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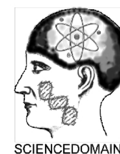

Qualitative Characterization of Solvent and Cooked Extracts of *Tribulus terrestris L.* Fruit

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Authors' contributions

This work was carried out in collaboration between all authors. Author KE designed the study and managed the review of manuscript. Author SSK performed the analysis and wrote the first draft of the author DB provided technical guidance. Authors KE and SSK managed the literature searches and author KE responded to reviewer's comments and finalized the draft. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Currently there has been an increased attention globally to identify antioxidant compound that are pharmacologically potent and have low or no side effects. As plants are source of natural antioxidants, much concentration has been given to plants. A variety of free radical scavenging antioxidants exists within the body in which many of them are derived from dietary sources like fruits, vegetables, etc. *Tribulus terrestris L.* fruit extract has an ancient tradition in folk medicine and in ayurveda as a diuretic, antiseptic, mood enhancer and anti-inflammatory agent. Though already few studies are available on antioxidative properties of *Tribulus terrestris,* yet no research has explored what happens to boiled or cooked extract of the sample. This was conceptualized in the present study with the hypothesis whether the extract can be incorporated into foods rather than as medicine. Hence, in this study, preliminary qualitative phytochemical analysis of *Tribulus terrestris* fruit was observed and also antioxidant activity of the methanol, ethanol, aqueous and cooked extract of *Tribulus terrestris* fruit was determined along with nitric oxide, superoxide and hydrogen peroxide scavenging assays. It was found that the cooked extract of *Tribulus terrestris* fruit too possessed greater percentage of inhibition activity when compared with other solvent extracts. Further, the results of preliminary

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phytochemical analysis revealed that the cooked extract was absent for tannins and glycosides which are generally considered as antinutritional factors. Further, quantification of various bioactive substances including saponins in *Tribulus terrestris* fruit extract may suggest whether it may be suitable for formulating as a functional food.

Keywords: Tribulus terrestris L.; fruit; polyphenols; phytochemical screening; antioxidants.

1. INTRODUCTION

The medicinal properties of several herbal plants have been identified and documented in a number of ancient literature and the use of herbal plants have been found to be effective in the treatment of various diseases. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [1].

The major groups of phytochemicals that may contribute to the total antioxidant capacity of plant include polyphenols and vitamins (C and E). Phenolic compounds can be nonnutrients [2]. Miller et al. [3] reported that phenol compounds are widely distributed in plants and are hydroxylated derivatives of benzoic acid and cinnamic acids and also suggested that phenolic compounds possess anti oxidative, free radical scavenging abilities and anticarcinogenic effects. Phenolic compounds including flavonoids are important in plant defence mechanisms against invading bacteria and other types of environmental stress [2,4].

While such plant flavonoids have long been recognized to possess anti-inflammatory, anti-allergic, antiviral and antiproliferative activities [2,4-7]. Djeridane et al, 2006 & Wong et al, 2006 reported that the antioxidant potential of medicinal plants may be correlated to the concentration of their phenolic compounds which include phenolic acids, flavonoids, anthocyanins and tannins [8,9]. These compounds are of great value in preventing the onset and/or progression of many human degenerative diseases [10]. The health-promoting effect of antioxidants from plants is thought to arise from their protective effects by counteracting reactive oxygen species [9]. Similarly, the antioxidants also help food products by delaying and inhibiting lipid oxidation and when added even to foods tend to minimize rancidity, retard the formation of toxic oxidation products, help maintain the nutritional quality and increase their shelf life [11].

Such antioxidants are also present in many of the plants and their products. As reported by Shanmugapriya et al. [12], *Tribulus terrestris*is a flowering plant of the Zygophyllaceae family, is native to warm temperature and tropical region. It can thrive even in desert climates and poor soil. *Tribulus terrestris*is used in folk medicine as tonic, aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic, litho-triptic and urinary anti-infectives [13]. Recently, anti-tumoural activity and effects on cardio vascular system have been stated by Tomova, Wu, Xie and Xu [14-17]. With these findings, it was conceptualized that *Tribulus terrestris* grown in Coimbatore District would also possess salubrious health effect. Hence, an effort was taken in the present study to prove that *Tribulus terrestris* fruit holds antioxidant capacity by conducting various preliminary qualitative analyses. There are few studies on the estimation of presence of phytochemicals and their free radicals scavenging properties have been described 8-11, yet no *In vitro* studies have reported about the cooked extract of *Tribulus terrestris* fruit. This was taken into consideration with an idea

of formulating foods in the future since people always consider and prefer as a functional food rather than consuming as herbal preparation or medicine. Based on these findings it may be further considered to quantify the positively responded components by qualitative screening to be later used for experimenting its activity against toxicity or malignancy in animal models.

