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UNDERSTANDING ANTI-CUMULATIVE TOXICITY EFFECT OF DOOSHIVISHARI AGADA THROUGH ANTI-OXIDANT AND METAL CHELATING POTENTIAL

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ABSTRACT

Dooshivisha, a concept explained in Ayurveda is attributed to the cumulative toxicity, where small amount of toxic principles get accumulated in the body over a period of time and start showing the toxic effects based on their nature and site of accumulation. One of the remedy suggested in classics is Dooshivishari Agada, comprising of nine ingredients and also used by many vaidyas of Ayurveda. Mode of action of Dooshivishari Agada has been evaluated in the present study. Anti-oxidant and Metal chelating potentials of Hydro-methanolic, Methanolic and Aqueous fractions of the formulation were evaluated using photometric method. Total phenolic content, Total anti-oxidant activity, DPPH radical scavenging activity, Hydroxyl radical scavenging activity, Lipid peroxidation assay and ferrous ion reducing assay were evaluated as per standard protocol. Methanolic fraction exhibited potent anti-oxidant and metal chelating potential compared to Hydro-methanolic and Aqueous fractions. Thus the present study is suggestive of anti-cumulative toxicity effect of Dooshivishari agada and may be further taken to the level of cyto-protective based clinical studies.

Key words: Dooshivisha, Agada, Anti-oxidant, Metal chelating, DPPH, Phenolics, Peroxidation, Hydroxyl

INTRODUCTION

Entering the twenty-first century, one of our greatest health threats is pollutants. We are constantly loading our body with small amounts of various toxins such as pesticides, herbicides, growth hormones and food additives. One more health hazard of present day is related with metal contamination leading accumulation of metals in the tissues. Natural or synthetic toxins because of their low toxicity index don't produce disease instantaneously rather accumulate and selectively targets cells or tissues producing cellular, metabolic diseases and even neoplastic disorders. In Ayurveda this phenomena is known as *Dooshivisha* and Signs and symptoms of such cumulative toxicity depend upon the part of the body such as *Amashaya, Pakwashaya* or in *Shareerika dhatu* (different tissue and cells) in which toxin get accumulated leading to different diseases.[1]

Toxicity can be to a cell (cytotoxicity), organ (organo-toxicity) or the word can be metaphorically used to describe toxicity to society at large. Toxicity can be measured by its effects on the target tissues. Indoor

and outdoor particle air pollution from exhaust smoke of vehicles or power plants can increase the risk of heart disease, bronchial asthma, COPD, lung cancer.[2] Toxins in water like minerals, metals, radio isotopes, pesticides, disinfectants can increase the risk of bone fracture, damage to brain, kidney, thyroid, teeth, reproductive system even causing carcinomatous growths.[3] Cumulative toxicity thus developed leads the body to succumb for oxidative stress by production of large amounts of free radicals such as superoxide anion radical ($\cdot O_2$), hydroxyl radical ($\cdot OH$), which has to be neutralized immediately. These molecules' are involved in cell injury and aging process. Anti-oxidant is the substance when present in low concentration (compared to that of oxidative substrate) significantly delays or prevents oxidation of that substrate i.e. it blocks the process of oxidation by neutralizing free radicals.[4] Vitamin C, Vitamin E, beta carotene, poly phenol, melatonin, selenium, lutien, lycopene, anthocyanins, glutathione and super-oxide dismutase are naturally found in food of animal and plant origin, which have been studied for their anti-oxidant property worldwide.[5] Certain plants in the form of their extracts are also subjected for anti-oxidant and metal chelating activities.[6]

There is a need for effective and safe anti-toxic formulation to combat the harmful effects of toxins ingested and exposed. Dooshivishari Agada is an Ayurvedic classical herbo-mineral formulation and is popularly prescribed by vaidyas for the management of skin ailments.[7] This formulation comprises of Pippali (*Piper longum* Linn.), Dhyamaka (*Cymbopogon martini* (Roxb.) Wats.), Jatamamsi (*Nardostachys jatamamsi* (D.Don) DC.), Lodhra (*Symplococus racemosa* Roxb.), Ela (*Elettariacardamomum* Maton.), Suvarchika (Salt petre), Kutannatum (*Oroxylum indicum* (L.) Benth. ex Kurz), Natam (*Valeriana wallichii*), Kushta (*Saussurea lappa* DC.), Yastimadhu (*Glycyrrhiza glabra* L.), Chandana (*Pterocarpus santalinus* L.) and Gairika (Red Ochre).[8]

In this work, Anti-toxic property of "Dooshivishari Agada (DA)" is studied by analyzing its anti-oxidant property with DPPH radical scavenging assay, Hydroxyl radical scavenging assay and its metal chelating effect with Ferrous ion chelating ability.

