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Phytochemical estimation and evaluation of antioxidant activity of hydro-alcoholic and ethylacetate extracts of *Tribulus terrestris* linn. (gokhru) and *Solanum nigrum* linn (makoi)

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***Abstract*---**Tribulus terrestris L. and Solanum nigrum L. has been used traditionally since ages. To explore the phytoconstituents (qualitatively and quantitatively) and estimate antioxidant activity of hydroalcoholic and ethyl acetate extracts of fruits of Tribulus terrestris L. and Solanum nigrum. Different methods were adopted for phytochemical screening. Evaluation of antioxidant potential was achieved by total anti-oxidant capacity assay, hydrogen peroxide scavenging activity, DPPH radical-scavenging activity, Nitric oxide radical scavenging activity and reducing power assay. The qualitative estimation of fruits of Tribulus terrestris L and of Solanum nigrum L. showed the presence of various phytoconstituents likewise tannins, saponins, flavonoids and terpenoids. Quantitative estimation revealed the presence of flavonoids (5.01%w/w), saponins (0.70%w/w), and

terpenoids (0.013%w/w) in fruits of Tribulus terrestris L. whereas presence of flavonoids (10.46%w/w), saponins (11.02%w/w) and terpenoids (0.034%w/w) was found in fruits of Solanum nigrum L. It can be concluded that both the extracts of Tribulus terrestris L and Solanum nigrum L possess significant antioxidant activity.

Keywords---tribulus terrestris, solanum nigrum, phytoconstituents, anti-oxidant activity.

Introduction

Oxidative stress is one of the major factors for various ailments in humans. It occurs when the generation or formation of free radicals increases abruptly due to compromised mechanism of protective antioxidants in our body system. (García-Sánchez et al., 2020). Reactive oxygen species (ROS) includes hydrogen peroxide, superoxide, nitric oxide, oxygen–derived free radicals and oxidants, hydroxyl radicals and reactive nitrogen species (RNS) in body which are responsible for oxidative stress (Phaniendra et al., 2015). These species of free radicals have potential to damage DNA, lipids (lipid oxidation), protein (protein oxidation) (Brand, 2010; Fransen et al., 2012). Oxidative stress is found to be responsible in the pathogenesis of several ailments likewise hyperglycemia, hypertension, atherosclerosis, diabetes mellitus, cancer, cardiovascular diseases, alzheimer diseases, parkinsonism, obesity associated syndrome (Fransen et al., 2012; Rajendran et al., 2014; Liguori et al., 2018). Taken into account the involvement of oxidative stress in the pathogenesis of several disorders, inhibition of oxidative stress maybe a prospective in the management of disorders (Forman & Zhang, 2021).

Solanum nigrum belongs to family Solanaceae, commonly known as Black nightshade or Makoi (in Hindi) in India (Jain et al., 2011). In traditional medicine system, its aerial parts are used in treating snake bite, burns, stomach ulcers, whooping cough, rabies, asthma, conjunctivitis (Arunachalam et al., 2009; Nitish et al., 2011). The numerous phytoconstituents isolated from leaves and fruits of *Solanum nigrum* are found to be effective in treating hepatotoxicity and cytotoxicity (Omara, 2021; Patel et al., 2009). Moreover, phytoconstituents (saponins, flavonoids, glycosides) from *S. nigrum were* found to be beneficial as analgesic, anti-inflammatory, immunostimulant16, anti- diarrheal, anti-cancer, anti-microbial, anti-oxygenic (Rutala et al., 2013; Kalia et al., 2014; Padmashree et al., 2014).

