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In silico and *in vitro* Evaluation of Generic Medicines against DENV-2

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ABSTRACT

Acute febrile illness treatment based generic medicines are used for the assistance of symptoms of pain, inflammation and fever pain due to bacterial and viral infection. In our study aceclofenac, diclofenac, ascorbic acid, gallic acid, paracetamol, ibuprofen was evaluated anti-DENV-2 activity using in silico study against DENV-2 NS2B-NS3 and in vitro cytotoxicity and plaque formation assay against DENV-2. In our research all the medicines demonstrated the cytotoxicity value of 100µg/ml to Vero cell and were further analyzed for its impact on dengue viral propagation in a cell-based plaque forming assay. After finding it was stated that among all the medicines, ibuprofen and ascorbic acid showed 50% minimum effective and inhibitory concentration at 100-25µg/ml range after in silico study, pre-incubation and post-treatment assay which was statistically significant ($p<0.05$). But no generic medicines have been proved as prophylaxis activity. Therefore, our study suggested that ibuprofen and ascorbic acid (Vitamin C) could be the potential against dengue. For further combination research could be helpful for dengue patients after clinical trial. As per our suggestion, all the medicines could be reinvestigated against other dengue serotypes.

Keywords: *Dengue virus, Generic medicines, Molecular docking, Cytotoxicity assay, PRNT assay*

INTRODUCTION

Dengue virus (DENV) is spread to individuals by the *Aedes aegypti* and *Aedes albopictus* mosquitoes [1]. The disease is widespread in over 100 countries in the Americas, South-East Asia, the Eastern Mediterranean and the Western Pacific and causes up to 50 million infections annually [2]. DENV belongs to Flavivirus genus of Flaviviridae family and the DENV genome, a positive-sense single-stranded RNA, and its size is 10.7kb. It encodes a single poly-protein precursor that contains of three structural proteins (Envelope, Capsid and Membrane) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) which have significant characters in the host-pathogen interaction [3,4]. Patients with DENV infection are usually asymptomatic. But maximum patients shown acute febrile illness symptoms like fever, headache, vomiting, rash and joint pain [5]. Furthermore, few patients have additional development to life-threatening severe DENV infection like multiple organ failure, plasma leakage and severe bleeding [6]. The maximum number of DENV-2 cases, and most frequently identified, prevalent

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serotype infection outbreaks were reported globally, mainly in India to be a public cause of acute febrile fever [7].

We have no specific antiviral agents against DENV and only symptomatic treatments clinically, so effective anti -DENV drugs are needed to enhance disease or reduce disease severity and mortality during viral infection [8,9]. Many researchers reported the use of non-steroidal generic drugs (NSAIDs) like paracetamol, ibuprofen and aceclofenac for symptom control like high fever and joint pain as per standard protocol [10,11]. Appropriate dose of paracetamol is recommended for relieving the DF and ibuprofen, diclofenac should be avoided [12]. Current anti -inflammatory medicines such as acetylsalicylic acid, ibuprofen or related NSAIDs aggravate the bleeding effect and improve the condition of DHF [13]. For many years, ascorbic acid (Vitamin C) has been used to prevent many viruses and intracellular and improves immune purpose [14].

Based on the previous study, many researchers revealed that a derivative of gallic acid could be used as antibiotic, antiviral, and anticancer agent DENV [15]. One of the derivatives is isobutyl gallate (gallic acid derivative) have anti-inflammatory and antioxidant properties and have potential activity against Hepatitis C virus (HCV), RNA viruses, including influenza virus and poliovirus [16]. However, research regarding the potential of gallic acid and its derivatives as DENV antiviral is still limited. Therefore, in our present study, we intended to assess the antiviral effects of a series of anti–inflammatory medicines against infection with DENV-2 using *in silico* and *in vitro* screening assay.

