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ANTI-OXIDANT POTENTIAL OF METHANOLIC EXTRACT OF *TRIGONELLA FOENUM*, *TRACHYSPERMUM COPTICUM*, *NIGELLA SATIVA* AND THEIR COMBINATION IN 1:1:1 RATIO

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ABSTRACT: *Trigonella foenum (T. foenum)*, *Trachyspermum copticum (T. copticum)* and *Nigella sativa (N. sativa)* are important medicinal plants that need to be explored for their anti-oxidant potential. The aim of the present study was to evaluate the total anti-oxidant potential of methanolic extracts of *T. foenum*, *T. copticum*, *N. sativa* and the combination of these methanolic extracts in 1:1:1 ratio (Combination 1:1:1). The total phenolic content of the methanolic extracts of *T. foenum*, *T. copticum*, *N. sativa* and the Combination 1:1:1 was evaluated. Further the total anti-oxidant activity of the extracts was assessed by determining their total reducing capacity, DPPH radical scavenging activity and lipid peroxidation inhibition potential. The total reducing capacity was found to be 75.61, 115, 110.97 and 128.65 equivalents of ascorbic acid (µg)/mg for extract of *T. foenum*, *T. copticum*, *N. sativa*, Combination 1:1:1 respectively. The IC_{50} for DPPH radical scavenging activity obtained for *T. foenum*, *T. copticum*, *N. sativa* and Combination 1:1:1 was 2428.45, 316.18, 457.94 and 107.85 µg/ml respectively. The inhibition potential of lipid peroxidation was 3.26, 1.27, 1.62 and 0.94 µg/ml for *T. foenum*, *T. copticum*, *N. sativa* and Combination 1:1:1 respectively. The results of the present study suggest that *T. foenum*, *T. copticum*, *N. sativa* and Combination 1:1:1 provide a potential natural source of anti-oxidants, with combination 1:1:1 exhibiting the highest activity.

INTRODUCTION: Every cell constantly produces free radicals known as reactive oxygen species (ROS) as a natural by-product of aerobic metabolism which attack unsaturated lipids, DNA, proteins and other essential cellular molecules causing significant cellular damage [1].

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The production of these free radicals increases in response to exogenous sources such as UV radiations, environmental pollution and cellular dysfunction [1]. An innate antioxidant defence system comprising of enzymes, metal chelation, free radical scavenging activities etc. has been evolved in the human body. However, in event of the overwhelming production of radicals, a lack of equilibrium between free radicals and antioxidant defence mechanism leads to a condition called 'oxidative stress'. This oxidative stress is critical in ageing and the pathogenesis of various neurodegenerative, cardiovascular and chronic

diseases as Alzheimer's, Parkinson's disease, epilepsy, diabetes, arteriosclerosis etc [2]. The innate defence systems can be supported by antioxidant compounds consumed as foods supplements which may enrich its antioxidant status. Thus it is extremely important to find new and safe antioxidants from natural sources, plants being a prime source.

Trigonella foenum (*T. foenumm*) commonly known as Fenugreek (English) or Methi (Hindi) belonging to family Fabaceae is native to western Asia and extensively cultivated in India, China Morocco etc. The dried, oblong and yellowish brown seeds are used for its antidiabetic, anti-inflammatory and hypolipidaemic activity [3]. The major chemical constituents are diosgenin, trigonelline trigofoenosides A-G; trigoneosides etc [3].

Trachyspermum copticum ***(****T. copticum****)*** belonging to family Apiaceae is commonly known as Bishop's weed (English) or Ajowan (Hindi) [4]. It is native to Egypt and is cultivated in Iraq, Iran, Afghanistan, Pakistan and India. The dried ripe fruits are yellowish-brown in colour and possess agreeable aromatic flavour. The major chemical constituents of the drug are volatile oil mainly thymol, p-cymene, terpinene. It is used for gastrointestinal ailments, carminative and anthelmintic activities [4].