Tribulus Terrestris L. of family Zygophyllaceous, is a small prostrate shrub native to Mediterranean, subtropical and hot desert type conditions around the world (China, India, Spain, and Bulgaria). It is commonly known as puncture vine or Gokhru (in Hindi) in India and it is a well patronized medicinal herb in Indian and Chinese system of medicine. *T. terrestris* was found to be effective in treating numerous ailments of central nervous system, urogenital, musculoskeletal and gastrointestinal (Khare, 2007). The extracts of its aerial plant part are a profound source of various

therapeutically active phytoconstituents such as steroidal saponins, glycosides (Zheng et al., 2017), flavonoids (Kang et al., 2014), furostanol saponins (Hammoda et al., 2013), flavonoid glycosides (kaempferol, quercetin) (Dinchev et al., 2008) and these active secondary plant metabolites exhibit good pharmacological activity in sexual disorders, impotence and hormonal imbalances (Xu et al., 2009; Chen et al., 2012) .It was also found to be effective in treating other illnesses likewise cardiovascular diseases (Kang et al., 2014; Kamenov et al., 2017), microbial infections (Semerdjieva & Zheljazkov, 2019), cancer (Vala et al., 2014; Sisto et al., 2012) and helminthiasis (Angelova et al., 2013), renal diseases (Najafi et al., 2014; Saxena & Argal, 2015) and diabetes mellitus (El-Tantawy & Hassanin, 2007; El-Shaibany et al., 2015; Samani et al., 2016; Ercan & El, 2016; Momin et al., 2014).

Considering the wide range of activities of both the plants, the under-discussion study was designed to evaluate the phytochemical analysis of hydroalcoholic and ethyl acetate extracts of fruit of *S. nigrum* and fruits of *T. terrestris* as this part of the study is an integral part of further studies in which anti-obesity activity of these plants is proposed. Therefore, phytochemical screening was carried out along with antioxidant activity of hydroalcoholic and ethyl acetate extracts of fruit of *S. nigrum* and fruits of *T. terrestris*.

Materials and Methodology

Plant Collection, Identification and Preparation

The fruits of *T. Terrestris* and of *S. nigrum* were procured from nearby market of Ambala Cantt, Haryana, India. The authentication of both the samples was done by Plant Taxonomist of Department of Botany at Sri Venkateshwara University, Tirupati, India and the plant specimen vouchers are accessible in the University herbarium. The procured, authenticated fruit and fruits of both the plants was washed with running tap water 3 to 4 times and then dried in shade. After drying, fruits of *T. Terrestris* and fruits of *S. nigrum* were subjected to size reduction by using a grinder. The powder of plants was stored in air tight containers for further usage.

Preparation of extracts

100-gm of powdered fruit of *T. Terrestris* was extracted sequentially with Hydroalcoholic (60%) and ethyl acetate by soxhlation up to 72hrs. In a similar way, the 100-gm powder of fruits of *S. nigrum* was extracted. After extraction, filtration followed by concentrating of the extracts was done to obtain semisolid or dry mass by using rotary evaporator maintained at 40°C. The dried crude extracts were used to determine phytoconstituents (Qualitatively and Quantitatively) and *in-vitro* antioxidant potential.

Phytochemical Screening

Phytochemical evaluation is a qualitative approach to identify the type of

chemical constituents present in the crude plant extract. The methods opted for determination of alkaloids, fixed oils, steroids, phenolic compounds, volatile oils, fats, saponins, terpenoids, flavonoids, glycosides etc., are considered from standard procedures mentioned in Trease and Evans, Harborne, C.K Kokate and Khandelwal (Harbone, 1987; Nićiforović et al., 2010) as mentioned in Table 1: Phtyochemical Screening Methods.

