Research Article

Relative Toxicity of Leaf Extracts of *Eucalyptus globulus* and *Centella asiatica* against Mosquito Vectors *Aedes aegypti* and *Anopheles stephensi*

Savitha Sekhar Nair, Vinaya Shetty, and Nadikere Jaya Shetty

Centre for Applied Genetics, Bangalore University, J. B. Campus, Bengaluru, Karnataka 560056, India

Correspondence should be addressed to Nadikere Jaya Shetty; shetty_nj@yahoo.co.in

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The larvicidal activity of different solvent leaf extracts (hexane, diethyl ether, dichloromethane, and methanol) of *Eucalyptus globulus* and *Centella asiatica* against two geographically different strains of *Aedes aegypti* and *Anopheles stephensi* was investigated. The extracts were tested against the late third instar larvae of *Aedes aegypti* and *Anopheles stephensi*, and larval mortality was observed after 24 hours of treatment. LC_{50} and LC_{90} were calculated. The LC_{50} values of hexane extract of *Eucalyptus globulus* against the late third instar larvae of the BSN and JPN strains of *Aedes aegypti* and the DLC and KNG strains of *Anopheles stephensi* were 225.2, 167.7, 118.8, and 192.8 ppm, while those of the hexane extract of *Centella asiatica* were 246.5, 268.7, 50.6, and 243.5 ppm, respectively. The LC_{50} values of diethyl ether extract of *Centella asiatica* were 339.6, 134.5, 241, and 14.7 ppm. The hexane extracts of both plants and the diethyl ether extract of *C. asiatica* presented the highest potential for the control of *Aedes aegypti* and *Anopheles stephensi*. The present findings also reveal the necessity of assaying multiple strains of a species to fully comprehend the larvicidal efficacy of a compound.

1. Introduction

Apart from being a social nuisance, mosquitoes pose serious health threats to both men and animals considering that they are the principal vectors for many vector borne diseases including malaria, dengue, yellow fever, and Chikungunya [1, 2] in men and equine encephalitis, haemorrhagic septicaemia of buffaloes, and enzootic hepatitis in animals [3]. In some individuals, mosquito bites also result in acute systemic allergic reactions defined by the presence of one or more of the following: urticaria, angioedema, wheezing, dyspnea, hypotension, and decrease or loss of consciousness [4]. The mosquito, *Aedes (Stegomyia) aegypti* (Linn.), (Diptera: Culicidae) is the primary vector of dengue, yellow fever, and Chikungunya [2]. According to WHO, over 40% of the world's population is now at risk of dengue and there are 200 000 estimated cases of yellow fever, causing 30 000 deaths, worldwide each year. Malaria, on the other hand, a life threatening disease which caused an estimated 627 000 deaths in 2012 is transmitted exclusively through the bites

of *Anopheles* mosquitoes [5]. *Anopheles stephensi* Liston (Diptera: Culicidae) is a major vector in India as well as in some of the West Asian countries and has been shown to be directly responsible for about 40–50% of the annual malarial incidence [6, 7].

Mosquito control is an extensively researched topic considering that presently we do not have vaccines for malaria and dengue [8, 9] and, therefore it becomes necessary to check the spread of these diseases. Vector control methods, thus are of utmost prominence. The incessant use of chemical insecticides has often led to the disruption of natural biological control system and outbreak of insect species [10]. The selective pressure of conventional insecticides is enhancing the resistance of mosquito strains at an alarming rate [11], increasing the demand for new products that are eco-friendly, target-specific, and biodegradable. Plant-derived insecticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to men, and other advantages [12].

Earlier studies have shown the efficacy of *Eucalyptus* oils as a larvicide against mosquitoes [13–16]. In the year 2004, Yang et al. reported that *Eucalyptus* leaf oil, particularly some of its components such as 1,8-cineole, 1- α -terpineol, and (E)-pinocaveol, merits further study as lead compounds for the control of the human hair louse, *Pediculus humanus capitis* De Geer (Pediculidae) [17]. Constituents of the essential oil of *Eucalyptus camaldulensis* Dehnh and *Eucalyptus urophylla* S. T. Blake (Myrtaceae) α -phellandrene, limonene, p-cymene, c-terpinene, terpinolene, and α -terpinene have been shown to possess strong larvicidal effects against *Aedes aegypti* and *Aedes albopictus* [15]. The various components of *Eucalyptus* essential oil have been shown to act synergistically (and not additively) to bring the overall bactericidal activity [18].

