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Anti-Dengue and Safety Study of Novel Herbal Formulation- DENPAP® a Liquid Dosage Form

Dr. Maria Carina Cordeiro*, Sreedhar Pendyala**, Dr. Kotrappa Yankappa Mathur**
*Goan Pharma P. Ltd., 119 Natania, Near Dr Viaga's Bungalow, Behind PDA Colony, Porvorim, Goa 403 521, India.
**Liveon Biolabs Pvt. Ltd., # 46 & 47, Water Tank Road, KIADB Industrial Area, Antharasanahalli, Tumkur-572 106, Karnataka, India.

ABSTRACT

Dengue is a viral vector borne disease that has become a serious health problem worldwide and no treatment or vaccine is available for its prevention. The aim of the study was to evaluate the efficacy and safety of a novel herbal liquid formulation "DENPAP®". The toxicity study was performed to evaluate the oral acute toxicity of DENPAP® in Wistar rats as per OECD 425 guidelines: up and down procedure method. The efficacy study was carried out to identify the efficacious dose of DENPAP® for Anti-Thrombocytopenic effect in Chemical (Cylcophosphamide) induced Thrombocytopenia model in Sprague Dawley rats for a period of 14 days. The acute toxicity study was carried out in Wistar Albino rats at different doses for a period of 14 days and it was concluded that the LD50 value for DENPAP® was more than 2000 mg/kg. The efficacy study was carried out in Sprague Dawley rats for a period of 14 days and from this study it was concluded that the platelet counts of the animals increased in comparison to that of Cylcophosphamide induced Thrombocytopenia controls. From this study the LD50 for DENPAP® was obtained and the platelet count of animals in the group treated with DENPAP® increased after 3-5 days post dosing indicating its potential antithrombocytopenic effect.

Key Words: Dengue, DENPAP, Cylcophosphamide and Anti-Thrombocytopenia.

1. INTRODUCTION

1.1 Background

In recent years, the current dengue epidemic has become a focus of international public health awareness. Unlike malaria, which is more prevalent in remote areas, cases of dengue are distributed mostly in urban and sub-urban areas [1, 2]. This has made the epidemic more lethal as an outbreak is difficult to control due to highly populated areas in cities. Outbreaks of Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF) have been reported in India over the past four decades [3]. Dengue fever is caused by the arthropode-borne flavivirus named Dengue virus (DENV), transmitted by the Aedes aegypti and Aedes albopictus mosquito [4, 5]. It is a single stranded, positive-polarity, enveloped RNA virus that is transplanted in the cytoplasm as a single polyprotein and cleaved into three structural and seven nonstructural proteins [5]. To date, four antigenically related but distinct virus serotypes (DENV-1, 2, 3 and 4) have been identified as belonging to the genus Flavivirus in the Flaviviridae family [6-8]. Infection with one DENV serotype produces only specific antibody against that serotype. When antibody from the first infection is neutralized, secondary infections by other serotypes can cause more serious infection [9]. Although DENV-2 is known to be more lethal than other serotypes [10], some studies have revealed that primary infection with DENV-1 or DENV-3 always results in more dangerous disease than infection with DENV-2 or DENV-4 [7,11].

After a person is bitten, the virus incubation period varies between 3 and 14 days [7, 12], after which the person may experience early symptoms such as fever, headache, rash, nausea, and joint and musculoskeletal pain [7, 13]. This classic DF records temperatures between 39 and 40 °C and usually lasts

Corresponding Author:
Dr. Kotrappa Yankappa Mathur
Liveon Biolabs Pvt. Ltd., # 46 & 47, Water Tank Road, KIADB Industrial Area, Antharasanahalli, Tumkur-572 106, Karnataka, India.
Email: drmathur@liveonbiolabs.com Ph:+91 9686904909

5–7 days [10]. During this period, the virus may get into the peripheral blood stream and, if left untreated, can damage blood vessels and lymph nodes resulting in DHF with symptoms such as bleeding from the nose, gums or under the skin [12]. Most dengue infections are characterized by non-specific symptoms including frontal headache, retro-orbital pain, body aches, nausea and vomiting, joint pains, weakness and rash [13, 14]. Dengue infection results in a spectrum of disease ranging from a debilitating, self limited illness (dengue fever) to a life-threatening syndrome (DHF) [5].

