ejpmr, 2018,5(12), 517-526

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DETERMINATION OF EFFICACY AND MECHANISM OF ACTION OF ANTIOXIDANTS COMPONENTS FROM BLACK CUMMINS (*NIGELLA SATIVA*) AND SWALLOW ROOT (*DECALEPIS HAMILTONII*) USING *IN VITRO* BIOCHEMICAL AND CELL CULTURE ASSAY MODEL SYSTEMS

[1]*Vulli Venkata Rao, [2]Vempati Poornodai, [3]Nancy Bonareri Mitaki and [4]Suberu Safiu Adewale

[1]Sunrise University, Rajasthan, India.
[2]Biomedical Department, College of Medicine and Health Sciences, Dilla University, Ethiopia.
[3]Department of Biochemistry, Kampala International University in tanzania (KIUT), Daressalaam, Tanzania.
[4]Department of Biochemistry, Kampala International University- Health Sciences, Uganda.

***Corresponding Author: Vulli Venkata Rao**
Sunrise University, Rajasthan, India.

Article Received on 23/10/2018 **Article Revised on 13/11/2018** **Article Accepted on 02/12/2018**

ABSTRACT
Binding of phenolics including antioxidants to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action. This study was conducted to know the efficacy and mechanism of action of antioxidant components from black cumis (*Nigella sativa*) and swallow root(*Decalepis hamiltonii*) using *In vitro* biochemical cell culture assay model. Determination of binding ability of BCAE and SRAE indicated that all phenolic acids have the ability to bind to protein and DNA at varied levels. Tannic acid followed gentisic and gallic acid appear to bind strongly to both - a carrier protein– Human Serum Albumin and DNA. Binding of these components to target protein is implicated in enhancement of bioavailability and hence bioactivity. Interaction of phenolic acids thus is an important tool to predict the antioxidant potency *in vivo*.

KEYWORDS: *Nigella sativa, Decalepis hamiltonii.*

INTRODUCTION
Binding of phenolics including antioxidants to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action (Kragh-Hansen *et al*., 2002; Lin *et al*., 1987; Fichtl *et al*., 1991). The effect is especially significant for highly protein bound drugs, where only a small alteration in bound fraction can produce a profound change in the pharmacodynamically active free antioxidant concentration (Levy & Moreland, 1984; Rowland, 1980). Generally the bound antioxidant component to serum albumin serves as a storehouse and the unbound may be pharmacologically active (Seedher & Bhatia, 2006). Thus, the nature and magnitude of their interaction with serum albumin has important pharmacokinetic and pharmacodynamic implications. Fluorescence spectroscopy has been the most widely used spectroscopic technique for monitoring drug/antioxidant binding to plasma albumin because of its sensitivity, accuracy, rapidity and ease of use (Oravkova *et al.*, 1996; Epps *et al*., 1999). In the present investigation, interaction of the phenolics of black cumin aqueous extraxt-BCAE and swallow root aqueous extract-SRAE with human serum albumin has been studied using fluorescence spectroscopic technique. In addition, to understand the probable structure-function relationship interaction of pure phenolic acids with protein and DNA were also studied. Results have been discussed in terms of the binding parameter.

Also, phenolics bind to DNA leading to various biochemical changes (Labieniec & Gabryelak, 2006) including DNA-adduct formation, strand breaks etc. Nucleic acid bound to ethidium bromide - a polycyclic aromatic dye, exhibits marked increase in fluorescence and this phenomenon is used in the current study to study the interaction between DNA and isolated phenolics from black cumin and swallow root.

MATERIALS AND METHODS
Human Serum Albumin (HSA) - Phenolics interaction studies
Stock solution of human serum albumin was prepared to a concentration of 1.0 x 10-4 M in Tris-HCl buffer of pH 7.4 containing 100 mM sodium chloride. All the phenolic compounds were prepared to a concentration of 10 mg/100 mL in ethanol (95%) because ethanol has no fluorescence and does not affect the determinations. Crude water extracts of black cumin and swallow root

were prepared by extracting 1 g sample in 20 mL of distilled water. The concentrations of crude extracts, free, hydrolyzed and bound phenolics of swallow root were expressed in terms of gallic acid equivalents of phenol. All the stock solutions were then diluted with the buffer to obtain the actual assay concentration. All fluorescence measurements were made in a Shimadzu RF-5301PC Spectrofluorophotometer equipped with a xenon lamp source and 1.0 cm x 1.0 cm x 4.0 cm quartz cells. A series of assay solutions were prepared by adding 10 µL of stock solution of HSA and varied concentrations of phenolics (0.5-2.5 µg/mL) into each mark tube, respectively, and diluted to the mark of 1.0 ml with Tris–HCl buffer of pH 7.4. In each tube therefore, the total concentration of HSA was constant and the total concentration of phenolics were different. Tubes were mixed thoroughly and placed in the thermostat water-bath at 37^{o}C for 5 min, and transferred to the quartz cuvette and fluorescence emission spectra were recorded in the wavelength range 290–500 nm by exciting HSA at 280 nm using a slit width of 5/5 nm. Wavelength nearer to shift observed was recorded to understand the involvement of tryptophan/tyrosine residue in HAS.

