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SYNERGISTIC STUDY OF ANTIOXIDANT POTENTIAL OF DIFFERENT SPICES AND THEIR BIOACTIVE CONSTITUENTS

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ABSTRACT: Reactive oxygen species are associated with ageing process creating many health disorders such as Alzheimer, Parkinson, cancer, atherosclerosis and arthritis etc. These harmful actions of reactive oxygen species can be controlled to the some extent by using antioxidants which are present in plants, fruits, vegetables and spices. Turmeric, black pepper and clove are popular spices and have been used in traditional medicine. Their bioactive constituents i.e. curcumin, piperine and eugenol show many pharmacological impact on the nervous and neuromuscular system. Antioxidant activity of methanolic extract of above spices and their bioactive constituents have been evaluated synergistically by 1,1,-Diphenyl-2-picrylhydrazyl assay. Highest antioxidant potential is reported in mixture of curcumin and eugenol (97.38%) followed by methanolic extract of clove (95.91%) and mixture of methanolic extract of turmeric and clove (94.23%). Our studies clearly suggest that extract and their bioactive constituents individually does not shows as much as antioxidant potential as when they are mixed in definite proportion which may be due to synergistic effect.

INTRODUCTION: It is a well known fact that free radicals and other reactive species formed in living cells play an important role in origin of life and biological evolution. Free radicals can also cause lipid peroxidation.

Although there are some synthetic antioxidants like Butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) but all these are associated with some side effects.

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A majority of plant species have been investigated time to time for their use as antioxidants.

Dietary antioxidants which contain polyphenolic compounds, vitamin E and C, carotenoids are supposed to be the effective antiaging compounds and helps in prevention of oxidative stress related diseases. Recently antioxidant research is gaining interest day by day.

A tendency among modern consumers is the use of antioxidant obtained from natural sources, increased the interest in research. The general public, scientists, medical practitioners, nutritional and health experts are interested to know the antioxidant capacity of the food we eat, fruits, vegetable spices we consume. It has been observed that it is very difficult to separate each constituent

of the food and then test the antioxidant potential individually as it is not only time consuming but also a costly affair. Therefore scientists are interested in looking a convenient method to test the antioxidant capacity of compounds in food mixture. For this purpose, antioxidant activity of turmeric, clove, black pepper and their active principals; curcumin, eugenol, piperine and their synergistic combination has been tested by DPPH* assay.

Turmeric (*Curcuma longa*) belongs to family Zingiberaceae which are found throughout the tropics of Asia, Africa and Australia. It has wide range medicinal properties. It mainly contains curcumin, phenolics, terpenoids, flavonoids, which are present in its rhizomes. Very few investigations have been carried out in the aqueous principles.

The turmeric extract contains sufficiently high amount of curcumenoids (8.43%) and shows a very high antioxidant activity [1]. Proteins isolated from aqueous extract of curcuma species are heat stable and showed significant antioxidant activity [2]. The antioxidant activity of free and bound phenolics of turmeric extract prepared by treating with enzyme and without enzyme was analyzed and the enzyme assisted extract shows higher antioxidant potential than non-enzymatic extract [3].

Curcumin is the principal curcumenoid of the popular Indian spice turmeric which possess different medicinal properties [4, 5]. The other two curcumenoids are demethoxycurcumin and bis-demethoxycurcumin. Curcumin is as powerful antioxidant as vitamin C, E and β-carotene [6]. In another investigation curcumin and its synthetic bioconjugates are also reported as good antioxidant [7].

Black pepper (*Piper nigrum*) is a popular spice in oriental countries. Black pepper contains many antioxidants such as ascorbic acid, β-carotene, camphene, lauric acid, linalylautate, myristicin, palmitic acid, terpinen-4-ol, piperine and feruperine [8]. It has been observed that all the phenolic amides identified from black pepper possess significant antioxidant activities that are more effective than the naturally occurring antioxidant α-tocophenol and feruperine have antioxidant activity as high as the synthetic antioxidants bulylated hydroxyl anisole and butylated hydroxytoluene [9].