1.1 Objectives

1.1.1 The objectives of the present study is stated as follows

1) To analyse the preliminary qualitative phytochemicals of T*ribulus terrestris* fruit.
2) To observe *in vitro* antioxidant activity of Tribulus *terrestris* fruit.
3) To identify the differences in the qualitative phytochemical components and antioxidant activities between the selected solvent extracts and cooked extract of *Tribulus terrestris* fruit.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The healthy fruit samples of *Tribulus terrestris* plant were collected from Coimbatore District, Tamil Nadu, India. The fruits from *Tribulus terretris* were collected on 22.7.2013 from SIHS Colony of East Coimbatore which has an altitude of 431 meters above sea level. The fruit variety was authenticated by a qualified Taxonomist, at Botanical Survey of India and a voucher specimen (No.: BSI/SRC/5/23/2013-2014/Tech/1024) was deposited at the Tamil Nadu Agricultural University, Coimbatore. The Fresh fruits of *Tribulus terrestris were* collected and dried in the shade thereafter powdered and stored in a sterile air tight opaque container for further use, which was later taken into lab for screening tests.

2.2 Extraction of *Tribulus terrestris* Fruit

Aqueous, Methanol, ethanol and cooked extracts were prepared according to the methodology of Indian pharmacopoeia. Crushed coarse powder was subjected to exhaustive extractions using a soxhlet apparatus. Powder weighing 10g was extracted with 100ml of distilled water, methanol, ethanol and boiled distilled water (Boiled at 100ºC). Then the extraction was agitated vigorously for 3hours in an agitator and kept for 72hours. Then, all the four extracts were filtered through Whatman no.1 filter paper to remove the particles, in a Buchner funnel.

2.3 Phytochemical Analysis

Phytochemical analysis for major phytoconstituents of the fruit extract was undertaken using standard methods as described by Sani et al. [18]. The fruit extracts were screened for the presence of biologically active compounds like sugars, amino acids, proteins, phenols, steroids, etc.

2.4 Qualitative Phytochemical Analysis

2.4.1 Test for carbohydrates

2.4.1.1 Fehling's test

To 2ml of the fruit t extract, 1ml of a mixture of equal parts of Fehling's solution A and B were added. The contents were boiled for a few minutes. Formation of red or brick red precipitate indicated the presence of reducing sugar.

2.4.1.2 Benedict's test

To 0.5ml of the fruit extract, 5ml of Benedict's reagent was added and boiled for 5 minutes. Appearance of red precipitate showed the presence of reducing sugar.

2.4.1.3 Molisch's test

To 2ml of the fruit extract, 2 drops of freshly prepared 20% alcoholic solution of alpha-napthol were added, mixed and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of the violet ring at the junction of the soloutions and its disappearance on the addition of excess alkali solution indicated the presence of carbohydrates.

2.5 Test for Proteins

2.5.1 Million's test

To a small amount of the fruit extract 3ml of water and 1ml of Million's reagent were added. A white precipitate which turns red on heating was formed, which indicated the presence of proteins was formed.

2.5.2 Biurette's test

To 1ml of the fruit extract, 1ml of 10% sodium hydroxide solution was added and the resulting mixture was heated. To this a drop of 0.7% copper sulphate solution was added. Formation of purplish violet colour indicated the presence of proteins.

2.5.3 Ninhydrin test

To few ml of the fruit extract, was added 0.5ml of 0.2% of ninhydrin reagent, mixed thoroughly and heated in boiling water bath for 2 minutes. Appearance of purple colour indicated the presence of proteins and free amino acids.

2.6 Test for Oil and Fats

Small quantity of the extract was separately pressed between two filter papers. Oil strains on the paper indicated the presence of oils.

2.7 Test for Steroids

2.7.1 Leibermann-barchard test

To 1ml of the fruit extract, 2ml of the concentrated sulphuric acid were added followed by the addition of 2ml of acetic anhydride solution. Green colour developed turned blue, which indicated the presence of steroids.