Nigella sativa (*N. sativa*) belonging to family Ranunculaceae is commonly known as Black Cumin (English) or Kalonji (Hindi). The dried seeds are small, black, flattened, oblong, angular and funnel-shaped. The major chemical constituents present are nigellone, nigellicine, volatile oils (carvone, thymol etc.) etc. *N. sativa* is used as antimicrobial agent, liver tonic, antihypertensive and in skin disorders [5]. The total antioxidant activity of a plant material is the amalgamation of different antioxidant constituents present such as carotenoids, phenols etc. Phenolic constituents are the major antioxidant constituents [2]. The phytochemicals render their effects via different mechanisms such as radical scavenging, metal chelation, inhibition of lipid peroxidation, quenching of singlet oxygen etc [2]. Therefore, it is essential to assess the overall antioxidant capacity of the herb by several methods.

Accordingly, the aim of the present study was to determine the total phenolic contents and the antioxidant properties of methanolic extracts of *T. foenum*, *T. copticum* and *N. sativa*. The combination of these three extracts in 1:1:1 proportion has been previously reported to possess significant anti-diabetic and anti-hyperlipidemic activity [6]. Taking these experimental findings into consideration, the combination of all the three methanolic extracts in 1:1:1 ratio was also evaluated for its antioxidant potential.

MATERIALS AND METHODS:

Chemicals: DPPH was procured from Sigma–Aldrich (USA). 2-thiobarbituric acid (TBA) was procured from Loba Chemie Pvt. Ltd., Mumbai. Folin Ciocalteu Reagent and potassium ferricyanide were obtained from SD Fine Chemicals Ltd. (Mumbai, India). All other reagents used were of analytical grade and obtained from SD Fine Chemicals Ltd. (Mumbai, India).

Materials: The dried seeds of *T. foenum*, *N. sativa* and the dried fruits of *T. copticum* were purchased from local market. The identification and authentication of the above plant materials was done by Dr. Harshad Pandit, Reader, Department of Botany, Guru Nanak Khalsa College, University of Mumbai, Matunga, Mumbai, India. The dried material was ground to a coarse powder and kept in an air-tight container at room temperature until further use.

Extraction of plant materials: The individual powdered material (100 g) was defatted for 24 hours using petroleum ether (1000 ml). The defatted plant material was further subjected to soxhlet extraction using methanol as a solvent. The individual methanolic extracts of *T. foenum, N. sativa, T. copticum,* thus obtained were concentrated by solvent distillation and evaporating the solvent in a rotary evaporator (Superfit^{TM}) till constant weight. The extracts were dried in the oven and weighed to determine the yield of the extracts and stored in an air-tight container.

Total phenolic content: Total phenolic content of each extract was determined by the Folin–Ciocalteu method [7]. Briefly, 20 µl of extract solution was mixed with 1.16 ml distilled water and 100 µl of

Folin–Ciocalteu reagent, followed by addition of 300 µl of Na_2CO_3 solution (20%) after 1 min. The mixture was incubated at 40^0 C for 30 min and its absorbance was measured at 760 nm. Gallic acid was used as a standard for calibration curve. The phenolic content of the extracts were expressed as gallic acid equivalents (mg gallic acid equivalent per gm of extract) [7].

Total reduction capacity: The ability of extracts to reduce iron (III) was assessed by Oyaizu Method et al, 1986 [8]. 1 ml of different concentrations of extracts dissolved in methanol was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$, 10 g/l), the mixture was incubated at 50 °C for 30 min. After incubation, 2.5 ml of trichloroacetic acid (100 g/l) was added and the mixture was centrifuged at 1650g for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (1 g/l) and the absorbance was measured at 700 nm. The reducing capacity of the extract was expressed as equivalents of ascorbic acid (µg)/mg of extract [9].