Table 1
Phytochemical screening methods

S.NO	PHYTOCONSTITUENT	METHOD	INFERENCE
1	SAPONIN	Few ml of extract + 5ml distilled water, shaken vigorously for 3min.	Formation of foam indicates presence of saponins
2	ALKALOIDS	Few ml of extract + 1ml Dragendorff's reagent + 0.2ml acetic acid	Reddish brown precipitates indicate presence of alkaloids
3	TERPENOIDS &STEROIDS	Few ml of chloroform solution of extract + conc. Sulphuric acid, shaken vigorously	Red color on standing indicates terpenoids Lower layer turning yellow indicates steroids
4	GLYCOSIDES	Few ml of Chloroform solution of extract + 0.4ml glacial acetic acid + few drops of ferric chloride + 0.5ml conc. Sulphuric acid	Blue color formation indicates presence of glycosides
5	FLAVONOIDS	Few ml of extract + 1% ferric chloride (alcoholic)	Green color formation indicates presence of flavonoids
6	TANNINS	Few ml of extract + 1% gelatin solution + 10% sodium chloride solution	Precipitate formation indicated presence of tannins
7	CARBOHYDRATES	Few ml of extract + Molish's reagent + conc. Sulphuric acid alongside of test tube	Reddish violet ring indicates presence of carbohydrates
8	RESINS	Few ml of extract + water, shaking for few minutes	Formation of turbidity indicates presence of resins
9	PROTEINS	Few ml of aq. Solution of extract + 10 % sodium hydroxide solution, mixed well + 0.1% copper sulphate solution	Formation of violet or pink color indicates presence of proteins
10	AMINO ACIDS	3ml of extract + 3 drops of ninhydrin solution (kept on boiling water bath for 10 min)	Purple or bluish color appearance indicates presence of amino acids

11	FIXED OILS	Few drops of extract on filter paper	Staining of filter paper indicates presence of fixed oils

Quantitative estimation of phytoconstituents
Terpenoid content

A mixture of methanol and water (4:1) was used for maceration of 50gm of dried powdered plant material at 37° C for 24 hours. After completion of 24hrs, mixture was filtered and concentrated at temperature 40°C. 2M H_2SO_4 was added in concentrate and further was treated with $CHCl_3$. After extraction, separated hydro layer was separated and dried, residue was the terpenoidal content present in the sample and calculated as follows.

Percentage of terpenoid content=(weight of residue ÷ weight of sample taken)×100

Total Flavonoid Content

10 gm Plant material was extracted with 100 ml of 80% aqueous methanol at room temperature (in triplicates) for 24 hours, then filtered and processed for drying (on water bath) by transferring into crucibles or China dishes until a constant weight was attained. The dried constant mass is the flavonoid content present in the plant sample and was calculated as follows (Liu et al., 2013):

Percentage of total flavonoid = (weight of residue ÷ weight of sample taken)×100

Total Saponin determination

Grounded plant material (20gm) added in 100 ml of hydro-ethanol (20%) in a conical flask and then kept on hot water bath for 4 hours (maintaining temperature not exceeding 55°C. After 4hrs, mixture was filtered and residue was collected. The collected residue was re-extracted with 200ml of aqueous ethanol (20%) and both the extracts were combined and subjected to reduce up to 40ml. The concentrated 40 ml was poured into a separating funnel and treated with diethyl ether (20 ml) and then vigorously shaken. The layer formed in separating funnel is aqueous which was separated and discarded the other layer (ether). The purified separated aqueous layer was further fractionated with 60ml of n-butanol (repeatedly 3times). After this, the butanol fractions were combined and treated with 10ml aqueous sodium chloride (5%) 2 times. The fractions of butanol dried for attaining a stable weight percentage saponins content was calculated as follows (Sun et al., 2011):

% of Total saponin content=(weight of residue ÷ weight of sample taken)×100

Total Phenolic content (TPC)

The total phenol content present in extracts was determined by using slightly modified method of Folin-Ciocalteu reagent based spectrophotometric assay.

The extract solution (1mg/ml) was diluted with 46 ml of water followed by addition of 1ml Folin-Ciocalteu reagent and kept for 3 min and then, 2% w/v sodium carbonate solution was added into it. Afterwards, it was kept for 180 minutes with occasional stirring. The absorbance was measured at 760nm after generation of blue color. The phenolic compounds were expressed as µg of (GAE) Gallic acid equivalent (Saeed et al., 2012).

