THE APPLICATION OF ETHANOL-EXTRACTED *GLORIOSA SUPERBA* FOR METAPHASE CHROMOSOME PREPARATION IN MOSQUITOS

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Abstract. The application of ethanol-extracted *Gloriosa superba* for metaphase chromosome preparation in adult and 4th larva *Aedes aegypti* revealed that 0.5-8% ethanol-extracted *Gl. superba* solution could be used instead of 1% colchicine in Hanks' balanced salt solution. For adult mosquitos, the metaphase rates and average number of metaphase chromosomes per positive mosquito after intrathoracic inoculation with 1-2% ethanol-extracted *Gl. superba* solution were 100% and 11.8 (2-16) -12.6 (3-28) in females, and 80-90% and 16.5 (1-52) - 29.89 (1-72) in males, whereas the inoculation with 1% colchicine solution yielded 80% and 50% metaphase rates, and 18.25 (1-40) and 16.5 (2-53) average number of metaphase chromosomes per positive mosquito in females and males, respectively. For 4th stage larvae, the metaphase rates and average number of metaphase chromosomes per positive mosquito after incubation with 0.5-8% ethanol-extracted *Gl. superba* solution were 90-100% and 14.42 (1-65) - 64 (19-137), while incubation with 1% colchicine solution yield 100% metaphase rate and 10.9 (7-15) average number of metaphase chromosomes per positive mosquito.

INTRODUCTION

Metaphase karyotype study of eukaryotic organisms is one of the efficient, traditional techniques to differentiate and/or diagnose species, sibling species, subspecies, chromosomal abberration, etc. Consequently, the 0.005-1% colchicine solution has been used extensively for metaphase chromosome preparation in the cytogenetic study of a wide range of genera and species of plants (Vosa, 1973; Marks, 1976) and animals [protozoans (Yuh *et al*, 1997), helminths (Petkeviciute, 1996; Hirai *et al*, 2000) snails (Petkeviciute *et al*, 1995), insects (Baimai, 1977; Nunamaker *et al*, 1996)]. It has an effect on cell division by inhibiting spindle formation and/or microtubule polymerization, thus, mitosis is arrested in metaphase (Insel, 1996; Haraguchi *et al*, 1997).

Colchicine is an alkaloid of *Colchicum autumnale*, a plant that belongs to the Family Liliaceae and within this Family there are at least 11 genera comprising 21 species found indigenously in Thailand (Smitinand, 1980). Among 21 species of indigenous Thai plants, Dong Deung or *Gloriosa superba* has been recorded as the Thai medicinal herb used for treatment of arthritis. Recently, Sucharit *et al* (1995) were the pioneers who applied the Dong Deung (*Gl. superba*) for metaphase chromosome preparation in mosquito vectors. The authors declared that 2% water-extracted solution of fresh tubers could be used satisfactorily for metaphase chromosome preparation in *Aedes albopictus, Ae. aegypti, Ae. nivieus* subgroup, Ae. togoi, *Anopheles aconitus* complex, *An. dirus complex, An. maculatus* complex, *An. minimus* complex, *Mansonia bonneae* and *Ma. indiana*. Subsequently, Jitpakdi *et al* (1999) screened seven plant species of the Family Liliaceae, *ie* Waan Haang Chorakhe (*Aloe barbadensis* Mill.), dried leaf; Nomai Farang (*Asparagus officinalis* L.), dried root; Prongfaa

(*As. plumosus* Bak.), dried root; Saamsip (*As. racemosus* Willd.), dried root; Prik (*As. sprengeri* Regel), dried root; Dok Mai Cheen (*Haemerocallis flava* L.), dried flower; and Dong Deung (*Gl. superba* L.), dried rhizome and three plant species of the Family Agavaceae, *ie,* Maak Phuu Maak Mia (*Cordyline fruticosa* Goppert), dried rhizome; Chan Daeng (*Dracaena loureiri* Gagnep.), dried wood; and Waan Ngaa Chaang (*Sansevieria cylindrica* Bojor.), dried rhizome for metaphase chromosome preparation in adult mosquitos using an inoculation technique. The results indicated that the filtrate of 1% solution of dried *Gl. superba* rhizome soaked in 0.85% sodium chloride solution (NSS) could be used instead of 1% colchicine in Hanks' balanced salt solution (HBSS) for metaphase chromosome preparation of adult females and/or males of *Ae. aegypti*, *Culex quinquefasciatus*, *Toxorhynchites splendens* and *An. vagus*. As an adjunct to the previous studies, we report herein the benefit of ethanol-extracted *Gl. superba* rhizome for metaphase chromosome preparations in both 4th stage larvae and adult mosquitos.

