MOLECULAR CHARACTERIZATION OF HIRUDIN IN *POECILOBDELLA VIRIDIS (BLAANCHARD)* BY USING R- HIRUDIN

DESHMUKH S.S.
Department of zoology, shivaji science college, congress nagar, Nagpur (MS) India.
Corresponding Email : shitaldeshmukh@sscn.in

ABSTRACT : Disinfected leeches, *Poecilobdella viridis* were allowed to fed on the blood of healthy human volunteers (males and females) to know the biochemical alterations in human blood in response to injected leech saliva, which contains hirudin. Using various tools of bioinformatics, characterization of hirudin was investigated. It is found that a segment of hirudin between amino acid numbers 10 to 30 is antigenic in nature and this epitope contains a sequence, " GQNLCLCEGSNVCGQGNKCIL". The average antigenic prospensity for hirudin is found to be 1.0245. In the predicated region of the epitope beta sheet is seen indicating high amphipathic nature of hirudin. Radial immunodiffusion assay was carried out first by using r hirudin as antigen and it was found that hirudin is antigenic. Flexibility information which is useful in identification of potential antigenic sites, shows four potential antigenic sites between amino acid number 10 to 35. The predicted epitope (10 to 30) lies in this region only. This region showed four motifs, out of which three motifs are MHC class I related and motif is B cell related. The present results and the characterization of hirudin from various tools of bioinformatics strongly indicate the antigenic nature of hirudin and hence it is suggested that utmost precaution should be taken during application leech with respect to size and number of leeches so that adequate amount of leech saliva is administered in venous blood.

Key words : *Poecilobdella viridis*, characterization, RIA, antigen, sites

INTRODUCTION:

In recent years, doctors have make use of leeches to restore blood circulation in grafted or severely injured tissue mostly in surgery and trauma care, when blood accumulates and causes trouble. Leeches can be used to reduce the swelling of any tissue that is holding too much blood.

Surgeons worked out a method for stitching bisected arteries and veins together under a microscope, thus making it possible to reattach severed tissue and to transplant skin flaps. However, many of these operations failed because of a problem called venous congestion, inadequate blood drainage from the reattached or transplanted tissue. It is fairly easy to rejoin severed arteries that carry blood into the finger, says plastic surgeon Jeffery Friedman of Houston's Baylor college of medicine, but it is difficult to find and reconnect the veins that drain blood from finger as a result, even the most skilled and careful surgeon may not be able to link all the veins, and blood will begin to pool within the finger due to which clot may form resulting into cut off of blood flow into the finger, eventually killing it. Swelling and blue or purple colour signals venous congestion. When these symptoms appear, nothing is as effective as leeches, says Donald Mackay of Pennsylvania tate University College of medicine who has been prescribing leeches since 1988.

The benefits of the treatment lie not in the amount of blood that the leeches ingest, but in the anti-blood clotting (anticoagulant) enzymes in the saliva that allow blood to flow from the bite for up to six hours after the animal is detached, effectively draining away blood that could otherwise accumulate and cause tissue death. Zongli Huet al, (2009).

Leeches are used in the treatment of breast surgery. Haas G. in 1925 in the town of Giessess, Germany performed the first human hemodialysis in the history of medicine and hirudin served as the anticoagulant.

In 1994, a woman's scalp was ripped off when her hair was pulled into moving machinery. Doctors performing microsurgery in the University of Southern California, reattached the scalp, but the area of tissue swelled with congested blood. With no safe alternative available, the surgeons applied leeches, one at a time for eight days, to suck up the stagnant blood and allow proper healing . While the scalp healed, new capillaries formed in the scalp wound, and healthy circulation eventually is formed.

Leeches are commonly used to treat mental illness, inflammatory process to nephritis, laryngitis, eye disorders, brain congestion, tumors, skin disease, gout and whooping cough and even in obesity. A common treatment for headache is to apply several leeches and allow them to draw blood. They are also used for tonsillitis and piles by securing a leech with thread and lowering it into the patient's throat and allowing it to feed on the swollen glands (Chowkhande *et al.*, 1999). Research in US showed that a chemical derived from the leeches could help in reducing the death and heart attacks in people suffering from coronary heart disease. Leech saliva and its components have lead to the "treatment of cardiological and hematological disorders".

