



Evaluation of *in vitro* antioxidant and anti-atherogenic properties of selected *Siddha* polyherbal decoctions

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ABSTRACT

This research work explains the antioxidant and anti-atherogenic effects of selected *Siddha* polyherbal decoctions such as *Nilavembu kudineer*, *Kabasura kudineer*, *Notchi kudineer* and *Adathodai kudineer*. Even though all the above decoctions have been used for fever, cold, cough, bronchitis, dyspho-nea and body pain in *Siddha* system of medicine, their antioxidant and anti-atherogenic properties were not investigated scientifically. We have analyzed the polyphenolic content, antioxidant and anti-atherogenic properties of decoction of the above-mentioned herbal formulations. The toxicity of the decoctions was also performed in PMBC. In the investigated formula-tions, *Notchi kudineer* showed higher level of total phenolic content (560 mg GAE/100 g), followed by *Adathodai kudineer* (260 mg GAE/100 g), *Nilavembu kudineer* (150 mg GAE/100 g) and *Kabasura kudineer* (90 mg GAE/100 g). Similarly, *Notchi kudineer* exhibited strong antioxidant activity in terms of radical scavenging potential against DPPH (IC-50: 2.12 mg/L), followed the *Adathodai kudineer* (IC-50: 2.27 mg/L), *Nilavembu kudineer* (IC-50: 4.48 mg/L) and *Kabasura kudineer* (IC-50: 9.29 mg/L). In toxicological study all the decoctions of selected formulations didn't show any toxicity. Similarly, *Nilavembu kudineer* showed maximum inhibition of lipid peroxidation when compared to other herbal decoctions. Among the investigated *Siddha* for-mulations, *Nilavembu kudineer* was found to possess high antioxidant and anti-atherogenic potentials and hence it could be further investigated as anti-atherogenic drug using *in vivo* model.



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INTRODUCTION

There are so many medical systems in India and *Siddha* System is one of them which originated in South India. In this system, medicines are classi-fied into internal and external medicines and in each type there are 32 forms are available. *Kudineer* or *Kasayam* is one among them (Thyagaraja R, 2004) which is decoction in English. As per *Siddha* liter-atures generally decoctions are prepared by boil-ing the fresh or dried drugs with the addition of specified volume of water till the water is reduced to one fourth and then filtered (The *Siddha Formu-lary of India*, 1992). Shelf life of decoction is only three hours (Murugesamudalier, 1988). The general dosage of decoction is for infants 2.5 ml to 5 ml (Upto

one year), Toddler 5 ml (1 – 3 yrs), Preschooler 20 ml (3 – 5 yrs), Grade-schooler 30 ml (5 – 12 yrs) and Adults , 60 - 100 ml (Above 12 yrs) ([The Siddha Formulary of India, 1992](#); [Mahadevan and Vanmathi Palraj, 2016](#)).

In the modern world cardiovascular complications are common due to abnormal deposits of lipids, cholesterol, and plaque build ups. It is the well known cause of morbidity and mortality in the grown up countries ([Prabha et al., 2013](#)). In this disease plaque builds up inside the arteries. Early pathological alterations, the oxidized Low Density Lipids gathers in the damaged cells and other phagocytes called fatty streaks are seen on the interior surface of blood vessels and lymphatic vessel surface of the aorta and arteries of heart. They are collected of lipid sedimentation in the macrophages (fat deposits on blood vessel walls) in the intima of these vessels. Whether they develop to higher lesions depends on hemodynamic forces, such as blood pressure and the plasma levels of atherogenic lipoproteins. Development of the is the thickening, hardening and loss of elasticity of the walls of arteries process leads to the next stage, in which fibrous plaques form ([Thomas, 1984](#)). Oxidized low-density lipoprotein makes a dangerous position in the beginning position of atherosclerosis, while the formation of a blood clot is one of the most recent serious pathological consequences of this ailment ([Ferdowsian and Barnard, 2009](#)). Hyper lipidemia is the very important cause linked with cholesterol-lipid, calcium deposits in arterial, others being high blood pressure, smoking, diabetes mellitus and other factors. Food habits are an important cause for the avoidance of cardiovascular diseases ([Lusis, 2000](#)).