Materials and Methods:

Plant material

The ingredients of DA were collected from the local market of Mysuru city Karnataka state, India. The collected drugs were authenticated by comparing with the voucher specimens maintained at Department of Dravyaguna and Rasshastra of JSS Ayurveda Medical College, Mysuru, Karnataka, India. All ingredients were mixed in equal proportions (w/w) and pulverized using a mini pulverizer in to a coarse powder (Image 1). Three types of extracts (Hydro alcoholic, Methanolic and Aqueous extracts) were prepared using standard protocol (Image 2)[9]**.** All the extracts were subjected for following tests successively.

Step 1 (Standardization of Dooshivishari agada powder and extract)

A) Organoleptic studies (For powder and Extract)

- Color

- Odor
- Texture
- Taste

B) Basic physico-chemical tests[10]

- Total ash
- Acid insoluble ash
- Alcohol soluble extractive
- Water soluble extractive

Step 2 (Anti-oxidant and Metal chelating potential of Doshivishari agada)

Extracts were subjected for below mentioned analytical procedures using standard protocol.

1. Total phenolic content
2. Total anti-oxidant assay
3. DPPH radical scavenging assay
4. Hydroxyl radical scavenging assay
5. Lipid peroxidation assay
6. Ferric ion reducing assay

ANTIOXIDENT ACTIVITY[11,12]:

A. Free radical scavenging activity by DPPH Method

Free radical scavenging potentials of the extract will be tested against a methanolic solution of α,α-diphenyl-β-picryl hydrazyl (DPPH). Antioxidants reacts with DPPH and convert it to α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. The change in the absorbance produced at 517 nm has been used as a measure of antioxidant activity.

B. antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The green color is measured at 695 nm. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

C. Hydroxyl radical scavenging activity

Phenylhydrazine in solution has been shown to produce hydroxyl radicals. Hydroxyl radical scavenging will be measured by studying the competition between deoxy-*d*-ribose and sample extracts for hydroxyl radicals produced by Phenyl hydrazine. The extent of deoxy-*d*-ribose degradation will be measured by Thiobarbituric acid reactive substances (TBARS) method of Ohkawa et al .

D. Lipid peroxidation assay

In this assay, peroxidation of egg phospatidylcholine liposomes is induced by $FeCl_3$ and ascorbic acid as reducing agents. $^{\cdot}OH$ radicals generated by mixing Fe^{3+} and ascorbate attack the egg phospatidylcholine liposomes. This leads to the formation of (Malondialdehyde) MDA and other aldehydes, which form a pink chromagen with TBA absorbing at 532nm.

E. Reduction of ferric ions[13]

Fe^{+2} reacts rapidly with 1, 10-*O*-phenanthroline and forms red colored complex which is exceptionally stable. This complex has a strong absorption in the visible spectrum at a wavelength of 510 nm. Extracts reacts with Fe^{3+} to reduce and convert it to Fe^{2+}. The degree of coloration indicates the reduction potential of the extracts. The change in the absorbance produced at 510 nm has been used as a measure of Ferric ions reducing activity. The reduction is measured taking Sodium dithionite instead of the extract and considered as equivalent to 100% reduction of all the ferric ions present.

Results:

Final product obtained after thorough mixing of individual ingredients was red in

color with visible fibrous content, emitting fragrant aroma, bitter and salty in taste and amorphous texture.

Hydro-methanolic fraction was red in color and having sticky nature with little precipitate, which need to be mixed thoroughly before preparing test samples. Methanolic fraction was clear red in color, consisting sticky texture with characteristic odor. Aqueous fraction was dark brown in color with less sticky content.

Anti-oxidant and Metal chelating activities were analyzed using photometric method measuring peak absorbance.