In-vitro antioxidant assays
Total antioxidant capacity (TAOC)

An aliquot of 0.3ml extract (different concentrations 50-400 µg/ml) was added in 3ml mixture of reagents (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). This reaction mixture was placed into incubator maintaining temperature 95°C for 90 minutes. The mixture was cooled up to room temperature and was proceeded for absorbance at 695nm against blank. Ascorbic acid was used as standard and values for TAOC were calculated as ascorbic acid equivalents (Abu et al., 2017).

DPPH radical-scavenging activity

Different concentrations of extract were prepared (0.1-5 µg/ml). To 3ml of extract, 1ml of 0.1mM solution of DPPH (2, 2-diphenyl-2picryl-hydrazil) in ethanol was added. This mixture of extract and DPPH was incubated in dark for 30 minutes and then absorbance was recorded at 517nm. Test was done in triplicates considering Ascorbic acid as standard. The result was expressed as percentage DPPH scavenging effect and estimated by using the following formula (Awah & Verla, 2010).

$$\text{"DPPH radical scavenging activity} = \{\text{Absorbance(blank)} - \text{Absorbance(sample)}\} \div \text{Absorbance(blank)} \times 100\text{"}$$

Hydrogen peroxide (H_2O_2) scavenging activity

Ascorbic acid was used as standard. Different concentrations (10-320 µg/ml) were prepared and 1ml from these were added in 2.4ml of 0.1M phosphate buffer (PBS) of pH 7.4, followed by addition of 0.6ml of H_2O_2 solution (40mM) and it was vigorously shaken and kept at ambient temperature for 10 minutes. The absorbance was recorded at 230nm. The Hydrogen peroxide scavenging activity was estimated according to the following (Kitts et al., 2000).

$$\% \text{ inhibition} = 1-(A1-A2) \div Ao \times 100$$

Where, Ao= absorbance of control (water instead of sample),
A1= absorbance of sample
A2=absorbance of sample in Phosphate Buffer Solution only.

Nitric oxide radical scavenging activity

A mixture of (1%) sulphonilamide, (2%) H_3PO_4, and Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride) was freshly prepared and labelled as solution AB. 3ml of extract (different concentrations *i.e.,* 10-320 µg/ml) was added in 5mM of sodium nitroprusside in phosphate buffer and was incubated at 25°c for 2.5hours, afterwards treated with solution AB which was freshly prepared. Ascorbic acid was used as standard. The absorbance was recorded at 546nm. % Inhibition was calculated as (Jawad et al., 2013):

$$\% \text{ inhibition} = (Ao\text{-}At) \div A0 \times 100$$

Where, Ao= absorbance of the control (blank without extract)
At=absorbance of extract.

Reducing power assay

In this method, different concentrations *i.e.,* 10-320 µg/ml of extracts were prepared. 2.5ml of extract was added in equivalent amounts of sodium phosphate buffer (200mM), 1% $C_6N_6FeK_3$, mixed properly and incubated at 50°C for 20 minute and small amount (2.5ml) of trichloroacetic acid (10%w/v) was added, mixed and subjected to centrifugation for 8min at 1000rpm. After centrifugation, upper layer was separated out and 5ml of deionized water was added along with addition of 1ml $FeCl_3$ (0.1%) and absorbance was estimated at 700nm wavelength. This procedure was done in triplicate and mean values ±SD was calculated. The EC_{50} values were calculated from concentration absorbance graph (Bendary et al., 2013).

Results and Discussion

Percentage Yield and qualitative phytochemical evaluation

The % yield in case of hydroalcoholic extract of *T. terrestris* was found to be 2.55%w/w and in case of ethyl acetate extract, % yield of *T. terrestris* was obtained to be 0.78%w/w. The percentage yield in hydroalcoholic extract of *S. nigrum* was found to be 23.77%w/w and in case of ethyl acetate extract, percentage yield of *S. nigrum* was obtained to be 3.32% w/w. The qualitative estimation of both the plant extracts showed presence of various phytoconstituents as depicted in table 2.