Hirudin has many advantages over the commonly used anticoagulant such as heparin. Hirudin does not interact with other blood proteins or the thin epithelial lining of blood platelets and, unlike heparin, can act upon bound thrombin. The most important feature of hirudin as a therapeutic is that it is a weak immunogen and thus is very unlikely to provoke an adverse reaction in patients during treatment (Markward *et.al.*,1982;1983). Hirudin is a strong thrombin inhibitor that does not need a cofactor for its activity. It does not require the action or the presence of antithrombin.

MATERIALS AND METHODS:

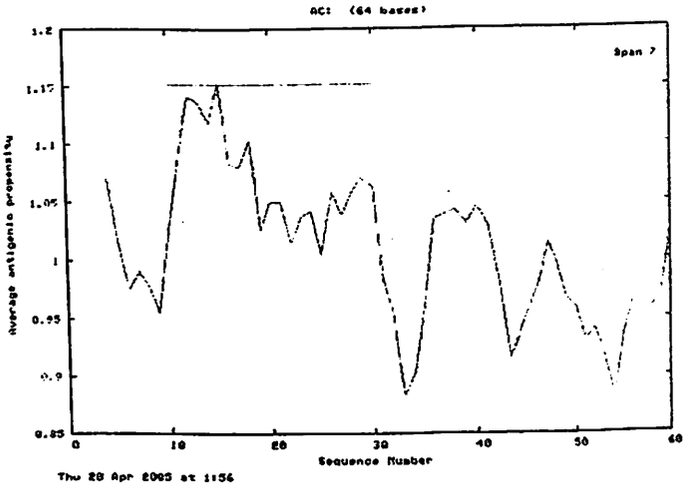
In the set of experiments four leeches were applied to each volunteer until sated and there after immediately hematological parameters were studied.

In one set of experiment r- Hirudin procurred from Dr. Jurgen Hofirger NAMOS (Nanotechnology of Biomimeties on surfaces), Germany. It was used as antigen to test its immune reaction *in vitro*. For this 5 volunteers were applied leeches (one each) for different duration's and antibodies against the leech saliva were allowed to form in volunteers for 96 hr. and then venous blood from the volunteers was removed after 96 hrs of leeching. Serum was separated, which contain antibodies against natural hirudin and other ingradients in leech saliva. The serum was used for immunoelectrophoresis, by Radial Immuno Diffusion Assay.

RESULTS AND DISCUSSIONS

VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEK
NQCVTGEGTPKPQSHNDGDFEEIPEEYLQ

The sequence is 65 residues long Average antigenic propensity for this protein is 1.0245



There is 1 antigenic determinant in hirudin sequence:

n	Start Position	Sequence	End Position
1	10	GQNLCLCEGSNVCGQGNKCIL	30

Fig. 1.1- Antigenic plot for sequence

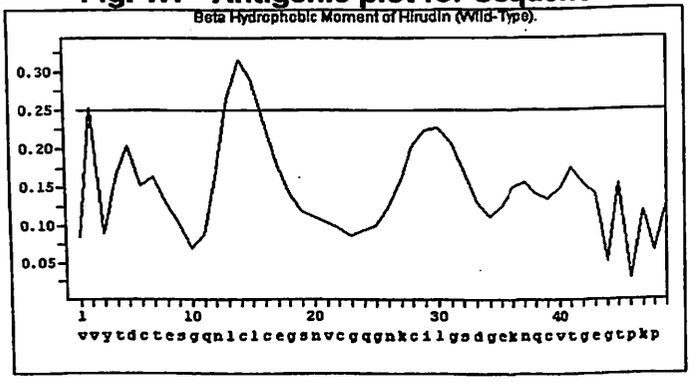
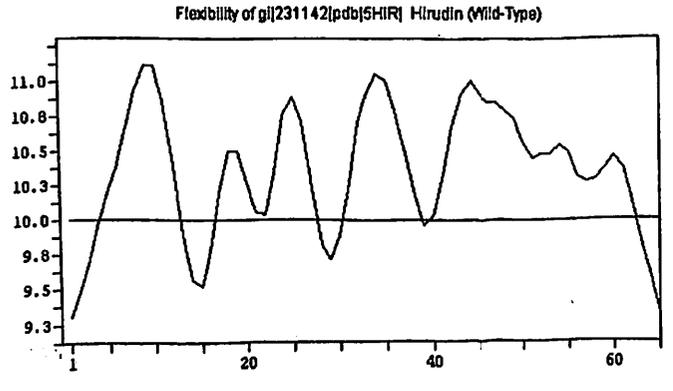
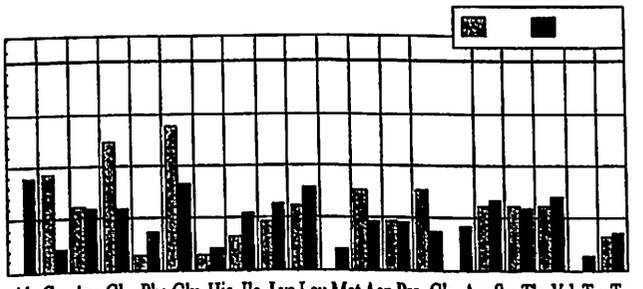