A.Total Phenolic content (Graph 1)

Total phenolic content was expressed in terms of micro grams equivalent to gallic acid/mg. sample 2 (Methanolic fraction) had maximum phenolic content (697.524) followed by Hydro-methonolic fraction (602.286) and Aqueous fraction (401.333)

B. Total Anti-oxidant assay (Graph 2)

Total anti-oxidant activity was expressed in terms of Ascorbic acid equivalent value in mg/g of extract. Methanolic fraction exhibited maximum anti-oxidant potential (30.41) followed by Hydro-methanolic fraction (25.92) and Aqueous fraction (19.18)

C. DPPH radical scavenging assay (Graph 3)

All fractions showed scavenging capabilities for DPPH radical. IC_{50} values were compared with the value of Ascorbic acid. Methanolic fraction had low IC_{50} value (32.65) showing maximum DPPH radical scavenging potential followed by Hydro-methanolic fraction (178.29) and Aqueous fraction (216.87).

A.Hydroxyl radical scavenging assay (Graph 4)

Methanolic fraction exhibited hydroxyl radical scavenging activity to the higher extent compared to other two. In lower concentrations, both aqueous and hydro-methanolic fractions had similar activity, but gradually, hydro-methanolic fraction showed increased level of activity but again coincided with aqueous fraction in higher concentration.

B. Lipid peroxidation assay (Graph 5)

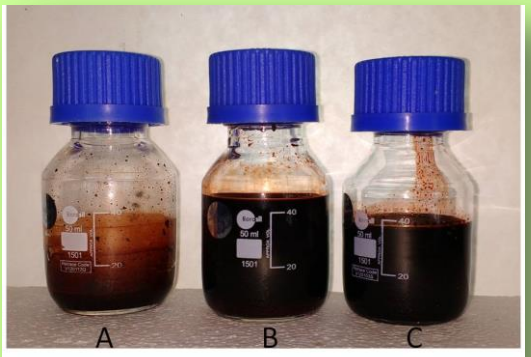
Methanolic extract prevented lipid peroxidation strongly compared to other fractions in dose dependent manner. Although Hydro-methanolic fraction exhibited more activity in lower concentration, Methanolic fraction's activity rose steeply and almost equivalent to hydro-methanolic fraction's potential in mid range and was little more in higher concentrations. Though aqueous fraction exhibited considerable potential in preventing lipid peroxidation, it was far lesser than former two.

A.Reduction of Ferric ions (Graph 6)

Extracts react with Fe^{3+} to reduce and convert it to Fe^{2+}. Coloration indicates the reduction potential of extracts. As per the photometric values, Methanolic fraction exhibited marked reduction potential compared to other two. Though the increase in activity is not observed in initial concentrations, steep increase was observed in mid and higher concentrations of Methanolic fraction. With respect to hydro-methanolic and aqueous fractions, hydro-methanolic fraction exhibited little higher reduction potential compared to aqueous

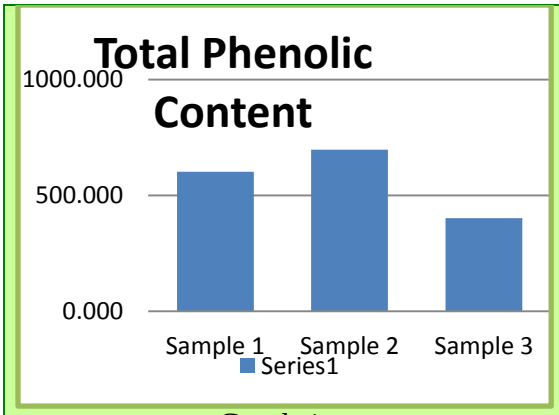
fraction, but both showed increase the activity in concentration dependent manner.