DNA - Phenolics interaction studies

Calf thymus DNA sufficiently free of protein was used. A260/A 280 of 1.8-1.9 were considered for the assay. DNA concentrations were determined spectrophotometrically with an extinction coefficient of 6600 M-1 at 260 nm (Song *et al*., 2002). All the experiments were carried out in 5 mM Tris-HCl buffer pH 7.0 containing 50 mM NaCl. DNA and ethidium bromide were dissolved in buffer at a concentration of 3 and 1 µg/mL, respectively. Phenolics concentration ranged was between 0.5-2.5 µg/mL. The isolated phenolics were added to ethidium bromide bound to calf thymus DNA and the intensity of fluorescence of ethidium bromide was measured. Fluorescence spectra was recorded using an excitation wavelength of 478 nm and the emission range set between 480 and 850 nm using a slit width of 5/5 nm.

RESULTS

HSA-phenolics interaction studies

Results from HSA interaction studies indicate that the changes occurred in the environment of tryptophan residues which is dependent on the applied phenolic compounds. As shown in Figure 1-3 nearly all the phenolic compounds and extracts tested showed HSA binding except but to varying extent with a variable range of 30-200 fold or over the maximum quencher – tannic acid with a KSV of 3.178 x 106 M-1. Next best phenolic acid for binding was ferulic acid with a KSV of 0.084 x 106 M-1 followed by HMBA (0.068 x 106 M-1), syringic (0.059 x 106 M-1), vanillic (0.053X106 M-1) caffeic (0.050 x 106 M-1), p-hydroxybenzoic (0.043 x 106 M-1), procoumaric (0.039 x 106 M-1), gentisic (0.035 x 106 M-1), cinnamic (0.024 x 106 M-1) vanillin (0.019X106 M-1) and gallic acid (0.016 x 106 M-1).

Stern-Volmer constants of the qenching of tryptophan fluorescence by phenolics and spice crude extracts is shown in Table 1. It is evident from the results that, HSA fluorescence was strongly quenched by tannic acid (KSV – 3.178 x 106 M-1) and moderate quenching was observed in BCAE (KSV – 0.249 x 106 M-1), and swallow root bound phenolic acid extract (SRBP, KSV – 0.203 x 106 M-1). Low quenching was observed for SRAE (KSV – 0.008 x 106 M-1), vanillin (KSV – 0.0.019 x 106 M-1) and gallic acid (KSV – 0.016 x 106 M-1). However, vanillic acid even though indicated fluorescence quenching initially, but increased the fluorescence of HSA molecule at higher concentrations.

Interestingly, BCAE is constituted by 77% tannic acid and ~ 23% by protocatechuic acid. BCAE showed the second highest binding next to standard tannic acid (Table 1), suggesting that BCAE - phenolics particularly tannic acid may contribute to the binding. The same may therefore circulated effectively *in vivo* via binding to HSA. Generally HSA being the carrier protein, increased binding efficiency also indicate the increased bioavailability which may potentiate the effect *in vivo*.

In order to understand the probable binding of SR phenolics in SRAE, SRAE-characteristic compounds such HMBA, hydroxyl benzoic acid, vanillin and vanillic acids were also examined for their binding ability with HSA. Figure 3 provides the fluorescence emission spectra of different phenolic fractions of SR, which are constituted by different phenolic acids in a definite proportion (Table 2). Data together with the calculation of Stern-Volmer constant indicate that SRBP showed binding more or less equivalent to that of BCAE with a KSV of 0.203 X 106M-1 with a good correlation coefficient (r) of ~ 0.998 suggesting a stronger binding ability to HSA. SRHP and SRFP showed ~ 35 - 38 fold less binding than SRBP. SRFP appear to be less effective in binding than SRHP since the r value for SRHP is 0.977 which is acceptable than SRFP which showed an r value of ~ 0.67.

In order to understand the interaction with the constituents present in the extract, in the current study attempts were made to determine the binding efficiency based on the Stern-Volmer constant of individual constituent in pure form and relative percent contribution was calculated for BC and SR extracts. Data is presented in Table 2. Figure 4 has been plotted to depict the expected ability of these extracts in comparison with standards.

At equal phenolic concentration, BCAE showed more binding (80%) considering tannic acid as 100%, followed by SRFP (79%), SRAE (60%), SRBP (42%) and SRHP (30%). Increased efficiency in SRFP and SRAE could be due to the presence of HMBA, p-HBA and vanillic acid. Although similar levels and slightly higher levels of HMBA and vanillic acid were found in SRBP, ~ 2-fold reduction in activity could be due to

associated gallic acid which is a poorer quencher than gentisic acid which was found in SRFP. It should be noted that expected binding efficiency depicted in figure 4 varies with the actual experimental binding as depicted in Table 1. SRFP which was one of the best binder to HSA is almost equivalent to that of tannic acid (79%) when compared to SRBP (42%), SRBP showed stronger binding – 38 fold better than SRFP the extract. However, there was no alteration in BCAE. Data thus may suggest that phenolic constituents may interact themselves and may exhibit profound synergistic or antagonistic effects. Therefore biopotency of the antioxidant extract depends not only on the efficacy of antioxidant activity as estimated *in vitro*, but also on its bioavailability and biodistribution via binding to a carrier protein like HSA and their synergistic or antagonistic effect within themselves.