2.7.2 Salkowki's test

To 2ml of the fruit extract 1ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Red colour produced in the chloroform layer, indicated the presence of steroids.

2.8 Test for Thiols

To 0.5ml of the fruit extract enough ammonium sulphate was added to saturate the solution, 2-4 drops of 5% sodium nitroprusside was then added followed by one or more drops of concentrated nitric acid. Transient magenta colour developed in the presence of thiols.

2.8.1 Test for alkaloids

2.8.1.1 Dragendorff's test

To 0.5ml of the fruit extract, 2ml of HCl were added. To this acidic medium, 1ml of Dragendorff's reagent was added. Formation of red precipitate indicated the presence of alkaloids.

2.8.1.2 Wagner's test

To 1ml of the fruit extract was acidified by adding 1.5% v/v of HCl and a few drops of wagner's reagent. Formation of brown precipitate indicated the presence of alkaloids.

2.8.2.3 Meyer's test

To 1ml of the fruit extract few drops of Meyer's reagent was added, formation of yellow cream precipitate indicates the presence of alkaloids.

2.9 Test for Flavonoids

2.9.1 Alkaline reagent test

To a few drops of fruit extract, few drops of sodium hydroxide solution were added. Intense yellow colour was formed which turned to colourless on addition of few drops of dilute HCl, indicated the presence of flavanoids.

2.9.2 Shinoda's test

To 0.5ml of the fruit extract, 5-10 drops of concentrated HCl and small pieces of magnesium were added and the solution was boiled for few minutes. Magenta colour was produced after few minutes which indicated the presence of flavanoids.

2.9.3 Zinc hydrochloride test

To the fruit extract, a pinch of zinc dust and concentrated HCl were added. Appearance of magenta colour after few minutes indicated the presence of flavanoids.

2.10 Test for Phenols

2.10.1 Ferric chloride test

To 1 ml of the fruit extract, 2ml of distilled water followed by drops of 10% aqueous ferric chloride solution were added. Formation of blue or green or violet colour indicated the presence of phenols.

2.10.2 Lead acetate test

1ml of the fruit extract was diluted with 3 ml of distilled water and to this few drops of 1% aqueous solution of Lead acetate was added. A yellow precipitate was formed which indicated the presence of phenol.

2.10.3 Libermann's test

To small quantity of the fruit extract, added 0.5 ml of 20% sulphuric acid followed by the addition of few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue or green when made alkaline with aqueous sodium hydroxide solution.

2.11 Test for Saponins

2.11.1 Foam test

To the fruit extract a few drops of sodium bicarbonate solution were added. Shaken vigourously and kept for 3 minutes. A honey comb like froth was formed indicated the presence of saponins.

2.11.2 Haemolysis test

A suspension of RBC in normal saline was treated with the small amount of leaf decoction, Haemolysis was observed which occurred due to the presence of saponins.

2.12 Test for Glycosides

2.12.1 Legal's test

To the fruit extract, 1ml of sodium nitroprusside solution was added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour confirmed the presence of glycosides.

2.12.2 Keller killiani test

A small amount of the fruit extract was dissolved in 1 ml of water and then aqueous sodium hydroxide solution was added. Formation of yellow colour indicated the presence of glycosides.

2.13 Test for Tannins

2.13.1 Ferric chloride test

To 1ml of the extract, few drops of 5% aqueous ferric chloride solution was added. A bluish black colour which disappeared on addition of few ml of dilute sulphuric acids forming yellowish brown precipitate indicated the presence of tannins.

2.13.2 Lead acetate test

To 5ml of the extract a few drops of 1% solution of lead acetate was added. A yellow precipitate was formed indicating the presence of tannins.

2.14 Evaluation of Antioxidant Activity by *In vitro* Techniques Nitric Oxide Radical Scavenging Activity

Sodium nitroprusside (5µM) in standard phosphate buffer solution was incubated with different Concentration of the test extracts dissolved in standard phosphate buffer (0.025M, pH 7.2) and the tubes were incubated at 25ºC for 5hr. After 5h, 0.5ml of incubation solution was removed and diluted with 0.5ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water). The absorbance of chromophore formed was read at 546nm. The control experiment was also carried out in similar manner, using distilled water in the place of extracts. The experiment was performed in triplicate and % scavenging activity was calculated using the formula 100− [100/blank absorbance × sample absorbance]. The activity was compared with ascorbic acid, which was used as a standard 19.