Image 1
Dooshivishari Agada Compound

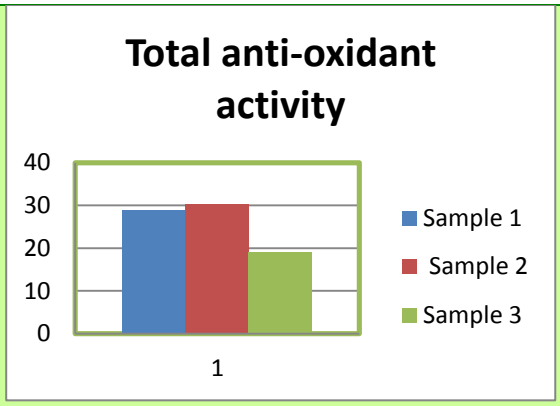


A: Hydro-methanolic fraction, B: Methanolic fraction, C: Aqueous fraction

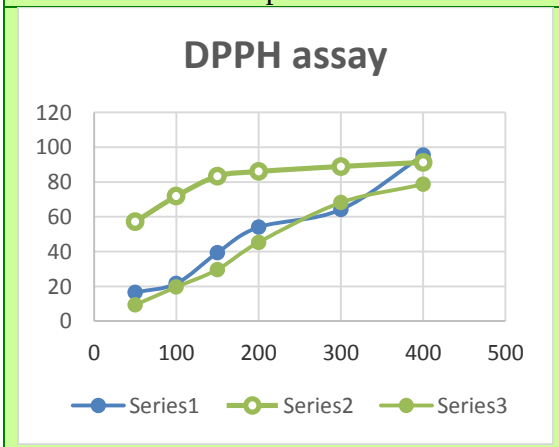
Image 2
Different fractions of D.A



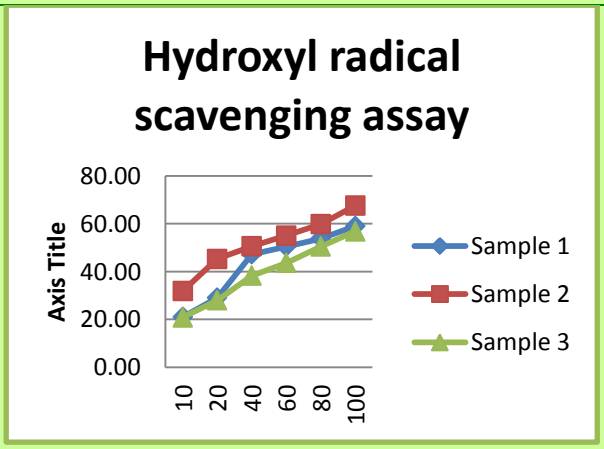
Graph 1



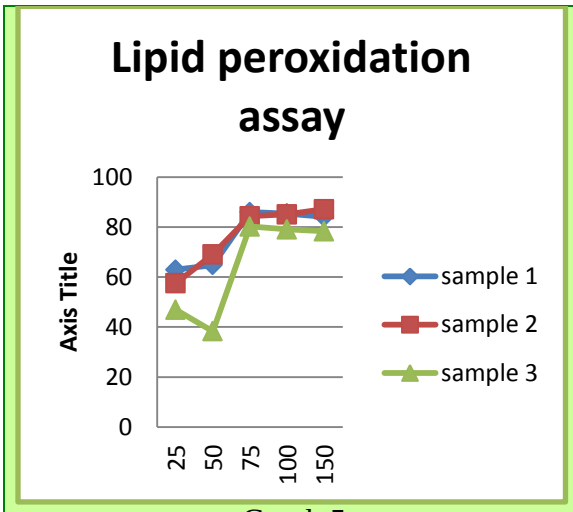
Graph 2



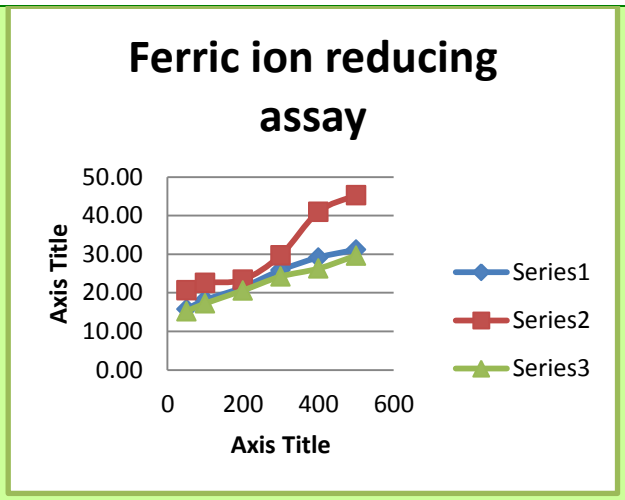
Graph 3



Graph 4



Graph 5



Graph 6

DISCUSSION:

Dooshivisha (cumulative toxicity) may be any pollutant contaminated food and air in small amounts leads to pathological conditions in the long run. Such contaminants may be in the form of pesticides, fungicides, synthetic drug molecules, coloring agents or metallic agents in food and air. Though many anti-toxic preparations are in the present day practice, studying them on the basis of modern science becomes essential for evidence based clinical practice. *Dooshivishari agada* being such anti-toxic preparation was taken into account during present study based on possible Anti-oxidant and metal chelating capabilities and hence justified on the basis of previous studies undertaken to prove cyto-protective and organ protective studies.