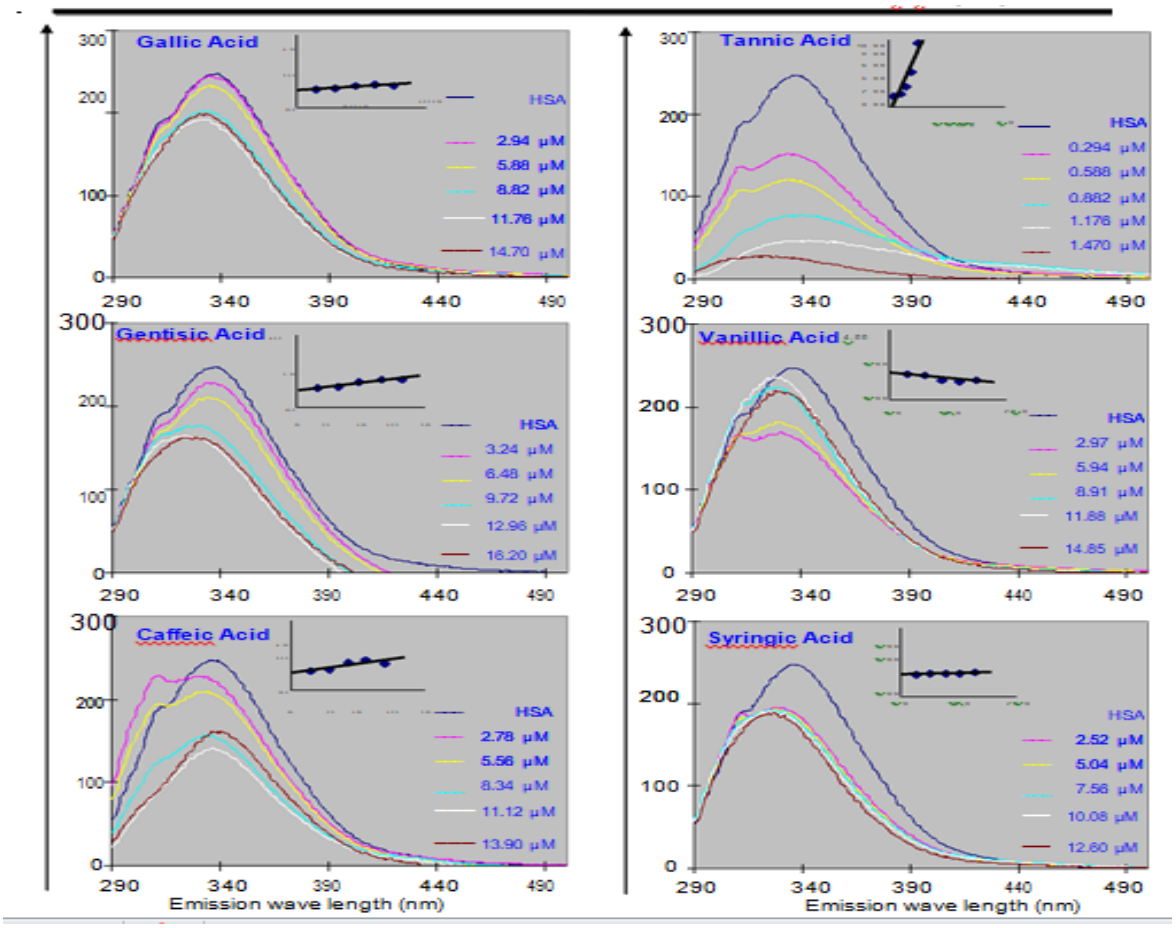

Figure 1: Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F^o/F).

