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Clastogenic effect of *Picrorhiza kurroa* rhizome extract on cultured human peripheral blood lymphocytes

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ABSTRACT

Introduction: The *Picrorhiza kurroa* rhizome has a long history of use in Indian Ayurvedic and Chinese system of medicine for the treatment of a wide spectrum of diseases. Today it is viewed as an important therapeutic target in both Western and Eastern medicinal systems. This work was aimed to study the clastogenic effect of *Picrorhiza kurroa* rhizome extract on cultured human peripheral blood lymphocytes.
Methods: Hydroalcoholic extract of rhizome was prepared and mammalian chromosomal aberration test was conducted using cultured human peripheral blood lymphocytes. The study was performed in two independent phases where the human peripheral blood lymphocytes were exposed to various of the extract in absence and presence of metabolic activation system for a continuous and short duration.
Results: *Picrorhiza kurroa* rhizome extract did not induce chromosome aberration up to 2500 µg/mL in final culture concentration in the presence (1% v/v) and absence of metabolic activation.
Conclusion: *Picrorhiza kurroa* rhizome extract is completely safe to be used as a medicine since it manifest its healing effects without causing genotoxicity.

Implication for health policy/practice/research/medical education:
The genotoxicity of *Picrorhiza kurroa* rhizome extract was studied in the human peripheral blood lymphocytes. The results suggest that it is a safe plant to be used as a medicine since it manifest its healing effects without causing genotoxicity.
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Introduction

Picrorhiza kurroa Royle ex. Benth belongs to Scrophulaceae family and is commonly called Kutki or Kutka. It is a well-recognized herb in the Ayurvedic and Chinese traditional medicine. It is a wonder herb with hepatoprotective (1-4), anticholestatic (5), antioxidant (6), antidiabetic (7) and immune-modulating properties (8-10). The leaves, root and rhizome of *Picrorhiza* are effective in numerous ailments namely liver and upper respiratory tract illnesses, chronic diarrhea, malaria, jaundice, epilepsy, paralysis, rheumatoid arthritis and skin diseases (11-14). In rats with malaria, *Picrorhiza* reestablished depleted glutathione levels helping to conserve a normal oxidation-reduction balance (15).
Additionally, *Picrorhiza* extract has been found to be useful in dealing with viral hepatitis symptoms as it has favorable anti-hepatitis B surface antigen potential (16).
Nowadays plants or plant extracts have important therapeutic applications in both Western and Eastern medicinal systems. However, some of them are potentially toxic, mutagenic or carcinogenic. Thus, to evaluate potential genotoxicity of the standardized hydro-alcoholic extract of *Picrorhiza kurroa* rhizome, mammalian chromosomal aberration test was conducted on cultured human peripheral blood lymphocytes.

Material and Methods

Material

Picrorhiza kurroa rhizomes were procured from Patanjali Natural Coloroma Pvt Ltd and stored in ambient conditions for further study. The other solvents and chemicals were purchased from Sigma-Aldrich, India.

Extraction

The rhizomes of the plant were dried in shade for about 3 weeks and ground using a mixer to a coarse powder. Using a soxhlet extraction method, the powder of dried rhizome was processed with petroleum ether (40-50°C) for 18 h in

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order to remove fat and unwanted components. The treated powder was further processed with hydro-alcoholic solution (60:40) by using the same extraction procedure for 18h. The extract was evaporated to dryness in a rotary flash evaporator at a temperature not exceeding 60°C, then stored in an air tight container.

Test system

Human venous blood from healthy adult donors lacking current viral infections was collected and used for the study. The study was conducted in accordance to the procedures and recommendations of OECD Guideline 473, updated and adopted July 1997.

The study was conducted in two independent phases:

Initial Chromosome Aberration Assay (Phase I)

Confirmatory Chromosome Aberration Assay (Phase II)

Based on the results of solubility, it was tested up to the highest concentration 2500 µg/mL (both phases of the experiment). Dose range finding study was conducted as an integral part of the chromosomal aberration assay. A range of soluble dose concentrations ranging viz., 2500, 1250, 625, 312.5, 156.3, 78.1, 39.1, 19.5, 9.8 and 4.9 µg/mL was applied in the initial chromosome aberration assay.

In the first phase of the experiment, the proliferating human lymphocytes (72 ± 2 hours after the initiation of whole blood culture) were exposed at various doses of the Test Sample (4.9, 9.8, 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250 and 2500 µg/mL) in the absence and presence of metabolic activation system (1% v/v) for a short duration (about 4-hour exposure).