2.15 Superoxide Radical Scavenging Activity

Superoxide radical ($O2^-$) was generated from the photo reduction of riboflavin and was deducted by nitro blue tetrazolium dye (NBT) reduction method [20]. Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne et al (1975) [21]. The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5mM NBT) solution, 0.2ml of EDTA (0.1M EDTA), 0.05ml riboflavin (0.12mM) and 2.55ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was

illuminated for 30 min and the absorbance at 560nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples and % scavenging activity was calculated using the formula 100− [100/blank absorbance × sample absorbance].

2.16 Hydrogen Peroxide Scavenging Activity

The ability of the *Tribulus terrestris* extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. [22]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100µg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40mm). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging assay of *Tribulus terrestris* extracts and standard compounds were calculated. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged.

$$\% \text{ Scavenged } [H_2O_2] = [(AC - AS)/AC] \times 100$$

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of *Tribulus terrestris L.* extracts or standards.

3. RESULTS AND DISCUSSION

The screening of fruit extract for therapeutic attributes, has been carried out with the help of preliminary phytochemical analysis. Phytochemical screening is of principal in recognizing new sources of therapeutically and industrially value added compounds possessing medicinal implications, to utilize the naturally existing resources [18]. However, the solvent which is used for extraction makes the difference in qualitative outcome with each other. Different types of solvent play an important role in extractability of different phytochemicals. Thus, various extractions with different solvents also furnish clarity to identify which solvent will yield better outcome. Nevertheless, in the present study other than aqueous, methanol and ethanol solvents, decisively cooked extract was also employed in order to determine whether real time cooking precisely yields same antioxidative properties. If this is assessed, then it may suggest in the real sense, what it could yield qualitatively at household application (at Kitchen) rather than at *In vitro* lab level.

The phytochemical characteristics of *Tribulus terrestris* fruit are summarized in Table 1. The results revealed the presence of medically active compounds in the plant studied. It could be seen that proteins, steroids, thiols, alkaloids, flavanoids, phenols, glycosides and tannins were highly present in the ethanolic extract which are similar to the previous reports of Shanmugapriya et al. [12]. Such bioactive and phytoconstituents may be responsible for various pharmacological actions of this plant part like antibacterial, anticancer, antiulcer, cardioprotective and chemoprotective activities as suggested by Chhabra et al. [23]. Proteins, steroids, thiols, alkaloids, flavanoids, phenols, saponins, glycosides, tannins were slightly present in the methanolic extract. Whereas, in aqueous extract only thiols, alkaloids and flavanoids were slightly present. Similarly in cooked extract, phytochemical constituents like proteins, steroids, thiols, alkaloids, saponins, phenols and flavanoids were present in slighter amounts. Thus, it is imperative that presence of phenols and flavanoids in the

cooked extract may also render significant favourable activity against oxidative stress or inflammatory conditions. On the other hand, cooked extract was also absent for tannins and glycosides which are generally considered as antinutritional factors.

Table 1. phytochemical analysis of *Tribulus terrestris L.* fruit

Phytochemical constituents	Result of qualitative tests			
	Aqueous extract	Ethanolic extract	Methanolic extract	Cooked extract
Carbohydrates	-	+	-	-
Proteins	-	++	+	+
Oil and Fats	-	-	-	-
Steroids	-	++	+	+
Thiols	+	++	+	+
Alkaloids	+	++	+	+
Flavonoids	+	++	+	+
Phenols	-	++	+	+
Saponins	-	+	+	+
Glycosides	-	++	+	-
Tannins	-	++	+	-

++ = Highly presen,t + = slightly present, - = Absent

Lakshmi Devi et al. [24] in their study showed that the methanol extracts of *Tribulus terrestris* and *Pongamia pinnata*, were possessing less superoxide scavenging activity than the standard ascorbate. Whereas, in the present study methanol extract of *Tribulus terrestris* fruit showed a higher percentage of inhibition (57.90%) against superoxide radicals than the standard ascorbic acid (38%) at lower concentration of 0.2ml. Similarly, from the Fig. 1 it is understood that the cooked extract of *Tribulus terrestris* fruit lead to a significant percentage inhibition (79%) against superoxide radicals as compared with the standard (62%).