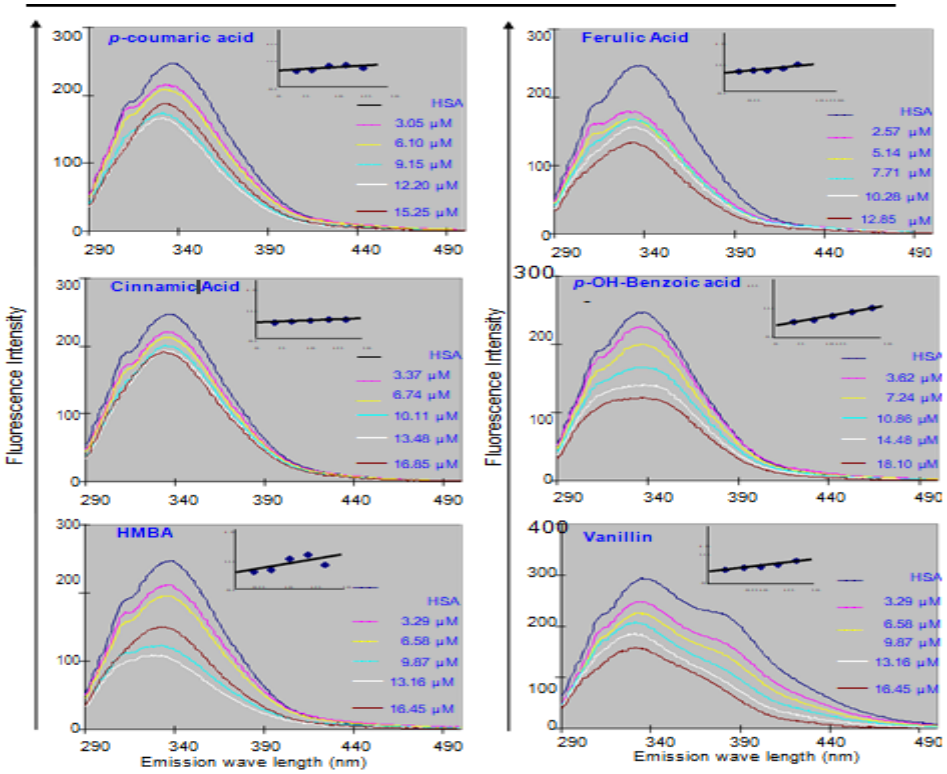

Figure 2: Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F^o/F).

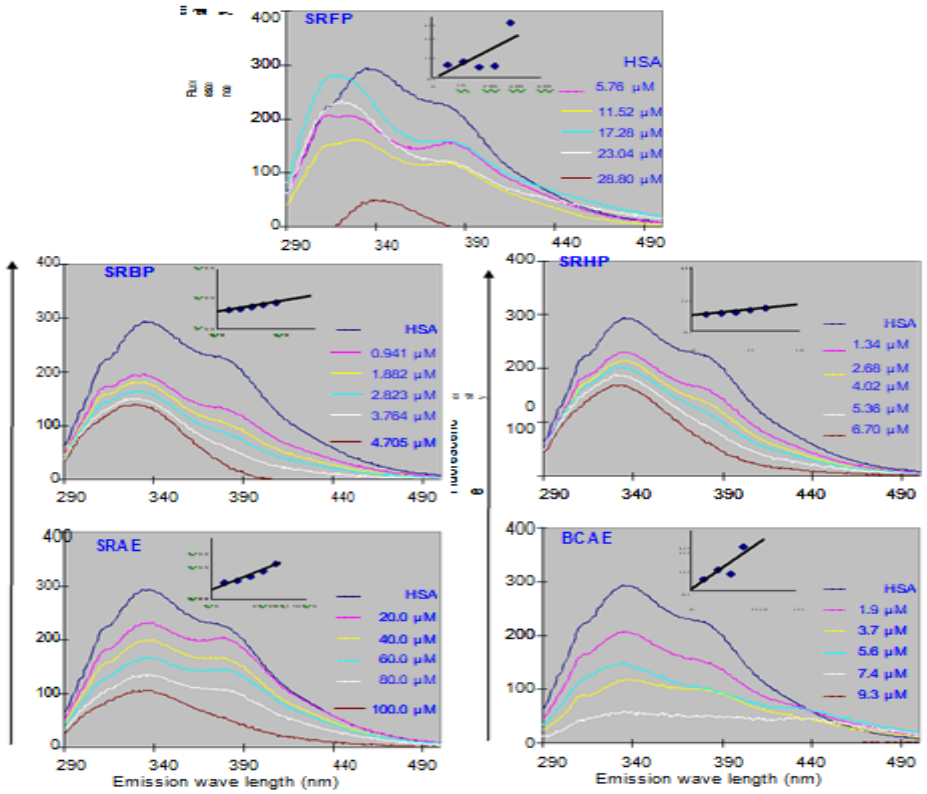

Figure 3: Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F^o/F.

Table 1: Stern-Volmer constants of the quenching of tryptophan fluorescence by phenolics and spice extracts.

Sample	Stern-Volmer Constant, Ksv (M^{-1})	Regression coefficient (r)
Tannic acid	3.178 x 10_6	0.931
BCAE*	0.249 x 10_6	0.836
SRBP*	0.203 x 10_6	0.998
Ferulic acid	0.084 x 10_6	0.909
HMBA	0.068 x 10_6	0.642
Syringic acid	0.059 x 10_6	0.988
SRHP*	0.058 x 10_6	0.977
Vanillic acid	0.053 x 10_6	0.894
SRFP*	0.053 x 10_6	0.670
Caffeic acid	0.050 x 10_6	0.828
p-HBA	0.043 x 10_6	0.994
p-coumaric acid	0.039 x 10_6	0.689
Gentisic acid	0.035 x 10_6	0.945
Cinnamic acid	0.024 x 10_6	0.983
Vanillin	0.019 x 10_6	0.981
Gallic acid	0.016 x 10_6	0.912
SRAE*	0.008 x 10_6	0.981

* Concentrations are expressed as gallic acid equivalent (GAE) phenol.

Table 2: Contribution of different phenolic components to total HSA fluorescence quenching in black cumin and swallow root extracts.

