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A Preliminary Biochemical Screening of *Spermacoce Hispida* Linn Seeds in Fenton Induced Toxicity in Chicken Smooth Muscle

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Abstract:
Spermacoce hispida (family; Rubiaceae) is widely used as an Ayurvedic folk medicine. The present study is focused on preliminary phytochemical and cytoprotective effect of Spermacoce hispida and was investigated against Fenton mixture induced oxidative stress in isolated chicken ileum preparation. The seed of Spermacoce hispida was extracted with different organic solvents in increasing order of polarity. The preliminary phytochemical investigations were determined using hydroalcoholic extract of Spermacoce hispida. In biochemical- pharmacological activity, we evaluate the effect of hydro alcoholic extract of Spermacoce hispida in oxidative stressed ileum preparation invitro. Group I tissues received vehicle. Group II tissues were oxidatively stressed with Fenton mixture. Group III and IV tissues were treated with two graded doses of naraginine after the administration of Fenton mixture. Group V, VI and VII tissues were superfused with three graded doses of hydro alcoholic extract of Spermacoce hispida with Fenton mixture. The toxicity of Fenton mixture was objectified by significant difference of malondialdehyde (TBARS), Reduced glutathione (GSH), Glutathione peroxidase (GPx) and catalase, ($p<0.05$) in oxidative stressed tissues. On preconditioned tissues with extract (10, 20 and 40mg), the oxidative stress was decreased with increase in antioxidants level. This effect of extract was found to be dose dependent. Based on the present findings Fenton mixture induced toxicity in isolated superfused tissues was significantly antagonized by the antioxidant activity of Spermacoce hispida extract.

Key words: *Spermacoce hispida, Fenton, oxidative stress, antioxidant*

1. Introduction

Oxygen radicals in the body are reactive, short lived and play a role in most major health problems (1). A number of radicals are formed in biological systems via a range of different processes. The oxygen containing radicals involved in "oxidative stress" together with some non radical species are collectively called Reactive Oxygen Species (ROS) and are often toxic intermediates in different metabolic processes. Examples of ROS known to induce damage *in vivo* are H_2O_2, organic hydroperoxides, HOCl, NO, O^{2-}, alkoxyl radicals and the hydroxyl radical. ROS can be derived from numerous sources *in vivo* including normal respiration, photochemical reactions and enzymatic reactions. A large number of enzymes have been shown to be capable to generate ROS and include the cytochromes P450, various oxidases, peroxidases, lipogenases and dehydrogenases (2).

One of the most hazardous radicals is the extremely reactive hydroxyl radical, with an almost diffusion limited half life of about one nanosecond. The main source of hydroxyl radicals *in vivo* is probably the so called Haber–Weiss reaction, where O^{2-} reduces Fe^{3+} to Fe^{2+} and in that way initiates the Fenton reaction between Fe^{2+} and hydrogen peroxide (2).

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH$$

In our study we use this Fenton mixture to produce free radicals in chicken ileum *invitro*. There is an emerging interest in the use of naturally occurring antioxidants for their therapeutic usage (3). Particularly, flavanoids are considered as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cardiovascular dysfunction, and inflammatory disorder (4). The World Health Organization (WHO) estimates that 80 percent of the world's population presently uses herbal medicine for some aspect of primary health care (5).

Spermacoce hispida is a perennial plant belonging to the family Rubiaceae. *T*raditionally it has been used for the treatment of various diseases. Recent research have shown that it also has antioxidant efficacy, antihyperlipidemic effect and anti-inflammatory activity (6). The plant extract is given as an astringent in hemorrhoids and gall stones and seeds are used in diarrhea and dysentery. A decoction of the herb is used in the treatment of headache (7). It has a wide variety of application not only in the Allopathy, but also in the Siddha System of Medicine. The choornam and lehyam of this plant is helpful in reducing the overweight (obesity), bloody diarrhea, internal heat, venereal diseases. The roots are dried and powdered and given along with cow's milk daily twice for conditions like urinary infections, oligurea, etc. Recently it is found that this herb contains Calcium and Phosphorus in abundance hence administration of this drug in form of chooranam or kudineer (decoction) is recommended in conditions like bone diseases, fractures etc (8). This herb has been found to have innumerable uses in folk medicine but none of these have been scientifically proved so far.
Therefore, our aim is to test the antioxidant effect of different concentrations of seed extract of *Spermacoce hispida* in toxicity induced smooth muscle preparation using fenton mixture.

2. Materials and methods

2.1. Collection of plant materials
The plant material was identified and authenticated and dried seed powder was collected from ABT Botanical Conservation, Salem district, Tamilnadu, India.

