RESEARCH ARTICLE

In-vitro antioxidant and GC-MS analysis ethanolic extract of poly herbal drug

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ABSTRACT: Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human against infections and degenerative diseases. Current research is now directed towards natural antioxidants originated from plants due to safe therapeutics. Poly herbal drugs is used in Indian traditional medicine for a wide range of various ailments. To understand the mechanism of pharmacological actions, antioxidant properties of the Poly herbal drugs extract were tested using standard in vitro models. The ethanolic extract of Poly herbal drugs exhibited strong scavenging effect on superoxide, nitric oxide radical and reducing power radical scavenging assay. The free radical scavenging effect of Poly herbal drugs extract was comparable with that of the reference antioxidants. The data obtained in the present study suggests that the extract of Poly herbal drugs have potent Invitro antioxidant and Anti Diabetic activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage.

Keywords: Antioxidant, Free Radicals, GC-MS, Poly herbal Drug.

INTRODUCTION

Indian medicinal plants and many herbal formulations belonging to the traditional systems of medicine like Ayurveda have been investigated on various aspects of disease curing drugs. Side effects and expenses associated with allopathic drugs have provoked the need for research into drugs which are without the side effects, especially those belonging to the traditional systems of medicine (Jose and Kuttan, 2000) Research emphasis has been directed towards herbal drugs either in single or in combination having specific diagnostic and therapeutic principles. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals (Kunchandy & Rado, 1990) currently, polyhedral formulations are employed for the treatment of various types of diseases, such as respiratory diseases, cancer, acquired immunodeficiency syndrome (AIDS), diabetics and ulcers in order to achieve enhanced therapeutic effects.

In view of above information the present study has been undertaken to assess the invitro antioxidant and anti-diabetic activity of polyherbal formulation containing drugs from different herbs viz Cassia auriculata, Mimosa pudica, Mangifera indica, Cyperus rotundus and Asparagus racemosus.

MATERIALS AND METHODS

Plant collection

Cassia auriculata, Mimosa pudica, Mangifera indica, Cyperus rotundus and Asparagus racemosus were collected. The plants were dried in open air and then kept in an oven at 60°C for some time, then grinded into a fine powder by using electric blender and stored in clean labeled airtight bottles.

Preparation of the plant extract

Hundred grams of plant powder was subjected to hydroalcoholic extraction (Distilled water: Ethanol = 2:1) by hot percolation method through soxhlet apparatus. Thereafter extract was dried using rotary evaporator and dried extract was put through a process of standardization. The percentage yield of the extract was determined and was found to be 12%.

Antioxidative Activity:

Scavenging of DPPH radical

This assay (Yokozawa et al., 1998) based on the measurement of the scavenging ability of antioxidant test extracts towards the stable radical. The free radical scavenging activity of methanol and aqueous extracts of Poly herbs were examined invitro using DPPH [1, 1 Diphenyl, 2-picryl-hydrazyl] radical. The test extracts were treated with different concentrations from a maximum of 250 µg/ml to minimum of 4µg/ml. The reaction mixture consisted of 1ml of 0.1mM DPPH in methanol, 0.95 ml of 0.05 M Tris-HCL buffer (pH-7.4),
1 ml of methanol and 0.05 ml of herbal extract. The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding extracts. The experiment was performed in triplicate and percentage of scavenging activity was calculated using the formula 100- [100/blank absorbance X sample absorbance]. The blank was also carried out in similar manner, using distilled water in the place of extracts. The activity was compared with ascorbic acid, which was used as a standard antioxidant.

**ABTS radical cation decolourisation Assay**

ABTS (54.8 mg) was dissolved in 50 ml of distilled water to 2 mM concentration and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before use (Re and Pellegrini, 1999). To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 734 nm. The assay was performed in triplicate.

**Scavenging of Nitric Oxide**

Sodium nitroprusside (5 µM) in standard phosphate buffer solution (Sreejayan Rao, 1997) was incubated with different concentration of the methanol and aqueous extracts dissolved in standard 0.025 M phosphate buffer (pH-7.4) and the tubes were incubated at 250°C for 5 hours. After Hours, 0.5 ml of incubation solution was removed and diluted with0.5 ml of Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% aphthylethylenediamine dihydrochloride in water). The absorbance of chromophore formed was read at 546 nm.

**Metal chelating activity**

The chelation of ferrous ions by extracts was estimated by method of Dinis et al. (Dinis et al., 1994). Briefly, 50 µl of 2 mM FeCl2 was added to 1 ml of different concentrations of the extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine–Fe 2+ complex formation was calculated as [(A0- As)/ As] × 100, where A0 was the absorbance of the control, and As was the absorbance of the extract/ standard. Na2EDTA was used as positive control.