Currently available hypolipidemic drugs (statins) have been associated with numeral side effects. Atherosclerotic medicines statins give adverse effects like exacerbation of hemorrhoids, vitamin A and D deficiencies, cardiac arrhythmias, respiratory problems, vertigo, severe itching, tingling, nausea, vomiting, abdominal pain and diarrhea ([Fitzakerley, 2005](#)). One research article explained the adverse effect of the statins, thereby, managing lipid related disorder may be cost effective, have undesired adverse effects, painful to the patients or not easily accessible. Use of Niacin can go ahead to skin problems, liver toxicity and in birth defects also. Mixed therapy of statins and fibrates reasons a noticeably bigger risk of myopathy and rhabdomyolysis. In the observation of these setbacks, it is required to expand agents that are effective, inexpensively available and with minimum side effects as the herbal medical intervention ([Jorum and Machocho,](#)

[2016](#)). Same information was noted in a Siddha review article of [Kanakavalli et al. \(2014\)](#). The hypo lipidemic drugs created one more major problem that is renal failure ([Gupta et al., 2013](#)). But herbals have a most important task in anti-hyper lipidemic activity and recommend that the lipid reducing action is decided through stimulating of liver cholesterol biosynthesis and decrease of lipid absorption in the intestine ([Kanakavalli et al., 2014](#)).

Traditional herbal medicines are used for treating and curing of so many health problems and as a nutraceutical. Toxicological study and research assist to survive secured and expect advantage from man-made and herbal medicines while avoiding adverse effects. So many research works proved the safety efficacies of natural remedied. Very few information is available about the toxicity of herbal products. Some plants produce toxic constituents for defense purposes. There phytochemicals of alkaloids, flavonoids, terpenoids and saponins which can play the role of signalling molecules, neuro peptided and hormones in humans. So many heavy metals are also present in herbs like lead, cadmium, mercury and arsenic there may also be responsible for toxicity ([Sharwan et al., 2015](#)).

The confidence that bad cholesterol that may also be responsible for the coronary heart diseases and later atherosclerosis is a primary precept of modern medicine. Medicines expected at decreasing serum low-density lipoproteins cholesterol are now considered to be a vital factor of any effort to avoid cardiac problems. A prospective contributory role in cardiovascular complications have been noticed for oxidized low density lipoproteins, but this form of bad cholesterol explains that it is not in association with serum levels of native LDL. Somewhat, particular oxidation inhibitor level is to be an important factor influencing serum absorptions of oxidized LDL ([Colpo, 2005](#)).

Antioxidants can decrease the process of oxidative damage all over the body. In common an antioxidant is a simple molecule that reduces the oxidation of other molecules. Tocopherol (Vitamin E), ascorbic acid (Vitamin C), Selenium and tetraterpenoids (carotenoids) such as beta carotene, lycopene and lutein are some of the antioxidants which are present in our foods and plant materials ([Elakkiyaa et al., 2017](#)). This research work explains the antioxidant and anti-atherogenic effects of selected Siddha poly herbal decoctions namely Nilavembu kudineer (NVK), Kabasura kudineer (KSK), Notchi kudineer (NCK), Adathodai kudineer (ATK) ([Kuppusamudaliyar and Uthamarayan, 2009](#); [Kup-](#)

pusamymudaliyar, 1987; Bhavani, 2015). Even though these medicines have several therapeutic uses and few research studies have been conducted on their therapeutic potential of the anti-oxidant, anti-analgesic and inflammation reducing effects are not yet revealed in water extract of the selected herbal formulations. This research paper revealed with the in vitro antioxidant and anti-atherogenic properties of selected Siddha polyherbal decoctions.

MATERIALS AND METHODS

Preparation of the drug samples

Raw ingredients of NVC, KSK, NCK and ATK were procured from local herbal market, Thanjavur, Tamilnadu, India and identified and authenticated in the NABL accredited lab of CARISM, SASTRA University. All the ingredients were powdered coarsely using a lab mill and then mixed according to formulation given in the text of [The Siddha Formulary of India \(1992\)](#).

Decoction preparation

Coarse Powdered sample of herbal formulations 30 g was mixed with 500 ml of water and boiled still it reduce to 125 ml. Then the contents were filtered and the filtrate was kept under the deep freezer twelve hours and lyophilized. The lyophilized dry extract was re-mixed in distilled water at a stock concentration of 10 mg/ml and used for the testing shown in Figure 1.