Fig. 1.2- BEETA HYDROPHOBIC MOMENT OF HIRUDIN

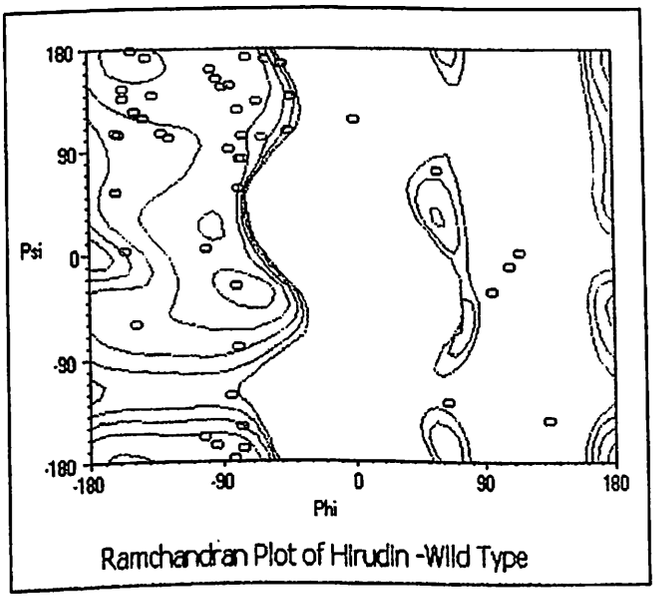



```
gi|2311421 VVYTDCTESGQNLCLCEGSNVCGQGNKCILGSDGEKNQCVTGEGTPKPQSHNDGDFEEI 59
 Consensus CBBBCCCCCCCCBBBBCCCBBBCCCCBBBBBCCCCCBBBBBCCCCCCCCCCCCCCCCC 59
Chou-Fasma BBBBBBCCCCCCBBBBCCCCCCCCCCBBBCCCCCCCBBBBCCCCCCCCCCCCCCHHH 59
   Garnier BBBBCCCCCCCBBBBBCCCCCCCCCCBBBBCCCCCCCBBBBCCCCCCCCCCCCCCHHH 59
  Homology CBBBCCCCCCCCBBBBCCCBBCCCCBBBBBCCCCCBBBBBCCCCCCCCCCCCCCCCC 59
Hydro Hone BBBBBBCCCCCCBBBBCCCCCCCCCCCBBBCCCCCCCBBBBCCCCCCCCCCCCCCHHH 59
Published  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC 59
Computer H CCCCCCCCCCCCCCCCCCCCCCCCCCCCBBBCCCCCCCCBBBCCCCCCCCCCCCCCC 59

gi|2311421 PEEYL                                                    64
 Consensus CCCCC                                                    64
Chou-Fasma CCHHH                                                    64
   Garnier HHHHH                                                    64
  Homology CCCCC                                                    64
Hydro Hone HHHHH                                                    64
Published  CCCCC                                                    64
Computer H CCCCC                                                    64

Sequence: gi|231142|pdb|5HIR| Hirudin (Wild-Type)
```

Fig. 1.4- Hirudin Secondary structure prediction



Ala Cys Asp Glu Phe Gly His Ile Lys Leu Met Asn Pro Gln Arg Ser Thr Val Trp Tyr



Conformation	Phi	Psi	Omega	Residues per turn	Translation per residue
Antiparallel beta	-139	+135	-178	2.0	3.4
Parallel beta	-119	+113	180	2.0	3.2
alpha helix	-57	-47	180	3.6	1.5
3-10 helix	-49	-26	180	3.0	2.0
Xi-helix	-57	-70	180	4.4	1.15
Polyproline I	-83	+158	0	3.33	1.9
Polyproline II	-78	+149	180	3.0	3.12
Polyproline III	-80	+150	180	3.0	3.