DPPH free radical scavenging assay: The ability of extracts to scavenge DPPH radicals was determined by Brand-Williams *et al*., 1995 with slight modifications [10]. Briefly, 1 ml of a 200 µM methanolic solution of DPPH was mixed with 1 ml of different concentrations of extract solution in methanol. The mixture was then vortexed vigorously and incubated at room temperature in the dark for 20 min. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

$$\% \text{ DPPH scavenging} = \frac{\text{Abs (Control)} - \text{Abs (sample)}}{\text{Abs (Control)}} \times 100$$

Where, A= Absorbance at 517 nm

***In-vitro* lipid peroxidation activity:**

Thiobarbituric acid reactive substances (TBARS) Assay: [11] The potential of the extracts to inhibit the cellular damage by peroxidation of membrane lipids due to oxidative stress was evaluated in terms of thiobarbituric acid reactive substances (TBARS).

Preparation of rat heart homogenate (10%w/v): Healthy male wistar rats were used for the experiment. The animals were sacrificed and hearts were perfused with normal saline. Hearts were isolated and homogenized in ice cold phosphate buffer saline using a Teflon homogenizer. All the experimental procedures and protocols used in the study were reviewed and approved by the Institutional Animal Ethics Committee, which is registered under the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India (IAEC Approval No: UICT/PH/IAEC/0807/6).

Method: Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution to 3 ml of the 10% w/v heart homogenate and incubated for 30 min at room temperature. In case of test samples, 0.2 ml of different concentration of the extracts (*T.foenum*- 1-10 mg/ml, *T. copticum*- 1-2 mg/ml, *N.sativa*- 1-2 mg/ml, Combination 1:1:1-0.4-1.4 mg/ml) was added to tubes containing 0.1 ml of the heart homogenate, 0.1 ml of Sodium dodecyl sulphate (8.1% w/v), 0.75 ml of 20% acetic acid and 0.75 ml of 0.8% Thiobarbituric acid aqueous solution. In case of control sample, distilled water was added in same amount instead of the test extract solution. Individual assay controls were used, to avoid color interference of the extracts.

Volume in each tube was made up to 2 ml with distilled water and then heated on a water bath at 95^0C ± 1^0C for 60 min. After heating, the volume in each tube was made up to 2.5 ml with distilled water. 2.5 ml of butanol: pyridine mixture (15:1) was added in each tube. The reaction mixture was vortexed and centrifuged at 4000 rpm for 10 min. The organic layer was then removed and absorbance was read at 532 nm against the blank using a UV spectrophotometer.

The percent inhibition of lipid peroxidation was calculated by following formula:

$$\% \text{ Inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Sample)}}{\text{Abs (Control)}} \times 100$$

Where, Abs = Absorbance at 532 nm

RESULTS

Extraction of plant materials: The yield and organoleptic parameters for methanolic extracts of *N.sativa, T. copticum* and *T. foenum* are shown in **Table 1**. The amount of extractable components is expressed as percentage by weight of dried plant materials.

TABLE 1: YIELD AND ORGANOLEPTIC PROPERTIES OF METHANOLIC EXTRACTS OF *T. FOENUM, T.COPTICUM* AND *N. SATIVA*

Parameter	Methanolic extract of *T.foenum*	Methanolic extract of *T.copticum*	Methanolic extract of *N.sativa*
Color	Yellowish brown	Greenish brown	Blackish brown
Odor	Characteristic spicy	Agreeable aromatic	Slightly aromatic
Consistency	Semisolid	Syrupy liquid	Semisolid
Yield	16.2 %	15.5 %	4 %

Total phenolic content:

The total phenolics data for methanolic extracts of *N. sativa, T. copticum, T. foenum* as well as combination 1:1:1 is shown in **Table 2**. The amount of total phenolics (gallic acid equivalents) is expressed as percentage by weight of dried extract. The combination 1:1:1 ratio showed highest phenolic content followed by methanolic extract of *T. copticum, N. sativa* and *T. foenum*.

TABLE 2: TOTAL PHENOLIC CONTENT OF METHANOLIC EXTRACTS OF *T.FOENUM, T. COPTICUM, N. SATIVA* AND COMBINATION (1:1:1).