Centella asiatica is mostly studied for its medicinal properties. Very few studies have documented its potential as a biopesticide. A study has suggested that the leaf extract of *C. asiatica* is promising as a larvicide and adult emergence inhibitor against *Culex quinquefasciatus* Say (Culicidae) [19]. Shukla et al. [20] isolated and studied the growth inhibitory effect of 2 α , 3 β , 6 β , 23- α tetrahydroxyurs-12-ene-28-oic acid, a triterpenoid glycoside against larvae of *Spilarctia obliqua* Walker (Arctiidae).

Numerous studies have examined the variations in larvicidal efficacies of plant extracts tested against different mosquito species; however, we were not able to find any reports of the larvicidal efficacies of plant extracts tested against different strains of mosquitoes of the same species. Susceptibility studies involving strains from different locations could be beneficial to gauge the efficacy of the drug being studied and as a prelude to investigations involving the inherent resistance mechanisms of the vectors to the drugs.

Different solvent types can significantly affect the potency of the extracted plant compounds and there is a difference in the chemoprofile of the plant species [21]. This is because polar solvents extract polar constituents of the plant, while nonpolar solvents extract the nonpolar constituents of the plant. Hence, the best results will be observed using the plant extract of the solvent whose polarity matches that of the molecules in the plant responsible for insecticidal activity (if any). Additionally, extracts or pure compounds derived from specific solvents can influence the bioactivity, probably because of the active components which are present in large quantities [22].

In the present study, we have investigated the larvicidal activity of the hexane, diethyl ether, dichloromethane, and methanol extracts of the leaves of *Eucalyptus globulus* Labill (Myrtaceae) and *Centella asiatica* (Linn.) Urban (Apiaceae) against two geographically different strains of *Aedes* (*Stegomyia*) *aegypti* (Linn.) (Diptera: Culicidae) and *Anopheles stephensi* Liston (Diptera: Culicidae).

2. Materials and Methods

2.1. Mosquito Culture. Four strains which consisted of laboratory colonies of *Aedes aegypti* collected from J. P. Nagar (JPN) (12.9120°N; 77.5930°E) and Basaveshwaranagar (BSV) (12.9867°N; 77.5386°E) and *Anopheles stephensi* collected from Dollars Colony (DLC) (13°2'30"N; 77°34'3"E) and

Kengeri (KNG) (12.9100°N; 77.4800°E) were used for this study. Larvae collected from the said locations of BBMP region, Karnataka, India, during the months March–April 2012, had been reared continuously in the insectary at the Centre for Applied Genetics, Bangalore University, following the method of Shetty [23].

2.2. Collections of Plant Materials. The leaves of *Eucalyptus globulus* and *Centella asiatica* were collected from Bangalore city (11°1'48"N; 77°2'36"E; elevation: 399 m), Karnataka, India, in July 2013, and were authenticated at the Department of Botany, Bangalore University, Bangalore, India.

2.3. Preparation of Plant Extracts. The leaves (2 kg each) were air dried in shade for 15–30 days. The dried leaves were then powdered mechanically using a commercial electrical stainless steel blender. One kg of powdered leaves was extracted successively by maceration using nonpolar to polar solvents, namely, hexane, diethyl ether, dichloromethane, and methanol. In each solvent, the plant material was soaked for 48 h at 35°C and filtered twice using Whatman number 1 filter paper to obtain the extract, and to the residue the same solvent was added again. The procedure was repeated twice to obtain maximum extract. The extracts were concentrated at reduced temperature using a rotary vacuum evaporator and stored at a temperature of 4°C. One gram of the concentrated plant extract was dissolved in 100 mL of 1 : 1 acetone : dimethyl sulfoxide (DMSO) and considered as 1% stock solution. From this stock solution, varying concentrations of each extract was prepared and these solutions were used for larvicidal bioassay. All chemicals used in this study were of extra pure grade and were obtained from Sisco Research Laboratories PVT. Ltd., India.