Fig . 1.6 Ramchandran Plot of Hirudin

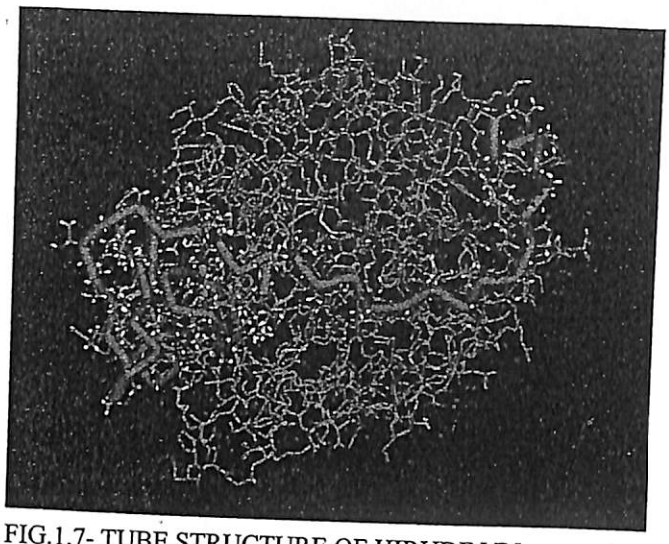


FIG.1.7- TUBE STRUCTURE OF HIRUDIN IN THROBIN-HIRUDIN COMPLEX.