MATERIALS AND METHODS

Ethanol-extracted *Gl. superba*

The dried rhizomes of *Gl. superba* were purchased in Chiang Mai Province, northern Thailand. Approximately, 1.5 kg of dried, powdered rhizome was macerated with 5 liters of 95% ethanol at room temperature for 2 days. The mixture was suction filtered through a funnel and residue was reextracted with 95% ethanol for 2 times. The filtrate obtained was combined and evaporated by using a rotary evaporator at 60ºC, lyophilized and kept at -20ºC. Working solution was prepared by diluting the ethanolic extract with NSS to an appropriate test concentration. For temperature stability tests, the experiments were divided into two sets, *ie* the working solution was heated at 121ºC for 15 minutes using an autoclave before use and the stock of lyophilized ethanol-extracted was frozen at -20ºC for 8 months prior to prepare the working solution.

Chromosome preparation

For adult mosquitos: Metaphase chromosomes were prepared by using the improved technique of Choochote *et al* (1992). Newly emerged males and females of laboratory-reared *Ae. aegypti* were inoculated intrathoracically with 0.3 and 0.5 µl of 0.25-8% ethanol-extracted *Gl. superba* solution, respectively. One percent colchicine solution (1g colchicine alkaloid USP in 100 ml of HBSS) and NSS were used as the positive and negative control, respectively. The inoculated mosquitos were held in an insectary at 27 ± 2ºC, 70-80% RH for 3 hours and then dissected in a small drop of 1% hypotonic sodium citrate solution on a siliconized slide. Reproductive organs were excised from the last abdominal segment. The organs were left in 1% hypotonic sodium citrate solution for 10 minutes and then transferred to a small drop of Carnoy's fixative (equal part of 45% acetic acid and 95% ethanol) on a siliconized slide for 2 minutes, then a drop of 60% acetic acid was added, the organs teared and mixed well with dissecting needles. A drop of cell suspension then was placed on a clean microscopic slide on a warming plate at about 45-50ºC. The droplet of cells was released slowly from the Pasteur pipette to form a circular trail of monolayer cells. The dried slides were stained with 10% Giemsa in phosphate buffer pH 6.8 for 30 minutes, rinsed with deionized water, air-dried at room temperature and mounted in Permount (Fisher, Fairlawn, NJ, USA).

For 4th stage larvae: The techniques for metaphase chromosome preparations were mainly followed the techniques as described above except the early 4th stage larvae of *Ae. aegypti* were incubated with 0.25-10% ethanol-extracted *Gl. superba* solution, 1% colchicine in HBSS, and NSS for experiments, positive and negative controls, respectively, and larval brains were dissected, fixed, smeared and stained with 10% Giemsa.

Slides were examined for metaphase rates and number of metaphase chromosomes per 2,000 cells counted under a compound microscope using an oil immersion objective.

RESULTS

Details of metaphase rates and the average number of metaphase chromosomes per positive adults and larvae are shown in Table 1 and Table 2, respectively. Pictures of metaphase chromosomes of ovary, testis and 4th stage larval brain ganglion after treatment with 1% colchicine solution and 1% ethanol-extracted *Gl. superba* solution are illustrated in Fig 1.

