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Review

Mechanistic approach of drug discovery from natural substances for thrombocytopenia: A major cause of death in dengue fever patients

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Abstract

Dengue hemorrhagic fever has been one of the most important resurgent tropical diseases in the past 17 years. It is caused by dengue virus (DENV), belongs to family Flaviviridae and characterized as single stranded RNA virus with four serotypes. Proteins and viral entry are typical targets for developing new molecules. The advances in discovery of Dengue virus Nonstructural (NS) Protein complexes, RNA 5' triphosphatase and NS5 methyltransferase/RNA dependent RNA polymerase enzymes serve as potential targets for developing dengue virus related drugs using plant sources. High mobility group box 1 (HMGB1), emerging molecular mechanism underlying the regulation of pathogen-associated molecular patterns (PAMPs)-induced HMGB1 secretion, helpful in future for developing strategies to come with new drugs. Dengue virus relies on conformational changes in envelop protein E, to fuse the viral lipid membrane with endosomal membrane. There are no approved antiviral drugs or vaccines to combat dengue infection, although dengue vaccines have entered Phase 3 clinical trials. Drug discovery and development efforts against DENV and viral pathogen must overcome specificity, efficacy, safety and resistance challenges. As a consequence, the search for new antidengue agents from medicinal plants has assumed more urgency than past. Current review discusses about symptoms, efficient vector control strategies, pathogenesis, diagnosis, ongoing therapies, and its management and prevention using herbal drugs. The demand for plant based medicines is growing as they are generally considered to be safer, non toxic and less harmful than synthetic drugs. Current studies show that natural products represent a rich potential source of new antidengue compounds.

Key words: Dengue virus, dengue virus nonstructural protein, vaccines, high mobility group box 1, antidengue plants

1. Introduction

1.1 Dengue fever-A growing serious healthcare concern

Dengue/dengue hemorrhagic fever has been one of the most imperative resurgent tropical diseases in the past 17 years, with escalating geographic allocation of viruses and mosquito vectors, amplified incidence of epidemics, the growth of hyperendemicity and the manifestation of dengue hemorrhagic fever in new areas (Gibbons, 2010). The prevalence of this infection has augmented in tropics and subtropics of the world (Libraty *et al*., 2002; Yamada *et al*., 2003). It is projected that nearly one third of the world inhabitants are at threat of acquiring dengue infection with 100 million cases of dengue fever (DF) and half million dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), about 12,000 deaths occur worldwide yearly (Figure 1) (Gubler, 1998; Wang *et al*., 2000; Koraka *et al*., 2001, Leong et al., 2007). Dengue, the mosquito borne infection caused by four serotypes (type 1-4) of dengue virus. Stern forms (DHF/DSS) of dengue infection are non-predictable and deficient in awareness of the clinical features chiefly plasma leakage can lead to delayed identification of DHF and DSS. The existing epidemiologic inclination underscores the magnitude of dengue infections and there is necessity for appropriate comprehension of presentation patterns, diagnosis, treatment and long-term improvements for disease regulation and scrutiny of dengue infections. Some antiviral agents are available to treat dengue infection at the moment. The attempt to control dengue vector and introduction of safe and effective vaccine remain the important preventive measure (Callahan *et al*., 2001).

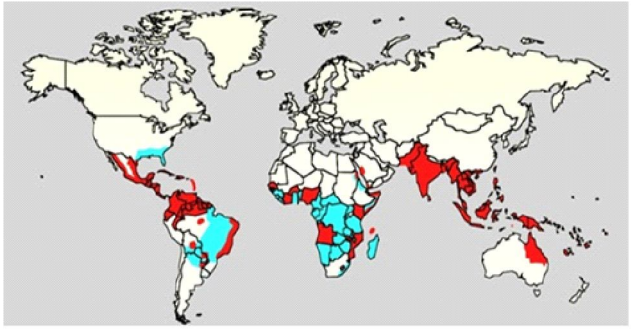

Figure 1: Worldwide prevalence of Dengue (2013) affected by *Aedes aegypti* flavivirus in epidemic (red) and non epidemic region (blue).

Source: WHO DengueNet (www.who.int/globalatlas).

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The geographical distribution of disease has greatly expanded some 7.5 to 8 billion people, live in area where dengue viruses can be transmitted. A pandemic in 1998 where 4.5 million cases of dengue fever and DHF were reported from 56 countries, however, only some cases reported in WHO (WHO, 2002; Muhamad *et al*., 2010). It is estimated that each year, 50 million infection cases occur with 7,00,000 cases of DHF and at least 20,000 deaths among children, fatalities could be twice as high (Figure 2).

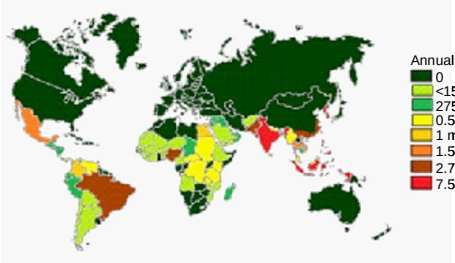

Figure 2: Dengue epidemiology in the world (2007-2013)
Source: WHO DengueNet (www.who.int/globalatlas).

The epidemiology of dengue fever in the Indian subcontinent has been very complex and has considerably changed over almost past six decades in terms of rampant strains, affected geographical locations and sternness of disease (Figures 3, 4, 5).

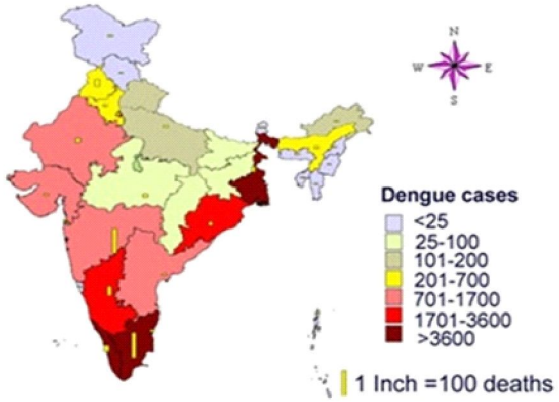

Figure 3: Dengue epidemiology in India (2013) showing number of death reported in a country in 2013
Source: National Vector Borne Disease Control Programme data

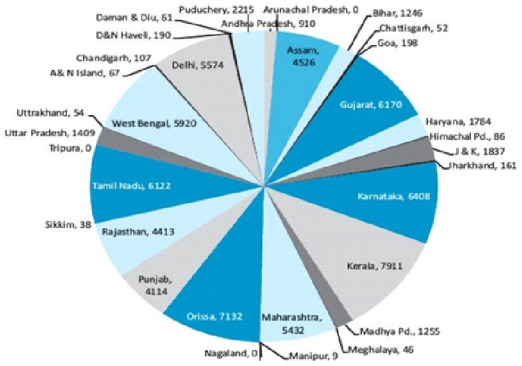

Figure 4: Distribution of dengue cases in Indian states in 2013
Source : National Vector Borne Disease Control Programme data