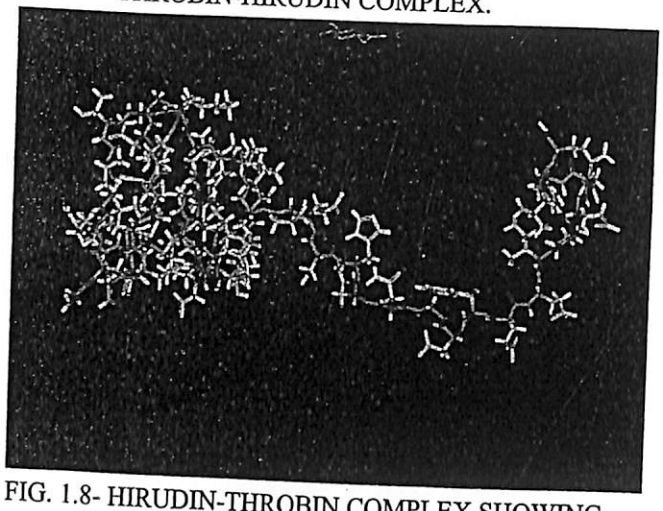


FIG. 1.8- HIRUDIN-THROBIN COMPLEX SHOWING SIDE CHAIN OF HIRUDIN

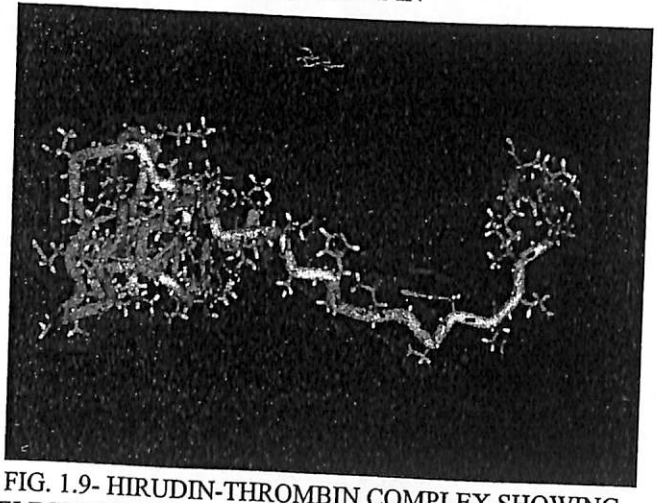


FIG. 1.9- HIRUDIN-THROMBIN COMPLEX SHOWING ELECTRON DENSITY MAP OF HIRUDIN MOLECULE

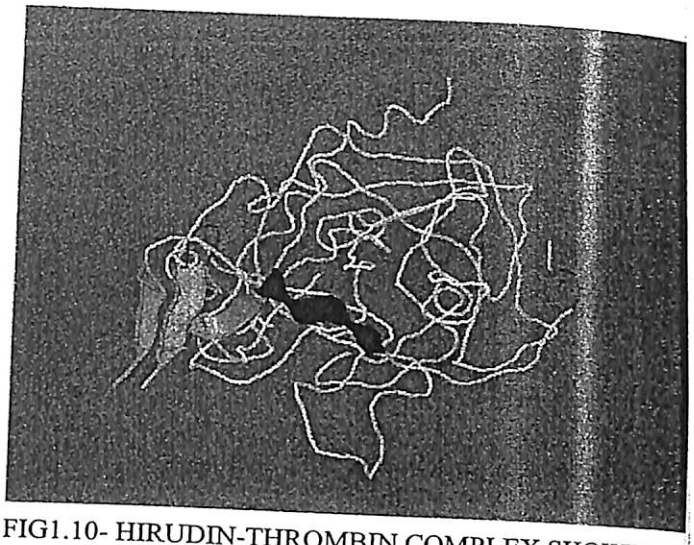


FIG1.10- HIRUDIN-THROMBIN COMPLEX SHOWING BEETA SHEET.

The production of anti hirudin antibodies raise the question as to whether the generation of anti-hirudin antibodies interferes with the anticoagulant activities of r-hirudin. The radial immunodiffusion assay in the present investigation demonstrated *in vitro* binding of the human anti hirudin antibody to r hirudin. However, the hematological investigation indicated that the antibodies against hirudin have no influence on the thrombin inhibition property of hirudin as the clotting time was found to be increased in all the volunteers tested. However, the experimental results of prothrombin time demonstrated that prothrombin time was not affected indicating that there are no binding sites of hirudin on prothrombin. To confirm this, help from various Bioinformatics sites is taken. Before predicting the epitope of hirudin let us briefly see the molecular characterization of hirudin.

There are three disulfide linkages located with-in the first 39 N- terminal residues of naïve hirudin. Hirudin contains a highly acidic N- terminal segment. There are 5 acidic amino acids, 4 glutamin and one Tyrosine SO_3H, within the last 9C-terminal residues.One of the likely reactive sites of hirudin, is a lysin residue flanked by two prolins. The part of molecule of hirudin shows hydrophilicity on kyte Doolittle scale and hydrophobicity on Hopp-Wood scale. These scales show 3 hydrophobic domains in a particular region. Removal of the acidic c-terminal amino acids of naïve hirudin results into loss of hirudin inhibition activity. Hirudin contains 3 molecules of lysine and 6 molecules of cysteine per molecule.The fragment between 14 to 22 (clcegsnvc) forms 4 motifs and they share the □ sheets.The epitope region is in □ sheet region.The motif map shows 4 motifs in epitope region of which three motifs are MHC class I related and one motifs is B̄cell related.The molecule shows high beta hydrophobic moment (Fig 1.2). The ratio of hydrophilicity to hydrophobicity is 1.54298. Percentage of hydrophilic amino acids is 53.0612 and that of hydrophobic amino acids is 46.9388. The estimated molecular weight of hirudin is 6969.57. Atomic composition is carbon (287) hydrogen (446),

Nitrogen (80), Oxygen (110) and sulfur (6) thus total number of atoms is 929. The details of amino acid composition is given in table is and fig. 1.5 it indicates that hirudin is glycerin rich with a frequency of 0.138. The isoelectric point is 3.82623. The hirudin N-terminus is globular and very tight because of the presence of the three disulfide bridges and its C-terminus is rather light with numerous amino acid residues. The hirudin N-terminal sequence is known to interact with the catalytic site of thrombin. In this context amino acid residues 52 to 56 are predicted to be extremely important for the link between hirudin and thrombin.

The above characterization is taken from DS gene protein sequence site after running the hirudin sequence. The purpose was to give the proof for our results of hirudin strong antegenicity of hirudin observed in radial immunodiffusion assay in the present investigation.