Observations on the slide preparations of ovaries and testes of *Ae. aegypti* after intrathoracic inoculation with 0.3-0.5 µl of 0.25-8% ethanol-extracted *Gl. superba* solution revealed that the 0.5-4% and 0.25-4% solutions provided non-significant differences in the average number of metaphase chromosomes per positive female [6.88 (2-25) - 12.6 (3-28)] and male [7.71 (4-14) - 29.89 (1-72)] when compared to the yield by using 1% colchicine solution [18.25 (1-40) in female, 16.5 (2-53) in male]. The 0.25% solution provided a very low yield of metaphase chromosomes in adult females, with 1 (1) metaphase chromosome per positive female. Likewise, the 8% solution

Table 1
Metaphase rates of *Ae. aegypti* after intrathoracic inoculation with 0.3-0.5 µl of NSS, 1% colchicine solution, and 0.25, 0.5, 1, 2, 4, 8% ethanol-extracted *Gl. superba* solutions, the rates were expressed as at least one metaphase chromosome set per 2,000 cells examined per mosquito ovary or testis.

Experiments	Sex[a]	No. mosquitos having metaphase chromosome(s) of				Average No. chromosomes per positive mosquito (range)
		0	1-10	11-40	>40	
NSS (control)	Female	1	9	-	-	2.33(1-4)
	Male	3	7	-	-	1.57(1-2)
1% colchicine solution	Female	2	3	5	-	18.25(1-40)
	Male	5	4	1	-	16.5(2-53)
Gl. superba						
0.25%	Female	7	3	-	-	1(1)[d]
	Male	3	5	2	-	7.71(4-14)NS
0.5%	Female	1	6	3	-	8.56(3-16)NS
	Male	-	4	6	-	12.9(5-22)NS
1%	Female	-	3	7	-	11.8(2-16)NS
	Male	1	2	6	1	29.89(1-72)NS
1%[b]	Female	-	4	6	-	13(8-22)NS
	Male	1	2	5	2	32.56(2-69)NS
1%[c]	Female	-	3	7	-	15.6(4-27)NS
	Male	-	-	7	3	33.4(11-79)NS
2%	Female	-	4	6	-	12.6(3-28)NS
	Male	2	4	3	1	16.5(1-52)NS
4%	Female	1	8	1	-	6.88(2-25)NS
	Male	1	6	3	-	7.67(2-19)NS
8%	Female	-	-	-	-	-
	Male	-	-	-	-	-

[a]Ten mosquitos for each sex; [b]Stock frozen at -20ºC for 8 months; [c]Heat at 121ºC for 15 minutes; NS, $p > 0.05$; [d] $p < 0.05$ (*t*-test, two sided)

Table 2
Metaphase rates of 4th stage larvae after incubation with NSS, 1% colchicine solution, and 0.25, 0.5, 1, 2, 4, 8, 10% ethanol-extracted *Gl. superba* solutions. The rates were expressed as at least one metaphase chromosome set per 2,000 cells examined per mosquito brain.

Experiments[a]	No. mosquitos having metaphase chromosome(s) of				Average No. chromosomes per positive mosquito (range)
	0	1-10	11-40	>40	
NSS (control)	8	12	-	-	1.42(1-4)
1% colchicine solution	-	9	11	-	10.9(7-15)
Gl. superba					
0.25%	2	17	1	-	3.77(1-12)[d]
0.5%	1	11	7	1	14.42(1-65)NS
1%	-	10	5	5	25.35(1-85)[d]
1%[b]	-	9	5	6	24.9(1-97)[d]
1%[c]	-	10	5	5	22.2(1-79)[d]
2%	1	2	13	4	31.74(2-89)[d]
4%	-	-	5	15	64(19-137)[d]
8%	1	15	1	3	16.16(1-96)NS
10%	-	-	-	-	-

[a]Twenty 4th stage larvae for each experiment; [b]Stock frozen at -20ºC for 8 months; [c]Heat at 121ºC for 15 minutes; NS, $p > 0.05$; [d], $p < 0.05$ (*t*-test, two sided)

could not be applied for metaphase chromosome preparation because all of the ovarian and testicular cells were degenerated after 3-hour-period of inoculation. Statistical analysis of the average number of metaphase chromosomes per positive female and male mosquitos in temperature stability test, *ie* 1% solution prepared from stock frozen at -20ºC for 8 months [female: 13 (8-22), male: 32.56 (2-69)] and 1% solution heated at 121ºC for 15 minutes [female: 15.6 (4-27), male: 33.4 (11-79)] with 1% normal working solution [female: 11.8 (2-16), male: 29.89 (1-72)] manifested non-significant differences.