Many phyto-pharmacognostic and analytical studies have been carried out on *Dooshivishari Agada* previously[14,15]. Anti-oxidant activity comprises of many protocols such as DPPH, Hydroxyl radical scavenging assay and Lipid peroxidation assays apart from total anti-oxidant activity.

The hydroxyl radical is an extremely reactive free radical formed in biological system and can exert great damage in almost every molecule found in living cell[16]. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly and can cross cell membranes rapidly. Reaction of Hydrogen peroxides with copper and ferrous ions yields hydroxyl radical and may be the origin of cell toxicity[17]. Lipid peroxidation is defined as oxidative destruction of poly-unsaturated lipids. In biological systems, malondialdehyde is very reactive species and takes part in damaging liver cells[18]. Even heavy metals can exert oxidative stress on cells. Meatals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological molecules and this hypothesis is supported by studies carried out on cultured cells and animals using nucleobase products typical for the oxygen attack on DNA[19]. Hence studying Ferric ion reducing assay was also thought of in the present study.

No study was carried out with all above protocols to prove the anti-oxidant potential and hence adapted in the present study. Various fractions like, hydro-methanolic, Methanolic and aqueous extractions were taken in to consideration to assess activity of compound based on solubility, which may help in phyto-pharmaceutical studies.

Color of *Dooshivishari agada* may be due to *Raktachandana* and *Gairika* having bright red colour. Ingredients like *Kushtha, Bhustrana* and *Ela* had characteristic aroma rendered fragrance to the compound preparation. Color of hydro-methanolic and methonolic factions corresponded with color of compound and may be due to the solubility of pigments of *Raktachandana* in methanol, where as aqueous fraction had only dark brown color.

Phenolics especially tannins and flavonoids have been reported to possess strong anti-oxidant activity and potent capacity to donate rapidly a hydrogen atom to free radicals.[20,21] So the anti-oxidant activity of the extracts and fractions may be attributed to the concentration of phenolic compounds in them. In the present study, Methanolic fraction contained maximum phenolic compound followed by hydro-methanolic and aqueous fractions. Even total Anti-oxidant activity, DPPH radical scavenging activity, Hydroxyl ion scavenging activity, Lipid paraoxidation prevention and Ferric ion reduction activities followed the same pattern depending upon the phenolic concentration among fractions. In all above mentioned assays, Methanolic fraction exerted potent activity compared to other fractions due to high phenolic content.

Studies show that supplementation of anti-oxidants along with chelating agents prove to be best compared to monotherapy with chelating agents alone and shown to improve removal of toxic metals from system as well as better and faster clinical recoveries in animal models. Hence the ferric ion reduction capacity of compound *Dooshivishari agada* may be attributed to the presence of group of phenolic and allied compounds functioning as natural anti-oxidants[22].

Ayurvedic perception of treatment is always remained as multi-dimensional approach. Plants having different activities are logically mixed together and used as one preparation. *Ela (Elettariacardamomum* Maton.*), Lodhra (Symplococus racemosa* Roxb*)* and *Gairika (Red ochre)* are used in *Visha chikitsa*. *Dhyamaka (Cymbopogon martini* (Roxb.) Wats.*)* and Salt petre are *mootrala dravyas. Kushtha* (*Saussurea lappa* DC.) and *Shyonaka (Oroxylum indicum* (L*)* are *Vatakaphahara dravyas*. *Tagara (Valeriana wallichii)* and *Jatamansi (Nardostachys jatamamsi* (D.Don) DC.*)* act on *manasika bhava*. *Yashtimadhu (Glycyrrhiza glabra* L.) and *Rakta-chandana (Pterocarpus santalinus)* are potent *Pittahara Dravyas. Pippali (Piper longum* Linn) is *sroto-shodhaka* due to its *ushna, teekshna* and *kledakara guna*. Thus the ingredients of *Dooshivishari Agada* not only act on *Tridosha* level but also on *manas* and help in removal of *vishakari tatwa* from the body.

CONCLUSION:

Ayurvedic anti-toxic compound Dooshivishari agada has a very good anti-oxidant and ferric ion reduction potential.

Methanolic fraction exhibits potent activity in all aspects of the anti-oxidant models compared to aqueous extract. Due to its powerful ferric ion reducing capabilities, the formulation may be further tried to treat heavy metal toxicity as mono or combination therapies.

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