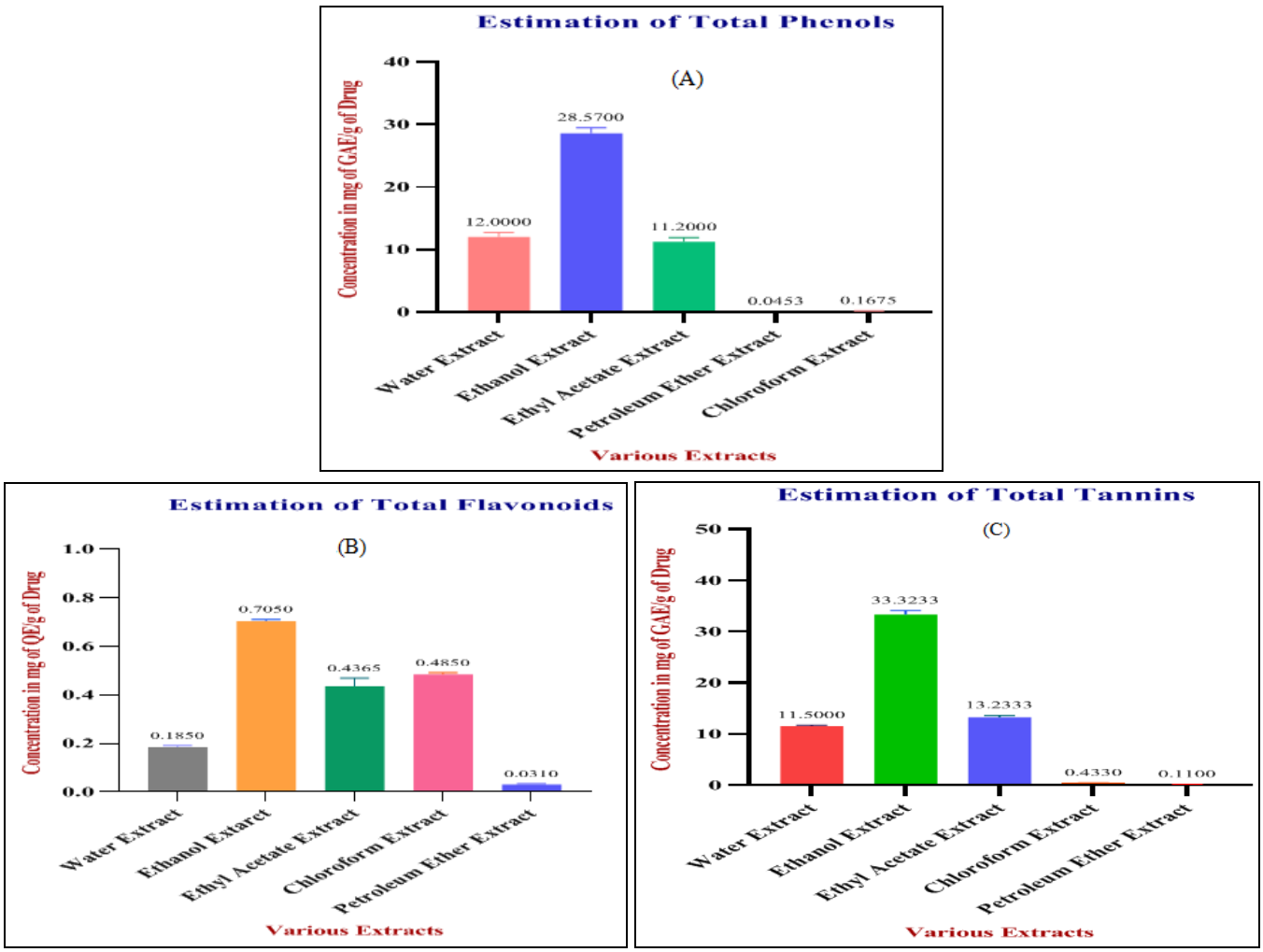


FIG. 2: QUANTITATIVE ESTIMATION OF TOTAL (A) TOTAL PHENOL (B) TOTAL FLAVONOID (C) TOTAL TANNIN CONTENT IN VARIOUS SOLVENT EXTRACTS OF D5 *CHOORANAM*