Total phenol content

The total phenolic content of extracts was estimated according to the method of [Singleton et al. \(1999\)](#). Suitably diluted sample (100 μ l) was taken with 250 μ l of Folin's-Ciocalteu reagent and 1000 μ l of 5% of Na_2CO_3 was added and incubated for 30 min in dark. Then the absorbance was measured at 720 nm using Spectrophotometer. A calibration curve was prepared using standard gallic acid (16 – 100 mg/L; $y = 0.0094x - 0.0585$; $R^2 = 0.9939$) and used to calculate the total phenolic content of the extract and the results were expressed as gallic acid equivalents (mg GAE / 100 g sample).

Antioxidant activity

Antioxidant activity of the extracts was evaluated using DPPH radical scavenging assay ([Sánchez-Moreno et al., 1998](#)). In the presence of antioxidant compounds, the purple coloured DPPH (2,2-diphenyl-1-picryl-hydrazyl) is reduced to yellow colored DPPH-H. DPPH was prepared by taking 3 mg in 100 ml of methanol and the absorbance at 515 nm was adjusted to 0.78 using methanol. Different concentrations of the extract was taken (100 μ l)

along with 900 μ l of DPPH and incubated in dark for 30 mins at room temperature and absorbance was measured at 515 nm. A control with solvent instead of sample was performed for each extract. Based on the absorbance values, the antioxidant activity was calculated using the formula: $(\text{Abs. Control} - \text{Abs. Test} / \text{Abs. Control}) \times 100$ and results were expressed on percentage basis.

Toxicity assay

Peripheral blood mononuclear cells (PBMC) were separated from the blood of healthy adult volunteers by taking heparinized blood with equal volume of Histopaque isolation medium. Blood was added slowly through walls of the tube and centrifuged at 1,400 rpm for 30 min. The upper plasma layer was removed and buffy layer was collected in a new falcon tube and washed with PBS (pH 7.4) twice. PBMC cells were collected by centrifugation at 2000 rpm for 10 min and re-suspended in RPMI medium, counted and distributed in 96-well plate and maintained under aseptic conditions ([Jamuna et al., 2017](#)). Different concentrations of extract were loaded in each well and incubated for 3 h at 37° C in an incubator. Then 20 μ L of 5% MTT reagent ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added in each well and incubated for 2 h at 37° C. Then 100 μ L of Acidic isopropanol (0.05 M HCl in Iso-propanol) was added and shaken for 30 min on a plate shaker in the dark to dissolve the formazan crystals. The absorbance was measured at 590 nm and the percentage of cell viability. From the results, we understood that the studied sample was not toxic to PMBC at the selected concentration range and hence we have used the same concentrations for anti-proliferative activity.

Anti-atherogenic activity

The anti-atherogenic property of different solvent extracts will be evaluated in macrophage cell model according to the method described by [Aviram et al. \(2008\)](#). The PBMC cells were incubated with LDL and herbal extracts and LDL oxidation was induced chemically by adding 1 mM CuSO_4 . The extent of LDL oxidation was determined after 2 h of incubation by analyzing the lipid peroxidation ([Botsoglou et al., 1994](#)). Cell lysate was homogenized by taking the cell pellet with 5 ml of PBS. Then 0.3 ml of this homogenate and 0.3 ml of the samples were taken and incubated at room temperature for 10 min. Then 1.5 ml of TBA reagent (0.375g was dissolved in 10% TCA) was added and kept in water bath at 80°C for 15 min. This was centrifuged at 2000 rpm for 10 min and the supernatant was collected and its absorbance was measured at 540 nm