Observations on the slide preparations of brain ganglia of 4th stage larva of *Ae. aegypti* after incubation with 0.25-10% ethanol-extracted *Gl. superba* solution indicated that 0.5-8% solution could be used instead of 1% colchicine solution for metaphase chromosome preparation since they provided the higher average number of metaphase chromosomes per positive larva [0.5%: 14.42 (1-65), 1%: 25.35 (1-85), 2%: 31.74 (2-89), 4%: 64 (19-137), 8%: 16.16 (1-96)] than 1% colchicine solution [10.9 (7-15)]. Statistical analysis of the average number of metaphase chromosomes per positive mosquito among various dilution series (0.5-8%) of ethanol-extracted *Gl. superba* solution and 1% colchicine solution exhibited non significant differences in the 0.5% [14.42 (1-65)] and 8% [16.16 (1-96)] solutions. The 0.25% solution provided the unsatisfying outcomes, whereas the 10% solution could not be used for metaphase chromosome preparation because all of the 4th stage larval brain-ganglia were degenerated after 3-hour-period of incubation. Statistical analysis of the average number of metaphase chromosomes per positive larva in temperature stability test, *ie* 1% solution prepared from stock frozen at -20ºC for 8 months [24.9 (1-97)] and 1% solution heated at 121ºC for 15 minutes [22.2 (1-79)] with 1% normal working solution [25.35 (1-85)] manifested non significant differences.