**GC –MS Analysis**

**Preparation of extract**

Rhizome powder of *Curcuma caesia* Roxb were shade dried. 20 g of the powdered tubers were soaked in 95% ethanol for 12 h. The extracts were then filtered through Whatmann filter paper No.41 along with 2 gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with 95% ethanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phyto components of the plant material used. 2 µl of these solutions was employed for GC/MS analysis (Merlin et al., 2009).

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min iso thermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

**Identification of Components**

Identification was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The spectrum of unknown components was compared with the version, 2005, software, Turbo mass 5.2. This is done in order to determine whether this plant species contains any individual compound or group of compounds, which may substantiate its current commercial and traditional use as an herbal medicine. Further it helps to determine the most appropriate methods of extraction these compounds (Karthikeyan Ravichandran et al., 2015).
RESULTS AND DISCUSSION

DPPH assay is being used widely as a preliminary test which provides information on the reactivity of test compound. This method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen donating antioxidant (AH) due to the formation of non-radical form DPPH-H. Antioxidant reacts with DPPH which is a stable free radical and is reduced to the DPPH-H and as a consequence the absorbance decreased from the DPPH˙ radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds present in the poly herbal extract in terms of hydrogen donating ability. (Burits, and Bucar, 2000)

DPPH assay is a simple and acceptable method to evaluate the anti-oxidative activity of compounds (Chen and Ho, 1995). DPPH is a stable free radical, which when encounters proton donors such as antioxidants, the radicals get quenched, absorbance gets reduced and thus used to measure the antioxidant activity of specific compound or plant extract (Koleva et al., 2002; Qureshi et al., 2010).

The DPPH radical has been widely used to test the potential of compounds as free radical scavengers of hydrogen donors and to investigate the antioxidant activity of plant extracts. The DPPH free radical scavenging activity of plant extract may be due to neutralization of DPPH radical either by transfer of hydrogen or of an electron (Shimada et al., 1992).

The Poly herbal mixture exhibited a significant dose dependent inhibition of DPPH activity. In this study, at 100 μg /ml, the extract showed highest inhibition of DPPH activity shown in (Table1;Figure 1). The results of DPPH-free radical scavenging assay suggest that the Poly herbal mixture extract is more capable of scavenging free radicals.

Table 1: DPPH radical scavenging activity

| Concentration (μg/ml) | Poly herbal Drug | Std (ascorbic acid) |
|-----------------------|------------------|--------------------|
| 10                    | 20.83 ± 1.93     | 24.73 ± 1.84       |
| 20                    | 40.46 ± 2.55     | 48.64 ± 3.39       |
| 30                    | 53.54 ± 3.09     | 64.64 ± 3.80       |
| 60                    | 71.34 ± 1.91     | 77.23 ± 5.12       |
| 100                   | 85.02 ± 3.05     | 92.09 ± 4.36       |

ABTS⁺ Activity

ABTS⁺ is a blue chromophore produced by the reaction between ABTS and potassium per sulfate. Previous studies have suggested that the total polyphenolic content of the plant extracts are positively correlated to the scavenging activities and reducing potential of an extract. Our result shows that scavenges pre-generated ABTS⁺ radicals Addition of polyherbal extracts to this pre-formed
radical cation reduced it to ABTS in a concentration dependent manner (Table 2; Figure 2). These results were compared with those obtained with ascorbic acid, which indicates that the extract is a potent antioxidant. This is measured in terms of inhibition of generation of $\text{O}_2$. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent disease. (Auudy et al., 2003).

Natural phenolics exert their beneficial health effects mainly through their antioxidant activity by decreasing oxygen concentration, intercepting singlet oxygen, preventing 1st chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalysts, decomposing primary products of oxidation to non-radical species, and breaking chains to prevent continued hydrogen abstraction from substances. (Sharma et al., 2012).

Table 2: ABTS Assay

| Concentration (µg/ml) | Poly herbal extract | Std          |
|----------------------|---------------------|--------------|
| 50                   | 12.49 ± 1.14        | 14.69 ± 1.14 |
| 100                  | 21.96 ± 1.14        | 19.95 ± 1.14 |
| 250                  | 28.31 ± 1.6         | 26.22 ± 1.6  |
| 500                  | 37.66 ± 1.27        | 36.65 ± 1.27 |
| 1000                 | 47.29 ± 0.84        | 45.20 ± 0.84 |

No Activity

Nitric oxide, a gaseous free radical and is relatively less reactive. But its metabolic product peroxynitrite, formed after reacting with oxygen is extremely reactive and directly induce toxic reactions such as SH group oxidation, protein - tyrosine nitration, lipid peroxidation and DNA modification (Moncada et al., 1991; Yermilov et al., 1995). Nitric oxide has been demonstrated to participate in the beta cell damage during STZ- induced diabetes (Duran Reges et al., 2004). Nitric oxide plays an important role in various inflammatory processes. Sustained level of production of this radical is directly toxic to tissues and contribute to vascular collapse associated with septic shock, whereas chronic exposure of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Tylor et al., 1997).