Recently oil of black pepper was tested for antioxidant activity by DPPH*, $ABTS^+$ and β-carotene bleaching method and it shows antioxidant potential [10, 11].

Piperine is the main bioactive constituent of black pepper and it is found to act as a hydroxyl radical scavenger at low concentrations. Recently nano encapsulated black pepper (with hydroxypropyl beta-cyclodextrin) were synthesized and enhanced antioxidant activity was found[12]. It has also been observed that piperine enhances antioxidant activity of curcumin[13].

Clove (Syzygium aromaticum) is a member of family Myrataceae. These are the flower buds which resemble irregular nails. Good quality clove buds contains 15-20% essential oil which is dominated by eugenol (70-85%), eugenyl acetate (15%) and β-caryophyllene (5-12%) which together make up 99% of the oil[14]. The other constituents are methyl amyl ketone, methylsalicylate, α and β-humulene, benzaldehyde, β- ylangene and chavicol.

Clove essential oil has the highest antioxidant capability compared to any essential oil, perhaps one of the highest known for a food or supplement. It has been included in some longevity formulae for this reason. Clove and eugenol possess strong antioxidant activity which is comparable to the activities of the synthetic antioxidants, BHA and pyrogallol [15].

Essential oil from clove leaf possesses scavenging activity against DPPH* radical at concentration lower than the concentration of eugenol, butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA). It also shows a significant inhibitory effect against hydroxyl radicals and acts as an iron chelator [16, 17]. Eugenol inhibits 5-lipoxygenase activity and leukotriene - C_4 in human PMNL cells [18].

Recently the antioxidant potential of methanolic extract of Syzygium aromaticum is determined by DPPH* method and it shows very high radical scavenging activity, it also exhibit high chelating ferrous ion ability [19].

MATERIALS AND METHODS: Turmeric, black pepper and clove were purchased from the local market. Chemicals such as: curcumin, piperine, eugenol and gallic acid were purchased from CDH Pvt. Ltd. New Delhi, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH*) was purchased from HiMedia Pvt. Ltd. Mumbai, India and Methanol AR was purchased from Loba Chemie Pvt. Ltd. Mumbai, India. The absorbance of all sample were recorded by UV- Visible spectrophotometer (Systronic, Model No. 1302). The experiments were performed in the Department of Chemistry, CMP Degree College (Constituent College of University of Allahabad) and Department of Biochemistry, SHIATS, Allahabad during July 2013 to December 2013.

A. **Preparation of methanolic extract:** Commercially available turmeric, clove and black pepper (10 g) was refluxed separately with 50 ml of methanol on heating mantle for 20 minutes. The extract was filtered and then solvent was evaporated in rotary evaporator under pressure to obtain in powder form. Now the antioxidant potential of methanolic extracts of above medicinal plants and their active principals were evaluated by DPPH* assay.

B. **1, 1,-Diphenyl-2-picrylhydrazyl assay (DPPH* Assay):** The hydrogen atom or electron donating ability of the plant extract was determined from bleaching of purple colored methanol solution of DPPH* [20-22], gallic acid was used as standard. This spectrophotometric assay uses the stable radical DPPH* as a reagent. The procedure involves measurement of decrease in absorbance of DPPH* at its absorption maxima of 517 nm. DPPH* was prepared at a concentration of 0.002%.

The stock solutions of the extracts were prepared in methanol (1 mg/10 ml). Different volume (1.0, 0.5, 0.25, 0.125 and 0.062 ml) of extracts was taken in separate test tube and volume was made up to 2 ml with methanol. Now 2ml of DPPH* solution was added in each test tube and kept in dark for 30 minutes. The same procedure was followed for gallic acid as well. Later optical absorbance was recorded at 517 nm using UV- Visible spectrophotometer.

Methanol with DPPH* was used as a control. All the samples were tested in triplicate. The formula used for the calculation is:

% Inhibition of DPPH* activity = (A - B / A) X 100

Where A = absorbance of control; B = absorbance of sample.