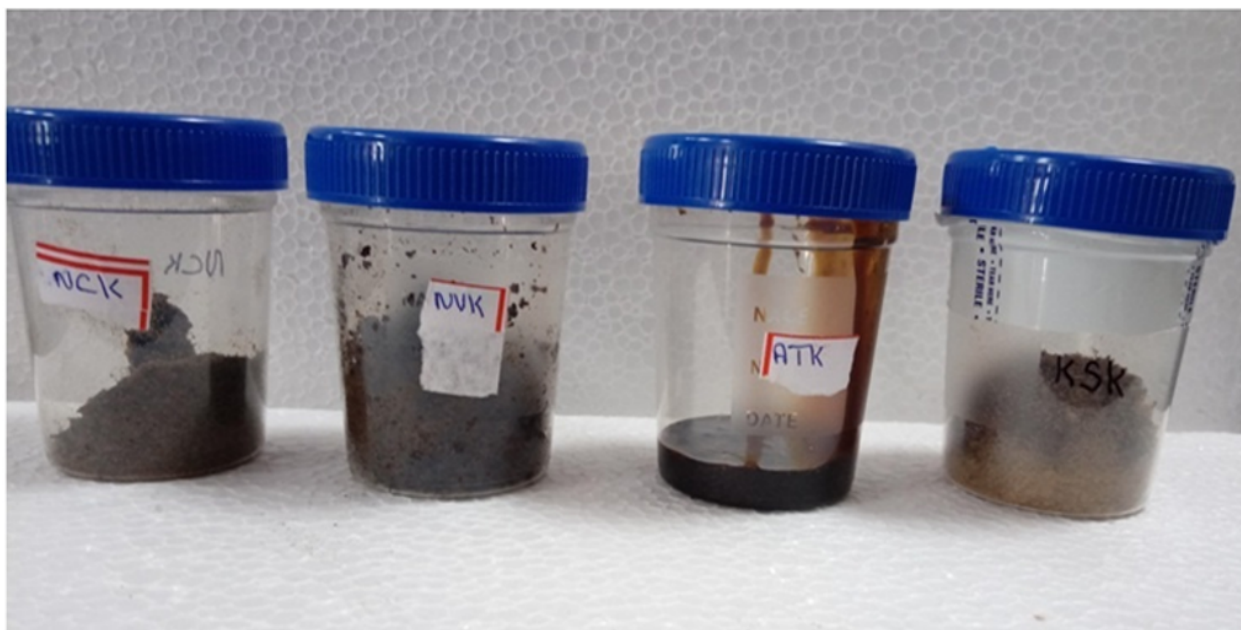


Figure 1: Lyophilized aqueous extract of Herbal decoctions (NCK - Notchi kudineer; NVK - Nilavembu kudineer; ATK- Adathodai kudineer and KSK- Kabasura kudineer)