Total flavonoid, total phenol, and total tannin content of various solvents extracts of D5 *Chooranam* were calculated against the standard curve with R^2 value of 0.997, 0.998, and 0.999 respectively. The estimation of phytochemicals in D5 *Chooranam* extracts revealed that total phenols, total flavonoids, and total tannin content was found to be present in high concentration in ethanol extract compared with the other extracts. Followed by ethanol extract, water extract was found to contain more phenol whereas it has lesser tannin and flavonoid content. Besides ethanol and water extract, appreciable amount of flavonoids and tannins were present in ethyl acetate extract of D5 *Chooranam* also. Whereas the chloroform extract of the study drug has exhibited decreased level of the phytoconstituents like phenol, flavonoid, and tannin, than water and ethyl acetate extract. Meanwhile, petroleum ether extracts notably contain least quantity of phenols, flavonoids, and tannins than above mentioned other four solvent extracts. Contrast to the estimation of other phytoconstituents, alkaloid content was quantified directly in D5 *Chooranam* without preparing any extract by gravimetric method of Harborne (1973). 0.323g of alkaloid was found to be present in 5 gram of drug and it was graphically presented in **Fig. 3.**

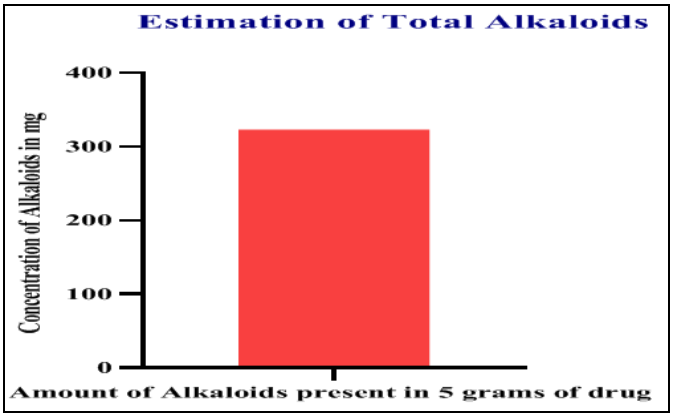


FIG. 3: QUANTITATIVE ANALYSIS OF ALKALOID CONTENT IN D5 CHOORANAM

Antioxidant Potential of D5 *Chooranam*: D5 *Chooranam* extracts were analyzed for the antioxidant potential using various *in-vitro* assays, like nitric oxide radical scavenging assay, Superoxide radical scavenging assay, Hydroxyl radical scavenging assay and total reducing assay. It was observed that various concentrations of drug extracts ranging from 10-1500µg/ml have scavenged the free radicals in dose specific manner in different methods. All the assays were done in triplicates.

Nitric Oxide Radical Scavenging Assay: Nitric oxide scavenging potential of D5 *Chooranam* extracts showed a variable level of inhibitory activity in a dose specific manner. For this assay, Ethanol extract, Ethyl acetate extract, Water extract and Aqueous extract were used in the concentration ranging from 20-100µg.

Whereas petroleum ether and chloroform extract were tested with 50-250µg concentration as they did not show any scavenging activity at lower concentration. The percentage of inhibition of various drug extracts were calculated against standard compound ascorbic acid using formula as mentioned under methods.