Table 2
Qualitative analysis of various extracts of *T. terrestris* and *S. nigrum*

S. No	Phytoconstituent	*Tribulus terrestris*		*Solanum nigrum*	
		Hydroalcoholic	Ethyl acetate	Hydroalcoholic	Ethyl acetate
1.	Saponin	+	+	+	+
2.	Alkaloid	-	+	-	-
3.	Terpenoid	+	+	+	-
4.	Steroid	+	+	+	+

5.	Glycosides	-	+	+	+
6.	Flavonoids	+	-	+	+
7.	Tannins	+	+	+	+
8.	Proteins	+	-	+	-
9.	Carbohydrates	-	-	+	+
10.	Amino acids	+	-	+	-
11.	Fixed oils	-	+	+	-

Key: +ve: Present, -ve: Absent

Quantitative estimation of Phytoconstituents

The amount of flavonoids, saponins and terpenoid was found to be (5.01%w/w), (0.70%w/w) (0.013%w/w) respectively in *T. Terrestris*. Similarly, the quantified amount of flavonoids, saponins and terpenoids in *S. nigrum* was found to be 10.46%w/w, 11.02%w/w, 0.034%w/w respectively. It is evidently from previous studies on phytoconstituents that these possess good antioxidant properties (Côté et al., 2010; Aryal et al., 2019).

Total phenol content (TPC)

These compounds tend to possess antioxidant potential owing to the presence of -OH and these phenolic moieties are found to promote the synthesis of native antioxidant molecules in body system (Aliyu et al., 2013). Various findings have suggested that, phenolic compounds perform antioxidant activity by several mechanisms such as peroxide decomposition, free radical inhibition, metal inactivation and oxygen scavenging thus inhibiting oxidative stress. TPC in hydroalcoholic extract of *T. terrestris* fruits was estimated to be 43.6 mg/g GAE (Gallic acid equivalent) and for ethyl acetate extract of *T. terrestris* fruits was evaluated to be 13.06 mg/g GAE. Whereas, the TPC in hydroalcoholic extract of *S. nigrum* fruits was obtained to be 64.01 mg/g GAE and ethyl acetate extract of *S. nigrum* fruits was estimated to be and 22.07 mg/g GAE (fig.1). The total phenol content was found to be more in hydroalcoholic extracts of both the plants and hence can be considered to possess good anti-oxidant potential.

Total antioxidant capacity

This assay reflects the existence of antioxidant molecules which are soluble in water as well as fat (Kedare & Singh, 2011). Total antioxidant capacity of hydroalcoholic extract of *T. terrestris* fruits was estimated to be 36.16µM/g AAE (ascorbic acid equivalent) and of ethyl acetate extract of *T. terrestris* fruits was estimated to be 24.7916µM/g AAE. Total antioxidant activity of hydroalcoholic extract of *S. nigrum* fruits was found to be 42.59 µM/g AAE and ethyl acetate extract of *S. nigrum* fruits was estimated to be 27.83µM/g AAE. (fig. 2).