2.4. Larvicidal Bioassay. Larvicidal activity of each extract derived from the leaves of *Eucalyptus globulus* and *Centella asiatica* against the two strains of *Ae. aegypti* and *An. stephensi* was assessed by using a slightly modified version of the WHO standard method [24].

Initially, the mosquito larvae were exposed to a broad range of test concentrations to determine the activity range of each extract. Based on the results of preliminary screening, batches of 25 late third instar larvae were added to 300 mL wide mouth disposable bowls containing serial concentrations (6–9 concentrations, yielding between 10% and 95% mortality in 24 hrs) of each plant extract made up to 250 mL by volume using tap water. The test was carried at a temperature of 25 $\pm$ 2°C and relative humidity of 75 $\pm$ 5%. The numbers of dead larvae were counted after 24 hours of exposure and percentage mortality was calculated for each test as follows:

Percentage Mortality

$$= \frac{\text{Number of dead larvae/pupae}}{\text{Number of larvae introduced}} \times 100. \quad (1)$$

The final percentage mortality was calculated from the average of three replicates. Solutions containing tap water and 1 : 1 acetone : dimethyl sulfoxide, but without the plant sample,

served as controls. The control mortalities were corrected by using Abbott's formula [25].

2.5. Statistical Analysis. The average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90} and other statistics at 95% fiducial limits of upper confidence limit (UCL) and lower confidence limit (LCL), and Chi-square values were calculated using the SPSS 12.0 (Statistical Package of Social Sciences) software (Finney, 1971). The Chi-square values were considered significant at $P < 0.05$ level.

3. Results

The larvicidal efficacies of hexane, diethyl ether, dichloromethane, and methanol extracts of both *Eucalyptus globulus* and *Centella asiatica* were established for two different mosquito strains of each of the above said species from the Bangalore, Karnataka. The data were recorded and statistical data including regression equation, LC_{50} , LC_{90} , LCL, UCL, and Chi-square values were calculated and presented (Table 1). No mortality was observed in the control.

All plant extracts showed varied level of toxic effects with species after 24 hours of exposure, while the methanol extract demonstrated very low larvicidal efficacy in the case of both of the plants.

3.1. *Eucalyptus globulus*. Among the *An. stephensi* strains, the DLC strain displayed the highest susceptibility to the hexane extract (LC_{50} 118.8 and LC_{90} 450.4 ppm) followed by the diethyl ether extract (LC_{50} 464.1 and LC_{90} 800.1 ppm). The KNG strain of *Anopheles stephensi*, on the other hand, showed the highest larval mortality in the dichloromethane extract (LC_{50} 162.0 and LC_{90} 582.4 ppm), followed by the hexane extract (LC_{50} 192.8 and LC_{90} 827 ppm). The BSN and JPN strains of *Ae. aegypti* were most susceptible to the hexane extract (LC_{50} 225.2, 167.7 and LC_{90} 423.6, 304.0 ppm, resp.). The methanol extract was the least effective in all cases. A higher level of tolerance was recorded in the BSN strain of *Ae. aegypti* to the methanol extract (LC_{50} 1839.0 and LC_{90} 3235.9 ppm).

3.2. *Centella asiatica*. The hexane extract produced the highest mortality in the case of the BSN and JPN strains of *Ae. aegypti* (LC_{50} 246.5, 268.7 and LC_{90} 593.1, 1606.8 ppm, resp.), and also the DLC strain of *An. stephensi* (LC_{50} 50.6 and LC_{90} 328.8 ppm), the KNG strain of *An. stephensi* however was most susceptible to the diethyl ether extract (LC_{50} 14.7 and LC_{90} 118.0 ppm). Again, the methanol extract was the least potent in all cases. The BSN strain of *Ae. aegypti* displayed a higher level of tolerance (LC_{50} 776.5 and LC_{90} 3853.4 ppm) upon treatment with the methanol extract.

4. Discussion

The leaf extracts of *Eucalyptus globulus* and *Centella asiatica* showed an overall moderate larvicidal effect when tested against different strains of *Ae. aegypti* and *An. stephensi*.