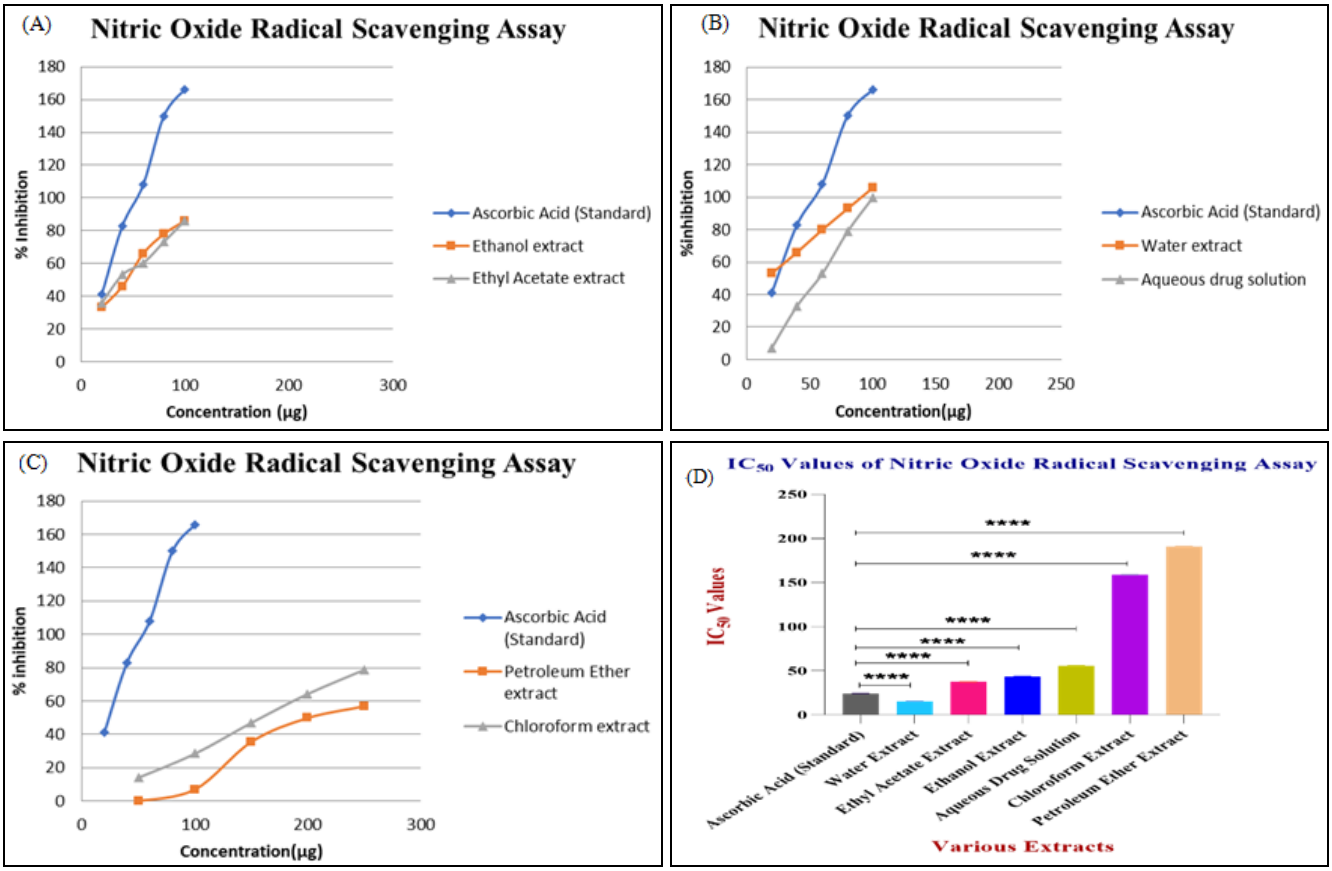


FIG. 4: NITRIC OXIDE RADICAL SCAVENGING POTENTIAL OF (A) ETHANOL AND ETHYL ACETATE EXTRACTS OF D5 *CHOORANAM*. (B) WATER EXTRACT AND AQUEOUS SOLUTION OF D5 *CHOORANAM*. (C) PETROLEUM ETHER AND CHLOROFORM EXTRACT OF D5 *CHOORANAM*. (D) IC50 VALUE OF SEVERAL D5 *CHOORANAM* EXTRACTS. (ASCORBIC ACID WAS USED AS STANDARD)

The IC_{50} value of water extract was found be 15.5µg/ml, which was lesser than that of ascorbic acid (standard) IC_{50} value (24.29µg/ml). From this result, it was evident that D5 *Chooranam* acts as a strong nitric oxide radical scavenger even than the standard ascorbic acid. The result was also statistically significant ($P<0.001$) and it was presented in **Fig. 4A, 4B, 4C & 4D**.

The IC_{50} value of other extracts like ethyl acetate, ethanol, and aqueous solution was found to be 37.53 µg/ml, 43.61 µg/ml, and 55.68µg/ml respectively. Higher IC_{50} values were observed for chloroform extract and petroleum ether extract (158.86 µg/ml and 190.81 µg/ml) respectively and hence they have least nitric oxide radical scavenging activity.

All the results were statistically significant with a P value less than (0.001).