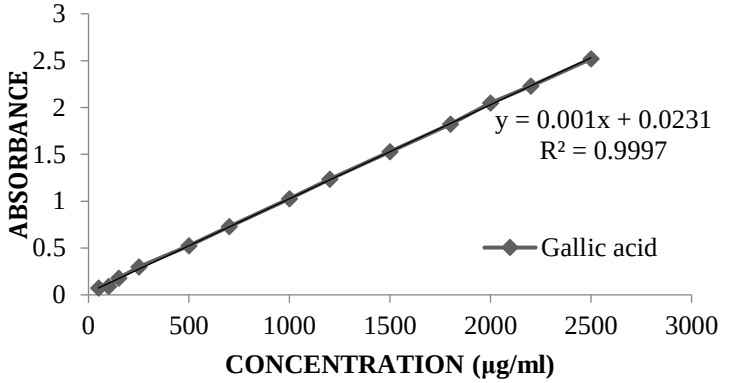

Figure 1. Standard curve for Gallic acid for estimation of total phenol content

DPPH scavenging activity

It is one of the reliable, easy and precise method for evaluation of ability of antioxidants to scavenge free radicals responsible for oxidative stress, and it is also useful in quantification of antioxidants (Razali et al., 2019). At 5µg/ml, DPPH scavenging activity of hydroalcoholic extract of *T. terrestris* fruits was found to be 72.65±0.13 % and for ethyl acetate extract it was obtained to be 77.65±0.16 % whereas, that of ascorbic acid was found to be 84.98±0.11 %. IC_{50} of hydroalcoholic extract of *T. terrestris* fruits was found to be 2.62 µg/ml and of ethyl acetate extract is 2.03 µg/ml whereas IC_{50} of ascorbic acid was obtained to be 1.58 µg/ml.

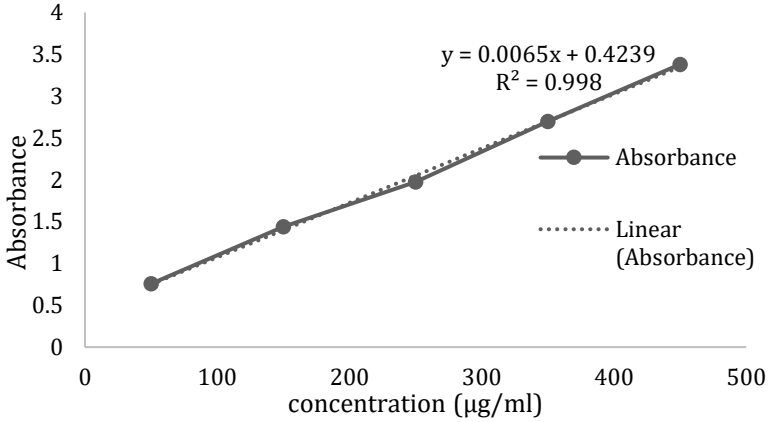

Figure 2. Standard curve of ascorbic acid for estimation of Total Anti-oxidant capacity

At 5µg/ml, DPPH scavenging activity of hydroalcoholic extract of *S. nigrum* fruits was found to be 72.32 ± 0.11 % whereas in case of ethyl acetate extract, it was found to be 74.65±0.14 %. IC_{50} of hydroalcoholic extract of *S. nigrum* fruits was found to be 2.40 µg/ml and for ethyl acetate extract found to be 2.06 µg/ml (fig.3). These estimations indicate that both these plants can be advantageous in oxidative stress management (Farooq et al., 2019).

Hydrogen peroxide scavenging activity

It is a type of free radical, whose higher concentrations are toxic for normal functioning of cells in our body, hence these should be scavenged by suitable antioxidants (Benslama & Harrar, 2016). Anti-oxidant potential of hydroalcoholic extract of *T. terrestris* fruits was obtained to be 74.62±0.61 % and of ethyl acetate extract of *T. terrestris* fruits against hydrogen peroxide radical was estimated to be 72.55±0.79 % at 320 µg/ml, whereas that of ascorbic acid was 89.49±0.19 %. IC_{50} of hydroalcoholic and ethyl acetate extract of *T. terrestris* fruits was found to be 114.94 µg/ml and ethyl acetate extract was estimated to be 121.00µg/ml whereas IC_{50} of ascorbic acid was found to be 80 µg/ml. Anti-oxidant activity of hydroalcoholic extract of *S. nigrum* fruits against hydrogen peroxide radical was estimated to be 75.15±0.31 % and ethyl acetate extract was evaluated to be 71.65±0.79 % at 320 µg/ml. IC_{50} of hydroalcoholic extract of *S. nigrum* fruits was evaluated to be 120.93 µg/ml and of ethyl acetate extract of *S. nigrum* fruits was estimated to be 126.92µg/ml accordingly (fig.4). The IC_{50} values of the extracts of *T. terrestris* were less than *S. nigrum* which indicate that *T. terrestris* has better hydrogen peroxide radical scavenging as compared to *S. nigrum.*