Materials and Methods

Inflammatory responses play crucial roles in viral virulence and pathogenesis and contribute to dengue disease severity. So, in this study, we screened 6 anti -inflammatory medicines through *in silico* and *in vitro* approach.

In silico Study

From the protein databank (PDB) (www.rcsb.org/pdb/), the crystal structure (PDB ID: 2FOM) of the DENV-2 NS2B-NS3 was acquired and for active site detection study of DENV-2 NS2B-NS3, CASTP (Computed Atlas of Surface Topography of Proteins) was used. Based on doctors' suggestions and review of literature, the acute symptoms based generic medicines' structure were collected from Pubchem database. The drug-likeness score and Lipinski's rule of five (Ro5) of generic medicines were investigated by molinspiration online server. Molecular Docking calculations were carried out with Auto Dock 4.0 software against NS2B-NS3 protease receptor of DENV-2 (PDB: 2FOM) and for visualization chimera software was used [17,18].

In vitro Study

Cell Viability Assay

MTT [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide)] assay was performed to evaluate the cytotoxicity of generic medicines according to the manufacturer's instructions. Percentage survival of cells after treatment was determined through this assay using Graph Pad Prism 5 software [19].

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Pre-Incubation Assay
Vero cells were plated in 96-well plates (20,000 cells/ well), a day in advance. After adsorption with a serially diluted DENV-2 (MOI=1) virus solution for 2 hour and at 4^{o}C overnight (12 hour) in 100µl (non-cytotoxic concentrations) volume, the solution was replaced with (DMEM+2% FBS) and used to infect Vero cells (3 wells for each concentration at 200µl/well) in the 96-well plate and 150µl methyl cellulose was used after 2 hour of adsorption in the incubator (37^{o}C, 5% CO2). This assay was planned to find the capability to block DENV-2 from inflowing susceptible cells with negative control (absence of DENV-2 with cell) and positive control (absence of generic medicines with DENV-2). The cells were stable and marked with crystal violet solution containing 1% crystal violet, NaCl, and 2% formalin [19,20].

Post-Treatment Assay
Vero cells in 96 well plates (20,000 cells/well) were infected with DENV-2 (MOI = 1) without pre-incubating with generic medicines. After 2 hours of adsorption, the virus inoculum was enunciated, the monolayer washed with 1X PBS, and then fed with complete medium containing the generic medicines (corresponding non-cytotoxic concentrations).

After 2 hours of contact, the monolayer was removed and covered with growth medium containing methylcellulose and plaques were settled after 48 days. This assay was planned to measure the ability of generic medicines to inhibit DENV-2 within the infected cell [19,20].

Protective Assay
Vero cells in 96 well plates (20,000 cells/well) were treated with the various non-cytotoxic concentrations of generic medicines for 12 hours. Post treatment, the cells were washed away twice with 1X PBS and infected with DENV-2 (MOI = 1) for 2 hours. After 2 hours of adsorption, the mix was enunciated, the monolayer was washed with 1X PBS and overlapped with growth medium containing methylcellulose and plaques were established after 48 days [19].

Statistical Analysis
Data obtained from three independent experiments are presented as the mean ± standard deviation (SD). Three or more sets of data were analyzed by one-way ANOVA with statistical significance was set at $P < 0.05$.

RESULTS

***In silico* Study**
The best energy binding affinity score was -0.48 kcal/mol to -6.10 kcal/mol of generic medicines like aceclofenac, diclofenac, ascorbic acid, gallic acid, paracetamol, ibuprofen against DENV-2 NS2B-NS3 protein (PDB: 2FOM) (Table 1 and Figure 1).

Pharmacophore properties of these medicines showed the molecular properties and drug-likeness score. According to Ro5 (HBA/ HBD value up to 10 and 5, respectively; MW <500, Log P <5 and TPSA <140 Å, violation=0) and drug-likeness score results showed that aceclofenac, diclofenac, ascorbic acid, gallic acid, paracetamol, ibuprofen could be used as drug further (Table 2).