Superoxide Scavenging Assay: Superoxide scavenging potential of D5 *Chooranam* extracts were studied against standard curcumin. Percentage of radical scavenging activity and IC_{50} values of D5 *Chooranam* extracts against superoxide radicals were depicted in **Fig. 5A-E.** Ethyl acetate extract showed the excellent superoxide scavenging activity with a low IC_{50} value of 49.41µg/ml. It clearly depicted that its activity is almost near to the standard Curcumin (IC_{50} = 15.26 µg/ml). Followed by Ethanol extract, Aqueous extract, water extract and Chloroform extract was found to possess appreciable superoxide scavenging activity with IC_{50} value of 72.63 µg/ml, 87.30 µg/ml, 96.52 µg/ml, and 150.91 µg/ml respectively.

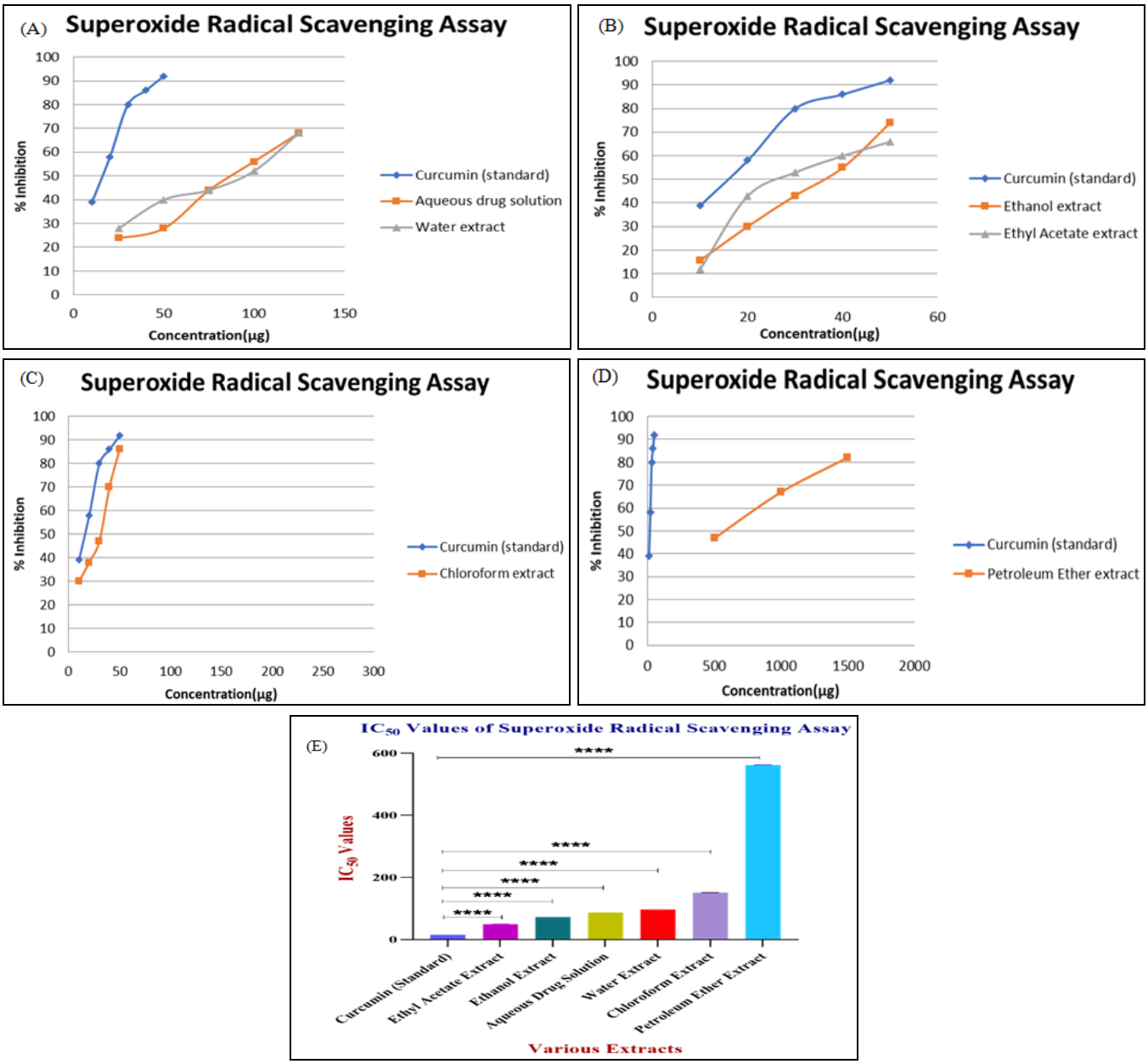


FIG. 5: SUPEROXIDE RADICAL SCAVENGING ASSAY OF (A) AQUEOUS SOLUTION AND WATER EXTRACT. (B) ETHANOL AND ETHYL ACETATE EXTRACT (C) CHLOROFORM EXTRACT (D) PETROLEUM ETHER EXTRACT (E) IC_{50} VALUE OF VARIOUS D5 *CHOORANAM* EXTRACTS. (CURCUMIN WAS USED AS STANDARD)