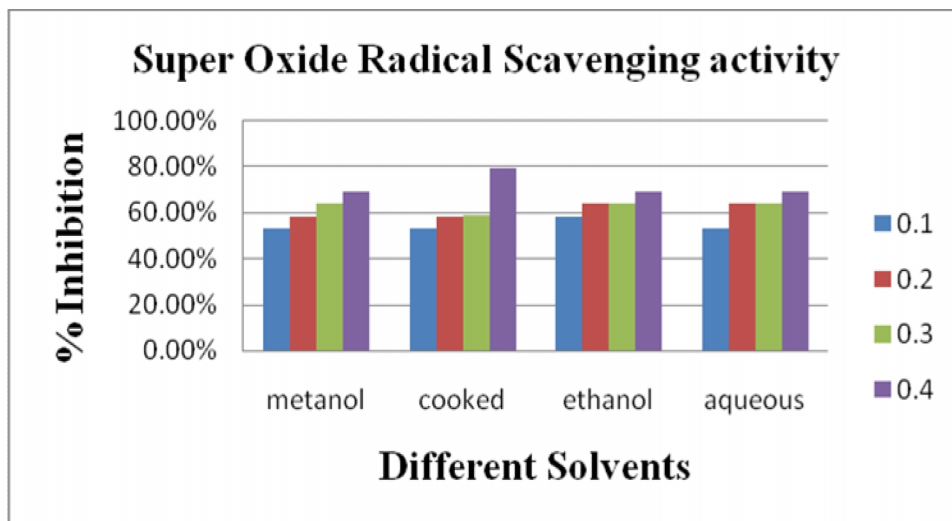

Fig. 1. Study on super oxide scavenging activity of *Tribulus terrestris L.* fruit methanol extract

Gopalakrishnan et al. [25], conducted a study in the superoxide scavenging activity of ethanolic extract of *Mollungo nudicaulis* and they found out that the plant posses equal superoxide scavenging activity as compared with the standard ascorbate. However, the results of the present study revealed that the superoxide scavenging activity of ethanolic extract of Tribulus *terrestris* fruit posses greater radical scavenging action against the standard ascorbate in all four concentration (0.1-0.4ml). Similarly, the superoxide scavenging activity of aqueous extract of *Tribulus terrestris* fruit is a good superoxide radical scavenger and efficiency of *Tribulus terrestris* fruit is high compared to ascorbic acid.

The scavenging ability of water, ethanol, methanol and cooked extract on Nitric oxide is shown in Fig. 2. However, scavenging activity is observed in the cooked extract of the *Tribulus terrestris* when compared with the methanol, ethanol and aqueous extracts.

Results show that the scavenging activity of *Tribulus terrestris* extract posses significant free radical scavenging action against nitric oxide (NO) induced release of free radicals at the concentration between 0.1-0.4μ/ml, showing 15-85% of nitric oxide inhibition respectively in cooked extract, 2-45% in aqueous extract, 22-68% in methanol and 3-35% in ethanolic extract compared to ascorbic acid used as standard.

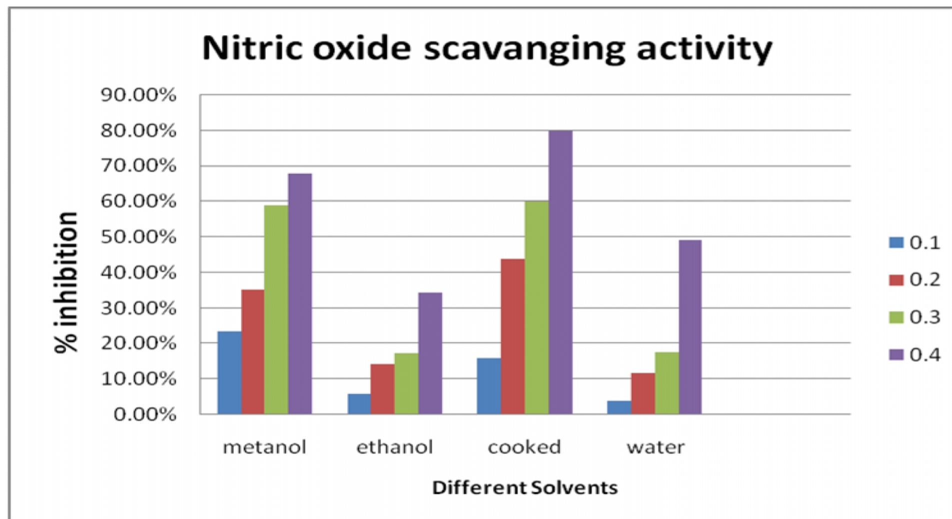

Fig. 2. Study on nitric oxide scavenging activity of *Tribulus terrestris(l)* fruit extract

