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Assessment of secondary metabolites: *in vitro* antiarthritic and antihemolytic potential of various extracts of garlic

Sonia Johri*, Nasir Khan, Sarita Tiwari, Lovie Shakyawar

Department of Life Sciences, ITM University, Gwalior;
***Email:** soniajohari@itmuniversity.ac.in*

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ABSTRACT

Background & Aim: *Allium sativum* commonly known as garlic is an herb used over years to prevent from various ailments such as cardiovascular diseases, cognitive disorders and also enhances the immune system. The aim of the present study is to study the *in vitro* antiarthritic and antihemolytic potential of garlic in aqueous and methanolic extracts. Fourier transform infrared spectroscopy was performed to study the presence of functional groups.

Experimental: The study involved detection of primary and secondary metabolites in *allium sativum* i.e garlic. *In vitro* antiarthritic and heat induced haemolytic assay was evaluated so that it could be used against haemolytic diseases such as malaria, swine flu etc. FTIR spectra were performed to detect the presence of functional groups.

Results: Natural products due to their immense bioactivity, finds applications against various diseases without having side effects**.**

Recommended applications/industrie: This herb is very easily accessible and is edible it can be used as a pharmaceutical agent. Bioactive constituents can be isolated and explored for further activity.

1. Introduction

Herbal medicines are used as prophylactic and therapeutic agents against hepatic, renal, cardiovascular as well as inflammatory diseases, by modulating risk factors such as hypertension, high blood cholesterol, thrombosis, and preventing other chronic diseases associated with ageing (Rahman 2001, Tanaka *et al*., 2006, Rahman, 2003, Neil *et al.*, 1994).

Secondary plant metabolites play a very important role and has bioapplications. Garlic (*Allium sativum*) which plays a very important dietary and medicinal role for centuries finds therapeutic uses which include beneficial effects as antibiotic, anticancer, anti-inflammatory, hypoglycemic, and hormone-like agents. Garlic extracts have been used to treat

infections for thousands of years. It's typical pungent odour and antibacterial activity is due to active principle allicin, which is produced by enzymatic (alliin lyase) hydrolysis of alliin after cutting and crushing of the cloves. Pharmacological effects of garlic are attributed to the presence of pharmacologically active sulfur compounds including diallyl sulfide, diallyl disulfide, allicin, and dipropyl sulfide. These compounds have been known to increase the activity of enzymes involved in the metabolism of carcinogens (Fisher *et al*., 2007), as well as anti-inflammatory effects *in vitro* and *in vivo (*Sabayan *et al*., 2007; Murugavel and Pari, 2007; Park *et al.*, 2005; Lang *et al.*, 2004; Son *et al*., 2006; Chang *et al*., 2006). Thiacremonone, a novel and major sulfur compound (0.3%) in garlic, has higher anti-oxidant properties compared with other sulfur compounds. Allicin is an odorless sulfur-containing chemical derived from the amino acid cysteine which is further broken down to a compound called Ajoene that inhibits blockage in blood vessels from clots and atherosclerosis (Hwang *et al*., 2007).

World Health Organisation (WHO) reported that about 80% of the world's population depend mainly on traditional medicine and the traditional treatment involve mainly the use of plant extracts. Natural antioxidants are the secondary metabolites of the plant and do not have side effects when taken *in vivo* (Chen *et al*., 1992; Walton and Brown, 1999). Many plants contain wide variety of free radicals scavenging molecule such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites Phenolic compounds which are important secondary metabolites have redox properties which neutralizes the free radicals thereby quenching singlet and triplet oxygen decomposing peroxides as an important antioxidant. (Osawa, 1994). Tannins also find applications as a potential agent in variety of disease states (Packer *et al.*, 1999).

2. Materials and Methods

2.1. Plant Collection

Fresh garlic cloves were collected from local market of Gwalior, M.P.

2.2. Preparation of garlic powder

1000g of garlic cloves were peeled and cut in to small pieces and shade dried. The dried garlic was ground to a fine powder.

2.3. Preparation of methanolic extract

50 g of powder was weighed and sequentially extracted with methanol and water respectively in soxhelet apparatus for 72 h. After solvent extraction it was evaporated to obtain a powdered extract for various biochemical analysis (Kumar *et al*., 2012).

2.4. Preparation of aqueous extract

50g of powder was weighed and extracted with distilled water in soxhelet apparatus for 72 h. After solvent extraction it was evaporated to obtain a powdered extract for various biochemical analysis (Kumar *et al*., 2012).

2.5. Phytochemical analysis of primary metabolites

Preliminary phytochemical screening of the extract was performed for the presence of basic primary metabolites like carbohydrates, proteins and lipids (Plummer, 1987*)*

2.5.1. Carbohydrates. Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used for Molisch's test and Fehling's test for the presence of carbohydrates and reducing sugars respectively.

2.5.2. Proteins.

Biuret's test: To 1 ml of test extract, 4% of sodium hydroxide solution and few drops of 1% copper sulphate solution were added. Formation of a violet red colour indicated the presence of peptide bond.