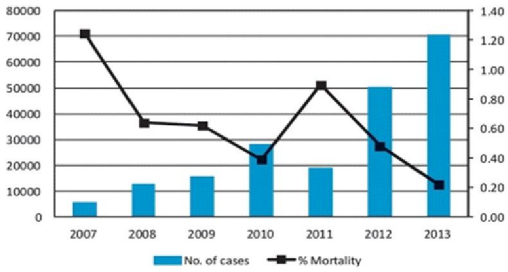

Figure 5: Total dengue cases reported to NVBDCP (left axis) and percentage mortality (right axis) in India, 2007-2013

1.2 Dengue virus and its serotype

Dengue (also called "break bone fever"), an important infectious disease that is caused by the dengue virus, (flaviviridae) of the genus flavivirus (Wang *et al*., 2002). The flaviviruses are surrounded by a spherical lipid envelope. The dengue virus is a single stranded RNA virus, approximately 11 kilo bases with an icosahedral nucleocapsid covered by a lipid envelope (Callahan *et al*., 2001). The dengue virus has four intimately related but dissimilar serotypes, DEN 1 to DEN 4; within which are several genotypes. The virion is composed of 3 structural proteins (known as core, membrane and envelope) and 7 non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5), proteins (Guzman and Kori, 1996). As the infection with dengue virus 1 provides lasting immunity, however, there is no cross shielding immunity to other dengue viruses, therefore, all dengue virus types may infect a person living in an endemic area. Dengue is understood to be an urban disease. The cycle of infection was maintained by viruses that use the mosquito (*A. aegypti*) as a vector to infect the human host, in turn which serves as sources of viral amplification. The *A. aegypti* is a small highly domesticated, black and white tropical insect that prefers to feed on humans during the daytime. There are two peaks of biting activity; early morning for 2 to 3 h. and in the afternoon for several hours before dark. It breads in artificial containers in and around homes. Female *A. aegypti* feeds on several persons and may transmit dengue virus to many persons in short course of time (Koraka *et al*., 2001; Hahn *et al*., 2001; Raja *et al*., 2009; Rodenhuis-Zybert *et al*., 2010).

1.3 Dengue virus structure

1.3.1 Viral structural proteins

E protein

The DENV E (envelope) protein, found on the viral surface, is important for the primary attachment of the viral particle to the host cell. Dengue virus is transmitted by a mosquito, *Aedes*. Several molecules interaction is through viral E protein ICAM 3-grabbing non-integrin (Navarro-Sanchez *et al*., 2003), CD 209 (Tassaneetrithep *et al*., 2003), Rab 5 (Krishnan *et al*., 2007), GRP 78 (Jindadamrongwech *et al*., 2004), and the mannose receptor (Miller *et al*., 2008) encompass essential factors mediating attachment and viral entry (Perera *et al*., 2008).

prM/M protein

The DENV prM (membrane) protein, important for formation and maturation of the viral particle, consists of seven antiparallel β-strands stabilized by three disulfide bonds (Perera *et al*., 2008). The glycoprotein shell of the mature DENV virion consists of 180 copies each of the E protein and M protein. The pr peptide linked with the E protein until the viral particle is released into the extracellular environment. This pr peptide acts like a cap, covers the hydrophobic fusion loop of the E protein until the viral particle has exited the cell (Perera *et al*., 2008).

1.3.2 Virus nonstructural protein

NS1 protein

The first nonstructural protein is a 48,000 molecular weight glycoprotein, hydrophilic, water-soluble, monomeric glycoprotein. NS1 may remain intracellular, be transported to the plasma membrane, or secreted from the cell. Secretion of large amounts of NS1 may be restricted to infected mammalian, not mosquito cells.

NS2 protein

The NS2 coding region consists of two hydrophobic proteins, NS2a (MW-20,000) and NS2b (MW-14,500). It is required for proper proteolytic processing of the C terminus of NS1.

NS3 protein

The serine protease is DENV NS3 (MW-70,000; hydrophilic protein), as well as RNA helicase and RTPase/NTPase. The protease domain consists of six β-strands arranged into two β-barrels formed by residues 1-180 of the protein. The catalytic triad (His-51, Asp-75 and Ser-135), found between two β-barrels, and its activity is dependent on the existence of the NS2B cofactor. This cofactor wraps around the NS3 protease domain and becomes part of the active site. The remaining NS3 residues form the three sub-domains of the DENV helicase. A six-stranded parallel β-sheet bounded by four α-helices make up sub-domains I and II, and sub-domain III is composed of 4 α-helices surrounded by three shorter α-helices and two antiparallel β-strands (Perera *et al*., 2008).

NS4 protein

Similar to proteins encoded in the NS2 region, NS4a (MW-16,000) and NS4b (MW-27,000) are hydrophobic proteins.

NS5 protein

The DENV NS5 protein is a 900 residue peptide with a methyltransferase domain at its N-terminal end (residues 1-296) and a RNA-dependent RNA polymerase (RdRp) at its C-terminal end (residues 320-900). The methyltransferase domain consists of an α/β/β sandwich flanked by N-and C-terminal subdomains. The DENV RdRp is similar to other RdRps containing palm, finger, and thumb subdomains and a GDD motif for incorporating nucleotides (Figure 6) (Perera *et al*., 2008).

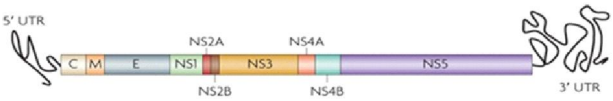

Figure 6: Dengue virus genome structure [encodes three structural (capsid [C], membrane [M], and envelop [E] and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) protein]
Source: Tomlinson *et al*., 2009.

Since DENV serotype has been divided into four categories and hence it has been differentiated by target genome position in the sequence of 5' to 3' targeting 3' NCR of the viral genome: (a) DEN-1 target genome position at 10469-10667 (199 bp); (b) DEN-2 at 10449-10659 (211 bp); (c) DEN-3 at 10289-10506 (218 bp); (d) DEN-4 at 10289-10517 (229 bp).

1.4 Antiviral agents

There are many compounds present in the universe that can be employed against viral diseases (Baranisrinivasan *et al*., 2009; Momtaz and Abdollahi, 2010; Vignesh *et al*., 2011). Squalamine, a compound obtained from dogfish shark and sea lamprey has the persuasive antiviral activity against many viruses including dengue (Zasloff *et al*., 2011). Concentration dependent protective effects on human endothelial cells against dengue had shown *in vitro*. In today's time, nearly thirty seven licensed antiviral drugs are present, yet no reliable antidengue drug is present (De Clercq, 2004; Czeizel *et al*., 2006). But, dengue propagation can be prohibited by targeting the susceptible sites of dengue life cycle using the small drug molecules (Wilder-Smith *et al*., 2007). According to Schul *et al*. (2007), the use of antiviral drugs after acute dengue infection can extensively cause the diminution in virus particles and virus-caused disease (Table 1). They further proposed that AG129 mice model is a suitable object to study the antidengue drugs. Another chemical compound NITD008, analogue of adenosine, also showed the potential antiviral activity against many vector-borne viruses especially dengue (Yin *et al*., 2009). The dengue virus titer and virus-caused disease reducing ability is shown in both *in vitro* and *in vivo* studies. Moreover the antiviral drug should be checked on the basis of its origin, cost, cytotoxicity, purification, *etc*. (Selisko *et al*., 2007). Many natural antidengue drugs derived from plants were rejected in past due to their difficult extraction and cytotoxic effects. Thus, the progress in antidengue drugs has many hurdles, but their development should be checked on critical basis.