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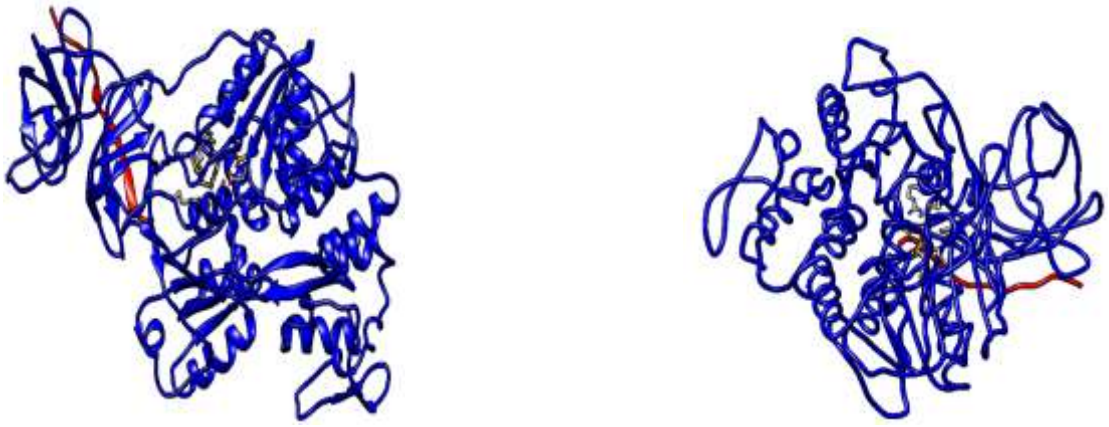
Table 1: Docking Results of Generic Medicines against DENV-2 NS3-NS2B

Ligand	Estimated Free Energy of Binding (kcal/mol)	Estimated Inhibition Constant, K_i
Aceclofenac	-4.49	514.82 µM
Diclofenac	-4.38	4.60 µM
Ascorbic acid	-5.83	53.27 µM
Gallic acid	-5.20	153.85 µM
Paracetamol	-4.94	240.14 µM
Ibuprofen	-6.10	33.63 µM

Table 2: Calculation of Molecular Properties of Generic Medicines

Ligand	miLogP	TPSA	MW	nON	nOHNH	nviolations	Drug-likeness score
Aceclofenac	4.43	75.63	354.19	5	2	0	0.67
Diclofenac	4.47	49.33	296.15	3	2	0	0.86
Ascorbic acid	-1.40	107.22	176.12	6	4	0	0.84
Gallic acid	0.59	97.98	170.12	5	4	0	0.07
Paracetamol	0.68	49.33	151.16	3	2	0	0.08
Ibuprofen	3.46	37.30	206.28	1	0	4	0.96



(a) **(b)**

Fig. 1: 3D image of a) Ibuprofen b) Ascorbic acid after Molecular docking

Molecular docking showed that ibuprofen and ascorbic acid could be the best inhibitors against DENV-2 NS2B-NS3 protease

Cell Cytotoxicity

Cell cytotoxicity of 6 generic medicines was determined using MTT assay in Vero cell. Percent cell survival cells was determined and non-cytotoxic concentrations of generic medicines were evaluated. It was observed that all the medicines were non-cytotoxic below 100µg/ml (Figure 2).