The toxicity of NO increases greatly when it reacts with superoxide radical, producing the highly reactive peroxynitrite (ONOO⁻) anion (Huie and Padmaja, 1993). Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, alzheimer’s and arthritis. Oxygen reacts with the excess NO to generate nitrite and peroxo nitrite anions, which act as free radicals. (Hashem et al., 2007).
From results of Nitric oxide method, it proved that poly herbal drug extract is equally effective as anti-oxidant compared to the ascorbic acid (Table 3; Figure 3). These compounds compete with oxygen to react with NO and thus inhibit the generation of the nitrite and peroxy nitrite anions. This results indicates that, the extract might contain compounds capable of inhibiting nitric oxide and offers scientific evidence for the use of the drug in the indigenous system in treatment of various diseases.

### Table 3: Nitric oxide radical scavenging activity

| Concentration (ug/ml) | Poly herbal extract | Std. Ascorbic acid |
|-----------------------|---------------------|--------------------|
| 10                    | 22.02 ± 0.18        | 26.02 ± 1.1        |
| 20                    | 26.78 ± 0.86        | 28.51 ± 1.40       |
| 50                    | 31.73 ± 1.76        | 32.67 ± 2.51       |
| 100                   | 41.4 ± 1.86         | 44.61 ± 1.80       |
| 150                   | 56.98 ± 0.55        | 58.16 ± 1.68       |

**Iron chelating activity**

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxy radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Thus, the chelating effect of the coexisting chelators can be determined by measuring the rate of color reduction. The formation of the ferrozine–Fe²⁺ complex is interrupted in the presence of aqueous extract of poly herbal extract indicating that have chelating activity (Table 4; Figure 4).

Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxy radicals (Hashem et al., 2007). Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that forms bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion (Cao G et al., 1996). Thus, *poly herbal extract* demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity.

### Table 4: Iron Chelating Activity

| Concentration (ug/ml) | Poly herbal extract | Std ascorbic acid |
|-----------------------|---------------------|------------------|
| 10                    | 28.12 ± 1.73        | 33.85 ± 1.94     |
| 20                    | 36.52 ± 1.17        | 38.70 ± 1.58     |
Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS is a method that combines the features of Gas-Liquid Chromatography (GLC) and Mass Spectrometry (MS) to identify different substances within a test sample. Free radicals play a crucial role in the development of tissue damage in pathological events. The extraction method presented is simple, rapid and inexpensive, with reduced solvent consumption. GC-MS method used for the analysis of the obtained extracts can be an interesting tool for testing the amount of some active principles in herbs used in cosmetic, drugs, pharmaceutical or food industry. The acidic fractions were silylated and subjected to GC-MS investigation. It is evident from the Table 7 that all fractions have a complex chemical composition. Some of the GC-MS peaks remained unidentified, because of lack of authentic samples and library data of corresponding compounds.

Table 5: GC-MS analysis of ethanolic extract of poly herbal combined drug

| S. No. | Compounds | Retention Time (Min.) | Peak Area % | Molecular Formula | MW |
|--------|-----------|-----------------------|-------------|-------------------|----|
| 1.     | Butane1,1-diethoxy-3-methyl- | 3.37 | 0.43 | C₈H₁₉O₂ | 160 |
| 2.     | Diglycerol | 3.73 | 4.11 | C₆H₁₈O₃ | 166 |
| 3.     | benzenacetaldehyde | 4.44 | 0.55 | C₆H₁₀O | 120 |
| 4.     | Propane,1,1,3-triethoxy - | 4.76 | 1.15 | C₆H₁₈O₁ | 176 |
| 5.     | 4h-pyran-4-one,2,3-di-hydro-3,5-dihydroxy6-methyl | 5.94 | 0.83 | C₆H₈O₄ | 144 |
| 6.     | Benzoifuran,2,3-dihydro -(coumaran) | 6.97 | 3.97 | C₆H₄O | 120 |
| 7.     | resorcial | 8.17 | 5.26 | C₆H₁₀O₂ | 110 |
The major phytochemical compounds identified were 3-o-methyl-d glucose, ergosterol, Ercosta-7,22-dien-3ol,(3a,22e)-, resorcinol, n-hexadecanoic acid.

Phenolic compounds have also been known as antioxidant agents, which act as free radical terminators and have shown medicinal activity as well as exhibiting physiological functions. It was reported that compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effects of this extract.