Table 1: Antiviral agents used in management of dengue

S.No	Antiviral agent	Mode of action	Active against	Strain used	Reference
1.	2´ C methyl deaza-adenosine	Nucleoside inhibitor	Anti flaviviral	DENV and HCV	Migliaccio *et al*., 2003
2.	NITD008	Adenosine nucleoside prodrug	Anti flaviviral	↓ DENV *in vitro* and *in vivo*	Yin *et al*., 2009
3.	NITD203	Adenosine nucleoside prodrug	Anti flaviviral	↓ DENV, YFV, WNV, HCV	Chen *et al*., 2010
4.	1OAN1 and DN57opt	Peptide	-	Block viral cell binding, ↓ ADE *in vitro*	Costin *et al*., 2010
5.	Tetracycline	-	-	DENV-2, YFV-17D *in vitro*	Yang *et al*., 2007
6.	Doxorubicin	-	-	DENV-2, YFV-17D *in vitro*	Kaptein *et al*., 2010
7.	CD-14 monocyte receptor, clathrin medited endocytosis	RNAi	↓ Virus replication	Prevent DENV-2 entry and replication in human monocytes	Alhoot *et al*., 2011
8.	CM-10-18 and ribavirin	Broad spectrum nucleoside analog	↓ DENV infection	↓ DENV-2 viremia	Chang *et al*., 2011

1.5 Types of dengue syndrome

Dengue viruses are arboviruses, capable of infecting humans, and causing disease. These infections may be asymptomatic or may lead to (a) classical dengue fever, or (b) dengue hemorrhagic fever without shock, or (c) dengue hemorrhagic fever with shock. Dengue fever is a self-limiting disease and represents the majority of cases of dengue infection. An incidence of *Aedes aegypti* and *Aedes albopictus* together with movement of dengue virus of more than one type in any particular area tends to be associated with outbreaks of DHF/DSS.

Classical Dengue or break bone fever is an acute viral infection, caused by at least 4 serotypes (1, 2, 3 and 4) of dengue virus (Figure 7). It may occur epidemically or endemically. Epidemic is unstable and starts during rainy season when procreation of vector, *i.e., Aedes aegpyti* is abundant, temperature also play important role in conduction of virus when kept at 26°C, fail to transmit DEN-2 virus. *Aedes aegypti* is the main vector. Dengue outbreaks have also been attributed to *Aedes albopictus*, *Aedes polynesiensis*, and several species of *Aedes scutellaris* complex. Dengue hemorrhagic fever is a dangerous form of dengue fever, caused by supplementary infection with more than one dengue virus. The severe disorder is thought to be due to double infection with dengue virus-the first probably sensitizes the patient, while the second produces an immunological catastrophe. DSS have fever of 2-7 days along with warning signs including severe plasma leakage leading to shock and fluid accumulation with respiratory distress, severe bleeding, severe organ impairment of one of the organ include liver (liver: AST or ALT e" 1000; CNS: *e.g.*, seizures, impaired consciousness; heart: *e.g.*, myocarditis; kidneys: *e.g.*, renal failure) (Table 2). The heamagglutination inhibition (HI) test is generally used as the main serological test; using detection of IgM by antibody-capture ELISA as a reliable diagnostic test for both primary and secondary dengue infections (Table 3). In dengue fever and blood transfusion, paracetamol administration has been one of the important tools for treatment of dengue hemorrhagic fever and dengue shock syndrome as shown in Table 4.

During early infancy, severe dengue develops after a primary dengue virus infection. There has been a medical examination that severe dengue throughout the first year of life is seen only in chubby infants. Libraty and his co-workers (2015) examined the relations between the advancement of severe dengue and adipose tissue accumulation patterns. It was observed that adipose tissue contains two potential targets for dengue viral infection, *i.e.*, adipocytes and adipose tissue macrophages. During the first year of life, total body adiposity and visceral adipose tissue stores were at their highest levels (Libraty *et al.*, 2015).

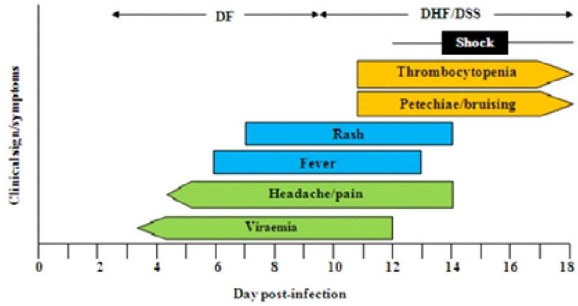

Figure 7: Generalized time course of the events associated with DF, DHF and DSS

The incubation period before the development if signs of infections generally range from 4 to 7 days.

Table 2: Description schedule for dengue fever

S.No.	Content	Syndrome		
		Classical dengue fever	**Dengue hemorrhagic fever**	**Dengue shock syndrome**
1.	Definition	Starts during rainy season when breeding of vector *A. aegypti* is abundant, temperature 26°C.	Is severe form of dengue fever, caused by one or more infection with more than one dengue virus.	Has dengue fever for several days with multiple severe warning sign
2.	Pathology	The mosquito becomes infective by feeding on patient from day before onset to the 5th day (viraemia stage). Followed by remission of few hours to 2 days (biphasic curve)	One dengue virus infection first sensitizes the patient while other produce immunological catastrophe.	
3.	Incubation period	3 to 10 days	4 to 6 days	2 to 7 days
4.	Symptoms	Chill, high fever, intense headache, muscle and joint pain, within 24 hours-retro orbital pain eye pressure photophobia	High fever Facial flushing, headache, tenderness at right costal margin, generalized abdominal pain, mucosal bleeding, increase in liver size	Severe plasma leakage shock Fluid accumulation with respiratory distress, Severe bleeding Severe organ impairment
5.	Other complications	Extreme weakness anorexia constipation altered taste sensation colic pain, abdominal tenderness dragging pain in inguinal, region sore throat, general depression rashes	Febrile convulsion Severe neutropenia Severe thrombocytopenia	Seizures Impaired consciousness Myocarditis Renal failure Severe neutropenia Severe thrombocytopenia
6.	Laboratory diagnostics	Widal test Complete blood count	Cytokines level Thrombocytopenia (100.00/mm^3 or less) Haemoconcentration (20%)	Liver: AST or ALT $\geq$ 1000 CNS Heart Kidney