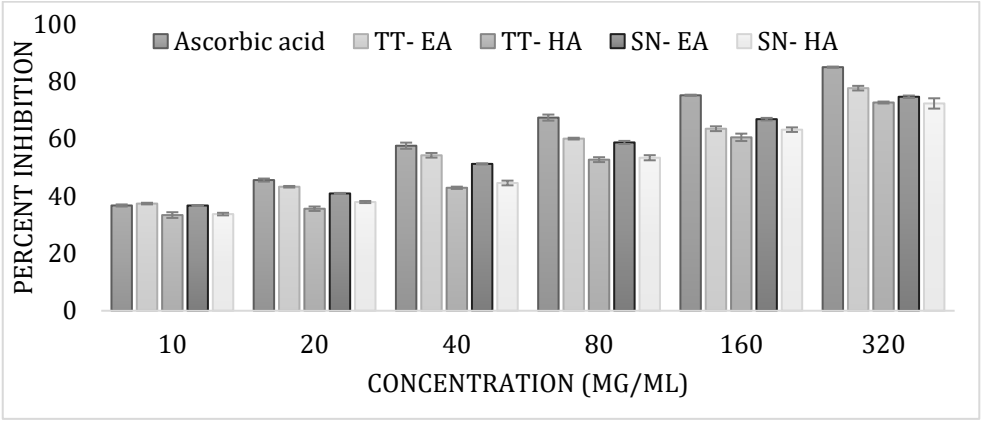

Figure 3. DPPH radical scavenging activity of hydroalcoholic and ethyl acetate extract of *T. terrestris* and *S. nigrum*

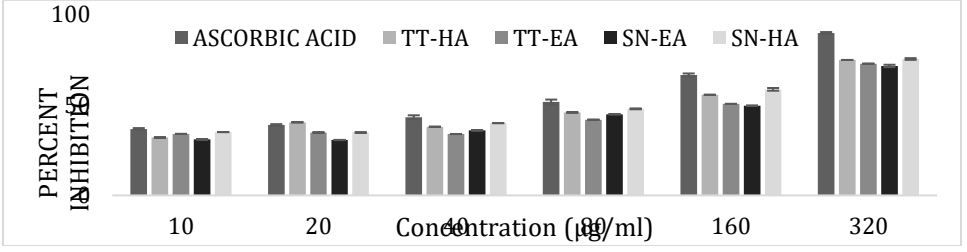

Figure 4. H_2O_2 activity of hydroalcoholic and ethyl acetate extract of *T. terrestris* and *S. nigrum*

Nitric oxide scavenging activity

This method involves the reduction in NO release from sodium nitroprusside as a consequence of competition between NO and scavengers for oxygen. Anti-oxidant activity of hydroalcoholic and ethyl acetate extract of *T. terrestris* fruits against nitric oxide radical was found to be 72.08±0.19 % and 71.04±0.22 % respectively at 320 µg/ml, whereas antioxidant activity of ascorbic acid was found to be 79.74±0.12 % at 320 µg/ml. IC_{50} of hydroalcoholic extract of *T. terrestris* fruits was found to be 95 µg/ml and ethyl acetate extract of *T. terrestris* fruits 89.13µg/ml. IC_{50} of ascorbic acid was obtained to be 52.26 µg/ml. Anti-oxidant activity of hydroalcoholic extract of *S. nigrum* fruits against nitric oxide radical was found to be 72.06±0.17 % and 76.04±0.12% respectively at 320 µg/ml. IC_{50} of hydroalcoholic extract of *S. nigrum* fruits was found to be 95.183 µg/ml and for ethyl acetate extract of *S. nigrum* fruits was obtained to be 88.80µg/ml (fig.5).