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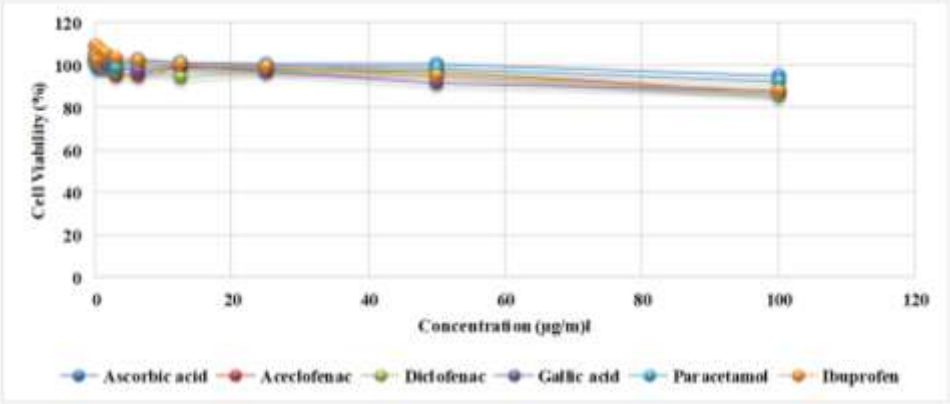

Fig. 2: MTT based cell cytotoxicity assay of Generic Medicines

MTT based cell cytotoxicity assay for the generic medicines used in the study. It was observed that all the medicines were non-cytotoxic between 100µg/ml till 0.781µg/ml, these concentrations were used for all the further *in vitro* bioassay. PRNT bioassay developed to evaluate anti-viral activity of the generic medicines

Antiviral Therapy

Further, three types of bioassays were developed to classify the concentration of generic medicines for possible DENV-2 inhibitory activity using plaque forming assay to determine no. of plaques formed at MOI= 1. It was found that the virus used for the study was $3x10^8$ pfu/ 100µl in concentration.

Effect of Pre-incubation Assay with Generic Medicines

The type-1 assay was measured to identify the ability of generic medicines to block DENV-2 from entering susceptible cells. It was observed that generic medicines were able to resist the development of plaques when used in the concentrations between 100 and 6.25µg/ml and $p<0.05$ which was statistically significant (Figure 3).

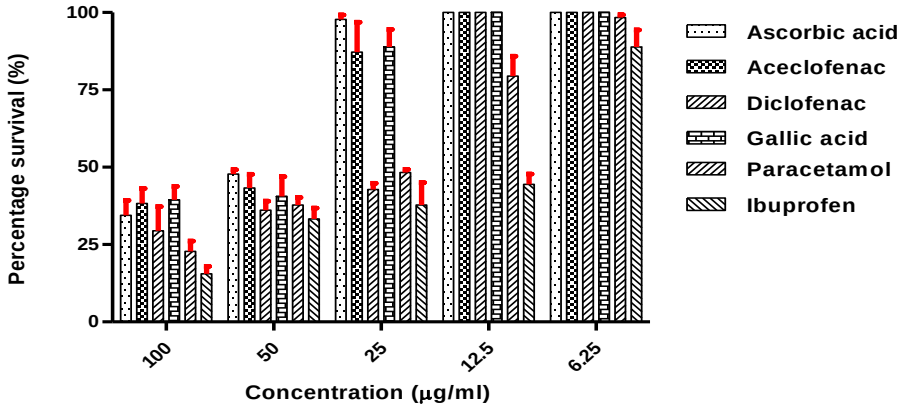

Fig. 3: Pre-incubation assay of generic medicines against DENV-2

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Pre-incubation of generic medicines with dengue virus: Ibuprofen and ascorbic acid were found to have inhibit dengue entry into the susceptible cells and may affect post entry replication steps at concentrations range of 100 and 6.25µg/ml and statistically significant ($p<0.05$)

Effect of Post-treatment assay with Generic Medicines

The type-2 assay was considered to assess the ability of generic medicines to inhibit DENV-2 within the infected cell. The generic medicines were used in the study neither inhibited DENV-2 replication post entry nor reduced the number of plaques formed when used in the concentrations between 100 and 12.5µg/ml and $p<0.05$ which was statistically significant (Figure 4).