RESULTS: The antioxidant value of methanolic extracts of different plants, their bioactive constituents and various mixtures were evaluated by DPPH* assay (**Table 1**).

TABLE 1: ANTIOXIDANT POTENTIAL OF METHANOLIC EXTRACTS OF DIFFERENT PLANTS, THEIR BIOACTIVE CONSTITUENTS AND VARIOUS MIXTURES

S. No.	Methanolic extracts of different plants, their bioactive constituents and various mixtures	% Antioxidant activity (by DPPH* assay)
1	Curcumin	75.23
2	Turmeric (meth. ext.)	73.98
3	Eugenol	66.43
4	Clove (meth. ext.)	95.91
5	Piperine	14.05
6	Black pepper (meth. ext.)	44.64
7	Curcumin: Eugenol (1:1)	97.38
8	Turmeric: Clove (meth. ext.) (1:1)	94.23
9	Curcumin: Piperine (1:1)	86.67
10	Turmeric: Black pepper (meth. ext.) (1:1)	59.29
11	Piperine: Eugenol (1:1)	90.23
12	Black pepper: Clove (meth. ext.) (1:1)	92.16
13	Curcumin: Eugenol: Piperine (1:1:1)	90.00
14	Turmeric: Clove: Black pepper (meth. ext.) (1:1:1)	89.73
15	Gallic acid (Stand.)	95.41

The curcumin and methanolic extract of turmeric show antioxidant potential with 75 and 73 % DPPH* inhibition (**figure 1**) while eugenol, methanolic extract of clove (**figure 2**) and piperine, methanolic extract of black pepper (**figure 3**) exhibited antioxidant activity with 66.43, 95.91 and 14.05, 44.64% respectively as compared to gallic acid (standard). The mixture of curcumin and eugenol (1:1), methanolic extract of turmeric and clove (1:1) (**Figure 4**) showed antioxidant potential with 97.38, 94.23%, while curcumin and piperine, methanolic extract of turmeric and black pepper (1:1) (**Figure 5**); piperine and eugenol, methanolic extract of black pepper and clove (1:1) (**Figure 6**); curcumin, eugenol and piperine, methanolic extract of turmeric, clove and black pepper (1:1:1) (**Figure 7**) exhibited antioxidant activity with 86.67, 59.29; 90.23, 92.16 and 90.00, 89.73 % in that order.