Compared to the other extracts of D5 *Chooranam*, Petroleum ether extract exhibited the minimal level of superoxide radical scavenging activity with very high IC_{50} value. (561.91µg/ml). Results of all these

assays were statistically significant with P value less than 0.001.

Hydroxyl Radical Scavenging Assay: The hydroxyl radical scavenging potential and IC_{50} of various solvent extracts of D5 *Chooranam* was presented in the **Fig. 6A-D.** Despite the results of other assays, Petroleum ether extract, and Ethyl acetate extract exhibited high hydroxy radical scavenging activity with an IC_{50} value of about 25 µg/ml for each which is less than the IC_{50} value of BHT (125 µg/ml). Results of this study demonstrated the strong hydroxyl radical scavenging potential of D5 *Chooranam*.

In addition, ethanol extract and water extract showed the satisfying level of radical scavenging activity with an IC_{50} value of 62.5µg/ml and 70µg/ml respectively. Whereas, chloroform extract exhibited low scavenging activity with very high IC_{50} value (500 µg/ml).

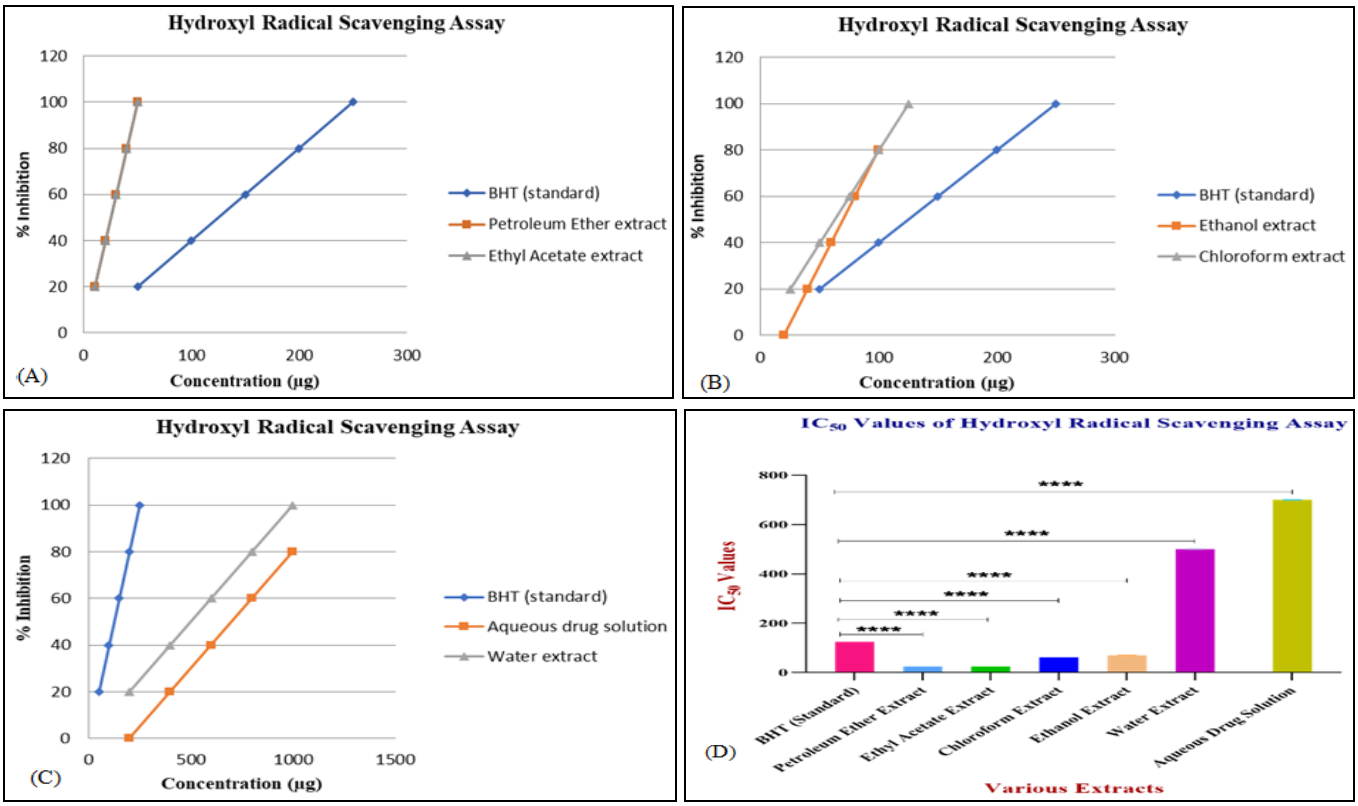
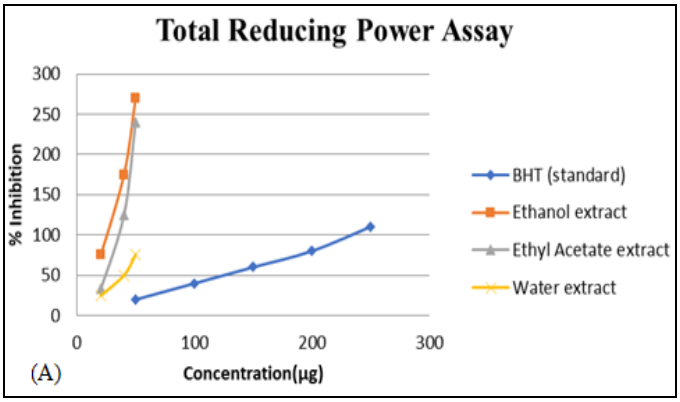