2.2. Extraction Of Plant Material
The powdered plant material was then extracted with 70% ethanol by cold percolation method. The extract was concentrated *in-vaccuo*. The brown colored residue was used for the following experiment. The extract was dissolved in physiological salt solution (PSS) on the day of experiment. The ethonolic extract have been selected for further biochemical pharmacological evaluation.

2.3. Chicken Ileum
Chicken ileum was collected from slaughter house near Amrita, Edapally in Kerala, India.

2.4. Chemicals Used
Alcohol, Methanol, Chloroform, Diethyl Ether, Petroleum Ether, Ammonia, Iodine solution, H_2SO_4, 2% NaOH, dil. HCl, NaOH Solution, Methylene blue, Ninhydrin solution, Dragondroff's reagent, Dilute $FeCl_3$ solution. Physiological Salt Solution (Tyrode Solution) contained (in gm/L) Nacl - 8.0, $NaHCO_3$ - 1.0, Glucose - 1.0, $NaH_2 Po_4$ - 0.05, KCl- 0.2, $MgCl_2$- 0.1, $CaCl_2$- 0.2), Fenton mixture (Ferrous sulfate, Sod EDTA, Di potassium hydrogen orthophosphate was purchased from Nice chemicals, Kerala.

2.5. Extractions And Yield Of Extracts
The coarsely powdered plant drugs were subjected to extract with different solvents. The extract was then concentrated *in-vaccuo* and the % of yield were calculated. The same extract was used for further studies. The concentration of the major phytoconstituents in the extracts depends on the % yield of the extracts.

2.6. Qualitative Analysis
The constituents of the extract were tested for the presence of alkaloids, flavonoides, carbohydrates, sterols, glycosides, saponins, cardiac glycosides, anthroquinone glycosides, saponin glycosides, coumarin glycosides, proteins, aminoacids, tannins and phenolic compounds.

2.7. Superfusion Apparatus Set-Up
In this system the tissues were superfused in an inner tissue column with a coarse sintered glass filter disk sealed into the lower portion. A mixture of moistened O_2:CO_2 (95:5) was delivered by small diameter tubing to the lower portion of the chamber by the aerator. The basic requirements for isolated tissue preparation include provisions for temperature control by outer jacket containing water with adjustable thermostat and also controlled by oxygen and substrate delivery. A water-jacketed organ bath provides a stable and easily adjustable way of temperature control. Substrates and other nutrients that are required to sustain tissue function were provided via a physiological salt solution, similar in chemical composition to plasma. There are four inner columns for stabilization of tissues (9).

2.8. Selection Of Physiological Buffer Solution And Aeration Techniques
The buffer solution(s) chosen for the dissection and maintenance of the tissue is important, as it will affect the viability of the preparation and hence experimental protocol (10).

2.9. Artificial Solutions
Reagents used in the production of buffers have high quality to the equivalent of USP or Analytical Reagent grade. Tyrode is widely used physiological salt solutions. Tyrode's and its salt concentrations are often modified to resemble those in the donor's plasma.
A Tyrode solution (10) (pH 7.4; temperature, 37 ºC) was prepared to obtain the following final concentrations: 136 mMol sodium chloride, 5.0 mMol potassium chloride, 2.0 mMol calcium chloride, 11.9 mMol sodium bi carbonate, 0.98 mMol magnesium

chloride, 0.36 mMol Sodium dihydrogen phosphate and 5.55 mMol glucose which continuously was bubbled with air (11). The perfusion medium was filtered successively through filters, prior to use and was equilibrated with mixture of O_2: CO_2 (95:5) at 37 ˚C for 1 hour before and throughout the superfusion.

2.9 Preparation of fenton mixture

The method of preparation of Fenton mixture is similar to that reported earlier (12). About 13.9 mg FeSO4.7 H2O, 75 mg of sodium EDTA, and 30µl of 50% H_2O_2 were added to 10 ml of 0.1 M Dipotassium hydrogen orthophosphate solution and the reaction mixture was kept in a water bath at 40°C for 20 minutes. With continuous stirring and the solution was used as a source of hydroxyl free radical.