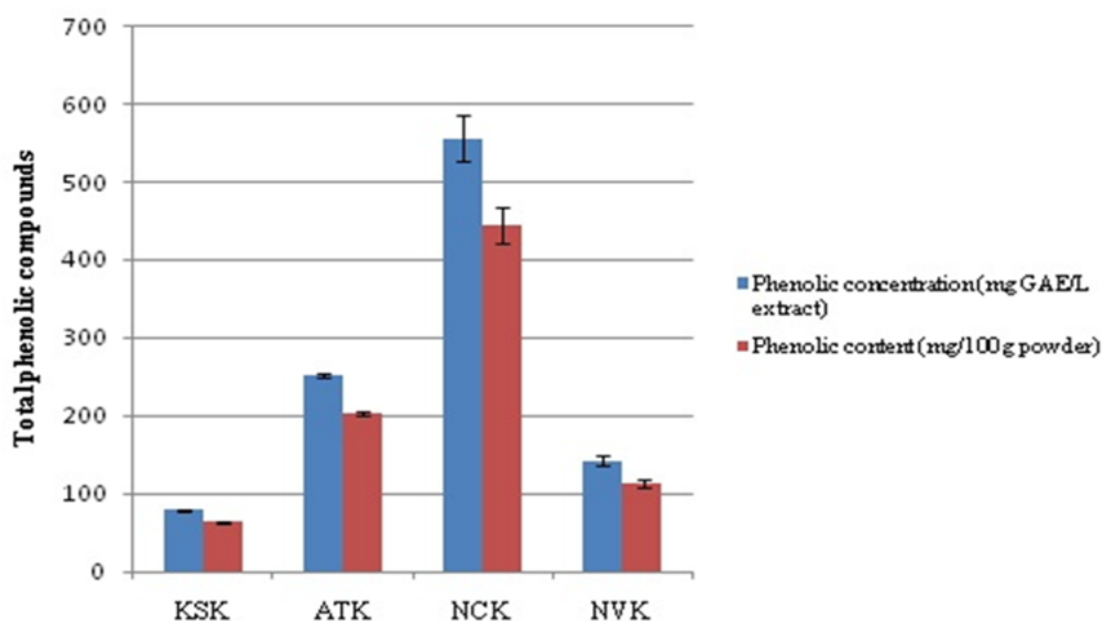


Figure 2: Total phenolic concentration of poly herbal decoctions

using an UV- Visible spectrophotometer. Based on absorbance, the MDA content of brain homogenate was calculated in nM / g tissue basis and the results were expressed as percentage inhibition of lipid per oxidation.

RESULTS AND DISCUSSION

NVK is composed of equal quantity of nine herbal ingredients of *nilavembu* (*Andrographis paniculata* Burm. f), *vettiver* (*Vetiveria zizanioides* L.), *vilamichamver* (*Plectranthus vettiveroides* Jacob), *san-*

thanam (*Santalum album* L.), *peipudal* (*Trichosanthes cucumerina* L.), *koraikizhangu* (*Cyperus rotundus* L.), *chukku* (*Zingiber officinale* Roscoe), *milagu* (*Piper nigrum* L.) and *parpatakam* (*Mollugo cerviana* L. Ser) (Nadkarni, 2005). It is prescribed for all types of fevers, detoxification of blood, liver and spleen from pathogenic endotoxins and also for body aches. The NVK is prepared by taking 25 g of powder with 500 ml of water and boiled till the decoction is concentrated to 125 ml (4:1 ratio). The warm decoction may be consumed 30 to 60 ml twice a day in empty stomach. Honey or Palm jaggery or sugar