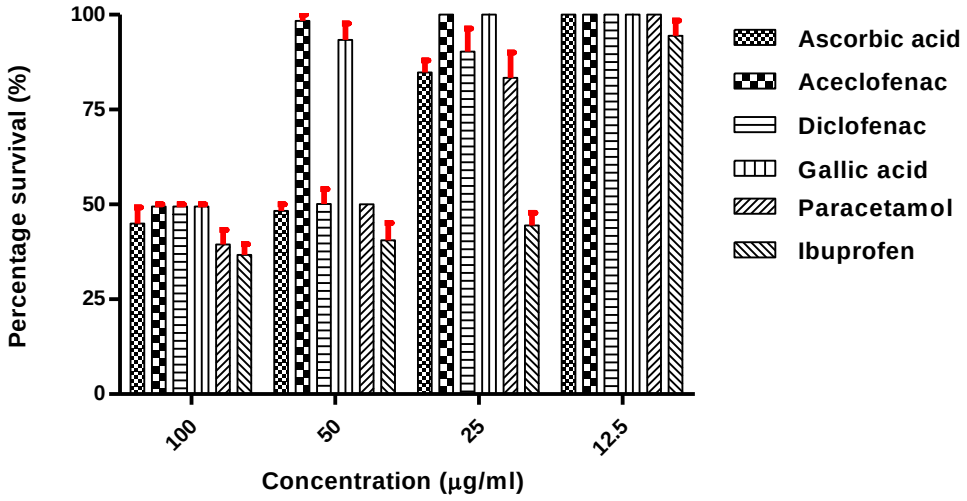

Fig. 4: Post-treatment assay of generic medicines against DENV-2

Effect of treatment with generic medicines on DENV infected cells. The best results showed that ibuprofen and ascorbic acid could inhibit DENV entry into the host cells at concentrations range of 100 and 12.5µg/ml and statistically significant ($p<0.05$)

Prophylaxis effect of generic medicines before DENV infections. The results depicted that none of the medicines showed any protective effect against DENV infections

Prophylaxis Activity of Generic Medicines

The type-3 assay was planned to screen protective effect of generic medicines against DENV-2. It was detected that none of the generic medicines was not able to decrease the number of plaque forming units to viewing the protective character.

DISCUSSION

Dengue infection is a dangerous mosquito borne disease in urban and rural area [2]. The supervision of DENV is fundamentally helpful and symptomatic. No specific treatment is available for dengue. NSAIDs can effects on bleeding and liver in dengue infection. Therefore, many countries are using widely generic medicines for the relief of symptoms of pain, inflammation and fever. Therefore, in India are approved non-prescription sale of ibuprofen, aceclofenac, diclofenac and paracetamol for symptoms based preliminary treatment for adult dengue patients. But these medicines are restricted for children, pregnant women and other

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patients due to side effects [21]. As per previous research evaluation many researchers reported on paracetamol, diclofenac, ascorbic acid was used in many hospitals with high dose for treatment of dengue, chikungunya and zika virus effected patients with high fever symptoms [22,23]. Therefore, safe and effective drugs that can improve fever and pain, decrease the risk dengue spreading with short duration are urgently needed. Till now there was no report of the antiviral study on generic medicines like ibuprofen, ascorbic acid, aceclofenac, diclofenac and gallic acid against DENV-2 using *in silico* and *in vitro* Vero cell infection model. This study highlights the need for generic drugs used in the management of dengue infection.

There were no research on molecular docking of generic medicines like aceclofenac, diclofenac, ascorbic acid, gallic acid, paracetamol and ibuprofen against DENV-2 NS2B-NS3 protein (PDB: 2FOM). Among all the medicines, ibuprofen (-6.10 kcal/mol) and ascorbic acid (-5.83 kcal/mol) were showing the best binding affinity results. The molecular properties and drug-likeness score of generic medicines predicted various properties according to Ro5 and drug-likeness score which indicated that aceclofenac, diclofenac, ascorbic acid, gallic acid, paracetamol, ibuprofen was found to have good drug likeness property. Our present study suggested that ibuprofen and ascorbic acid could be used further (Table 1, 2 and Figure 1).