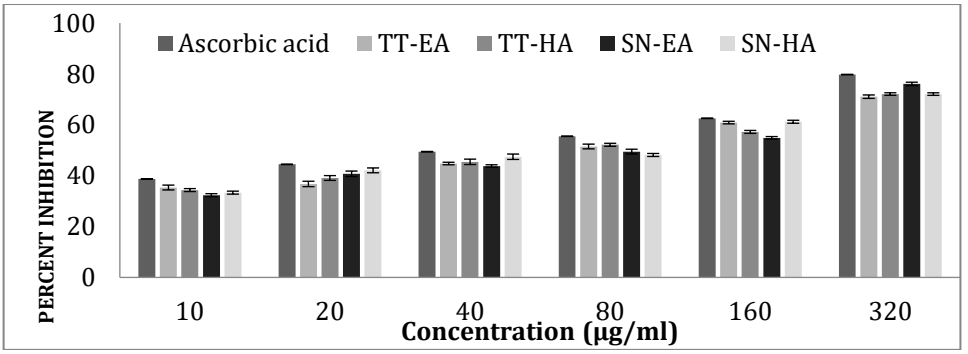

Figure 5. Nitric oxide activity of hydroalcoholic and ethyl acetate extract of *T. terrestris* and *S. nigrum*

Reducing power assay

The capacity of a compound to reduce itself is a significant possibility of its acting as a good antioxidant. The mechanism behind working of such compounds is that, they reduce Fe^{3+} to Fe^{2+}, which then create ferric-ferrous complex after reacting with ferric chloride. This can be observed at absorption maximum at 700nm. EC_{50} was calculated and was evaluated to be 36.16 µg/ml for hydroalcoholic extract of *T. terrestris* fruits and 24.79 µg/ml for and ethyl acetate extract of *T. terrestris* fruits whereas 11.70 µg/ml was estimated for ascorbic acid. EC_{50} was evaluated to be 42.59µg/ml for hydroalcoholic extract of *S. nigrum* fruits and 27.83 µg/ml ethyl acetate extract of *S. nigrum* fruits (fig.6). EC_{50} values of *T. terrestris* fruits were found to be less as compared to that of *S. nigrum* fruits, hence it can be stated that *T. terrestris* fruits has better reducing capability as compared to *S. nigrum* fruits.

Conclusion

It can be concluded from the results that Solanum nigrum contains a

comparable amount of phytoconstituents (flavonoids, saponins and terpenoids) and hydroalcoholic extracts of *T. terrestris* fruits and *S. nigrum* fruits have higher antioxidant potential as compared to their ethyl acetate extracts.

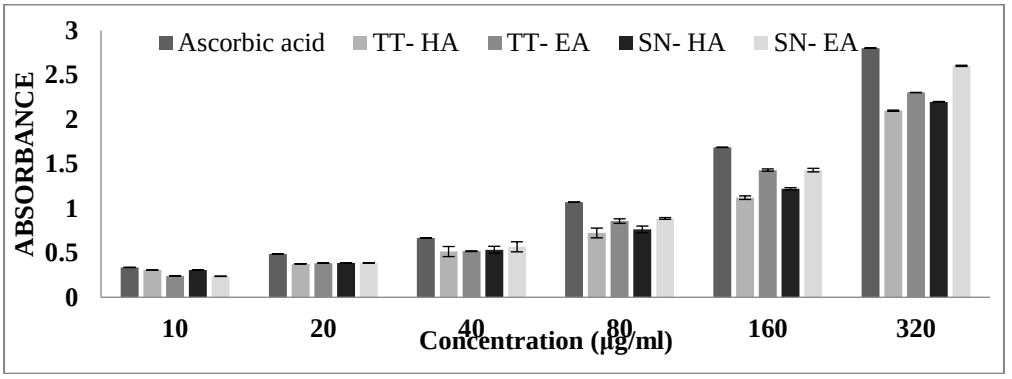

Figure 6. Reducing power assay of hydroalcoholic and ethyl acetate extract of *T. terrestris* and *S. nigrum*

Conflict of interest

Authors declare no conflict of interest.

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