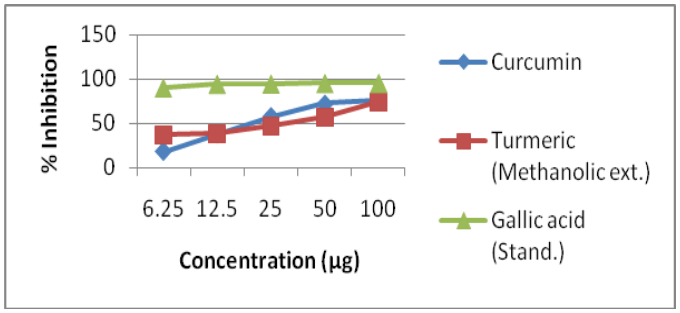

FIGURE 1: CURCUMIN AND METHANOLIC EXTRACTS OF TURMERIC

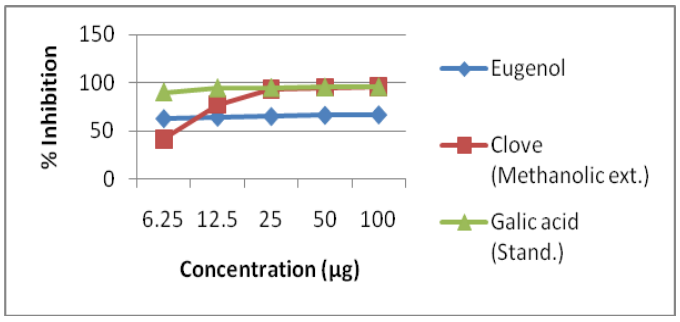

FIGURE 2: EUGENOL AND METHANOLIC EXTRACTS OF CLOVE

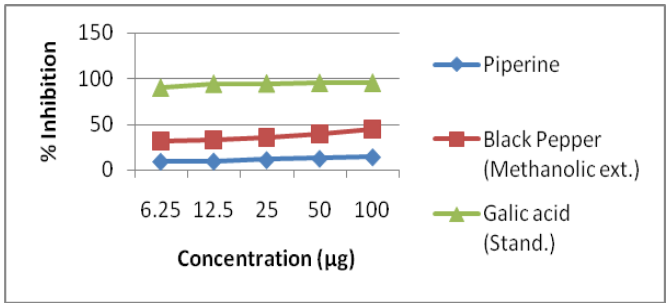

FIGURE 3: PIPERINE AND METHANOLIC EXTRACTS OF BLACK PEPPER

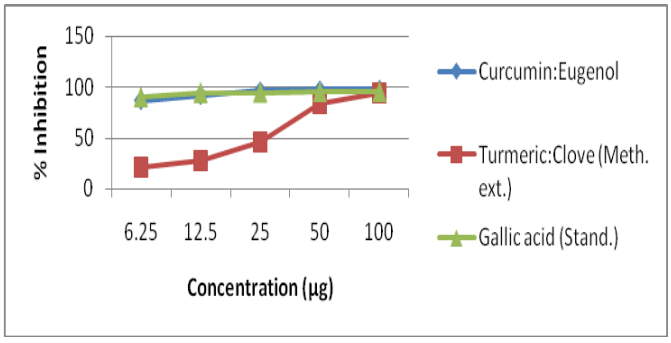

FIGURE 4: CURCUMIN: EUGENOL AND METHANOLIC EXTRACTS OF TURMERIC: CLOVE

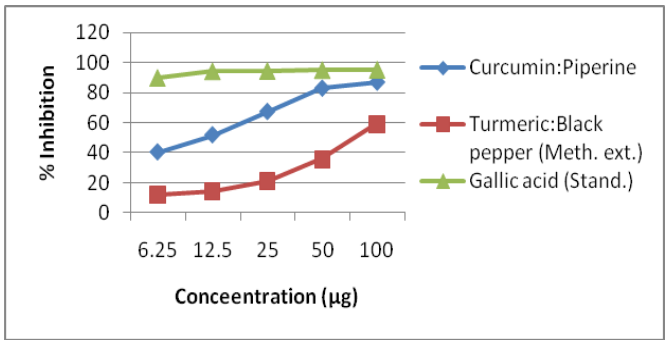

FIGURE 5: CURCUMIN: PIPERINE AND METHANOLIC EXTRACTS OF TURMERIC: BLACK PEPPER

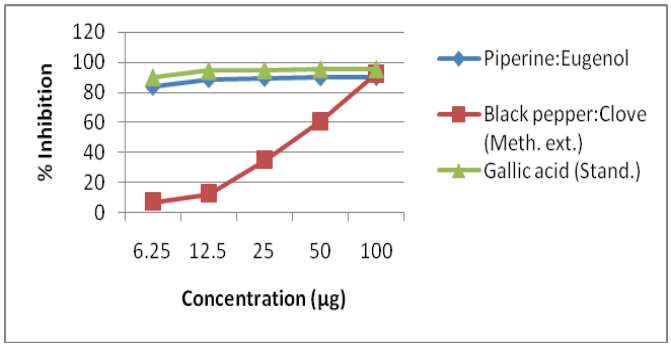

FIGURE 6: PIPERINE: EUGENOL AND METHANOLIC EXTRACT OF BLACK PEPPER: CLOVE

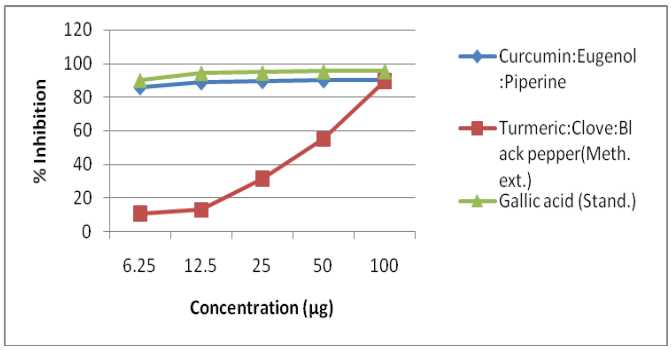

FIGURE 7: CURCUMIN: EUGENOL: PIPERINE AND METHANOLIC EXTRACT OF TURMERIC: CLOVE: BLACK PEPPER

DISCUSSION: The methanolic extract of clove showed higher antioxidant activity with 95.91% inhibition, in comparison to the methanolic extract of turmeric and black pepper with DPPH* % inhibition of 73.98 and 44.64. It is very interesting that the methanolic extract of clove has a higher antioxidant potential as compare to standard. Similarly, curcumin (principal constituent of the turmeric) showed higher antioxidant potential than eugenol and piperine, which are the active constituents of the clove and black pepper respectively. But when compared the antioxidant activity of the methanolic extracts with the principal constituents of the same plant, we found that curcumin is slightly more antioxidant activity as compared to methanolic extract of turmeric. However methanolic extract of clove and black pepper exhibited the higher antioxidant potential as compared to their active principal i.e., eugenol and piperine.

The mixture of methanolic extract of turmeric and clove (1:1), showed higher antioxidant activity with 94.23% inhibition in comparison to the mixture of black pepper and clove (1:1); turmeric, clove and black pepper (1:1:1); turmeric and black pepper (1:1) with DPPH* % inhibition of 92.16, 89.73 and 59.29 respectively. The bioactive constituents eg curcumin and eugenol (1:1) mixture exhibited higher antioxidant activity with 97.38% inhibition, which is followed by piperine and eugenol (1:1); curcumin, eugenol and piperine (1:1:1); curcumin and piperine, with 90.23, 90, and 86.67% respectively in comparison to gallic acid. It is very important to report here that the curcumin and eugenol synergistically showed higher antioxidant potential than the standard gallic acid.