Table 3: Summary of operating characteristics of dengue diagnostic method

Diagnostic methods	Diagnosis of acute infection	Time of result	Specimen	Time of collection after onset of symptoms	Facilities
Viral isolation and serotype identification	Confirmed	1-2 weeks	Whole blood, serum, tissues	1-5 d	Mosquito or cell culture facilities, BSL-2/BSL-3 laboratory, fluorescence microscope or molecular biology equipment
Nucleic acid detection	Confirmed	1 or 2 d	Tissues, whole blood, serum, plasma	1-5 d	BSL-2 laboratory, equipment for molecular biology
Antigen detection	Not yet determined Confirmed	1 d > 1 d	Serum Tissue for immunochemistry	1- 6 d NA	ELISA facilities Facilities for histology
IgM ELISA IgM rapid test IgG (paired sera) by ELISA, HI or neutralization test	Probable Confirmed	1–2 d 30 min 7 d or more	Serum, plasma, whole blood Serum, plasma,	After 5 d Acute sera, 1-5 d; convalescent after 15 d	ELISA facilities No additional supplies ELISA facilities BSL-2 laboratory for neutralization assay

Table 4: Treatment schedule for dengue

Condition	Drug	Dose	Frequency
Dengue fever	Paracetamol	500 mg	4 times daily
DHF grade 1 & 2	IV fluid 5% D/NSS In case of improvement IV fluid 5% D/NSS In case of no improvement IV 5% D/NSS	6 ml/kg/h Reduced to 3 mL/kg/h Increased to 10 mL/kg (1h) then reduced to 6 mL/kg/h Then to 3 mL/kg/h	Over 3 h For 6-12 h
DHF grade 3 & 4	IV fluid 5% D/NSS In case of improvement IV 5% D/NSS Blood transfusion 10 mL/kg/hr3 mL/kg/hr IV fluid 5% D/NSS	 20 mL/kg	Over 3 h For 24-48 h
DSS	Dopamine Epinephrine Norepinephrine	40 mg/mL; 80 mg/mL 0.1 to 1 µg/kg/min 0.1 to 2 µg/kg/min	2 times

1.6 Dengue fever associated thrombocytopenia

Thrombocytopenia (TCP) is defined as a reduction in the peripheral blood platelet count below the lower limit of normal (usually less than 150×10^9/L). Thrombocytopenia is associated with abnormal bleeding that includes spontaneous skin purpura and mucosal haemorrhages as well as prolonged bleeding after trauma. However, spontaneous haemorrhagic tendency becomes clinically evident only after severe depletion of the platelet count to level of about 20,000/µL.

Thrombocytes are small (1-4 µm in diameter), discoid, non-nucleate structures containing red-purple granules. The normal platelet count ranges from 150,000-400,000/µL and its life span is 7-10 d. Newly formed platelets spend 24-36 h in the spleen before being released into circulation but splenic stasis does not cause any injury to platelets normally.

The salient features of the ultra structure of the platelet are as under.

i) The mucopolysaccharide surface coat is the structural basis of platelet factor 3 and is important in platelet adhesion and aggregation as early events in thrombosis.

ii) Microfilaments and microtubules maintains the discoid shape of platelets.

iii) Granules perform important functions (Underwood, 1992).

The main function of platelets is the formation of haemostatic plug during normal haemostatic response, clot stability and retention as well as in vascular repair and antimicrobial host defense to vascular injury.

Thrombocytopenia may result from 4 main groups of causes:

i) Impaired platelet production

ii) Accelerated platelet destruction

iii) Splenic sequestration

iv) Delusional loss

1.7 Causes of thrombocytopenia

1. Impaired platelet production
   i) Generalized bone marrow failure in aneamias and cancers
   ii) Selective suppression of platelet production due to drugs (quinine, quinidine, sulphonamides, PAS, riphampicin, anticancer drugs, thiazid diuretics) and alcohol intake
2. Accelerated platelet destruction
   i) Immunologic thrombocytopenia in neonatal and post-transfusion, drug-induced, secondary immune thrombocyto penia, post infection, and lymphoma).
   ii) Increased consumption, *e.g*., DIC, TTP, gaint haemangio mas, microangiopathic haemolytic anaemia.
3. Splenic sequestration in splenomegaly
4. Dilutional loss due to massive transfusion of old stored blood to bleeding patients

Changes in thrombocytes by dengue virus-platelet interaction and the involvement of anti-DEN antibody to such changes were studied *in vitro* with the aim to explore a mechanism, how the DEN causes prominent thrombocytopenia characteristically seen in DHF. The results obtained showed that: (i) DEN antigen attached to human platelets without immune-mediated reaction, (ii) a decrease in platelet count was more markedly demonstrated by the binding of anti-DEN antibody on the DEN antigen associated with platelets than by the binding of the antigen-antibody complex on platelets, (iii) a modulation of endothelial cell by the infection of DEN to the cell was suggested as one of the causes of the thrombocytopenia (Funahara *et al*., 1987). Clinical manifestations of thrombocytopenia are not related only to the number of peripheral platelets in dengue infection, but its recovery is associated with clinical improvement. The level of platelets correlates with the vascular leakage of proteins and liver damage.

1.8 Complications associated to severe thrombocytopenia in patients with dengue are:

i) Dengue chorioretinitis and dengue-related ophthalmic complications

ii) Complications in pregnant patients

iii) Cardiac manifestations of dengue are uncommon but cardiac rhythm disorders such as atrioventricular blocks, atrial fibrillation, sinus node dysfunction and ectopic ventricular beats have been reported during episodes of DHF (Chuah, 1987; Veloso *et al*., 2003; Promphan *et al*., 2004).

iv) Neurological encephalopathy manifestation of dengue

v) Gastrointestinal manifestations of dengue are increasingly being identified and reported, such as hepatitis, fulminant hepatic failure, acalculous cholecystitis, acute pancreatitis, acute parotitis and febrile diarrhoea.

vi) Hepatic manifestations can be characterized by manifestations of acute hepatitis with pain in the hypochondrium, hepatomegaly, and jaundice with raised aminotransferase levels.

vii) Respiratory manifestations of dengue

viii) Lymphoreticular complications of dengue: Dengue virus antigen is found predominantly in cells of the spleen, thymus and lymph nodes. In DHF, lymphadenopathy is observed in half of the cases and splenomegaly is rarely observed in small infants.

ix) Musculoskeletal complications of dengue fever: Dengue fever has been described classically as break bone fever as it causes severe muscle, joint and bone pain. Rhabdomyolysis is not well characterized in DHF. There are a handful of case reports recognizing this complication (Gunasekera *et al*., 2000; Davis and Bourke, 2004).

1.9 Diagnosis

The heamagglutination inhibition (HI) test has been widely used as the main serological test; using detection of IgM by antibody-capture ELISA is a reliable diagnostic test both in primary and secondary dengue infections (Sa-Ngasang *et al*., 2006). A speedy increase in cytokines levels and chemical mediators during dengue disease might play a crucial role in inducing plasma leakage, shock and hemorrhagic manifestations (Sithiprasasna *et al*., 2004).