Among the four extracts studied, the hexane extracts of both plants and the diethyl ether extract of *C. asiatica* demonstrated higher potential for the control of strains of mosquito vectors, followed by dichloromethane. The methanol extract produced a comparatively less promising result. The effect of the extracts on larval mortality was dose-dependent, with increasing rates of larval mortality observed at increasing concentrations. The highest level of tolerance was observed in the BSN strain of *Ae. aegypti* towards the methanol extract of *E. globulus*.

Although the larvicidal efficacy of the extracts is not as promising as that of synthetic insecticides commonly in use today [26], the present results are comparable to those of earlier authors who worked on various plant extracts as larvicides against different mosquito species. The methanol leaf extract of *Clitoria ternatea* L. (Leguminosae) showed a dose-dependent larvicidal activity against *An. stephensi* with an LC_{50} value of 555.6 ppm [27]. In the case of *Morinda citrifolia* L. (Rubiaceae) the highest larvicidal activity was exhibited by the methanol extract of the leaf when compared to the hexane, chloroform, acetone, and water extracts; the LC_{50} in case of *An. stephensi* and *Ae. aegypti* was observed to be 261.96 and 277.92 ppm, respectively. The chloroform leaf extracts of *Nyctanthes arbortristis* L. (Oleaceae) demonstrated an LC_{50} value of 780.6 ppm, while the LC_{50} value of the methanol flower extract of the same plant was 679.4 ppm when tested against *An. stephensi*. Studies have also been carried out on the larvicidal potential of the essential oil extracted from the *Eucalyptus* species [13–15] and *E. globulus* in particular [28]. However, this is the first time report of the larvicidal efficacy of serial extracts of *Eucalyptus globulus* and *Centella asiatica* leaves using multiple solvents against different geographical strains of *Ae. aegypti* and *An. stephensi*. In the present study, strains of both species of mosquitoes were found to be highly susceptible to hexane leaf extracts when compared to the other solvent extracts.

Numerous authors have observed a converse relationship between extract effectiveness and solvent polarity where the efficacy increases with decreasing polarity [29–32]. Singh et al. [33] reported the LC_{50} values of the hexane extract of *Eucalyptus citriodora* Hook (Myrtaceae) against the IVth instar larvae of *An. stephensi* (69.86 ppm) and *Ae. aegypti* (91.76 ppm). Hexane extracts of other plants have also shown similar satisfactory larvicidal potency. LC_{50} of hexane leaf extract of *Citrus sinensis* (L.) Osbeck (Rutaceae) against early fourth instars of *Ae. aegypti* was found to be 446.84 ppm [34]. The LC_{50} of hexane leaf extract of *Murraya koenigii* (L.) Spreng (Rutaceae) against third instars *Culex quinquefasciatus* was found to be 963.53 ppm [35]. In the case of *Citrullus colocynthis* (L.) Schrad (Cucurbitaceae), however, the LC_{50} of the hexane extract was found to be 1451.29 ppm while that of the diethyl ether extract was found to be 503.39 ppm. This result is similar to that of the KNG strain of *An. stephensi* in our studies, where the diethyl ether extract of *C. asiatica* produced a better result than the hexane extract. Contrarily, the other *An. stephensi* strain that we worked with (DLC) exhibited higher susceptibility for the hexane extract against the diethyl ether extract.

TABLE 1: Effects of relative toxicity of solvent extracts (hexane, diethyl ether, dichloromethane, and methanol) of the leaves of *Eucalyptus globulus* and *Centella asiatica* against the late third larval stage of BSV and JPN strains of *Aedes aegypti* and DLC and KNG strains of *Anopheles stephensi*.