According to the study conducted by Onocho et al. [26], scavenging of hydroxyl radical generated from hydrogen peroxide by methanol extract of *Acalypha torta* had high scavenging activities when compared to the standard ascorbic acid, but in the present study the percentage inhibition was very low at the concentration of 0.1, 0.2 and 0.3µg/ml but at 0.4µg/ml concentration; instead of, it was found that there was an increase in percentage inhibition 59% against hydrogen peroxide radicals as compared with the standard 32%. Similarly, Tara et al. [27] studied the antioxidant activity of aqueous extract of seeds of *Cucumi callosis,* instead of hydrogen peroxide – scavenging activity was determined and the results indicated that the aqueous extract of *Cucumi callosis* instead of where exhibited the effective antioxidant activity, but from the results of the present study it could be concluded that the percentage of inhibition against hydrogen peroxide radicals was lower than that of the standard ascorbic acid, at concentration of 0.1, 0.2 and 0.3µg/ml. Whereas, there was a

significant inhibition 43.90% against hydrogen peroxide radicals as compared to the standard 32%. Likewise, from the Fig. 3 it is clearly evident that the hydrogen peroxide radical scavenging activity of cooked and ethanol extract of *Tribulus Terristris* had very low percentage inhibition at the concentration of 0.1, 0.2 and 0.3µg/ml but there was an increase in percentage inhibition at 0.4µg/ml when compared with the standard ascorbate.

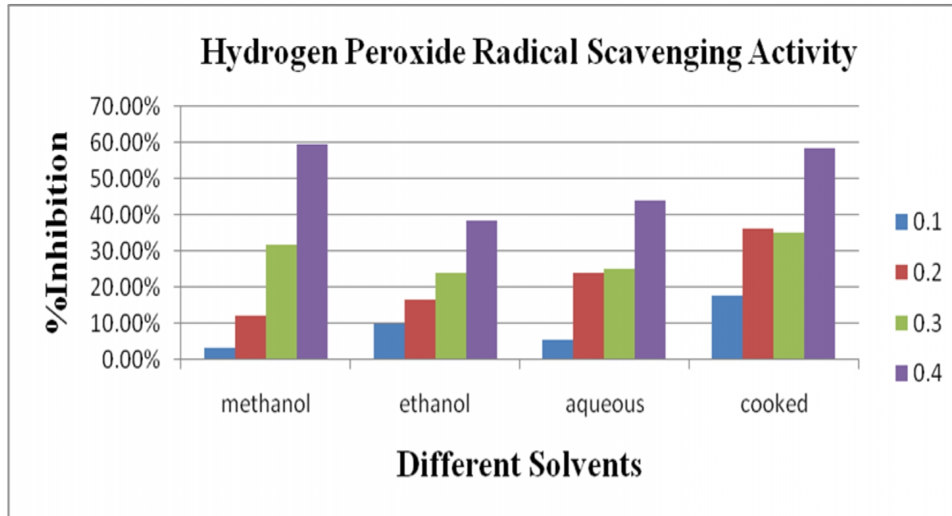

Fig. 3. Study on hydrogen peroxide scavenging activity of *Tribulus terrestris* fruit extract

4. CONCLUSION

The *Tribulus terrestris* fruit screened for phytochemical constituents was found to have the potentiality to possess various compounds that are vital for good health particularly rendering as an antioxidant source and with this attribute, it may also be considered to incorporate into various food products which would help to improve the health status of the consumers. As *Tribulus terrestris* fruit is generally dried and powdered and used as a pharmacognosy product for the treatment of various disorders, particularly as a diuretic, antiseptic, mood enhancer and anti-inflammatory agent. However, as most of the people may be reluctant to use it as it is raw as a medicine, would inturn gain momentum and be acceptable, If it is made into food products. Further, quantification of various bioactive substances including saponins from *Tribulus terrestris* fruit would add value to consider as formulated functional foods.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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