1.10 Drug targets

The objective of virtual screening is to discover new molecules that can connect to the targets and modulates the functions of biomolecules. Several investigations were conceded to discover the probable targets to treat viral infections. Proteins and viral entry are emblematic targets for developing new molecules. Viral entry inhibitors (Marijkem *et al*., 2011; Alen and Schols, 2012) and protein inhibitors are the major drugs to treat the viral diseases. Recent discoveries revealed that there is several protein and non-protein potential targets were discovered to develop antidengue agents. Various studies have investigated approaches restrain the viral particle entry. Membrane (M), and Envelope (E) proteins are the vital structural proteins which play an important role in virus entry into the cell. These are the one of the main targets to design and develop potential virus entry inhibitors. Non-structural (NS), proteins play a major function in the viral replication. NS Protein complex include NS3, NS2B, NS3 helicase/nucleoside triphosphatase, RNA 5' triphosphatase and NS5 methyltransferase/ RNA dependent RNA polymerase enzymes. All these NS proteins may serve as potential targets for developing dengue virus related drugs using *in silico* approach since they are required for viral replication. Both dengue NS1 antigen and serum IL-10 levels have shown association with severe clinical disease in acute dengue infection. The IL-10 has also been shown to suppress dengue specific T cell responses (Narmada *et al*., 2015).

1.11 Targets approaches for the treatment of thrombocy topenia

Cytokines and growth factors, such as interleukin-11 and thrombopoietin (TPO), play a key role in the production of platelets. A number of current clinical studies have provided substantiation, those pharmacologic agents that target megakaryocyte precursors and stimulate thrombopoiesis, and successfully reverse thrombocytopenia. Here, we review the decree of thrombopoiesis, the role of TPO, and a number of innovative compounds that stimulate platelet production by acting through the TPO receptor. Agents that stimulate TPO include the orally available nonpeptidic agonists eltrombopag and AKR-501, peptidic agonists AMG-531 and Peg-TPOmp, and small engineered antibodies (Afdhal and McHutchison, 2007). The chronic liver disease and hepatitis C

virus (HCV) patient recurrently experience thrombocytopenia that complicates the execution of the disease. Traditional therapy for thrombocytopenia consists of platelet transfusion, which can be linked with significant safety and economic issues. Consequently, efforts have been directed toward developing novel approaches for the treatment.

1.12 Possible natural substances for treatment of thrombocy topenia

There are number of compounds reported on the DENV antiviral activity of phytochemicals such as flavonoids, which are low molecular weight phenolic compounds found in different kinds of plants (Laille *et al*., 1998; Zandi *et al*., 2011). Recently, *in vitro* treatment of infected cells with quercetin resulted in 75% diminution of intracellular replication of DENV-2, and pinostrobin was exposed to inhibit DENV-2 NS2B/NS2B/NS3 protease in an *in vitro* study (Kiat *et al*., 2006). Another study reported inhibitory activity of several flavonoid derived compounds against DENV-2 in hepG2 cells, with a range of potency strengths of 72%-100% (Muhammad *et al*., 2010). Methanolic extracts of *Andrographis paniculate* and *Momordica charantia* showed 75%-50% antiviral inhibitory effect respectively, against DENV-1 replication in Vero cells (Table 5) (Tang *et al*., 2012).

Table 5: Herbal drugs for dengue

S.No.	Common name (Botanical name; family)	Parts/Type of extracts	Compound	Type of study/ mechanism	Type of strain / serotype	References
1.	Fagonbushes (*Fagonia indica*; Zygophyllaceae)	Whole plant Acetone extract	-	Antiviral	Dengue virus	Tonk *et al*., 2006
2.	Japanees orchid (*Gastrodia elata*; Orchidaceae)	Rhizome	D-glucan	Antidengue Interfere with virus adsorption in early stage of virus cycle	DENV-2	Tong *et al*., 2010
3.	*Meristiella gelidium*; Solieriaceae	Whole plant Polysacc extract	Polysaccharides Kappa carageenan (5)	Inhibit CPE infected Vero E6 cells	Dengue virus	Kadir *et al*., 2013
4.	Alligator weed (*Alternanthera philoxeroides*; Amaranthaceae)	Whole plant / Pet. ether extract	Coumarin	Antidengue, antiviral Inhibitory cytotoxicity in C6/36 cell lines	DENV serotype	Jiang *et al*., 2005
5.	Bakau (*Rhizophora apiculata*; Rhizophoraceae)	Whole plant / Pet. ether extract	-	Larvicidal inhibit DENV by inactivating viral particle activity	DENV-1	Renugadevi *et al*., 2012
6.	Desert date (*Balanites aegyptiaca*; Zygophyllaceae)	Whole plant/ Aqueous extract	-	Larvicidal inhibit CPE infected Vero E6 cells	-	Weisman and Chapagain, 2006
7.	Bitter melon (*Momordica charanthia*; Cucurbitaceae)	Fruit	-	Antiviral inhibit CPE infected Vero E6 cells	DENV-1	Kadir *et al*., 2013
8.	Black bean (*Castanospermum austral*; Fabaceae)	Stem bark/ Methanolic extract	Alkaloid Castanospermine (11)	Antidengue ER alpha glucosidase I inhibitorInhibitory infection of subset of envelop RNA and DNA virus *in vitro*	Dengue virus	Whitby *et al*., 2005
9.	Brown seaweed (*Cladosiphon okamuranus*; Chordariaceae)	Whole plant	Sulphated Polysacc Fucoidan (3)	Reduce infectivity by 20% as carboxy reduced fucoidan glucuronic acid converted to glucose attenuated inhibitory activity	DENV-2	Kadir *et al*., 2006
10.	Bushy matgrass (*Lippie alba*; Verbenaceae)	Whole plant	Essential oil		Dengue virus	Kadir *et al*., 2006
11.	Carrageen moss (*Chondrus crispus*; Gigartinaceae)	-	Sulphate polysaccharide	Antiviral inhibit virus entry	DENV-2	Talarico and Damonte, 2007
12.	Cat's claw (*Uncaria tomentosa*; Rubiaceae)	Stem barks	Pentacyclic oxindole alkaloid (12)	Antiviral Inhibit DENV-2 Ag detection in monocytes	DENV-2/ Dengue Ag+ cell	Reis *et al*., 2008