Botanical name	Solvents	Species	Strain	LC ₅₀ (ppm) (UCL-LCL)	LC ₉₀ (ppm) (UCL-LCL)	Regression equation	r	χ ²	df
<i>Eucalyptus globulus</i>	Hexane	<i>Ae. aegypti</i>	BSV	225.2 (210.9–263.5)	423.6 (400.4–598.9)	y = 4.6638x – 5.9716	0.9364	0.1902	7
			JPN	167.7 (152.1–186.7)	304 (266.3–400.9)	y = 4.9559x – 6.0248	0.984	0.0238	6
		<i>An. stephensi</i>	DLC	118.8 (88.1–151.2)	450.4 (373.8–953.3)	y = 2.2119x + 0.4106	0.9137	0.1123	7
			KNG	192.8 (154.7–255.1)	827 (655.2–1634)	y = 2.0242x + 0.3744	0.9378	0.1134	7
	Diethyl ether	<i>Ae. aegypti</i>	BSV	686.8 (630.8–756.4)	1216.7 (1073.7–1582.5)	y = 5.1546x – 9.6228	0.9594	0.0244	7
			JPN	318.8 (146.7–641.2)	2069.9 (1085.1–34888.5)	y = 1.5756x + 1.0554	0.9114	0.1811	6
		<i>An. stephensi</i>	DLC	464.1 (428.8–524.2)	800.1 (726.1–1050.7)	y = 5.4107x – 9.428	0.9382	0.0767	6
			KNG	318 (270.6–391.7)	951.9 (737.4–1618.7)	y = 2.688x – 1.7265	0.9279	0.0605	6
	Dichloromethane	<i>Ae. aegypti</i>	BSV	314.6 (276.1–385)	829.9 (677.1–1290.7)	y = 3.038x – 2.588	0.9846	0.0832	6
			JPN	362.2 (171.6–620.1)	2904.6 (1068.6–13925.1)	y = 1.4157x + 1.3773	0.889	0.1362	6
		<i>An. stephensi</i>	DLC	300.3 (256.3–394.2)	1235.4 (872.5–2283.9)	y = 2.0839x – 0.163	0.9747	0.1345	7
			KNG	162 (112.5–199.4)	582.4 (452.6–1323.4)	y = 2.3036x – 0.0899	0.9623	0.0536	7
<i>Centella asiatica</i>	Methanol	<i>Ae. aegypti</i>	BSV	1839.3 (1695.8–2049.7)	3235.9 (2930.2–4051.1)	y = 5.2171x – 12.032	0.9805	0.0504	7
			JPN	1467.9 (1281–1786.8)	3530.2 (2911.7–5612.3)	y = 3.3587x – 5.636	0.9644	0.0634	5
		<i>An. stephensi</i>	DLC	1263.3 (798.1–1817.3)	3614.3 (2988.3–12736.6)	y = 2.8038x – 3.696	0.8769	0.2807	7
			KNG	889.6 (764.7–1124.3)	2655.1 (2177.0–4907.4)	y = 2.6955x – 2.9496	0.9204	0.1292	6
	Hexane	<i>Ae. aegypti</i>	BSV	246.5 (213.1–307.1)	593.1 (527.3–1013.9)	y = 3.3561x – 3.027	0.9284	0.1366	5
			JPN	268.7 (174.4–374.7)	1606.8 (1082.1–5866.2)	y = 1.6479x + 0.9969	0.929	0.0583	5
		<i>An. stephensi</i>	DLC	50.6 (18.3–105)	328.8 (198.5–8730)	y = 1.5752x + 2.3154	0.8489	0.2479	6
			KNG	243.5 (221.1–274.2)	428.6 (362.7–584.5)	y = 5.2115x – 7.4372	0.9963	0.0055	5
	Diethyl Ether	<i>Ae. aegypti</i>	BSV	339.6 (258.1–472.3)	1599.3 (1173.6–3840.5)	y = 1.9022x + 0.1855	0.8489	0.0844	5
			JPN	134.5 (20.6–204.1)	770.8 (576.7–3191.7)	y = 1.6885x + 1.4054	0.9362	0.0463	5
		<i>An. stephensi</i>	DLC	241 (203.4–301.5)	630.7 (540.4–1116.2)	y = 3.063x – 2.2959	0.8489	0.1046	5
			KNG	14.7 (3–35)	118 (63.7–17122)	y = 1.4175x + 3.3433	0.8862	0.1491	5
	Dichloromethane	<i>Ae. aegypti</i>	BSV	339.7 (281–746.3)	2127.7 (1241.9–403246)	y = 1.6065x + 0.9337	0.9384	0.1913	5
			JPN	837.6 (777.9–1030.2)	1627.2 (1542.3–2558.8)	y = 4.4382x – 7.973	0.9	0.1722	5
		<i>An. stephensi</i>	DLC	236.8 (145.5–393.3)	3347.1 (1576.7–279272)	y = 1.1127x + 2.3581	0.9338	0.0533	5
			KNG	155.5 (20.9–295.5)	3022.9 (1732.5–33559.1)	y = 0.9933x + 2.8229	0.8881	0.0528	5
Methanol		<i>Ae. aegypti</i>	BSV	776.5 (590.99–1090.78)	3853.4 (2728.01–10164.21)	y = 2.1701x – 1.2027	0.8905	0.0966	5
			JPN	721.5 (369.54–1358.04)	2806 (1866.07–32489.71)	y = 2.1701x – 1.2027	0.8905	0.1928	5
		<i>An. stephensi</i>	DLC	476.8 (461.94–493.49)	587.9 (567.22–643.99)	y = 14.064x – 32.668	0.9832	0.0333	8
			KNG	177 (119.28–225.09)	559.9 (483.97–1260.17)	y = 2.5595x – 0.7538	0.9002	0.1107	5