Methanolic extract	mg Gallic acid equivalent /gm of extract
T.foenum	202.316 ± 8.20
T.copticum	322.872 ± 10.93
N.sativa	260.735 ± 11.82
Combination (1:1:1)	464.239 ± 17.39

Values are expressed as Mean ± SEM.

Total reduction capacity: Total reducing capacity was determined by the ability of extracts to reduce iron (III) to iron (II) and was compared with that of standard ascorbic acid. **Table 3** depicts the total reducing capacity of the test extracts and combination 1:1:1. The methanolic extracts of *N. sativa, T. copticum, T. foenumm* as well as combination 1:1:1 exhibited a concentration-dependent reducing capacity. Combination 1:1:1 showed highest total reducing capacity at all test concentrations followed by *T. copticum, N. sativa* and *T. foenum*.

TABLE 3: TOTAL REDUCTION CAPACITY OF METHANOLIC EXTRACTS *OF T.FOENUM, T. COPTICUM, N. SATIVA* AND COMBINATION (1:1:1).

Concentration (mg/ml)	Equivalents of ascorbic acid (µg)/mg of extract			
	T. foenum	*T. copticum*	*N. Sativa*	Combination (1:1:1)
1	39.63	57.19	53.53	69.51
4	46.34	74.02	56.34	92.19
10	75.61	115	110.97	128.65

Total reducing capacity is expressed as equivalents of ascorbic acid (µg)/mg of extract.

DPPH radical scavenging assay

DPPH radical scavenging assay is widely used to evaluate the free radical scavenging capacity of a compound [12]. The methanolic extracts of *N. sativa, T. copticum, T. foenum* as well as combination 1:1:1, showed a concentration-dependent DPPH radical scavenging activity as shown in **Table 4**. Combination 1:1:1 was found to be most active radical scavenger with the lowest IC_{50} followed by that of *T. copticum, N. sativa* and *T. foenum*.

TABLE 4: IC_{50} VALUES FOR DPPH FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS OF *T. FOENUM, T. COPTICUM, N. SATIVA,* COMBINATION 1:1:1 AND ASCORBIC ACID

Methanolic extract / Standard	IC_{50} value (µg/ml)
T.foenum	2428.45
T.copticum	316.18
N.sativa	457.94
Combination (1:1:1)	107.85
Ascorbic acid	61.67