2.10. Grouping Of Tissues

Groups	Stabilizaton 10 min	Drug 15 min	FM 15 min	Superfusion 15 min
I	Stabilizaton	No Drug	No FM	Superfusion
II	Stabilizaton	No Drug	FM	Superfusion
III	Stabilizaton	NIN I	FM	Superfusion
IV	Stabilizaton	NIN II	FM	Superfusion
V	Stabilizaton	SH I	FM	Superfusion
VI	Stabilizaton	SH II	FM	Superfusion
VII	Stabilizaton	SH III	FM	Superfusion

2.11. Procedure

Chicken (1.5 to 1.7 kg) was sacrificed by decapitation method and the ileum muscle was dissected as thin as possible and placed in a Petri dish containing frog Ringer solution. About 3 cm of ileum (4 pieces/ group) was mounted in an inner organ bath containing Tyrode solution at controlled (37±1°C) temperature. The tissue was maintained under a constant temperature. It was equilibrated for 15 min followed by NIN, SH and FM (Mentioned previous protocol). After this process, all group' tissues was homogenated and centrifuged at 2500rpm for 10 min. The supernant was analyzed for biochemical parameter like MDA, Gpx (13), GSH (14) and catalase (15).

2.12. Statistical analysis

Statistical analysis was carried out using SPSS software version 12.0. Values mentioned in the Tables are Mean ± SD. Significant difference was evaluated using One Way ANOVA with Duncan Multiple Range Test (DMRT). $p<0.05$ was considered as significant difference.

3. Result

The yield of each plant extract was calculated and the results are presented in Table-1. Extractive values are useful for the determination of adulterated drugs.

The data in Table-2 shows the preliminary phytochemical analysis. The dried powdered plant material was extracted successively using solvents like petroleum ether, methanol, benzene, chloroform and water in a cold percolation method. The extracts were then subjected to various qualitative tests using reported methods to determine the presence of various phytoconstituents.

The data in Table-3 shows the biochemical pharmacological results of seed extract of spermacoce hispida. TBARS was found to be increased in Fenton mixture perfused tissues (Group II) against normal tissues (Group I) ($p<0.05$). But on preconditioned tissues with extract the TBARS level was found to be decreased significantly and dose dependently (Group V, VI, & VII) ($p<0.05$,). This is corresponding to standard drug (Group III & IV).

The catalase level of Fenton mixture incubated tissues (Group II) was found to be decreased against normal tissues ($p<0.05$, Group I). But it was found to be elevated in extract preconditioned tissues (Group VI & VII) in dose dependently and significantly ($p<0.05$). No significant difference had been observed in extract preconditioned with low dose (Group V).

GPx and GSH level of oxidative stress induced tissues were found to be decreased against normal tissues ($p<0.05$, Group I). Extract preconditioned tissues were found to increase the level of both GSH and GPx significantly and dose dependently (Group VI& VII) ($p<0.05$). No significant difference in GSH and GPx level was observed in extract preconditioned with low dose (Group V). The biochemical enzyme like GSH, Gpx, MDA and catalase are almost the equivalent value of standard drug Naraginin.

4. Discussion

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, sterols *etc*. The successive root extracts of *Spermacoce hispida* have revealed the presence of alkaloids, flavonoids, glycosides, saponins and sterols. Thus the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development (16).

In our biochemical pharmacological study, oxidative stress is induced by Fenton's mixture to the isolated chicken ileum and compared with the extract *Spermacoce hispida* in three different doses. *Spermacoce hispida* is considered to be a highly effective antioxidant. However, it has been demonstrated that *Spermacoce hispida* has potent antioxidant properties (8). In this study, the isolated chicken gastro-intestinal tract tissue was homogenated and various biochemical parameters such as TBARS, glutathione, glutathione peroxidase and catalase were determined.

Glutathione peroxidases play a role in regulation of COX-2 and formation of prostaglandins via the arachidonic acid cascade (17). Glutathione peroxidases are a family of enzymes that mediate the elimination of hydro-peroxides in the gut (18 & 19). Glutathione peroxidase activity has also been linked to protection from colon cancer (20).

Oxidative stress in the smooth muscle environment, induced by Fenton's mixture leads to a decrease in glutathione peroxidase activity in the tissues treated with Fenton's solution alone. But the activity of glutathione peroxidase is significantly increased in the tissues treated with different doses of *Spermacoce hispida* in the ileum and this might be due to its antioxidant property.

GSH is involved either as a substrate in the cytosolic GSH redox cycle, or is able to directly inactivate free radicals and reactive oxygen species, which are known to be effective stress agents (21). Glutathione level decrease markedly in the fenton mixture treated organs. It causes the formation of free radicals in tissues. The formation of these radicals causes oxidative damage. The decrease in GSH in tissues leads to oxidative tissue damage (22). But the GSH level is significantly increased in the tissues treated with the extract along with fenton mixture. This shows that the drug administration restores the GSH activity due to its free radical scavenging property.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (23). Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. Catalase level is also increased in drug treated organs when compared to fenton treated organs. The decrease in catalase activity in fenton treated groups may be due to the involvement of catalase in scavenging of hydrogen peroxide free radicals mediated by fenton's solution (24 & 25).