There are currently no specific treatments for dengue fever [15]. Only standard treatment for management of fever is given, i.e., nursing care, fluid balance, electrolytes and blood clotting parameters [12]. Patients with dengue fever will be treated symptomatically, for example, sponging, acetaminophen [2], bed rest and oral rehydration therapy, and if signs of dehydration or bleeding occur the patients are usually hospitalized [10]. Aspirin should be avoided because it may cause bleeding [2]. Platelet count and Hematocrit should be measured daily from the suspected day of illness until 1–2 days after defervescence [2]. Current prevention of dengue by potential dengue vaccine and vector control is highly cost effective [15, 16]. In addition, mosquito control programs are the most important preventive method [10]. However, these are difficult to implement and maintain [17].

1.2 Epidemiology of Dengue

Current prevention of dengue by potential dengue vaccine and vector control is highly cost effective [15, 16]. In addition, mosquito control programs are the most important preventive method [9]. However, these are difficult to implement and maintain [17]. According to a World Health Organization (WHO) fact sheet dated December 2008, 80 % of the population in some Asian and African countries depends on traditional medicine as their primary health care due to economic and geographical constraints [18]. Up to 3.6 billion people are estimated to now live in tropical and subtropical areas where the dengue viruses have the potential to be transmitted [19, 20, 21]. Global estimates vary, but regularly approximate 50 million to 200 million dengue infections. Due to poor disease surveillance, low level of reporting, low case fatality rate, difficulties in diagnosis, and inconsistent comparative analyses, the true incidence and impact of dengue is likely significantly higher than that which is currently reported [19, 22, 23]. It is evident that dengue is now a worldwide concern; however, almost 75% of the global population exposed to dengue lives in Asia-Pacific [24, 25]. The World Health Organization (WHO) Western Pacific and SEA regions combined are attributed 75% of the global dengue disease burden [26, 21]. Due to the resurgence of dengue and its vector in the America over recent decades, Pan American Health Organization (PAHO) has once again launched an initiative targeting vector control and prevention in the region [26]. The 'Integrated Management Strategy for Dengue Prevention' is striving to reduce the disease and economic burden that dengue places currently on the America [24, 27]. All four dengue virus serotypes have been seen in Africa; however, DENV-2 appears to have caused most epidemics [28]. The Madeira Islands of Portugal have been in the midst of an outbreak since October 2012. This outbreak had resulted in 2164 cases by February 2013, with 78 imported cases from recent travelers to Madeira detected in 13 other countries throughout Europe [29, 30, 31]. In the Eastern Mediterranean region, dengue is classified as an 'emerging disease' [24]. Cases have only been officially reported to WHO for the last 2 decades, during which time three countries – Saudi Arabia, Pakistan and Yemen have had multiple outbreaks. There are some DENV-2 and DENV-3 genotypes found more commonly in the America which are known to be comparatively less virulent than Asian genotypes of the same serotype, as evidenced by reduced growth in both mosquitoes and culture [32-34].

Over the period of 2001-2010, researchers found in their study an annual average of 2.9 million cases and 5,906 deaths for the 12 countries analyzed (Bhutan, Brunei, Cambodia, East Timor, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Viet Nam). Tropical disease researchers have reported that in some Southeast Asian countries, national surveillance data substantially under-reported dengue cases. By analyzing data from prospectively followed cohorts with laboratory confirmation of dengue cases, the researchers found under-recognition of dengue cases by 8.7 fold in Thailand and 9.1 fold in Cambodia.

Throughout Southeast Asia, the epidemiology of dengue is changing with respect to age groups and population expansion beyond urban areas. For example, while dengue is generally considered to be a disease of early childhood (affecting individuals 2-15 years of age), increasing evidence exists that a shift to older age groups as well as infants as young as 1-2 months is occurring in endemic countries, including Thailand, Indonesia, and Singapore. The countries of Oceania contributed less than 0.2% of global apparent infections [35].

Increases of temperature may result in in-

creased survival and or migration of vectors into previously non-endemic geographic areas outside the tropics [33]. As the proliferation of Aedes mosquitoes is climate dependent, climate or meteorological factors can potentially provide useful information in predictive models. Weather variability has shown to be predictive of dengue activity [36-40]. Modern contributing factors to the rapid expansion of vector-borne communicable disease include globalization factors, such as travel and trade, associated with vector accommodating trends in modern human settlement and suitable climate conditions. Rapid urbanization and population growth have been identified as strong contributing factors to the increase of global dengue transmission and geographic expansion [19, 41]. These two factors, particularly in low and middle-income countries in tropical and subtropical regions, often precede the construction of necessary infrastructures for safe and comprehensive collection, storage and disposal of water [42]. Some studies suggest that rural dengue incidence can even surpass urban and semi-urban communities within the same region [43-45]. Sustainable vector control is one technical element of the Global Strategy for Dengue Prevention and Control, 2012–2020 [24]. In light of limited therapeutic strategies and the current lack of a vaccine, effective vector control methods are an essential component of the strategic direction to reduce dengue mortality and morbidity by 2020 [24, 26].