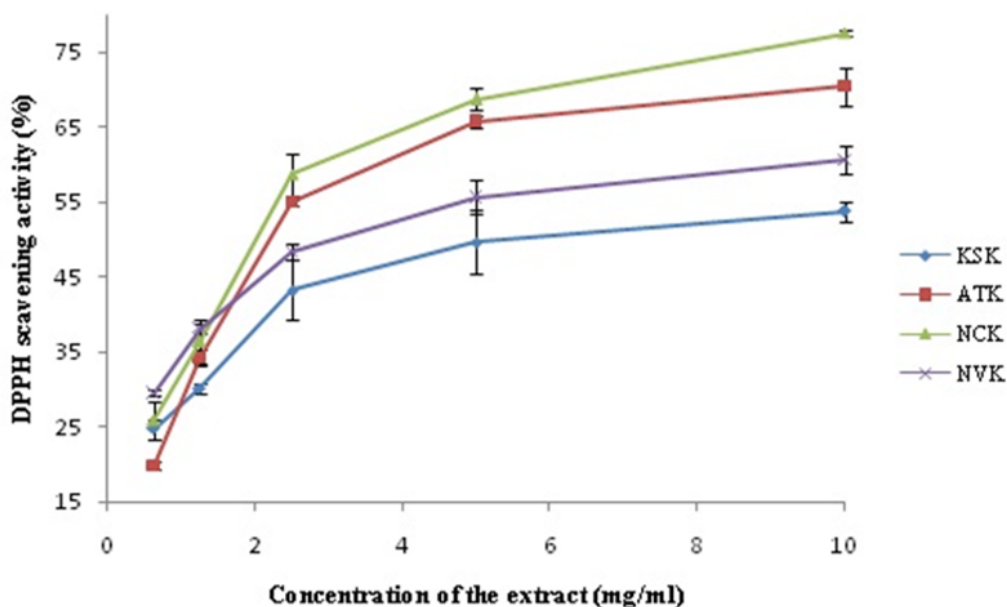


Figure 3: DPPH radical scavenging activity

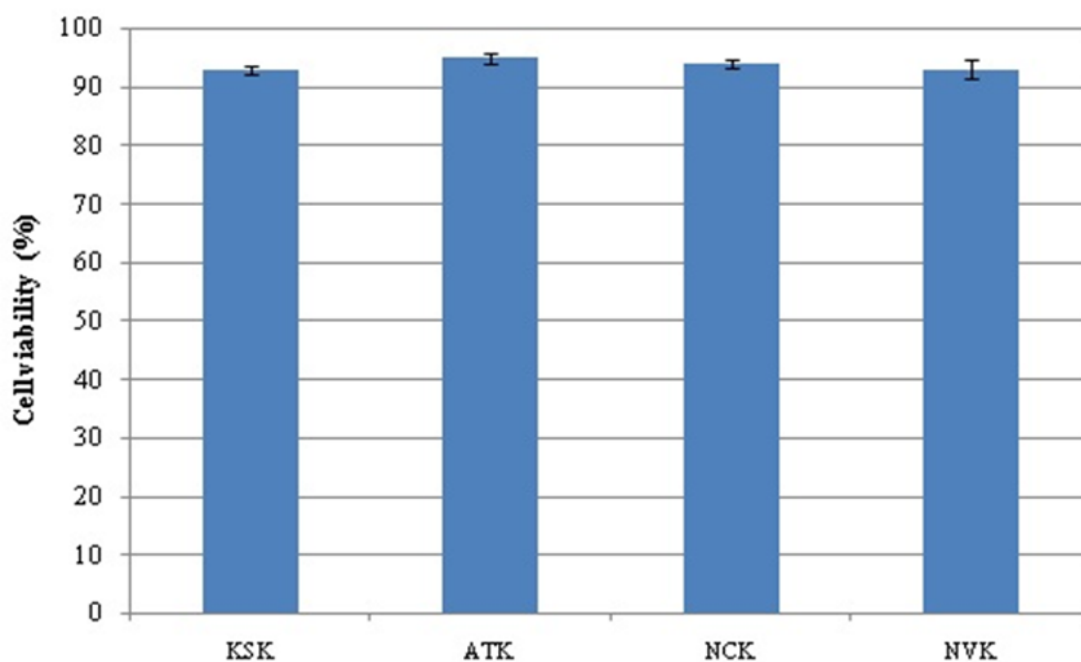


Figure 4: Toxicity results of herbal decoctions

candy can be used to enhance the taste. This drug is prescribed by Siddha medical practitioners as preventive and curative measure for treating various viral fevers like dengue, influenza, chikungunya and so forth (Narayanaswami, 1995; Shanmugam et al., 2017). Mahadevan and Vanmathi Palraj (2016) explained in their review article about the effectiveness of NVK in dengue management. In the same concept human clinical trial was conducted by Kala et al. (2014).

KSK consist of *Zingiber officinale*, *Piper longum*,

Syzygium aromaticum, *Tragia involucrate*, *Anacyclus pyrethrum*, *Hygrophilla auriculata*, *Terminalia chebula*, *Adathoda vasica*, *Coleus amboinicus*, *Saussurea lappa*, *Tinospora cordifolia*, *Clerodendron serratum*, *Andrographis paniculata*, *Sida acuta* and *Cyperus rotundus* (Narayanaswami, 1995; The Siddha Formulary of India, 1992). The KSK is prepared by taking 35 g of powder with 3 litres of water and boiling it till the decoction is concentrated to 250 ml (12:1 ratio). This decoction has the therapeutic effect of bronchitis, dysphonia, pneumonia, lungs infection and asthma. This decoction's standardiza-