We have observed increased levels of TBARS in fenton induced tissues. Reactive oxygen species (ROS) may attack any type of molecules, but their main target appears to be polyunsaturated fatty acids, which is the precursor of lipid peroxide formation (26). The decrease in the activities of antioxidant enzymes is in close relationship with the induction of lipid peroxidation (27). But the level of TBARS is significantly decreased in extract treated organs. *Spermacoce hispida* is one of the flavonoids to sequester metal ions and may contribute to their anti-lipoperoxidative property by preventing the formation of free radicals in the Fenton system. Flavonoids retain their free radical scavenging activities after forming complexes with iron ions and thus formation of metal ion chelates is also one of the antioxidant mechanisms of flavonoids (28).

The present study reveals that the seed extract of *Spermacoce hispida* exhibits satisfactory antioxidant effect against oxidative stressed induced tissues. *S. hispida* may react with free radical to neutralize their effect by donating electron thereby forming much less reactive radicals. Antioxidants such as superoxide dismutase, catalase and glutathione peroxide, which mainly act within the intracellular components, and low molecular mass antioxidant scavenging in the circulation that protect lipoproteins from oxidative modification in the extracellular fluid. This is the first report on antioxidant property of this plant against Fenton mixture induced oxidative damaged tissues. The results obtained by these methods provide some insight into the important factors responsible for the antioxidant potential and the mechanism of action.

5. Conclusion

In conclusion, hydroalcoholic extract of *Spermacoce hispida* seed has potential antioxidant activity against Fenton's reagent induced oxidative stress. The antioxidant activity of *Spermacoce hispida* extract might be helpful in preventing or slowing the progress of various oxidative stress- related diseases such as Coronary artery diseases, Cerebrovascular and peripheral vascular disease.

6. References

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Extractive Value (% Yield)					
Aqueous	**Chloroform**	**70% Ethanol**	**Hexane**	**85% Methanol**	**Pet. Ether**
2.80± 0.02	3.19± 0.02	3.91± 0.01	3.71± 0.02	3.06± 0.02	3.90± 0.03

Table 1: Extractive value of Spermacoce hispida
Mean ± SD

Phyto constituents	Aqueous	Chloroform	Ethanol	Hexane	Methanol	Pet. Ether
Alkaloids	-	-	+	+	+	+
Flavonoids	-	+	+	+	+	+
Carbohydrates	-	+	+	+	+	+
Saponins	+	+	+	-	+	+
Glycosides	+	+	+	+	+	+
Amino acids & proteins	+	-	+	+	+	+
Phytosterol	+	+	+	+	+	+
Fixed oils and fats	-	-	-	+	-	-

Table 2: Qualitative analysis of Spermacoce hispida
Note: + refers to Present and - refers to absent

GROUPS	MDA (nmoles of MDA formed/mg protein)	GPx (µmole of glutathione utilized/min/mg protein)	Catalase (µmole of H_2O_2 consumed/min/mg protein)	GSH (µg/mg protein)
I Normal	4.20 ± 0.60^{a}	7.18 ± 0.99^{c}	1.66 ± 0.23^{a}	4.28 ± 0.59^{b}
II DC	8.39 ± 1.16^{c}	4.36 ± 0.60^{b}	1.15 ± 0.17^{b}	2.08 ± 0.28^{ab}
III NIN 10 mg	4.55 ± 0.63^{a}	6.25 ± 0.86^{c}	1.57 ± 0.21^{c}	3.27 ± 0.45^{c}
IV NIN 20mg	3.56 ± 0.49^{a}	6.87 ± 0.95^{c}	1.66 ± 0.23^{a}	3.97 ± 0.55^{b}
V SH 10 mg	5.60 ± 0.77^{bc}	5.92 ± 0.82^{ab}	1.39 ± 0.19^{b}	3.14 ± 0.43^{c}
VI SH 20 mg	4.19 ± 0.58^{a}	6.67 ± 0.92^{c}	1.58 ± 0.21^{a}	3.82 ± 0.53^{c}
VII SH 40 mg	3.82 ± 0.53^{a}	7.03 ± 0.97^{c}	1.72 ± 0.23^{a}	4.36 ± 0.60^{b}

Table 3 Biochemical parameters

The statistical analysis was done by SPSS 12.0 software. Values are expressed as Means ± SD for 4 tissues in each group. Values not sharing a common superscript a, b, ab and c differ significantly at $p < 0$.