Ninhydrin test: To 2 ml of test extract, few drops of 0.25% of ninhydrin in acetone were added and heated in boiling water bath for 10 min. The formation of bluish purple colour indicated the presence of amino acid. (Kumar *et al*., 2012)

2.6. Phytochemical analysis of secondary metabolites

Preliminary phytochemical screening of the extract were performed for the presence of alkaloids, reducing sugars, proteins, flavonoids, tannins, phenols, phytosterol and saponins using the standard procedures (Kumar *et al*., 2012)

2.6.1. Alkaloids. Alkaloids were detected by using Wagner's test. To 1 ml of extract, 2-3 drops of Wagner's reagent was added. Appearance of reddish brown precipitate indicated the presence of alkaloids.

2.6.2. Saponins by Froth test. Extracts were diluted to 20 ml with distilled water and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

2.6.3. Steroids by Salkowski test. A few milligram of extract, 2 ml of chloroform and 2 ml of conc. sulphuric acid were added. Tubes were shaken and allowed to stand. A golden yellow red colour indicated the presence of phytosterols.

2.6.4. Tannins (ferric chloride test). Extract solutions were treated with 5% ferric chloride solution. Formation of blue colour indicated the presence of hydrolysable tannins and formation of green colour indicated the presence of condensed tannins

2.7. *In-vitro* anti-arthritic activity by inhibition of protein denaturation method

- The test experimental (0.5ml) consist of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0 .05ml of test solution (250 µg/ml).
- Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5%w/v aqueous solution) and 0 .05ml of distilled water.
- Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution (250 µg/ml).
- Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0.05ml 0f diclofenac sodium (250 µg/ml).

All the above solutions were adjusted to pH 6.3 using HCl (1N). The samples were incubated at 37°C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3minutes. After cooling, 2.5 ml of phosphate buffer was added to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm **(**Venkataraman *et al*., 2013).

The percentage inhibition of protein denaturation can be calculated as:

Percentage Inhibition = [100-(optical density of test solution – optical density of product control) / (optical density of test control)] ×100.

The control represents 100% protein denaturation. The results were compared with standard diclofenac sodium. The percentage inhibition of protein denaturation of different concentration was tabulated.

2.8. Heat induced haemolysis

Reaction mixture (2 ml) consisted of 1 ml test solution and 1 ml. of 10% RBC suspension. Saline was replaced with drug in test solution. Aspirin was taken as a standard drug. The tubes were incubated in a water bath at 56 ^{0}C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. Reaction was centrifuged at 2500 rpm for 5 minutes and OD of supernatant taken at 560 nm with UV- visible spectrophotometer (Okoli *et al.*, 2008).

2.9 **FTIR Spectroscopy**: The FTIR from 4000 to 400 cm^{-1} was recorded on a Perkin Elmer (spectrum 2) spectrometer. FTIR is used as a tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt *et al.*, 2007; Hazra *et al.*, 2007).

3. Results and discussion

Table 1 shows preliminary phytochemical analysis of primary and secondary metabolites. Results revealed that both methanolic and aqueous extract had higher content of alkaloids, flavanoids, saponins, protein and carbohydrates. The garlic cloves extract also had the same content. This was followed by tannins cardiac glycosides, carbohydrates, protein and reducing sugars.

Flavonoid is one of the main group of phenolic compound and widely distributed flavonoid, flavones and flavonols. Many flavonoids and related compound are reported to have strong antioxidative characteristics. Flavonoids and phenolics are the effective scavengers of the free radicals and chain breaking agents due to presence of hydroxyl group. Their presence indicates high analgesic and anti-inflammatory effects as a result of their membrane stabilizing ability against free radicals produced as a result of lipid peroxides and superoxides which causes cell membrane destabilization. They also possess anticancer, anti-diabetic, anti-aging properties and prevention of cardiovascular diseases. Tannins possess antiviral, antibacterial and antiparasitic effects and have the potential to fight against cancer. In the present study the pronounced antioxidant activity of *Allium sativum* was possibly due to its high phenolic and flavanoid content. Polyphenolic and flavanoid compounds contain conjugated ring structures and hydroxyl groups; which aids in providing an antioxidant status in cell free systems by scavenging singlet oxygen, superoxide

anion, lipid peroxy radicals, hydroxyl ions, nitric oxide ions, and stabilizing free radicals involved in oxidative processes (Shahidi *et al*., 1992; Dixon *et al.,* 2005).

Table 1. Preliminary phytochemical screening of extracts

Test	Aqueous extract	Methanol extract	Garlic clove extract
Tannins	+	-	-
Anthraquinone	-	-	-
Phlobatannins	+++	++	+
Cardiac glycosides	+	+	+
Carbohydrates (Molisch's test)	+	+	+
Proteins (Biuret test)	+	+	++
Reducing sugar(Fehling's test)	+	+	++
Alkaloids (Wagner's test)	++	++	++
Saponin (Foam test)	++	++	++
Steroids (Salkowski's test)	++	++	++
Tannins	+	+	+
Flavanoids	++	++	++

Table 2 depicts the *in vitro* antiarthritic activity by inhibition of protein denaturstion. The methanolic extract of garlic exhibited significant activity at 800µg/ml (63%) by inhibition of protein denaturation as compared with standard drug diclofenac sodium. The methanolic extract of garlic powder exhibits significant activity at 2000µg/ml (82.94 %) by inhibition of protein denaturation as opposed to the standard drug diclofenac sodium.