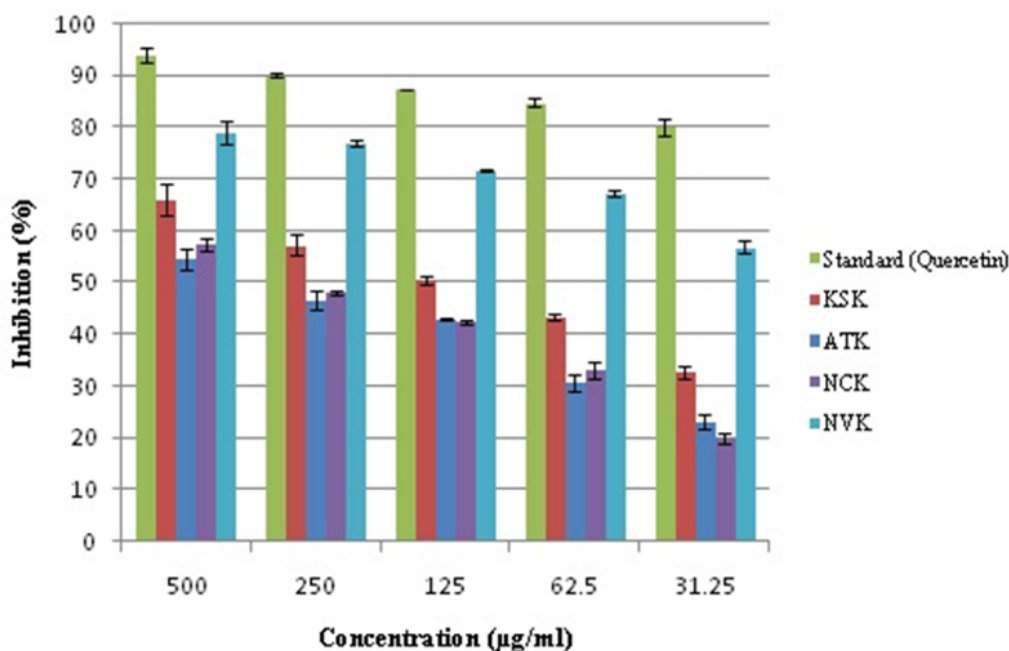


Figure 5: Anti-atherogenic activity results of herbal decoctions

tion work was carried out by [John et al. \(2015\)](#).

NCK has 10 g of Vitex negundo tender leaves, 10 g of black pepper, 5 g of garlic and 5 g of betel leaves. Crush the above drugs slightly, take 30 g and add $\frac{1}{2}$ litre of water. Boil this till reduced to 125 ml (4:1 ratio). This decoction is given for fever with severe cold, sneezing, head ache, head heaviness, dysphonia and body pain. A review paper of [Parameswaran et al. \(2012\)](#) noted that this decoction is also used for fever management and dengue fever preventive. Same concept was explained in another review of [Mahadevan and Vanmathi Palraj \(2016\)](#). In vitro activity was already reported in aqueous extract and the GC-MS was analyzed in ethanol extract. In this research work phytochemical investigations explained the existence of so many phyto-constituents namely they are triterpenoids which act as cancer preventive agents, steroids are used as antiarthritic and antiasthmatic drugs, flavonoids are polyphenolic molecules have antioxidant properties, tannins act as antilipidemic agents, saponins have antiseptic properties, vitamins, sugars have energy boosting effects, vanillin and ursolic acid ([Mahadevan and Vanmathi Palraj, 2016](#)).

Ingredients of ATK are Adathoda vasica (10 g), Glycyrrhiza glabra (2 g), Abies webbiana (2 g), Piper longum (2 g) and honey (10 g). Adathoda leaves were powdered and fry with honey. Added with $\frac{1}{2}$ litre water, boiled till reduce to 125 ml and filtered (4:1 ratio). Medically it is used for cough, fever, all type of lung disorder, asthma, tuberculosis, pneu-

monia and sinusitis ([Narayanaswami, 1995](#)). Its standardization work and therapeutic importance was explained by [Shanmugam et al. \(2017\)](#).

Total phenol content

Decoctions are the simplest, effective, easy digestive and fast curable natural medicine form. Phenolic compounds are present in almost all foods of plant origin, fruits, vegetables, and beverages are the major sources of these compounds in the human diet ([Hertog et al., 1993](#)). Decoctions are similar to medicated beverages. Phenolic compounds are known to exert preventive activity against infectious and degenerative diseases, inflammation and allergies via antioxidant, antimicrobial and proteins/enzymes neutralization/modulation mechanisms. Phenolic compounds are reactive metabolites in a wide range of plant-derived foods and mainly divided into four groups: phenolic acids, flavonoids, stilbenes and tannins. They work as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid oxidation ([Ozcan et al., 2014](#)). Figure 2 shows the total phenolic content of selected polyherbal decoctions. Along with this result NCK has the highest phenol content match with other decoctions.