FIG. 6: HYDROXYL RADICAL SCAVENGING ASSAY OF (A) PETROLEUM ETHER AND ETHYL ACETATE EXTRACT (B) ETHANOL AND CHLOROFORM EXTRACT (C) AQUEOUS AND WATER EXTRACT (D) IC_{50} VALUE OF VARIOUS D5 *CHOORANAM* EXTRACTS (BHT AS STANDARD)

Total Reducing Power Assay: D5 *Chooranam* extracts subjected to the total reducing power assay demonstrated various levels of reducing power activity. The results were statistically significant (P<0.001) presented in **Fig. 7A-C**. BHT was used as a standard drug.



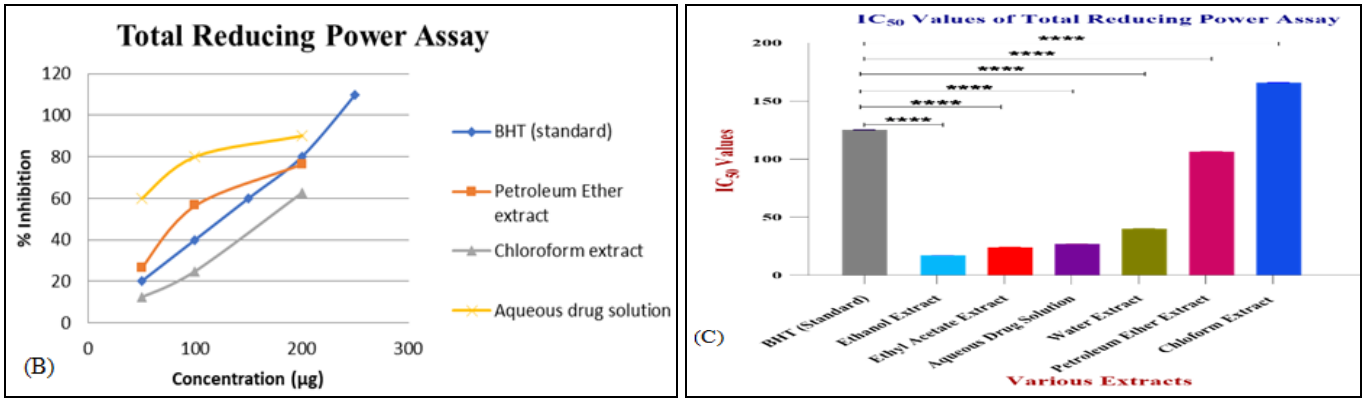


FIG. 7: TOTAL REDUCING POWER ASSAY OF (A) ETHANOL, ETHYL ACETATE AND WATER EXTRACT. (B) PETROLEUM ETHER EXTRACT, CHLOROFORM EXTRACT AND AQUEOUS SOLUTION (C) IC_{50} VALUE OF VARIOUS D5 *CHOORANAM* EXTRACT (BHT AS STANDARD)