13.	Chameleon plant (*Houttuynia cordata*; Saururaceae)	Whole plant, aerial stem, leaves Aqueous extract	Hyperoside (6)	Inhibit viral RNA replication inhibit action against DENV through direct inactivation of viral particle before infection of cells	RNADENV-2	Vijitttra *et al.*, 2012
14.	Chettaphangkhee (*Cladogynos orientalis*; Euphorbiaceae)	Whole plant / dichloromethane extract	-	Antidengue inhibit viral particle activity inhibiting CPE in Vero cells	DENV-2	Thirapanmethee *et al.*, 2011
15.	Chinese ginger (*Boesenbergia rotunda*; Zingiberaceae)	Rhizome	Flavonoid, cyclohexenyl, 4 hydroxy panduratin A (1), Panduratin A (2)	Antidengue inhibit DENV-2 NS3 protease *in vitro*	DENV-2 virus NS3 protein	Kiat *et al.*, 2006
16.	Chmberbitter (*Phyllanthus urinaria*; phyllanthaceae)	Aqueous and methanolic extracts	-	Antidengue Strong inhibitory activity against DENV-2 with more than 90% of virus reduction at MNTD	DENV-2	Lee *et al.*, 2013
17.	Gall oak (*Quercus lusitanica*; Fagaceae)	Seeds/methanol extract	-	Down regulation of NS1 protein expression in infected C6/36 cells	DENV-2	Sylvia, 2008
18.	Gatas-gatas (*Euphorbia hirta*; Euphorbiaceae)	Leaf; decoction/ ethanolic extract	-	Anti-thrombocytopenia	Platelet count enhancement	Apostol *et al.*, 2012
19.	Guava (*Psidium guajava*; Myrtaceae)	Leaves	Quercetin (13)	Antidengue, antithrombocytopenia inhibit formation of enzyme mRNA in virus	Platelet count enhancement	Kadir *et al.*, 2013
20.	Hempedu Burmi (*Andrographis paniculate*; Acanthaceae)	Leaves; methanolic extract	Flavonoids	Antiviral activity inhibit viral replication with 50% inhibition in CPE	DENV-1 serotype	Kadir *et al.*, 2013
21.	Holy basil (*Ocimum sanctum*; Labiatae)	Leaves	-	Antiherpes simplex virus activity inhibit CPE in Vero E6 cells	Dengue virus	Yucharoen *et al.*, 2011
22.	Krachai Dam (*Kaempferia parviflora*; Zingiberaceae)	Leaf and stem	-	Virucidal activity inactivate DENV-2 virus particle activity	DENV-2	Phurimsak and Leardkamolkarn, 2005
23.	Lemon grass (*Cymbopogon citrates*; Poaceae)	Whole plant	-	Antiviral inhibit CPE infected Vero E6 cells	DENV-1	Kadir *et al.*, 2013
24.	Lemon verbena (*Lippie citriodora*; Verbenaceae)	Whole plant	Essential oil	Antidengue, Antiviral inhibitory effect on DENV replication in Vero cells and has inacti-inactivated viral partcle activity.	DENV 1-4	Ocazionez *et al.*, 2010
25.	Lolot pepper (*Piper sarmentosum*; Piperaceae)	Leaf/Ethanol extract	Ascaricin	Larvicidal effective against early 4th instar larvae of *A. aegypti*	DENV-1	Chaithong *et al.*, 2006
26.	Long pepper (*Piper retrofractum*; Piperaceae)	Whole plant/ dichloromethane extract	-	Inhibitory action against DENV-2 infected cells by inactivating viral particle activity	DENV-2	Kadir *et al.*, 2013
27.	Marine eelgrass (*Zostera marina*; Zosteraceae)	Whole plant	Zosteric acid (10)	Antidengue inhibitory effect on DENV replication in LLC-MK2 cells	Dengue virus	Kadir *et al.*, 2013

28.	Neem (*Azidarachta indica*; Meliaceae)	Leaf Aqueous extract	-	Antiviral inhibit replication of virus by inhibiting CPE leading to absence of 511 bp	DENV-2	Parida *et al*., 2002
29.	Indian oleander (*Nerium indicum*; Apocynaceae)	Roots and leaves	-	Larvicidal	Dengue virus	Sharma *et al*., 2005
30.	Nirgundi (*Vitex negundo;* Lamiaceae)	Whole plant and leaf oil	-	Antiviral	Dengue virus	Ahmad *et al*., 2011
31.	Papaya (*Carica papaya*; Caricaceae)	Leaf/Aqueous extract	Flavonoids	Anti-thrombocytopenia / antiviral Inhibit NS2b /NS3 protease prevent DENV-2 viral assembly	Platelet count, WBC, neutrophils DENV-2	Ahmad *et al*., 2011
32.	Punarnava (*Boerhaavia diffusia*; Nyctaginaceae)	Stem	-	Anti-dengueInhibit viral replication	Dengue virus	Bharati and Sinha, 2012
33.	Red seaweed (*Cryptonemia crenulata*; Halymeniaceae)	Whole plant	Galactan (4)	Selective inhibitor of DENV-2 multiplication in Vero cells, Inhibitory effect increase with C2S 5 at both stages of adsorption and internalization	DENV-2	Kadir *et al*., 2013
34.	Red seaweed (*Gymno-gongrus griffithsiae*; Phyllophoraceae)	Whole plant	Kappa carageenan (5)	Antiviral inhibitory action against DENV-2 multiplication	DENV-2	Kadir *et al*., 2013
35.	Red seaweed (*Gymnogon-grus torulosus*; Phyllophoraceae)	Whole plant	Galactan (4)	Antiviral Inhibitory action against DENV-2 in Vero cells	DENV-2 but ineffective in DENV-1	Kadir *et al*., 2013
36.	Sea buckthorn (*Hippophae rhamnoides*; Elaeagnaceae)	Leaf extract	-	Antidengue maintain cell viability by inhibiting in plaque number after treatment of infected cellsIncrease TNF-α and α IFN-γ derivedhuman macrophage	DENV-2 Blood	Jain *et al*., 2008
37.	Yellow berried night shade (*Solanum xanthocarpum*; Solanaceae)	Whole plant	-	Effective against malarial vector	Dengue virus	Mohan *et al*., 2007
38.	Dodda vana mugali (*Spilanthes calve*; Asteraceae)	Flower head	-	Larvicidal	Dengue virus	Pandey *et al*., 2007
39.	Spotted sterculia (*Sterculia guttata*; Sterculiaceae)	Seeds/ Ethanol extract	-	Effective larvicidal	Dengue virus	Katade *et al*., 2006
40.	Sweet sagewort (*Artemisia annua*; Asteraceae)	Leaf / Decoction / Tea	Artemisinin (14)	Larvicidal	Dengue virus	Tonk *et al*., 2006
41.	Biota (*Thuja orientelis*; Cupressaceae)	Leaves and fruit	Essential oil	Larvicidal	Dengue virus	Sharma *et al*., 2005
42.	Velvet leaf (*Cissampelos pareira*; Menispermaceaee)	Methanolic extract of aerial parts	Cissampelo flavone (15)	Antidengue inhibit viral particle activity in Vero cells	DENV 1-4	Bhatnagar and Katiyar, 2010
43.	Whip vine (*Flagellaria indica*; Flagellariaceae)	Whole plant	-	Antidengue 45% inhibition *in vitro* in Vero cells	DENV-2	Thirapanmethee *et al*., 2011
44.	White Leadtree (*Leucaena leucephala, Mimosa scabrella, Tephrosia madrensis, Tephrosia crassifolia, Tephrosia viridiflora;* Fabaceae)	Seeds Seeds Leaves and flowers Leaves and flowers Leaves and flowers/	Galactomannans (7), Glabraine (8), 7-O- methyl glabranine (9)	Antiviral Inhibitory action against DENV *in vitro* in C6/36 cell, produce 100 fold decrease in virus titre of DENV-1Inhibitory effect on DENV replication in LLC-MK2 cells	Dengue virus	Wollinger *et al*., 2013

1.14 Potential of plant bioactive compounds to combat dengue

The isolated active products belong to various chemical classes such as sulfated polysaccharides, flavonoids, quercetin and natural chalcone compounds showed a wide range of activity against DENV. The chemical structures of fifteen of these different phytochemicals, isolated from 14 plants, are shown in Figure 8. These secondary metabolites of medicinal plants comprise a variety of compounds with a wide range of biological activities. There are reports on medicinal plants extracts and essential oils possessing potential to new antiviral properties. Many plant extracts in different solvents have been reported to exhibit activity against a vector of dengue fever.