When compared the antioxidant potential of mixtures of the methanolic extract of the plants and the mixtures of their active principals, we observed that mixture of curcumin and eugenol; curcumin and piperine; curcumin, eugenol and piperine, showed higher antioxidant potential as compared to their methanolic extracts i.e., turmeric and clove; turmeric and black pepper; turmeric, clove and black pepper respectively. However, piperine and eugenol was found to be slightly less antioxidant potential when compared with methanolic extract of black pepper and clove.

The antioxidant activity of turmeric extract is mainly due to the curcuminoids and the presence of two phenolic groups in curcumin is responsible for its high antioxidant activity. As far as the antioxidant activity of black pepper is concerned, it is due to the presence of phenolic amides. Its bioactive constituent piperine is a good hydroxyl radical scavenger.

High antioxidant activity of clove is due to the presence of eugenol, eugenol acetate and β-caryophyllene and the eugenol itself shows good antioxidant activity which is comparable to synthetic antioxidant like BHA and BHT. The highest antioxidant activity of curcumin and eugenol mixture is due to the presence of two free phenolic groups in curcumin and one free phenolic group in eugenol. These phenolic groups are good hydrogen donor making the compound highly antioxidant.

Piperine alone does not show good antioxidant activity, but when mixed with curcumin and eugenol it shows fairly high antioxidant activity which is a very good example of synergistic effect.

CONCLUSION: It has been observed that all the extracts and bioactive constituents have good antioxidant potential except the methanolic extract of black pepper and its active principal, piperine. However, all the mixtures exhibited very good antioxidant activity including the methanolic extract of black pepper and piperine when studied synergistically.

The most prominent antioxidant activity has been observed in the extract of clove; mixture (1:1) of extract of turmeric and clove; curcumin and eugenol as compared to all other samples.

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