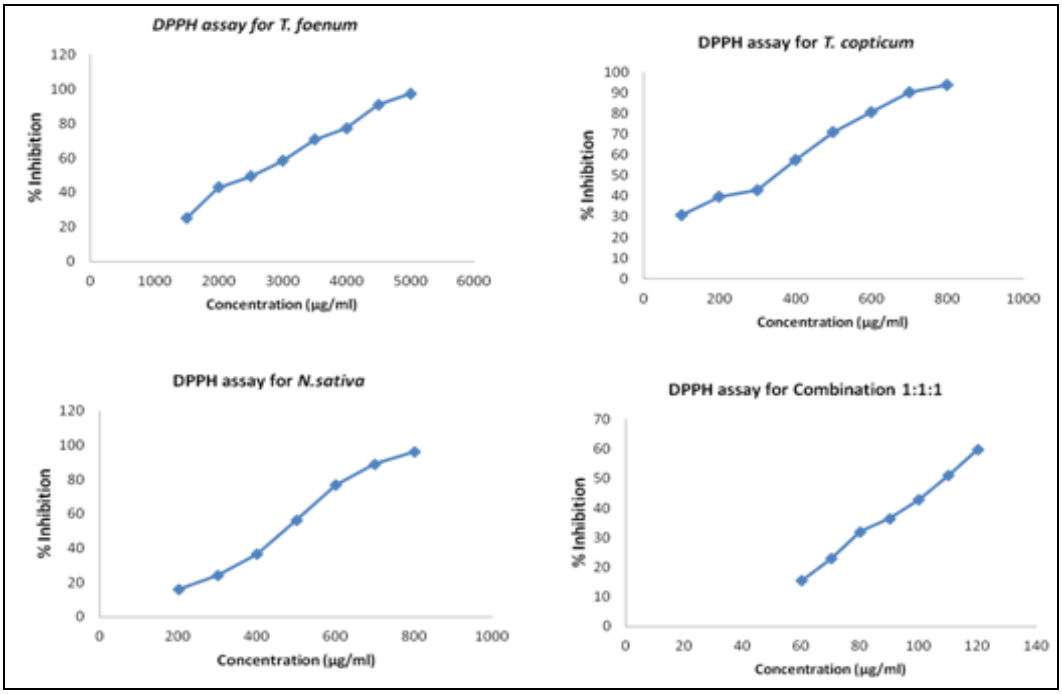

FIG. 1: DPPH FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS OF *T. FOENUM, T. COPTICUM, N. SATIVA,* COMBINATION 1:1:1

***In-vitro* lipid peroxidation activity – TBARS Assay:** In the present study, the ability of test drugs to inhibit the lipid peroxidation initiated in rat heart homogenate was evaluated using TBARS assay. In this assay the amount of thiobarbituric acid reactive substances (TBARS) formed by oxidation of rat heart homogenate was determined by measuring the absorbance at 532 nm and expressed as MDA equivalents. All the metahnolic extracts of *N. sativa, T. copticum, T. foenumm* as well as combination 1:1:1 were capable of preventing the formation of TBARS generated by ferrous sulphate in a concentration-dependent manner. The combination 1:1:1 was found to be most active followed by that of *T. copticum, N. sativa* and *T. foenum.*

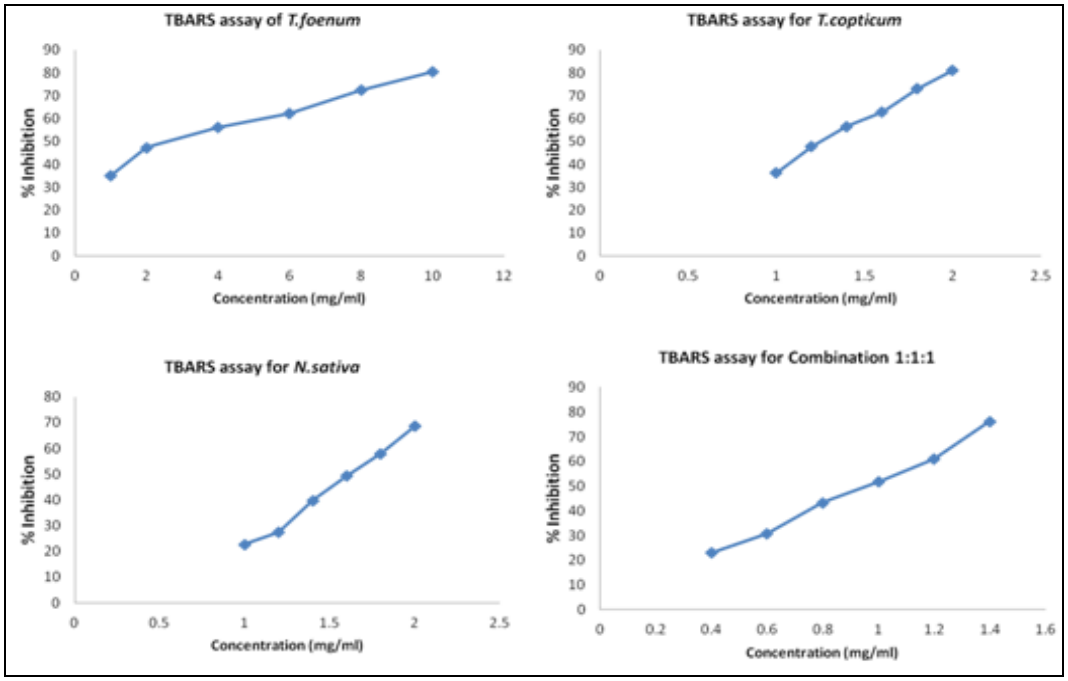

FIG. 2: TBARS ACTIVITY OF METHANOLIC EXTRACTS OF *T. FOENUM, T. COPTICUM, N. SATIVA,* COMBINATION 1:1:1

TABLE 5: IC_{50} VALUES OF METHANOLIC EXTRACT AND COMBINATION (1:1:1) FOR INHIBITION OF LIPID PEROXIDATION.