When compared to the other solvent extracts of D5 *Chooranam*, ethanol extracts showed the maximum reducing power activity with IC_{50} values of 17.05 µg/ml, and this was followed by Ethyl acetate extract, Aqueous extract, Water extract and Petroleum ether extract with an IC_{50} value of 24.05 µg/ml, 26.93 µg/ml, 40 µg/ml and 106.27 µg/ml respectively. Contrast to all other *in-vitro* assays, D5 *Chooranam* extracts exhibited good reducing power activity than that of standard drug, BHT (IC_{50}=125.06 µg/ml).

DISCUSSION: In the modern era, poly herbal preparations have gained greater importance than ever before, mainly due to their efficacy, less side effects and easy availability. As claimed by various research outcome, these polyherbal formulations are more advantageous than single herbal concentrations since they have enhanced synergistic actions of various ingredients. Plant produced. Plant produced secondary metabolites, are the main source of natural antioxidant that can eliminate the harmful reactive oxygen species. Antioxidants quench the action of free radicals, which are responsible for the etiology of various diseases particularly DM, neurodegenerative disorder, inflammation, and cancer. Free radicals are produced during the metabolic process leading to several clinical implications. Even though many synthetic antioxidants are available to deal the oxidative stress, due to the adverse effects research interest has been developing on the natural antioxidants. In the present study, D5 *Chooranam* a classical *Siddha* formulation was screened for phytoconstituents and also for its antioxidant potential to justify its therapeutic potential as an antidiabetic drug. In the drug extraction process, the yield of water extract was higher (7.67%) when compared to the other extracts of D5 *Chooranam*. In *Siddha* clinical practice D5 *Chooranam* was prescribed along with warm water as an adjuvant for diabetic patients. High yield of water extract of D5 *Chooranam* clearly depicted that the bioavailability of D5 chooranam at its therapeutic dose would be more under physiological conditions and thus it would be of much beneficial in combating Diabetes mellitus. Hence, the adjuvant (warm water) is a perfect choice for the absolute therapeutic benefit of D5 chooranam.

It is vital to monitor the phytoconstituents of D5 *Chooranam* to evaluate their efficacy and safety for therapeutic use. Phytochemical assessment of various solvent extracts of D5 *Chooranam* disclosed the existence of phenols, flavonoids, alkaloids, coumarins, tannins, saponins, quinones, carbohydrates, proteins, and cardiac glycosides. No previous studies about the presence of these phytoconstituents in the different solvent extracts of D5 *Chooranam* have been observed in the literature and hence this study is first of this kind. Phenol and flavonoid compounds are the plant secondary metabolites. They hold an aromatic ring with hydroxyl group and have been reported to possess anti-diabetic, cardio protective, antioxidants, anticancer, antibacterial, anti-inflammation, immune promoting and skin protection properties [16, 17]. In the quantitative analysis, presence of considerable number of bioactive compounds like phenols, flavonoids, tannins and alkaloids in ethanol extract exhibits the antioxidant potential of D5 *Chooranam* and their benefits on health. Reactive Oxygen Species (ROS) namely superoxides, peroxides, hydroxyl radicals

and singlet oxygen are the derivatives of oxygen which are continuously produced under physiological condition due to exposure of exogenous chemical and/or several endogenous chemical reactions that involves redox enzymes and bioenergetics electron transfer [18]. As a result of overproduction of ROS and/or insufficient antioxidant defence mechanism, there is an increase of ROS and which in turn leads to oxidative stress. Compared to the synthetic antioxidants, plants possess good antioxidant ability and are safer too. Various mechanisms like Inhibition of chain initiation, decomposition of peroxides, transition metal ion catalysts binding, reductive strength, and radical scavenging potential attributes to antioxidant activity. Extensive study on free radical scavenging potential provides the logical basis behind the unexplored properties like antioxidant activity of any natural product/drug.

In the present study, four different antioxidant methods for assessment of antioxidant activity have been studied. Various concentrations ranging from 20 - 100 µg/mL of D5 *Chooranam* were assayed for their antioxidant activity using different in vitro models. Extracts of D5 *Chooranam* presented notable level of scavenging activities against the superoxide radicals, nitric oxide radicals and hydroxyl radicals in a dose specific manner in the various methods.