This study highlights the presence of many metabolites in the poly herbal combined extract provide an overview of the different classes of molecules present that have led to their pharmacological activities. This study confirmed that the plant extract could be used for the treatment of various diseases. The GC-MS analysis of poly herbal combined extract showed the presence of various types of compounds in present. Compounds like 3-O-methyl-d glucose (C_{12}H_{20}O_5) to possess antitumor, oncogenic, and diabetogenic properties (Michael M et al.,1977) Ergosterol (C_{20}H_{30}O) to act a Anticomplementary activity, Anti-inflammatory, Nematicide, Insecticide, Hypocholesterolemic, Cancer preventive, Hepatoprotective (Dr.Duke's et al.,2011) Ercosta-7,22-dien-3ol,(3a,22e) - (C_{28}H_{46}O) used to treat Cancer prevention Anti-inflammatory Hypocholesterolemic, anti-hyper glycemic (Gowdhami et al.,2015) Resorcinol (C_{12}H_{36}O_3) having liver hepatopatology enzyme activity, anti-microbial activity (Dr.Duke's Phytochemical and Ethnobotanical Databases) n-hexadecanoic acid (C_{18}H_{36}O) act as nematicide, pesticide, hemolytic, S-alpha reductase inhibitor; Insecticide, Hypotropotective, Antihistaminic, Anticezemic, Antiacen, S- Alpha reductase inhibitor, Antiarthritic, Anticorony activities. (Lalitha Rani et al., 2009) phyto (C_{36}H_{46}O) to use in Antimicrobial, Anti-cancer, Anti-inflammatory Hypocholesterolemic, Nematicide, Anticorony, Antiarthritic, Hepatoprotective, Anti-androgenic (Gowdhami et al., 2015) d-asarinin (C_{18}H_{20}O_3) Cyto toxic activity (Gowdhami et al.,2015 Octadecanoic acid (C_{18}H_{36}O), Antifungal, Antitumour, Antibacterial(Lalitha Rani et al., 2009).

The plants sample on subjecting to GC-MS provides the result of different peaks determining the presence of seven different compounds. The molecular weight of these compounds is also known. By interpreting these compounds, it is found that this plant possess various therapeutically uses. This typical gas chromatogram shows the relative concentrations of various compounds getting eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in the plant. The numbers at various peaks are the retention time in minutes. The mass spectrometer analyzess the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearence of peaks at different m/z ratios (Kalimuthu et al., 2013).

These observations may be due to the nature of biological active components and the stronger extraction capacity of hydro ethanol could have been produced number of active constituents responsible for medicinal activity (Komal Kumar et al., 2011). The biological activities based on Dr. Duke's Phytochemical and Ethnobotanical Databases.

Our investigation through the present study revealed that the poly herbal combined extract is a reliable source of bioactive compounds like fatty acid esters, alcohols, hydrocarbons, alkanes, amines, terpenes, and sugars that justify the traditional usage of this plants As GC-MS is the first step towards understanding the nature of active principles (Ghorbani, et al., 2011), further investigation in this species is suggested for the development of novel drugs.

The investigation concluded that the stronger extraction capacity of ethanol could have been produced number of active constituents responsible for many biological activities. So that those might be utilized for the development of traditional medicines and

| Compounds                              | MW   | % R | Compounds |
|----------------------------------------|------|-----|-----------|
| 1,2,3-benzenetriol(pyrogallol)         | 198  | 2.07| C_{6}H_{12}O_{3} |
| Ergosterol                             | 352  | 3.77| C_{20}H_{30}O |
| 3-o-methyl-d glucose                   | 176  | 1.89| C_{12}H_{20}O_5 |
| n-hexadecanoic acid                   | 212  | 2.29| C_{18}H_{36}O_3 |
| 9,12-octadecadienoic acid (z,z)-      | 284  | 3.01| C_{20}H_{40}O |
| 1,3-benzodioxole,5,5\{(tetrahydro-1h,3h-furoi[3,4-c]furan-1,4-diyl)bis-[1s-(1a3aa,4a,6aaa)]- (synonym:d-asarinin) | 308  | 3.22| C_{28}H_{46}O_3 |
| 1,6-anhydro-a-d-glucopyranose(synonym:levoglucosan) | 202  | 2.15| C_{12}H_{20}O_5 |
| Naphthalene 1,2,3,4-tetrahydro-1,6dimethyl-4-(1-methylithy)-,(1s-cis)-(synonym:calamene) | 202  | 2.15| C_{12}H_{20}O_5 |

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further investigation needs to elute novel active compounds from the medicinal plants which may be created a new way to treat many incurable diseases.

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