As per our earlier *in silico* study, we reported the molecular docking study of aceclofenac against DENV-2 NS3 protease (PDB: 2VBC) with estimation of free energy of binding (-3.90 kcal/mol) [24]. Our study stated that 50μg/ml concentration with 43.33% and 100μg/ml concentration with 49.44% inhibition determined the pre-incubation and post-treatment effect of aceclofenac against DENV-2 which was statistically significant (Figure 3, 4).

Ibuprofen has moderately little risks for gastro-intestinal with safe and effective treatment of acute pain and fever. It is probably more effective than paracetamol as an antipyretic. But ibuprofen may inhibit with the anti-platelet effects and improve pain [25]. Our study exposed that 12.5μg/ml concentration with 44.44% and 15μg/ml concentration with 46% inhibition determined the pre-incubation and post-treatment significant effect of ibuprofen against DENV-2 (Figure 3, 4).

Paracetamol (known as acetaminophen) is suggested in the WHO dengue treatment guidelines for positive patients with high fever and joint pain symptoms. Cleef *et al.* (2016) reported dose-dependent inhibition of replicon-based assay, luciferase assay and cell culture assay in HeLa cell against DENV-1, 2 [26]. Also, Sood *et al.* (2015) reported that at 100 µg/ml viral inhibition concentration paracetamol has the anti-dengue activity against DENV-3 within the type-1 PRNT assay [27]. Our study revealed that 25μg/ml concentration with 48.33% and 50μg/ml concentration with 50% inhibition determined the pre-incubation and post-treatment effect of paracetamol against DENV-2 and $p<0.05$ which was statistically significant (Figure 3, 4).

As per the earlier study, Ramalingam *et al*. (2019) revealed that the orally treated vitamin C dengue patients from Kerala, Tamil Nadu and Madhya Pradesh have been shown the higher percentage of platelet counts in short time [28]. Lakhanpal *et al.* (2016) reported that vitamin C to all the age group patients of simple dengue fever led to rapid improvement in clinical features and early restoration of thrombocytopenia to increase platelet counts (>1,00,000/cmm) in all the

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age groups on day 5 and day 7 to the control group [29]. Chandra *et al.* (2013) revealed that Vitamin E and C supplementation may contribute to increase in platelet count and early recovery in dengue fever [30]. Also, per the previous study, vitamins such as D, E, and C have the ability to treat dengue disease [31]. Our study shown that 50μg/ml concentration with 47.77% and 50μg/ml concentration with 48.33% inhibition determined the pre-incubation and post-treatment with statistically significant effect of ascorbic acid (Vitamin C) against DENV-2 (Figure 3, 4).

Dewi *et al.* (2018) reported that isobutyl gallate (derivative of gallic acid) presented no cytotoxic properties against Huh7 cell at 167.19µg/ml concentration and demonstrated effective antiviral activity at 4.45µg/ml concentration [32]. Our study discovered that no cytotoxic effects against Vero cell with 50μg/ml concentration with 40.55% and 100μg/ml concentration with 49.44% inhibition determined the pre-incubation and post-treatment important effect of gallic acid against DENV-2 (Figure 3, 4).

CONCLUSION

Dengue fever is an acute febrile illness caused by the transmission of an arbovirus to humans by Aedes mosquitoes. Currently, there is no cure for dengue infections. Treatment comprises of supportive therapy with an emphasis on fluid management. The generic medicines could inhibit DENV-2 entry into the host cells. However, type-1 assay did not certainly expose if the generic medicines influenced the capacity to prevent the post entry step in the DENV-2 replication cycle. Hence, over the antiviral treatment ascorbic acid and ibuprofen were significantly more effective than other generic medicines. Though the dosage and use of ibuprofen still doubtable for dengue treatment due to side effects. Therefore, our findings suggest that ascorbic acid could be used for treatment of dengue. Because vitamin C is an efficient antioxidant and possesses anti-viral activity, very cheap medicine and accelerate the improvement of platelet level and low risk of bleeding complications during the early phase of viral infection. As a result, Vitamin C may be recommended to patients with dengue fever and other acute viral infections. Combination and clinical trial studies are needed in varied settings and larger populations as well as to assess safety and efficacy of particular doses.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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REFERENCES