Methanolic extract	IC_{50} value (µg/ml)
T.foenum	3.2616
T.copticum	1.2775
N.sativa	1.6196
Combination (1:1:1)	0.9438

DISCUSSION: Oxidative stress is implicated in ageing and pathogenesis of a number of ailments. Antioxidants from natural source can supplement and support the innate antioxidant defence system to counteract the oxidative stress [2, 13]. *T. foenum*, *T. copticum* and *N. sativa* are important medicinal plants widely utilized in traditional system of medicine [3, 4, 5]. The objective of the present study was to investigate the antioxidant potential of methanolic extracts of *T. foenum*, *T. copticum* and *N. Sativa*, their mixture in a 1:1:1 ratio i.e. combination 1:1:1and to uncover their mechanism of action. Accordingly, the three methanolic extracts and combination 1:1:1 were screened for their total phenolic content, total reduction capacity, DPPH radical scavenging activity and lipid peroxidation activity.

Phenolic compounds are important secondary metabolites of plants, majorly synthesized by the highly branched phenylpropanoid pathway. Polyphenols act as effective antioxidants in a number of chemical oxidation systems owing to their aromatic hydroxyl groups [14, 15]. In the present study, Combination 1:1:1 exhibited highest phenolic content followed by methanolic extract of *T. copticum*, *N. sativa* and *T. foenum*.

The total reduction capacity of the extract indicates its capacity to terminate the radical chain reaction initiated by free radicals, by donating electron and converting it into stable products. It demonstrates the antioxidant potential in terms of the reducing power of the extract [12, 15]. Combination 1:1:1 showed highest reducing power followed by methanolic extracts of *T. copticum*, *N. sativa* and *T. foenum* in a concentration dependent manner.

Free radicals, reactive oxygen species and nitrogen species are highly unstable and reactive species contributing to the oxidative stress [13]. In DPPH radical scavenging assay the ability of the extract to convert the purple coloured DPPH radical to colourless α, α diphenyl-β-picryl hydrazine is evaluated [15]. The degree of discolouration indicates the free radical scavenging activity of the extract. In the present study combination 1:1:1 possessed highest free radical scavenging potential followed by that of *T. copticum*, *N. sativa* and *T. foenum*.

The process of lipid peroxidation is a critical in pathogenic mechanism of free radicals. The reactive oxygen and nitrogen species attack the cellular membrane lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs). This leads to hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides further initiating a self propogating reaction, hampering the cellular viability. An antioxidant inhibiting the lipid peroxidation process is therefore of utmost importance [16]. In the present study combination 1:1:1 showed a better lipid peroxidation inhibition potential with a lower IC_{50} value followed by *T. copticum, N. sativa* and *T. foenum.*

Thus in the present study, combination 1:1:1 exhibited a better antioxidant potential followed by the methanolic extracts of *T. copticum, N. sativa* and *T. foenum* which correlates well with the total phenolic content of the respective extracts.

CONCLUSION: The present study demonstrates that antioxidant potential of methanolic extracts of *T. foenum*, *T. copticum*, *N. sativa* as well as combination of these three extracts in 1:1:1 ratio is contributed by their electron donating as well as free radical scavenging activity. These test extracts can also interfere and inhibit the lipid peroxidation process and prevent the cellular damage. The anti-oxidant potential correlates with the phenolic content of the extracts. The present study thus suggests that the *T. foenum, T. copticum, N. sativa* as well as combination of these three extracts in 1:1:1 ratio provide a potential natural source of antioxidants and benefit in the treatment of various disorders mediated by oxidative stress.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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