Nitric oxide (NO) is a radical produced by phagocytic cells and endothelial cells; it is involved in the management of several physiological processes [19]. Several diseases are associated with increased concentration of NO [20] and it plays substantial role in inflammatory process. Peroxynitrite anions are the free radicals produced during the reaction between oxygen and excessive nitrite, nitric oxide [21]. Nitric oxide can rapidly react in the physiological conditions to form nitrates, nitrites and s-nitrosothiols. These compounds play a crucial role in intermediating various xenotoxic effects such as DNA damage through peroxynitrite. In the present study, nitric oxide radical assay of D5 *Chooranam* was performed in all the 5 different solvent extracts. On comparative analysis with other extract, water extract of D5 *Chooranam* showed maximum nitric oxide radicals scavenging activity with IC_{50} value of 15.5µg/ml against the standard ascorbic acid whose IC_{50} value was found to be 24.29µg/ml. This outcome demonstrated that D5 *Chooranam's* antioxidant properties when combined with its adjuvant water. Oxidative enzymatic reaction such as autoxidation by catecholamine generates Superoxide anion from molecular oxygen [22]. Among free radicals Superoxide is a primary oxygen radical that is produced when an oxygen molecule receives one electron. The scavenging activity in relation to the superoxide radical is estimated in terms of suppression of generation of O_2^-. Superoxide anion acts as predecessors of hydroxyl radicals and singlet oxygen by initiating lipid peroxidation indirectly via superoxide and hydrogen peroxide [23]. The antioxidant nature of flavonoids is effectively elucidated via the scavenging of superoxide anion [24]. As D5 *Chooranam* is rich in flavonoids, it exhibits an appreciable scavenging action towards superoxide radicals.

Ethyl acetate extract exhibited maximum superoxide scavenging activity with half maximal inhibitory concentration value of 65.12 µg/ml compared to that of the standard curcumin with half maximal inhibitory concentration value of 15.26 µg/ml. The outcome of the study has showed the potential antioxidant behaviour of D5 *Chooranam*. Being a polar solvent, much water-soluble phytonutrients were present in the ethyl acetate extract of D5 *Chooranam* and hence the antioxidant capacity of therapeutic dose is justified.

In biological systems, hydroxyl radicals are formed as an extremely reactive species and have been responsible for free radical pathogenesis by damaging each and every molecule within any living cells [25]. This radical triggers DNA strand breakage by its capacity to join nucleotides and thus results in cytotoxicity, mutagenesis, and carcinogenesis. Additionally, this species is predicted as one of the lipid peroxidation process initiators and it also abstract H^+ ions from unsaturated fatty acids. In the current study, hydroxyl radical scavenging activity of D5 *Chooranam* extracts were compared with standard BHT. Among the other extracts, ethyl acetate and petroleum extract were found to be superior with same half maximal inhibitory concentration value of 25µg/ml, whereas standard BHT has IC_{50} of 125µg/ml. This indicates the excellent antioxidant activity of D5 *Chooranam.* Ethanol extract of D5

Chooranam exhibited excellent reducing power activity with IC_{50} value of 17.05µg/ml compared with standard BHT with IC_{50} value of 125.06µg/ml. which in turn represents potential metal chelating activity of D5 *Chooranam*. The presence of large amounts of phenols and flavonoids in D5 *Chooranam* was abundantly supportive of the observed antioxidant property. The strong antioxidant activity demonstrated by D5 *Chooranam* would possibly bring about the reduction of oxidative stress in diabetes patients.

CONCLUSION: Thus, our study findings demonstrate the antioxidant potential of the various D5 *Chooranam extracts.* Qualitative and quantitative assessment of various D5 *Chooranam* extracts showed the presence of significant number of various phytochemicals. *In-vitro* antioxidant analysis of D5 *Chooranam* also shows an antioxidative activity in most of the oxidative stress-related parameters. Though the individual ingredients of the study drug have been proved individually for diverse biological activity, the synergetic effect of those phytoconstituents in this formulation could be reason for its significant free radical scavenging activity. Hence, it can be concluded that D5 *Chooranam* has potent antioxidant activity against various free radicals which were generated during diverse metabolic reaction. Further detailed investigations are required to completely elucidate the cellular mechanism of D5 *Chooranam* on various disease conditions like DM which results from oxidative stress.

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