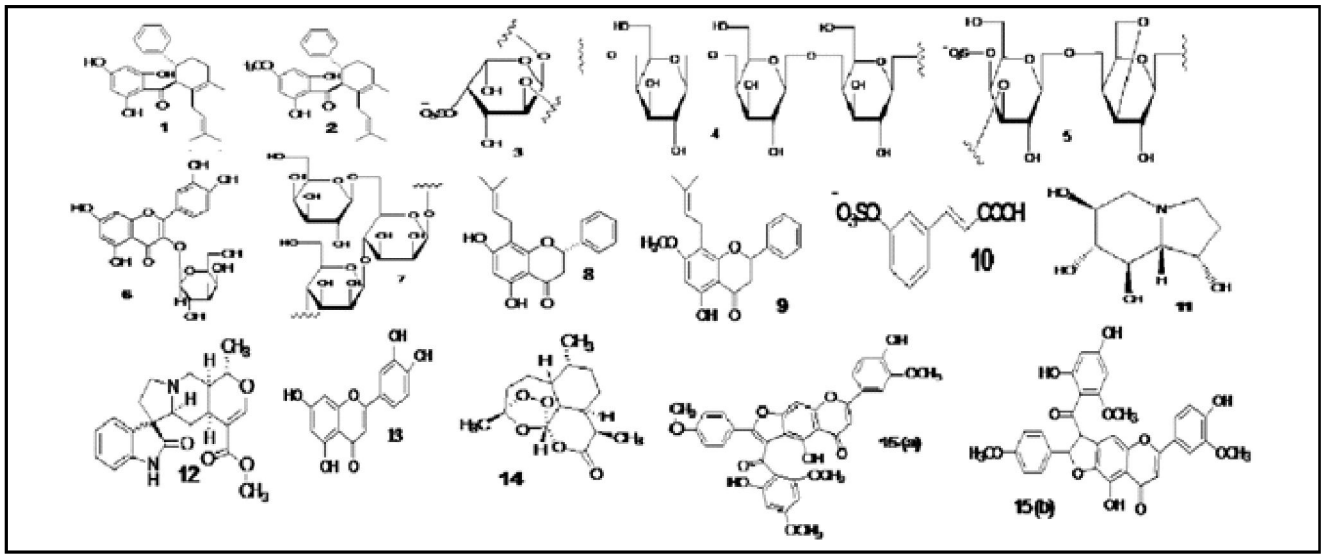

Figure 8: Structure of some potential compounds for treatment of dengue fever

1.15 Vaccinations

In 1944-1945, Sabin and Schlesinger (1945) prepared and tested the first live, attenuated (by serial mouse passage), dengue-1 virus vaccine. Vaccinated individuals experienced either no symptoms or a low-grade fever, with or without headache and malaise, lasting 24 h or less. All vaccines developed maculopapular rashes and petechiae. These efforts were unsuccessful. Hence, attempts to induce protection against experimentally transmitted dengue by inoculating human volunteers with unmodified virus by abnormal infection routes (*i.e.*, nasal instillation, dermal scarification, and instillation of conjunctival sacs) were made by Sabin (1952).

Table 6: Leading dengue vaccine candidates currently advanced to clinical testing

Vaccine strategy	Vaccine	Developer	Current status	References
Live attenuated yellow fever 17D/DENV chimeric vaccine	CYD vaccine (TDV) CCID50	Sanofi-Pasteur	Phase 3 trial with a tetravalent formulation in dengue endemic countries	Chambers *et al*., 1999; Guirakhoo *et al*., 2000; Lai and Monath, 2003
PDK cell passaged, live attenuated vaccine	-	WRAIR/GSK	Phase 2 trial with a tetravalent formulation in endemic countries	Chambers *et al*., 1999
Live attenuated D DENV Delta-30 mutation and intertypic	2prME/4/delta-30; 3prME/4/delta-30 Dengue 4/delta-30	NIH/Johns Hopkins	Phase ½ trials with monovalent formulations completed; tetravalent phase 1 initiated	Durbin and Whitehead, 2011)
DENV chimeric vaccines	$D1ME^{100}$	NMRC	Phase 1 with monovalent vaccine completed	Whitehead *et al*., 2003
Dengue prM-E DNA vaccine				
Recombinant 80% E subunit antigen vaccine	Vaxfectin-adjuvanted dengue DNA vaccine	Hawaii Biotech/ Merck	Phase 1 with monovalent vaccine initiated	Porter *et al*., 2012
Purified inactivated vaccine (PIV)	Dengue 2 adjuvanted with aluminum hydroxide Second generation JE PIV vaccine	WRAIR	Phase 1 with monovalent vaccine initiated	Putnak *et al*., 1996; Eckels and Putnak, 2003; Kaltenbock *et al*., 2009
Live attenuated chimeric DENV vaccine	DENVax	CDC	Phase 1 with monovalent vaccine initiated	Osorio *et al*., 2011; Brewoo *et al*., 2012

Abbreviations : DENV, dengue virus; PDK, primary dog kidney cells; WRAIR, Walter Reed Army Institute of Research; GSK, GlaxoSmithKline Biologicals; NIH, National Institute of Health; prM, premembrane envelope; NMRC, Naval Medical research Center; CDC, Centers of Disease Control and prevention

A tetravalent dengue vaccine has shown to be effective against symptomatic dengue in two phase III efficacy studies performed in five Asian and Latin American countries. Parameter inference was based on a Sequential Monte Carlo approach and used a legion version of the transmission mode (Morrison *et al*., 2010; Lanata *et al*., 2012). The two reference scenarios included are temporary cross-protection and combined cross-protection and cross-enhancement upon wild-type infection, following vaccination (Villar *et al*., 2015). Both scenarios were linked with differences in efficacy by serotype (Coudeville *et al*., 2015).

Knipl and Moghadas in the year 2015 developed a serotype-specific, vector-host compartmental model to evaluate the effect of vaccination in the existence of antibody-dependent enhancement and cross-protection, following recovery from primary infection. Vaccination can dramatically reduce the overall incidence of the disease. However, the length of vaccine-induced protection is shorter than the average lifetime of human population. Vaccination can potentially increase the incidence of severe infection of dengue haemorrhagic fever due to the effects of antibody-dependent enhancement. The degree and timelines for this increase depend strongly on the efficacy and duration of the vaccine-induced protection.

Two dengue virus type 2 vaccines produced in cell culture have been successfully tested for safety and immunogenicity. The dengue-2 PR-159/S-1 vaccine, which was derived by the selection of a stable virus clone after sequential passage in primary green monkey kidney cells, has been given to more than 145 volunteers and is a safe and moderately attenuated product. The vaccine strain used in the study had phenotypic markers of temperature, sensitivity, produced small plaques, and caused a reduced viremia in monkeys (Table 6) (Harrison *et al*., 1977; Eckels *et al*., 1980).