1.3 Diagnosis and Treatment of Dengue

Diagnosis of DV infection is routinely done by demonstration of anti DV IgM antibodies or by NS-1 antigen in patients' serum depending upon day of illness using ELISA kits (prepared by National Institute of Virology, Pune) [45]. The management of dengue virus infection is essentially supportive and symptomatic. No specific treatment is available. First report of existence of dengue fever in India was way back in 1946 [46]. Plant-based antiviral preparation promises a more potential alternative in combating Dengue disease. A number of natural compounds reported in traditional medicinal plants to have anti-dengue properties [47]. To date, 31 different species have been found to have the potential to treat dengue; some of these have not yet been investigated scientifically. In the Philippines, Euphorbia hirta, known locally as (tawa-tawa), is used in folk medicine to cure dengue fever by people in rural areas. While papaya leaf extract kills the bacterial infection that caused the fever, tawa–tawa extract prevents bleeding. In addition, unpublished research has found that Psidium guava leaves are a good way to increase platelets, thus helping to avoid bleeding. A water decoction of guava leaves contains quercetin, which acts to inhibit the formation of enzyme mRNA in the virus. Other plants which used are Andropogon citratum, Andrographis paniculata, Azidarachta indica, Carica papaya, Curcuma longa, Euphorbia hirta, Mimosa scabrella, Momordica charantia, Murraya koenigii Piper longum, Psidium guajava, Quercus lusitanica. The extract of all these plants have been tested against Dengue virus and has proved to be effective [48]. The active compounds present in these plants showed a wide range of activity against DENV. These include various chemical classes such as sulfated polysaccharides, flavonoids, quercetin and natural chalcone compounds [47].

2. MATERIALS & METHODS

The Herbal formulation DENPAP® was prepared and the formulation was assessed for microbial count, heavy metals, pesticide and phytochemicals before subjecting to safety and efficacy studies.

2.1 Study Design

2.1.1 Both the studies were approved by Institutional Animal Ethics Committee (IAEC) of Liveon Biolabs Pvt Ltd, Tumkur. The acute toxicity study was performed to evaluate the oral acute toxicity of Herbal Liquid Formulation DENPAP® in Wistar rats as per OECD 425 guidelines: up and down procedure method [49]. The study was conducted in Wistar albino rats aged 6-7 weeks and weighing about 145.1-151.2 g. The animals were acclimatized for a period of 5 days to laboratory conditions and were fed with ad libitum. The animals were exposed to 3 different doses i.e. 175, 550, 2000 mg/kg and were observed for a period of 14 days after dosing. All the animals were observed for changes in body weights, clinical signs & mortality and were subjected to detailed gross pathological examinations (Table 1).

2.1.2 The efficacy study was performed to evaluate the efficacious dose of Anti-Thrombocytopenic effect of Herbal Liquid Formulation DENPAP® in chemical induced Thrombocytopenic Sprague Dawley rats [50] and was carried out for a period of 14 days (Table 2). The study was performed in Sprague Dawley rats aged 8-10 weeks and weighing from 160.1 to 179.4 g. The animals were acclimatized for a period of 5 days to laboratory conditions and were fed ad libitum. The animals were administered orally with DENPAP® twice a day for a period of 15 days consecutively while Cylcophosphamide was administered by subcutaneous route daily for initial 3 days and ensured that the thrombocytopenia was established across all groups except G1. All the animals were observed for their

feed consumption, body weight, clinical signs, hematology & gross pathology.

3. RESULTS

3.1.1 Toxicity study

The acute toxicity study of DENPAP® in Wistar rats as per OECD 425 guidelines: up and down procedure method showed an increase in body weight wherein the percentage of body weight gain by animals exposed to a dose of 175, 550, 2000 mg/kg was recorded to be as 14.38%, 15.51% & 15.04 % respectively (Table 3). All animals were found to be normal and no mortality was observed. No gross pathological abnormalities were detected in any of the animals.

3.1.2 Efficacy study

The efficacy study of Anti-Thrombocytopenic effect of DENPAP® against Cyclophosphamide induced Thrombocytopenia in Sprague Dawley rats showed that there were no changes detected in body weights and no abnormalities were observed, however the weekly feed consumption rate decreased at G2 and G3 groups and this effect could be due to the Cylcophosphamide administration. There was a significant improvement in the mean platelet counts after a period of 3 days and further continued up to day 10 at G3-DENPAP® when compared to G2-Cylcophosphamide group (Table 4).