Antioxidant activity

The stronger association between oxidized LDL and cardiovascular disease suggests that a person's antioxidant status is a far more important determinant than LDL levels of the risk of developing advanced plaques ([Colpo, 2005](#)). Oxidative stress produces reactive free radicals and repairs the

basic molecules of body. Antioxidants may act as physical barriers to prevent Reactive Oxygen Species generation. The anti oxidants can stimulate the process of oxidation. At the present time, the need of natural anti oxidants looks to be increasing instead of artificial anti oxidants. Antioxidants are an important factor to maintain optimal cellular and human body health. Natural antioxidants help in avoiding the lifestyle diseases like carcinoma, diabetes mellitus and cardiac diseases. Other than vitamins there are rich sources of anti oxidants present in many medicinal plants (Kumar, 2014; Tanaka et al., 2016). The DPPH radical scavenging activity of different *Siddha* poly herbal decoctions were shown in the Figure 3. The estimate of the antioxidant activity by DPPH radical scavenging power has been broadly in use for different plant extracts. DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable radical, methanol solution of which has dark purple color with maximum absorption at 515 nm. Among the investigated herbal decoctions, NCK exhibited highest DPPH radical scavenging power, which is followed by ATK, NVK and KSK. NCK exhibited strong antioxidant activity in terms of radical scavenging potential against DPPH (IC-50: 2.12 mg/L), followed by the ATK (IC-50: 2.27 mg/L), NVK (IC-50: 4.48 mg/L) and KSK (IC-50: 9.29 mg/L).

Toxicity results

While ordinary cells are exposed to any toxic complex, they possibly will undergo necrosis due to loss of membrane reliability and rapid death occurs as a result of cell lysis. In this method, cells prevent dividing and increasing, or there is a decrease in cell viability, or can activate a genetic program to manage cell death (apoptosis). Necrotic cells undergo rapid swelling, loss of membrane integrity, shut down metabolism and tend to release their contents into the adjacent medium. The apoptotic cells undergo secondary necrosis and lyses by finishing the metabolism and by failure of membrane integrity (Nagaraj et al., 2017). The toxicity results of herbal decoctions were shown in the Figure 4. All four decoctions showed the percentage of above 92%. From the results, we concluded that the studied sample was not toxic to PMBC at the selected concentration range and hence we have used the same concentrations for anti-atherogenic activity.

Anti-atherogenic activity

Oxidation of low density lipoproteins and their increased uptake by macrophages is identified to result in the creation of foam cells, which are dangerous in the initiation of atherosclerosis through activation of inflammatory signaling cascades. Thus, powerful dietary antioxidants are getting attention

for the reversal of such pathological states. Epidemiological proof specifies that plants, which are rich in antioxidants and anti-inflammatory properties, are able to decrease cardiovascular pathological conditions (Shetty et al., 2014). One research work has proven this information through their experiment of ginger extraction *in vitro* study (Gunathilake and Rupasinghe, 2014). In addition there is one review article explains that the decoctions uses for this problem in Chinese herbs Liu et al. (2015). Figure 5 showed the anti-atherogenic activity results of herbal decoctions. In the present study's results are expressed that NVK has the highest anti-atherogenic activity nearly 80% followed by the KSK (68%), NCK (58%) and ATK (55%) respectively.

CONCLUSIONS

In this research work we have estimated the total phenolic content, antioxidant and anti-atherogenic activities of four common herbal decoctions such as *Nilavembu kudineer*, *Kabasura kudineer*, *Notchi kudineer* and *Adathodai kudineer*. Among the investigated four decoctions, *Nilavembu kudineer* was identified to contain notable polyphenolic content, antioxidant and anti-atherogenic properties. Hence, such indigenous drug with remarkable medicinal value could be further investigated using *in vivo* model to develop a natural, safe and plant-based anti-atherogenic drug.

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Abbreviations used

LDL: low-density lipoprotein. NVK: *Nilavembu kudineer*, KSK: *Kabasura kudineer*, NCK: *Notchi kudineer*, ATK: *Adathodai kudineer*, NABL: National Accreditation Board for Testing and Calibration Laboratories, PMBC: Peripheral blood mononuclear cells DPPH: 2,2-Diphenyl-1-picrylhydrazyl, GAE: Gallic acid equivalents; PBMC: Peripheral blood mononuclear cells, PBS: Phosphate buffered saline, RPMI medium: Roswell Park Memorial Institute medium, TCA: Trichloroacetic acid; TBA: Thio barbituric acid; IC-50: Inhibitory concentration-50.

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