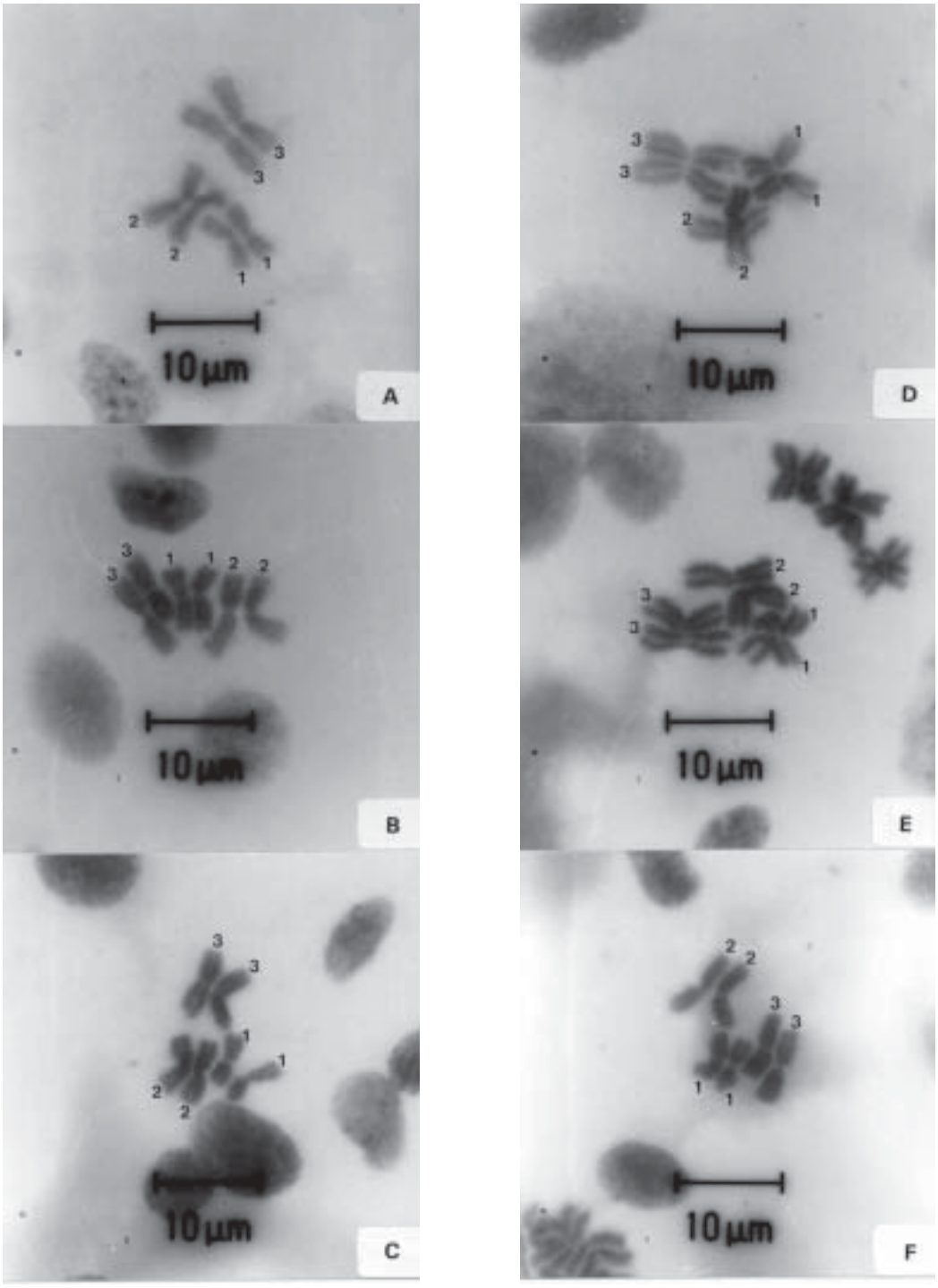

Fig 1- Metaphase chromosomes of adults and larvae of *Ae. aegypti* after treatment with 1% colchicine solution or 1% ethanol-extracted *Gl. superba* solution, stained with conventional Giemsa. Colchicine treatment: (A) Ovary chromosome. (B) Testis chromosome. (C) Brain chromosome. Ethanol-extracted *Gl. superba* treatment: (D) Ovary chromosome. (E) Testis chromosome. (F) Brain chromosome.

DISCUSSION

Currently, the application of indigenous, natural products has been one of the alternative ways to replace the synthetic and/or imported ones, therefore, the objective of present study is aimed to promote low cost, highly effective indigenous Thai plants as a natural source of colchicine-like substance(s) which can be prepared facilely and applied for routine cytogenetic study of wide-range genera and species of mosquito vectors and/or other organisms. Our present study showed that 0.5-8% ethanol-extracted *Gl. superba* solution could replace 1% colchicine in HBSS for metaphase chromosome preparation in both larva and adult *Ae. aegypti*. The application of ethanol-extracted *Gl. superba* solution for metaphase chromosome preparation in other mosquito genera and species (*Ae. lineatopennis*, *Ae. togoi*, *An. aconitus* complex, *An. peditaeniatus*, *An. vagus*, *Tx. splendens*), trematode (*Fasciola gigantica*) and lymphocyte cultivation for diagnosis of some genetic diseases also has yielded satisfactory results (unpublished data).

Formerly, the two pioneers, Sucharit *et al* (1995) and Jitpakdi *et al* (1999), have been declared the usefulness of *Gl. superba* for metaphase chromosome preparations in mosquitos. The former used 2 g of fresh tubers soaked in 100 ml of water as working solution and the incised, fresh tuber lost colchicine-like activity within one week after exposed to sunlight is the important drawback, whereas the latter used the filtrate of dried, powdered rhizome soaked in NSS. Our additional work has affirmed the low cost and high efficacy of ethanol-extracted *Gl. superba*. After ethanol extraction, approximate 1.5 kg of dried, powdered rhizome provided about 3 g of ethanol extract and it costed approximately US$ 19.35 (1.5 kg *Gl. superba* dried rhizome = US$ 7.5, 15 liters of 95% ethanol = US$ 11.85). The 3 g of ethanol extract could be prepared 300 ml of 10 μg/ml in NSS or HBSS and it saved approximately 35 times of synthetic and/or importable products (10 ml of 10 μg colchicine in 1 ml HBSS = US$ 22.5). Additionally, the stability of the ethanol extract after remaining frozen at -20ºC for 8 months prior to preparing the working solution or heating the working solution at 121ºC for 15 minutes gave satisfactory results. The optimal concentration of working solution should be calibrated depending on the genera, species and stages of mosquitos and/or other organism prior to routine use.

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