There are several reports indicating weak antegenicity of hirudin. However, in the present investigation, I have seen strong antigenic nature of hirudin and to support this, several above parameters were searched using various tools like Chou Fasma, Garnier, Hopp-wood. and several other tools. Antigenic plot for hirudin sequence was searched (Fig. 1.1) this is in accordance with Kolaskar and Tongaonkar method it found that amino acid number 10 to 30 form the epitope which is highly antigenic and the one and only one antigenic determinant. This epitope has a sequence, "GQNLCLCEGSNVCGQGNKCIL". The average antigenic prospensity for hirudin is found to be 1.0245. It is just a prediction by using different available tools.

Further hydrophobic movement of the hirudin molecule was studied for identifying amphipathic secondary structure i.e. helices and beta strands in the protein (Fig. 1.2) In the predicted region of the epitope beta sheet is seen indicating high amphipathic nature of hirudin. In the epitope, according to the Chas-Fasm secondary structure prediction method, LCLC sequence is having beta strand. This also supports the antigenicity of hirudin. Dodt J, et al, (1986).

Flexibility information is useful in identifying potential antigenic sites. Fig. 1.3 show four potential antigenic sites between amino acid number 10 to 35. The predicted epitope (10 to 30) lies in this region only.

Secondary structure production for hirudin was searched using tools like consensus, Chou Fasm, Garnier, Homology, Hydro Mome and is shown in fig. 1.4. The secondary structure prediction model also indicates the □ sheets in predicated epitopic region as given above.

In support of the secondary structure, Ramchandran plot for Hirudin is taken, which shows the large number of antiparallel □ sheets in Psi region (Fig. 1.6).

The densitometric analysis of all the fractions of immunological proteins indicated rize in g globulin fraction in the experimental blood samples after leeching. The densitometric graph shows four peaks indicating secretion of polyclonal antibodies. This finding in the present investigation indicates a complex nature of hirudin having many proteins with antigenic nature. Andreas Greinacher & Norbert Lubenow, (2001). This is because there is a long phylogenic distance between the annelid and a mammal.

REFERENCES:

Andreas Greinacher, Norbert Lubenow, (2001), " Cardiovascular Drugs -Recombinant Hirudin in Clinical Practice : Focus on Lepirudin." doi: 10.1161/01.CIR.103.10.1479. © 2001 American Heart Association, Inc.

Choukhande, M.S. ; R.U.Waghmare; S.C.Varshney (1999), "Leech application as an emergency treatment ' Inflamed piles". Cited form proceedings of National conference (antibacterial) ayurvedic medicines, held at New Delhi during 14-15 , 293-996.

Dodt J, Machleidt W, Seemüller U, Maschler R, Fritz H.,(1986), "Isolation and characterization of hirudin isoinhibitors and sequence analysis of hirudin PA".

Biol Chem, Hoppe Seyler. 1986 Aug;367(8) : pp.803-11. Haas, G. Versuche der Blutausw (1925), "Lebenden mit Hilte der Dialyse" . K; 4 (1) : 13-14.

Markward, F, G. Nowak ; J. Hoffmann (1983), "Comparative studies on thrombin inhibitors in experimental microthrombosis" . Thrombin Haemostar; 49 : 235-237.

Markward,F.(1982), "Vergleichende untersuchungen Zur antithrombotischen wirkung von Heparin and Hirudin. In : Haemostase, Thrombophile and Arteriosklerose". Van de Loo J. Asbeck F (ods). Schattauer, Stuttgart. New York: pp. 804-807.

U. Weydemann á P. Keup á M. Piontek, A. W. M. Strasser á J. Schweden, G. Gellissen á Z. A. Janowicz, (1995), " High-level secretion of hirudin by Hansenula polymorpha authentic processing of three different preprohirudins". Appl Microbiol Biotechnol (1995) 44:377Ð385 (Springer-Verlag 1995).

Zongli Hu, Ning Zhang, Feng Gu, Yong Li, Xiaojie Deng and Guoping Chen, (2009), "Expression, purification and characterization of recombinant targeting bifunctional hirudin in *Pichia pastoris*".

African Journal of Biotechnology Vol. 8 (20), pp. 5582-5588. online at http://www.academicjournals.org/AJB
ISSN 16845315 © 2009 Academic Journals