4. DISSCUSSION ON RESULTS

Dengue is a serious public health problem worldwide [35]. There are currently no vaccines available for the prevention and treatment of DENV infection [51]. DENV is a positive - stranded encapsulated RNA virus and is composed of three structural protein genes, which encode the nucleocapsid or core (C) protein, a membrane-associated (M) protein, an enveloped (E) glycoprotein and seven non-structural (NS) proteins [52]. DENV is a member of the genus Flavivirus and belongs to the Flaviviridae family [53]. The two component of viral serine protease, NS2B and NS3play a crucial role in viral replication as it is required for the synthesis of the polyprotein precursor prior to the assembly of the viral complex [54]. Thus NS2B-NS3 is considered to be a significant target for development of anti-dengue drugs.

Herbal medicines are the oldest remedies known to mankind. Herbs had been used by all cultures throughout history but India has one of the oldest, richest and most diverse cultural living traditions associated with the use of medicinal plants. Many polyherbal formulations are used for the treatment of number of diseases. Euphrobia hirta extracts in earlier study showed effective against thrombocytopenia a condition during dengue fever [55]. An herbal concoction of juice of papaya leaf, common neem and hill neem has been found to have anti-viral properties [56].

The LD50 for DENPAP® in the present study was more than 2000 mg/kg when dosed at different doses for a period of 14 days. Similarly the LD50 for methanolic extract of Tridax procumbens was found to be less than 2000 mg/kg B.Wt and more than 300 mg/kg B.Wt., during the 14 day study [57]. Also in another study the LD50 of polyherbal formulation for antiasthamatic activity was found to be 2262.7 mg/kg, p.o [58].

In the present study, the effect of DENPAP® administration in cylcophosphamide induced thrombocytopenic rat model showed that the platelet count improved after a period of 3 days (Table 4). Similarly, thrombocytopenia induced by Carboplatin was reversed on treatment with papaya leaf juice in a dose dependent manner with maximum effect after 7 days. The Active principles in papaya leaf juice were considered responsible for the antithrombocytopenic effect as also seen in the present study wherein DENPAP® was responsible for this effect [59]. In accordance with this study leaf papaya juice has also shown similar effect in hydroxyl-urea induced thrombocytopenia in rats [58]. Thus, this study indicates the anti-thrombocytopenic effect of DENPAP®.

5. CONCLUSION

5.1 From the results of the current toxicity study, there were no evident toxicological signs up to 14 days of observation and the LD50 value was found to be more than 2000 mg/kg B.wt when administered as a single dose at 3 dose levels i.e. 175, 550 & 2000 mg/kg B.wt as per acute oral toxicity study (OECD 425 Guidelines: UP-and-DOWN-procedure method).

5.2 The results of the evaluation of DENPAP® Syrup Formulations in chemical induced Thrombocytopenic Sprague Dawley rats for its Anti-Thrombocytopenic effect showed that the mean platelet counts of animals treated with formulation increased from day 3 to day 10 when compared to platelet count of G 2 group animals (Cyclophosphamide control) and the best results were seen after 3-5 days of dosing.

6. ACKNOWLEDMENT

The Intellectual property rights of the product: DENPAP® lies with Goan Pharma P. Ltd and has performed all the studies at Liveon Biolabs P. Ltd.

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List of Tables:

Table 1: Study design

No. of Groups	No. of Animals dosed at a time	Test Item	Starting Dose Level (mg/kg)	Sigma	Upper Bound Dose (mg/kg)	Maximum No. of Animals to Complete Limit Test if Upper Bound is Obtained
1	1	DENPAP®	175	0.5	2000	5

Table 2: Study design details with different groups

Group	Treatment	Dose (mg/Kg)	Route
G1	Control (Saline)	5 ml /kg	P.O
G2	Positive Control (Cyclophosphamide)	40	S.C
G3	Cyclophosphamide	40	S.C
	DENPAP®-2E 23.37%	721.30	P.O

Note: P.O-Per oral, S.C-Subcutaneous

Table 3: The body weight gain of animals at different doses: Acute toxicity test

Sr. No.	Dose (mg/Kg)	Body weight gain (%)
1	175	14.38
2	550	15.51
3	2000	15.04

Table 4: Platelet counts in different groups

Groups	Treatment	Platelet count-103 cells/µL			
		Day-3	Day-5	Day-7	Day-10
G1	Control (Saline)	722.3±62.25	744.8±91.56	887.7±295.06	852.8±198.55
G2	Positive Control -Cyclophosphamide (CP)	495.5±104.25	290.2±185.93	95.0±55.50	385.3±81.10
G3	CP + DENPAP®-2E 23.37%	613.8±42.80	384.2±32.62	222.2±63.85	676.0±321.72

n=8; values are mean ± SD