Sample	Contribution of phenolic components to the total fluorescence quenching (binding) capacity (in Units)				
	BCAE	SRAE	SRFP	SRHP	SRBP
Tannic acid	1341 (92%)	4940 (39%)	-	-	-
Ferulic acid	-	-	37 (>1%)	32 (3%)	46 (4%)
HMBA	-	7491 (58%)	1973 (22%)	490 (41%)	445 (34%)
Syringic acid	-	-	59 (>1%)	42 (4%)	14 (1%)
Protocatechuic acid	122 (8%)	-	278 (3%)	7 (>1%)	7 (>1%)
Vanillic acid	-	330 (3%)	566 (6%)	-	125 (10%)
Caffeic acid	-	-	53 (>1%)	28 (2%)	-
p-HBA	-	-	1994 (22%)	-	423 (33%)
p-coumaric acid	-	-	20 (>1%)	20 (2%)	63 (5%)
Gentisic acid	-	-	2289 (25%)	266 (22%)	-
Cinnamic acid	-	-	4 (>1%)	-	-
Vanillin	-	-	1706 (19%)	7 (>1%)	93 (7%)
Gallic acid	-	-	108 (1%)	298 (25 %)	76 (6%)

Numbers in the brackets indicate percent contribution to total fluorescence quenching

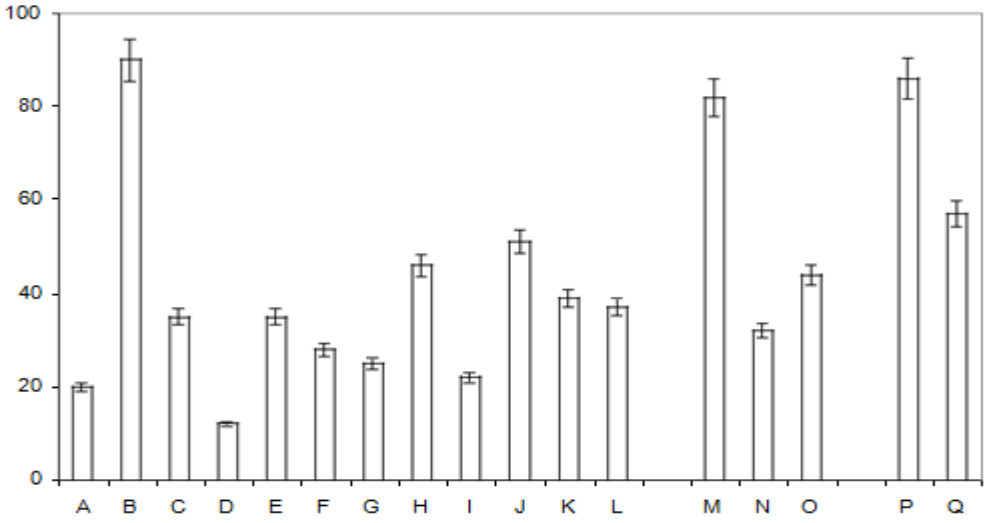

Figure 4: Percent fluorescence quenching (binding) of different phenolics at 2.5 µg/mL concentration.
A- Gallic acid; **B**- Tannic acid, **C**-Gentisic acid; **D**-Vanillic acid; **E**-Caffeic acid; **F**-Syringic acid; **G**-*P*-coumaric acid; **H**-Ferulic acid; **I**-Cinnamic acid; **J**-p-Hydroxybenzoic acid; **K**-HMBA; **L**-Vanillin; **M**- SRFP; **N**-SRHP; **O**-SRBP; **P**-BCAE; **Q**-SRAE. Values are mean ± SD.

DNA – phenolics interaction studies

Ethidium bromide emits intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It is reported that the enhanced fluorescence can be quenched by the addition of a DNA binding molecule. The quenching extent of fluorescence of ethidium bromide bound to DNA is used to determine the extent of binding between the second molecule and DNA (Liu *et al*., 2002). The emission spectra of ethidium bromide bound to DNA in the absence and presence of various isolated phenolics from black cumin and swallow root, free, hydrolyzed and bound phenolic acid extracts of swallow root and water extract of both the sources are given in Figure 5-7. The addition of these phenolics to DNA being in complex with ethidium bromide caused an appreciable reduction in emission intensity, indicating that the phenolics compete with ethidium bromide in binding to DNA.

At minimal phenolic concentration tannic acid had the highest binding capacity (24%) followed by *p*-coumaric acid (17%), caffeic acid (17%), gallic acid (15%), ferulic and cinnamic acid (14%). Hydrolyzed phenolic acid had stronger affinity for DNA with 14% followed by bound phenolic acid (13%) and free phenolic acid extract. The binding ability of SRWE, BCWE and *p*-HBA were almost negligible.

As noticed in protein-phenolic binding studies, tannic acid bound strongly to both protein and DNA. The degree of binding in one way may inhibit the molecule from getting oxidized and in that case it may protect. On the other hand its role in inhibiting the function of the bound component may not be ruled out. Further, the order of binding ability of BC extracts differ depending on the size of molecule either protein or DNA. The effect of phenolic acid in vivo in a given condition of disease may depend on the nature of the biomolecule circulating. This may to some extent responsible for contradictory effects of antioxidants *in vivo* when compared to that of *in vitro*.