1) Lt Col M.S. Mustafa, Col V. Rasotgi, Col S. Jain *et al.* Discovery of fifth serotype of dengue virus (DENV-5): a new public health dilemma in dengue control. Med J Armed Forces India. 2015; 70: 67-70.
2) Nivedita Gupta, Sakshi Srivastava, Amita Jain *et al.* Dengue in India. CENTENARY REVIEW ARTICLE. Indian Journal of Medical Research. 2012; 136(3):373-390

Journal of Advancement in Pharmacognosy
Volume 2 Issue 2, 2022
ISSN: 2582-8401

3) Samir Bhatt, Peter W. Gething, Oliver J. Brady *et al*. The global distribution and burden of dengue. *Nature*. 2013; 496:504-7.
4) Sophie Yacoub, Juthathip Mongkolsapaya, and Gavin Screaton *et al*. Recent advances in understanding dengue. F1000 Research. 2016; 5:1–10.
5) Srinivasa Rao Mutheneni, Andrew P Morse, Cyril Caminade *et al*. Dengue burden in India: recent trends and importance of climatic parameters. Taylor & Francis Emerg Microbes Infect. 2017; 6(8): e70.
6) Ramakrishnan SP, Geljand HM, Bose PN, Sehgal PN, Mukherjee RN *et al*. The epidemic of acute haemorrhagic fever, Calcutta, 1963; epidemiological inquiry. Indian J Med Res. 1964; 52:633–650.
7) Kumar Vikrama, B.N Nagpala, Veena Pande, Aruna Srivastava *et al.* An epidemiological study of dengue in Delhi, India. Acta Tropica. 2016; 153:21–27.
8) D'Elia RV, Harrison K, Oyston PC, Lukaszewski RA, Clark GC. Targeting the "cytokine storm" for therapeutic benefit. Clin Vaccine Immunol. 2013; 20:319 -27.
9) Kellstein D, Fernandes L. Symptomatic treatment of dengue: should the NSAID contraindication be reconsidered? Postgrad Med. 2019; 131:109-116.
10) K. Barupal, Vandana Gupta and Suman Ramteke. Preparation and characterization of ethosomes for topical delivery of aceclofenac. Indian J Pharm Sci. 2010; 72(5):582-586.
11) Mohamed Degwy, Saydia Tayel *et al. In vitro* and *in vivo* evaluation of aceclofenac lyophilized orally disintegrating tablets. IJPSR. 2012; 3(2):443-459.
12) Kaushik S, Kaushik S, Sharma V, Yadav JP. Antiviral and therapeutic uses of medicinal plants and their derivatives against dengue viruses. Phcog Rev. 2018; 12:177-85.
13) Fu-Kai Chuang, Shih-Ming Huang. Anti -inflammatory compound shows therapeutic safety and efficacy against flavivirus infection. Antimicrob. Agents Chemother. 2019: doi:10.1128/AAC.00941-19.
14) Thiberville SD, Moyen N, Dupuis-Maguiraga L *et al*. Chikungunya fever: Epidemiology, clinical syndrome, pathogenesis and therapy. Antiviral Res. 2013; 99: 345–370.
15) Mahmudah M. *In vitro* potency of gallic acid derivatives as an antiviral candidate of dengue virus serotype 2 and *in silico* study on NS5 protein program studi Magister Ilmu Biomedik. 2016: 45-50.
16) Misao U, Hisashi Y, Yukiko K, Masanori H, Tomihiko H and Hajime K. Antiviral effect of octyl gallate against DNA and RNA viruses Antiviral Res. 2007: 73 85–91
17) Mohd Adnan Kausar, Ali A, Qiblawi S. *et al*. Molecular docking-based design of Dengue NS5 methyltransferase inhibitors. Bio information. 2019; 15(6): 394-401.
18) Anubrata Paul, Arpana Vibhuti, Samuel Raj. Molecular docking NS4B of DENV 1-4 with known bioactive phyto-chemicals. Bio information. 2016; 12(3): 140-148.
19) Jaspreet Jain, Sujatha Sunil *et al*. Standardization of *in vitro* assays to evaluate the activity of polyherbal siddha formulations against chikungunya virus infection. Virus Dis. 2018; 29(1): 32-39.
20) Jaspreet Jain, Ankit Kumar *et al*. Antiviral activity of ethanolic extract of Nilavembu Kudineer against dengue and chikungunya virus through *in vitro* evaluation. Journal of Ayurveda and Intregrative Medicine. 2018; 1-7.
21) Simmons CP, Wolbers M, Nguyen MN *et al.* Therapeutics for dengue: recommendations for design and conduct of early-phase clinical trials. PLoS Negl Trop Dis. 2012; 6: e1752.