A recent comprehensive analysis of the human T cell response against wild-type DENV, suggested human lymphocyte antigen (HLA)-linked protective role for CD8(+) T cells (Azeredo *et al*., 2006a; 2006b). The characterization of CD8(+) T cell responses after live attenuated dengue vaccination showed responses in vaccines were readily noticeable and equivalent to natural dengue infection (Danko *et al*., 2011; Sabchareon *et al*., 2012; Lindow *et al*., 2013). Interestingly, broad responses to structural and nonstructural (NS) proteins were observed after monovalent vaccination. However, tetravalent vaccinations were, dramatically, focused toward the highly conserved NS proteins. Epitopes were highly sealed in a vast variety of field isolates and able to elicit multifunctional T cell responses (Weiskopf *et al*., 2015).

The CYD™, a live attenuated tetravalent vaccine (LATV) produced by Sanofi Pasteur. The multidose vaccine demonstrated protection against severe dengue, its overall efficacy was limited by DENV serotype, serostatus at vaccination, region and age (Tran *et al*., 2012). The National Institute of Allergy and Infectious Diseases have developed the LATV dengue vaccines, TV003/TV005. A single dose of either TV003 or TV005 induced seroconversion to four DENV serotypes in 74-92% (TV003) and 90% (TV005) of flavivirus seronegative adults and elicited near-sterilizing immunity to a second dose of vaccine administered 6-12 months later (Whitehead, 2015).

2. Future prospective

Following reinfection with a dengue virus of dissimilar serotype, severe disease is linked to high levels of antibody-enhanced viral replication early in illness which is followed by a surge of memory T-cell activation and a ‘storm’ of inflammatory cytokines and other chemical mediators. These compounds are released mainly from T cells, monocytes/macrophages and endothelial cells, and eventually cause an increase in vascular permeability. These advances underscored the fact that DHF/DSS pathogenesis is a complex, multifactorial process involving co-circulation of various dengue virus serotypes and the interaction of host and viral factors that influence disease severity. The constant search to define risk factors in liable populations, the new techniques of molecular virology and innovative approaches in vaccine design must be combined to achieve the ultimate objective of developing a safe and effective vaccine (Melino and Paci, 2007). Dengue virus nonstructural protein 5 (NS5) is a large multifunctional protein with a vital role in viral replication. The two nuclear localization sequences (NLSs) within the central region of dengue virus type-2 (DEN-2) NS5 (‘aNLS’ and ‘bNLS’) that are recognized by the importin alpha/beta and importin beta1 nuclear transporters, respectively. Site-specific mutations in the bipartite-type aNLS or bNLS region were introduced into a reporter plasmid indoctrinating green fluorescent protein fused to the N-terminus of DEN-2 NS5, as well as into DEN-2 genomic length complementary DNA. In contrast, mutations in either basic cluster of the aNLS decreased NS5 nuclear accretion and reduced virus production. It relates to the impaired ability of virus lacking nuclear-localizing NS5, as compared with wild-type virus expressing nuclear-localizing NS5, to reduce interleukin-8 production as part of the antiviral response (Pryor *et al*., 2007). High mobility group box 1 (HMGB1), an evolutionarily conserved protein, and is constitutively articulated in nearly all types of cells. The promising molecular mechanism underlying the supervision of pathogen-associated molecular patterns (PAMPs)-induced HMGB1 secretion. It may well be feasible to develop strategies that specifically satisfy damage-associated molecular patterns (DAMPs)-mediated inflammatory responses without compromising the PAMPs-mediated innate immunity for the clinical management of infection- and injury-elicited inflammatory diseases (Lu *et al*., 2014).

Antibody-dependent enhancement (ADE) of dengue virus (DENV) infectivity is thought to play a critical role in severe dengue disease. It occurs when pre-existing subneutralizing anti-DENV antibody (AB) produced from a primary infection encounters a DENV serotype different from that of the initial infection and forms immune complexes, which enable the efficient infection of Fcã receptor-bearing cells. However, the exact role played by ABs during a secondary infection of patients remains unknown. It was previously obtained a broadly cross-reactive neutralizing IgG1 human monoclonal anti-DENV envelope (E) Ab (HuMAb) D23-1G7C2-IgG1 from a DENV-infected patient having ADE activity. With the aim of being able to reduce the ADE activity, the Fc region of D23-1G7C2 was exchanged to generate ABs bearing each of the three other IgG subclasses (IgG2-4). In addition, N297A, a mutation known to reduce the affinity of the IgG1 Fc region for Fcγ receptors, was introduced into D23-1G7C2-IgG1. By contrast, in FcγRII-bearing K562 cells, the change to IgG2 increased ADE activity. Introduction of N297A mutation into D23-1G7C2-IgG1 resulted

in a marked reduction in ADE activity in both cell types. Compared to D23-1G7C2-IgG1, D23-1G7C2-IgG1-N297A was less protective in IFN-α/β/γ receptor knockout mice infected with a lethal dose of recombinant chimeric DENV, carrying prME of DENV-2 in Japanese encephalitis virus (Ramadhany *et al*., 2015).

In the dearth of a vaccine or any specific drug for its treatment, an early finding is considered obligatory to prevent any casualty. Recognition of viruses in human sera particularly in endemic areas is unwieldy, difficult and also not desirable. Therefore, as an alternative approach, finding of the dengue virus antigen in mosquitoes has provided a unswerving tool to (i) understand the types of viruses circulating in nature; and (ii) help in conniving vector-specific control strategies. A concoction of diagnostic techniques is currently available with some advantages or disadvantages. Traditionally, for virus isolations cell cultures and suckling mice have been employed. The mosquito cell cultures offer a good degree of specificity. Mosquito immunization techniques have been reported for detection and propagation of flaviviruses. Insect bioassays (Toxo-IFA) are generally unwieldy requiring special facilities and are not suitable for large-scale epidemiological surveillance. ELISA has been shown to be a quick and sensitive alternative to insect bioassays for monitoring arboviruses in wild populations. The current molecular diagnostic technology is reverse transcriptase polymerase chain reaction (RT-PCR) used for detecting virus infections in mosquitoes, which gives rapid results but is pricey and prone to contagion. Definite diagnosis of the impending dengue epidemic can be made using ELISA for virological surveillance system on dengue virus antigen in the mosquito vectors. Therefore, ELISA offers a potential tool and a convenient system and hence screened further by Toxo-IFA system for virus isolation. On the other hand, techniques like mosquitoes cell culture, mosquito inoculation (Toxo-IFA) and RT-PCR techniques can be employed for dengue virus amplification (Philip and Tyagi, 2006).

3. Conclusion

Current communication gives an insight of pathology, epidemiology, complications and diagnosis with special reference to natural drugs used and having better potential to be explored as new drug candidates for thrombocytopenia related dengue fever as found in *Carica papaya*, *Euphorbia hirta* and *Psidium guajava*.

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Conflict of interest

We declare that we have no conflict of interest

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