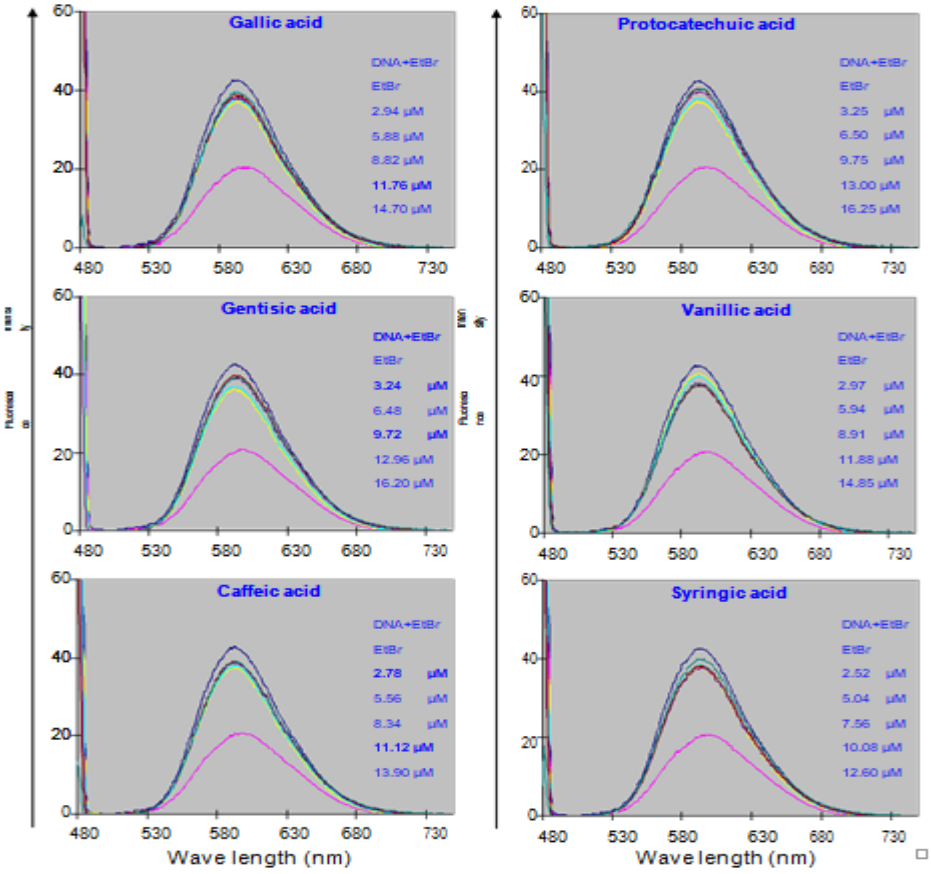

Figure 5: Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics, □exc= 478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml.

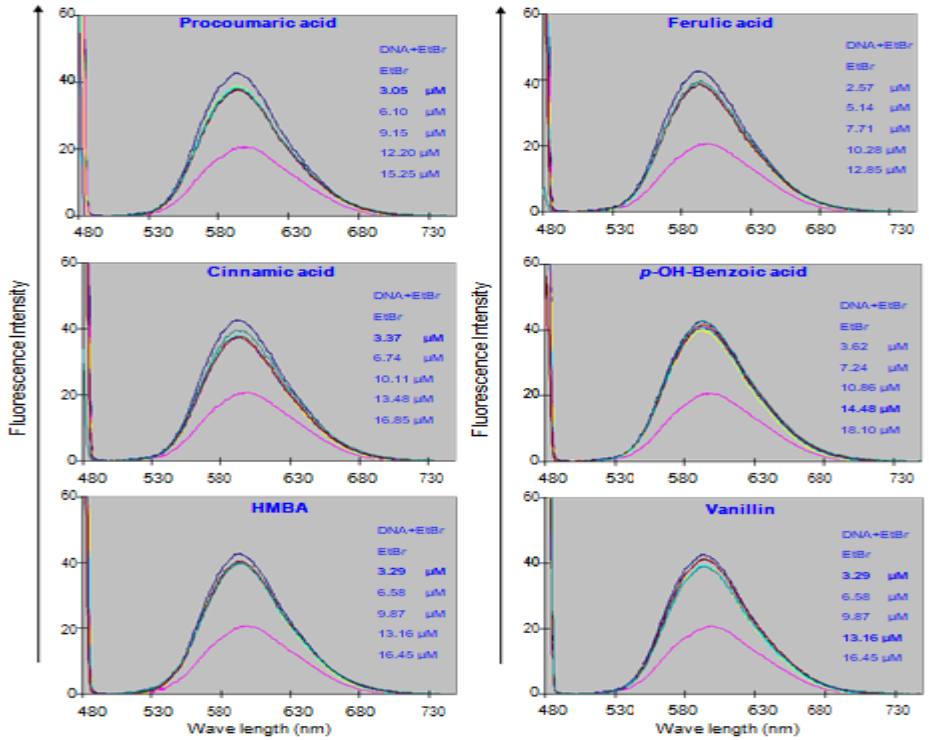

Figure 6: Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics and spice extracts, □exc=478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml.