Journal of Advancement in Pharmacognosy
Volume 2 Issue 2, 2022
ISSN: 2582-8401

22) Erna Kristin, Alfi Yasmina, Rizaldy Taslim Pinzon *et al*. Drug use pattern in dengue patients who visited hospitals and primary health center in Yogyakarta, Indonesia. J. Pharm. Sci. & Res. 2018; 10(8): 2065-2068.
23) Michael J. Gonzalez et al. High dose intravenous vitamin C treatment for zika fever. JOM. 2016; 31(1): 19-22.
24) Anubrata Paul, Arpana Vibhuti, Samuel Raj. *In silico* molecular docking analysis and inhibitory activity of aceclofenac against NS3 protein of DENV. DOI: 10.13140/RG.2.1.1574.2323.
25) David Kellstein & Luiz Fernandes. Symptomatic treatment of dengue: should the NSAID contraindication be reconsidered? Postgraduate Medicine. 2019; 131(2):109-116.
26) Van Cleef KW, Overheul GJ, Thomassen MC *et al*. Escape mutations in NS4B render dengue virus insensitive to the antiviral activity of the paracetamol metabolite AM404. Antimicrob Agents Chemother. 2016; 60(4):2554–2557.
27) Ruchi Sood, Rajendra Raut, Poornima Tyagi *et al*. *Cissampelos pareira* Linn: Natural source of potent antiviral activity against all four dengue virus serotypes. Plos one Neglected Tropical Diseases. 2015; 9(12): 1-20.
28) Kothai Ramalingam, Christina S Varghese, Chinchu Elias *et al*. A retrospective study on the effect of Vitamin C in the management of dengue fever in three different states of India. International Journal of Research in Pharmaceutical Sciences. 2019; 10(4), 2670-2673.
29) Lakhanpal G, Dange S V, Sharma S. Study of effect of vitamin C on thrombocytopenia in patients of simple dengue fever. International Journal of Medical and Applied Sciences. 2016; 5(2): 1-6.
30) Prasanna Chandra, Hemant Sharma, Akash Gupta. Role of antioxidant vitamin E and C on platelet levels in dengue fever. International Journal of Basic and Applied Medical Sciences. 2013; 3(1): 287-291.
31) Ahmed S., Finkelstein, J.L., Stewart, A.M., Kenneth *et al.* Micronutrients and dengue. American Journal of Tropical Medicine and Hygiene. 2014; 91: 1049-56.
32) B E Dewi, M Angelina *et al*. Antiviral activity of isobutyl gallate to dengue virus serotype 2 *in vitro*. IOP Conf. Series: Earth and Environmental Science. 2019; 251 (012018): IOP Publishing doi:10.1088/1755-1315/251/1/012018