The production of auto antigen (like Rheumatoid Factor) in certain arthritic disease is because of protein denaturation. Hence we can conclude that the methanolic extract of garlic are capable of controlling the production of auto antigen or functional groups (chemical bonds) present in an unknown mixture of plant extracts. (Gupta *et al.,* 2013)

Table 3 explains the antihaemolytic activity of the extract in the presence H_2O_2, where ascorbic acid was taken as a positive control. It was found out that when RBC cell were treated with H_2O_2 along with two different extract, marked reduction in the haemolysis was observed than that of cells treated with the toxicant alone. When cells were treated with the extract alone, no haemolysis was obtained explaining the non-toxic behaviour of the extracts on human RBC. Out of all extract, methanolic extract showed better results.

Table 2. Effect of aqueous and methanolic extract of garlic on protein denaturation.

Treatment	Concentration (µg/ml)	Inhibition(%)
Diclofenac sodium	200	89.20
	300	92.46
	500	92.50
	800	98.67
Methanolic Extract	200	33.00
	300	41.00
	500	50.00
	800	63.00
Aqueous Extract	200	15.00
	300	22.00
	500	28.00
	800	43.00

Table 3. Effect of garlic on heat induced hemolysis.

Treatment	Concentration (µg/ml)	Inhibition (%)
Aspirin	200	89.20
	300	98.67
	500	92.46
	800	92.50
Methanolic Extract	200	53.00
	300	50.00
	500	41.00
	800	33.00
Aqueous Extract	200	43.00
	300	28.00
	500	22.00
	800	15.00

Both methanolic and aqueous extracts demonstrated a significant inhibition of hemolysis *in vitro*. The inhibition effect shown by crude extracts of garlic at comparatively lower concentrations (37.5 µg/ml) was comparable with that of standard anti-hemolysis compounds such as aspirin. This experimental evidence indicates that aqueous and methanolic extracts could

have a potential therapeutic efficacy in disease processes causing destabilization of biological membrane. Tissue proteins are denatured causing production of autoantigens an arthritic disesases leading to inflammatory and arthritic diseases.

Table 4. FTIR frequency range and functional group present in the cloves of *Allium sativum*.

Frequency range (cm^{-1})	Functional groups
500-600	Bromo alkanes
600-800	Chloro alkanes
900-1000	Aromatic
900-1000	Alphatic amines
1000-1150	Fluoro alkanes
1500-1383	Nitro compound
2885-3000	Halogen compound

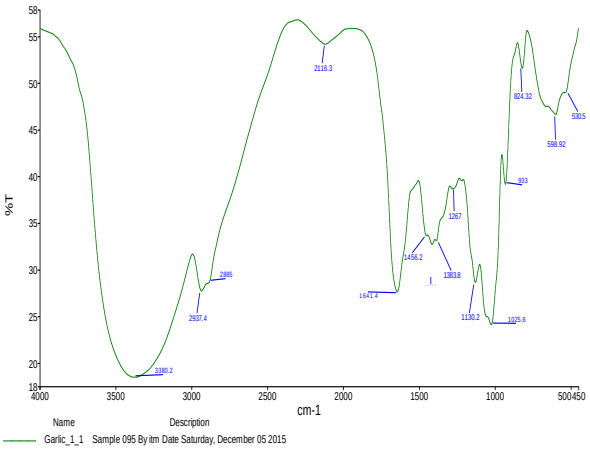

Fig. 1. FTIR spectra for the *Allium sativum* cloves extracts

The mechanism of action may be denaturation of tissue proteins which causes alteration in electrostatic, hydrogen, hydrophobic and disulphide boding. From the result of the present study it can be stated that *Allium sativum* is capable of controlling the production of auto antigens due to *in vivo* denaturation of proteins in rheumatic diseases. Protective effect on heat and hyotonic saline-induced erythrocyte lysis is known to be a very good index of anti- arthritic activity of any agent. Since the membrane of RBC is structurally similar to the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane (Brown and Mackey, 1968).

Table 4 and Figure 1 show the FTIR spectra for the Allium sativum cloves. In the present study FTIR spectroscopy was performed for easy and rapid determination and identification of various functional groups responsible for medicinal properties. IR spectroscopy is basically a vibrational spectrum. Presence of sulphur derivatives, polysaccharides, organic hydrocarbon, haloalkanes, aromatic compounds, aliphatic amines and nitro compounds are responsible for various medicinal properties of garlic.

4. Conclusion

The methanolic extract of garlic was more potential as compared to the aqueous extract. It possessed antiarthritic and antihemolytic potential and can be explored further.

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