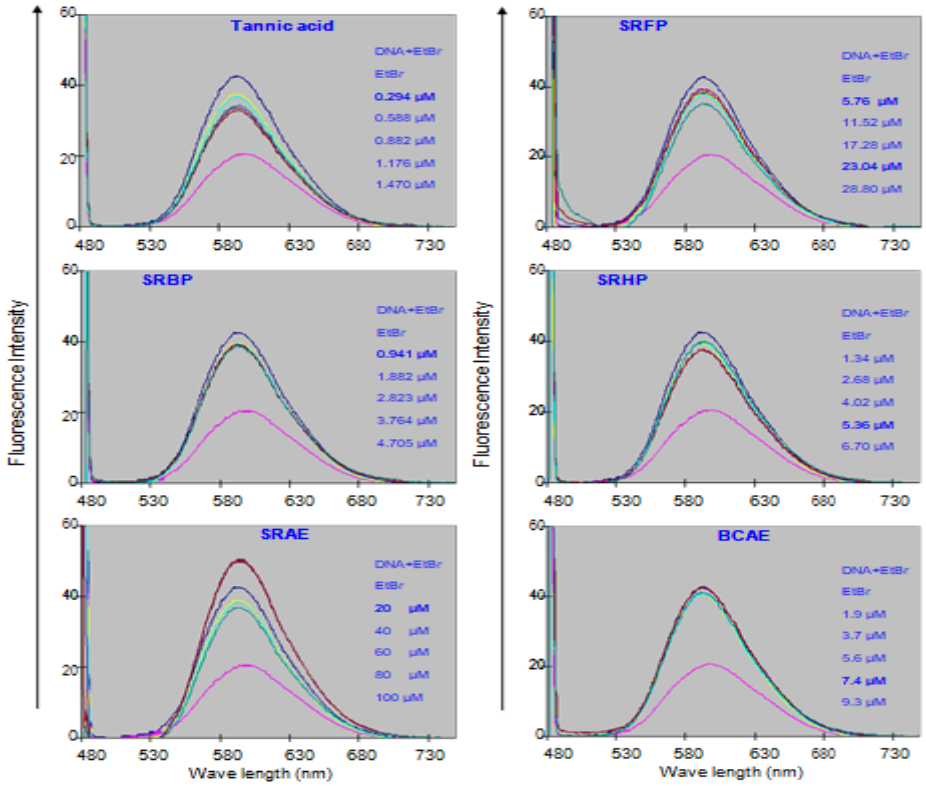

Figure 7: Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics, □exc= 478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml.

Table 3: Percent DNA binding of phenolic compounds and extracts of black cumin and swallow root.

Sample	% DNA binding
Tannic acid	46.72 ± 2.11
SRFP	35.28 ± 1.56
Gentisic acid	29.93 ± 0.98
Gallic acid	26.86 ± 2.08
SRAE	26.13 ± 2.03
Cinema acid	25.64 ± 2.01
Protocatechuic acid	24.18 ± 0.79
Caffeic acid	24.09 ± 1.58
SRHP	23.80 ± 1.66
p-coumaric acid	23.55 ± 1.02
Vanillic acid	23.21 ± 1.11
Syringic acid	22.77 ± 1.74
SRBP	20.00 ± 1.74
Ferulic acid	19.95 ± 1.02
Vanillin	17.18 ± 0.79
HMBA	13.92 ± 0.35
P-HBA	12.70 ± 0.68
BCAE	10.71 ± 0.19

Values are expressed as mean ± SD (n = 3).

DISCUSSION

HSA-phenolics interaction studies

Human serum albumin has been accepted widely as a principal transport protein for various drugs and phytonutrients including antioxidants and hence responsible for distribution of antioxidants through the circulation.

Phenolic antioxidants have been known for their therapeutic effects and have become an important component of nutraceuticals. One of the important mechanisms through which phenolic antioxidants exhibit bioactivity is via influencing longer half-life in the circulation and by avoiding themselves from degradation by drug degrading enzymes. Enhancement of bioactivity often has been attributed to its binding to human serum albumin. The most unique feature of serum albumin is its ability to reversibly bind to a wide variety of endogenous and exogenous bioactive compounds.

Human serum albumin (HSA) is a single-chain protein containing 585 amino acids and has an isoelectric point of 4.7. The protein has three homologous domains (I–III) and each of these is comprised of two sub domains (A and B). It contains a single tryptophan residue at position 214 in sub domain IIA (Kragh-Hansen *et al.,* 2002). The conformational changes of HSA were evaluated by the measurement of intrinsic fluorescence intensity of protein tryptophan residues at 296 nm before and after the addition of various phenolic compounds. Fluorescence measurements give information about the molecular environment in a vicinity of the chromophore molecules. Changes in the emission spectra of tryptophan are common in response to protein conformational transitions, subunit association, substrate binding or denaturation. Thus, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and their probable association reactions with phytonutrients (Tian *et al*., 2004).

Increasing concentrations of the isolated phenolic compounds, and crude extracts caused a linear reduction in the fluorescence of the tryptophan residues. The effect was strongest for tannic acid followed by bound phenolic acid extract and black cumin water extract. All phenolic acids studied decreased the fluorescence intensity significantly that their behavior could be compared to the quenchers.

Plant phenols are known to precipitate proteins and it is a well-known phenomena. The mechanism of this precipitation has been of interest for a long time, and hydrogen bonding and hydrophobic interactions have been reported as major binding modes. Hydrogen bonding between the phenolic hydroxyl group of plant phenols and the amide group of protein has been emphasized by the fact that phenol can be absorbed on the protein surface. They may interact with protein in reversible and irreversible ways leading to its conformational changes (Kawamoto *et al*., 1997). Thus tannic acid possessing the highest affinity to HSA is more effective in causing conformational modification of human serum albumin. Our data also indicated that although binding ability of BCWE is 31 fold higher than SRAE, increased abundance of phenolics 98 mg/g in SRAE as opposed to 3.2 mg/g in BCAE is evident. On ingestion of BCAE/SRAE extracts as used in traditional medicine the conformational changes are higher with SRAE. These changes influence the clearance rate and the bioavailability. During diseases more of binding of these antioxidants may inhibit oxidative damage to protein and DNA. This property hence may be useful in employing antioxidant as adjunct therapy during chemotherapy in cancer patients.