LC₅₀: lethal concentration that kills 50% of the exposed larvae, LC₉₀: lethal concentration that kills 90% of the exposed larvae, UCL: upper confidence limit, LCL: lower confidence limit, r: correlation coefficient, χ²: Chi-square significant at P < 0.05 level, and df: degree of freedom.

Even though our studies show methanol extracts to produce the least mortality, other plants such as *Morinda citrifolia* L. (Rubiaceae) and *Erythrina indica* Lam (Leguminosae) have been shown to produce greater susceptibility for their methanol extract when compared to their hexane extract [36, 37]. Therefore, the present study suggests that, in the case of *E. globulus* and *C. asiatica*, the compounds majorly responsible for the insecticidal activity are most probably in the nonpolar and intermediate range and are not polar.

There is probably also the result of the synergistic activity of the active compounds that influences the efficacy of the extract, against the physiological characteristics of each strain tested. For instance, dichloromethane and diethyl ether are solvents with very similar polarity and should typically elute compounds of the same class, and the results show that in some instances dichloromethane extracts prove that they have higher efficacy, while diethyl ether extracts show greater potency. Further studies involving larger number of strains and purified isolated constituents should reveal a clearer picture of interaction between the bioactive molecules and target species.

In the present investigation, it was also interesting to note that the variation in the response of different strains of mosquito of the same species to a specific plant extract is almost the same as the variation in the response of different strains of mosquito of different species to the same extract. For instance, the LC_{50} values for diethyl ether extract of *C. asiatica* against the *An. stephensi* strains KGR and DLC are 14.7 ppm and 241 ppm, while the LC_{50} values for the same extract against the *Ae. aegypti* strains JPN and BSV are 134.5 and 339.6 ppm. From this study, it is revealed that it is imperative to assay the extract against different geographical strains of the same species to ascertain the efficacy of a compound.

Earlier studies on different geographical strains of *An. stephensi* and *Ae. Aegypti* have reported varied levels of susceptibility to various insecticides commonly used in mosquito vector control [26, 38–43]. Unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavioral and physiological processes. Thus, there is very little chance of pests developing resistance to such substances. Identifying bioinsecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management [21]. In order to develop cost effective pesticides from the plant leaves used in the present study, it will be necessary to carry out further studies to determine the active compounds in these plants, their larvicidal efficacies, their individual and synergistic modes of action, the feasibility of large scale use, and stability of these active compounds under field conditions. Most studies using biological extracts have been tested against a single strain of a species. This is probably because one would not expect to observe large variations in the susceptibility of one strain to another. Unlike the varied resistance status observed in the case of synthetic insecticides, the biological extracts used in this study have not been used by municipal bodies as insecticides. Therefore, the observed variation in response

between different strains does not appear to have been caused by such a selection event, but rather by the intervention of biological and genetic factors, resulting in the change in the susceptibility to different plant extracts.

5. Conclusion

In the present study, the hexane extracts of both plants and the diethyl ether extract of *C. asiatica* demonstrated the highest potential for the control of strains of mosquito vectors. The results indicate that the compounds responsible for the insecticidal activity of both plants are most probably in the nonpolar and intermediate range. Also, the present findings show that it is necessary to assay multiple strains of a species to fully comprehend the larvicidal efficacy of a compound.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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