DNA – phenolics interaction studies

The interaction between DNA and phenolics isolated form the spice sources indicated higher binding ability for tannic acid compared to all other components used for the study. It is evident from the results that phenolic compounds which were simple in structure and aldehydes exhibited weak binding. The phenolic compounds which had almost similar structures at the aromatic ring showed similar binding ranging between 20-30%. These results suggest the possible role of hydroxyl groups in the process of intercalation. Earlier studies (Magdalena and Teresa, 2006; Whitley *et al*., 2003) have revealed the binding ability of tannic, gallic and ellagic acid to DNA. Tannic acid has been demonstrated to modify DNA bases by strongly binding to nucleic acid (Khan *et al.,* 2000). In our investigation, the ability of various other phenolic compounds especially hydroxybenzoic and cinnamic acid derivatives have been studied including the crude water extracts of *N. sativa* and *D. hamiltonii*.

Results from this study shows that the ability of majority of phenolic compounds to bind DNA is 50% lesser in comparison with tannic acid except for free phenolic acid extract which showed 25% lesser binding. The property of intercalation of these phenolic compounds appears to be related to the number of hydroxyls on the molecule (Ahmad *et al*., 1992), this fact can probably explain why phenolic compounds containing 1-3 hydroxyl groups demonstrates lesser affinity to DNA than tannic acid which has 21 hydroxyl groups. Thus the structural features of the used compound are important for its effect on a biomolecule such as DNA under appropriate conditions. The other possibility could be that binding to DNA is facilitated by the higher molecular size of tannic acid, which possibly gives rise to a greater hydrophobic character. Higher ionic strength does not inhibit the binding of tannic acid to DNA, suggesting that electrostatic interaction is not predominantly involved and possibly the binding is the result of a hydrophobic interaction (Goppelt *et al.,* 1981). Knowing that DNA is a highly organized macromolecular complex and its double helix provide a unique hydrophobic core of stacked bases (Jin *et al*., 1997), the last explanation

concerning the differences in affinity to DNA base pair between chosen chemicals seems quite probable. Moreover, the obtained results prove that the direct intercalation between phenolic compounds and DNA must be taken into account when evaluating the mechanism underlying the observed biological effects of these plant phenolic compounds.

REFERENCES

1. Ahmad, M. S., Fazal, F., Rahman, A., Hadi, S. M and Parish, J. H. 1992. Carcinogenesis, 13: 605-608.
2. Epps, D. E., Raub, T. J., Caiolfa, V., Chiari, A and Zamai, M. 1999. *The Journal of Pharmacy and Pharmacology*, 51: 41–8.
3. Fichtl, B., Nieciecki, A and Walter, K. 1991. Advances in Drug Research, 20: 117–66.
4. Goppelt, M., Langowski, J., Pingoud, A., Haupt, W., Urbanke, C., Mayer, H and Maass, G. 1981. Nucleic Acids Research, 9: 6115-27.
5. Jin, W. J., Wei, Y. S., Liu, C. S., Shen, G. L and Yu, R. Q. 1997. Spectrochim Acta Part A: Molecular and Biomolecular Spectroscopy, 53: 2701-7.
6. Kawamoto H, Mizutani K and Nakatsubo F. 1997. Phytochemistry, 46: 473-8.
7. Khan, N. S., Ahmad, A and Hadi, S. M. 2000. Chemico-Biological Interactions, 125: 177-189.
8. Kragh-Hansen, U., Chuang, V. T. G and Otagiri, M. 2002. *Biological and Pharmceutical Bulletin*, 25: 695–704.
9. Labieniec, M and Gabryelak, T. 2006. Toxicol *In Vitro*., 21: 146-56.
10. Levy, R. H and Moreland, T. A. 1984. Clinical Pharmacokinetics, 9: 1–9.
11. Lin, J. H., Cocchetto, D. M and Duggan, D. E. 1987. Clinical Pharmacokinetics, 12: 402–32.
12. Liu, J., Zhang, T., Lu, T., Qu, L., Zhou, H., Zhang, Q and Liangnian, Ji. 2002. *Journal of Inorganic Biochemistry*, 91: 269-276.
13. Oravkova, J., Bohs, B and Lindner, W. 1996. *Journal of Chromatography*: B, 677: 1– 28.
14. Rowland, M. 1980. Therapeutic Drug Monitoring, 2: 29–37.
15. Seedher, N and Bhatia, S. 2006. Pharmacological Research, 54: 77–84.
16. Song, Y. K., Billiar, T. R and Lee, Y. J. 2002. *American Journal of Pathology*, 160: 1069–1075.
17. Tian, J., Liu, X., Tian, X., Hu, Z and Chen, X. 2004. *Journal of Molecular Structure*, 691: 197-202.
18. Whitley, A. C., Stoner, G. D., Darby, M. V and Walle, T. 2003. Biochemical Pharmacology, 66: 907-915.