In the second phase of the experiment, the proliferating human lymphocytes (72 ± 2 hours after the initiation of whole blood culture) were exposed at diverse dose levels of the Test Sample (2500, 1500, 1000, 750 and 500 µg/mL) in the absence of metabolic activation system for a continuous treatment (up to 1.5 normal cell cycle length). One set of the proliferating human lymphocytes were exposed at various dose levels of Test Sample (2500, 1500, 1000, 750 and 500 µg/mL) in presence of metabolic activation system (1% v/v) for a short duration (about 4-hour exposure).

In both the phases (Experimental Phase 1 & 2) the treated cells were harvested at about 1.5 cell cycle length after treatment (22 hours). Mitomycin C and Cyclophosphamide were used as positive control substances for experiments without and with S9, respectively.

During harvesting of cell cultures, the cells were treated with metaphase arresting agent (Colchicine) 2 hours prior to harvesting. Later the cells were hypotonically treated, fixed in acetomethonol fixative and metaphase spreads were prepared by hanging drop method. The slides were stained with 5% Giemsa solution.

The metaphase cells were analyzed microscopically for the presence of chromosome aberrations if any, and a minimum number of 1000 cells per sample were screened randomly and number of metaphases was recorded in different fields to determine the mitotic index. The number of aberrant cells with one or more aberrations excluding gaps and the number of aberrant cells with one or more aberrations including gaps was recorded separately to calculate percent aberrant cells.

Results

Initial chromosome aberration assay

The initial chromosomal aberration was conducted with about 4-hour exposures to standardized *Picrorhiza kurroa* rhizome extract (Test Sample), in the presence and absence of S9 metabolic activation system. The assay was carried out with ten dose levels of the Test Sample viz., 2500, 1250, 625, 312.5, 156.3, 78.1, 39.1, 19.5, 9.8 and 4.9 µg/mL in the final culture. The result of mitotic indices at all the ten concentrations of the Test Sample was found to be comparable to the vehicle control group. The results of chromosome aberrations including gap and chromosome aberration excluding gap at various dose concentrations (2500, 1250, 625 µg/mL) did not reveal any induction of chromosome aberrations when compared to the vehicle control groups (Tables 1 and 2).

The results of the positive control, Mitomycin C (2.0 µg/mL; in the absence of metabolic activation) and Cyclophosphamide (60 µg/mL; in the presence of metabolic activation 1% v/v) significantly increased the number of structural chromosome aberrations when compared to vehicle control groups.

Therefore, it is clear from the results of the initial chromosomal aberration assay that *Picrorhiza kurroa* rhizome extract, in the presence and absence of S9 metabolic activation system does not induce chromosome aberrations at concentrations tested. However, the positive control groups showed an increase in the frequency of aberrant cells and thus demonstrated sensitivity of the test system.

Confirmatory chromosome aberration assay

The confirmatory assay was performed after the results of the initial trial were known. The clear negative result of the initial chromosome aberration assay was further confirmed for the evidence of clastogenicity, if any.

Assay without S9 activation-continuous exposure

Three days after culture initiation, the cultures were treated with the Test Sample, in the absence of metabolic activation at various dose concentrations (2500, 1500, 1000, 750 and 500 µg/mL) along with concurrent vehicle and positive controls. Incubation at 37± 2°C was continued until the time of harvest. The results of mitotic indices at all the five concentrations of the Test Sample were found to be comparable to the vehicle control group. No cytotoxicity was observed at any of the dose levels of the Test Sample, in the absence of metabolic activation. The results of chromosome aberrations including gap and chromosome aberration excluding gap at various concentrations (2500, 1500, 1000 µg/mL) did not reveal any induction of chromosome aberrations when compared to the vehicle control groups.

The results of the positive control, Mitomycin C (2.0 µg/mL) in the absence of metabolic activation showed a

Table 1. Mitotic Index observed in experimental phase 1 both in absence and presence of metabolic activation (10% v/v)

In presence of metabolic activation (10% v/v)			In absence of metabolic activation		
Dose levels (µg/mL)	**Mitotic index[a]**	**% Mitotic Index**	**Dose levels (µg/mL)**	**Mitotic index**	**% Mitotic index**
0.0 (VC)	13.052 ± 1.01	NA	0.0 (VC)	14.706 ± 0.73	NA
60 (CPA)	10.325 ± 0.73	79	2 (MMC)	8.345 ± 0.30	57
2500 (TS)	12.864 ± 0.43	99	2500 (TS)	13.339 ± 1.22	91
1250 (TS)	11.596 ± 0.45	89	1250 (TS)	14.782 ± 1.30	101
625 (TS)	11.97 ± 0.77	92	625 (TS)	14.222 ± 0.07	97
312.5 (TS)	11.511 ± 0.15	88	312.5 (TS)	14.179 ± 0.81	96
153.25 (TS)	11.692 ± 0.06	90	153.25 (TS)	13.951 ± 0.12	95
78.13 (TS)	11.785 ± 0.01	90	78.13 (TS)	13.493 ± 0.13	92
39.06 (TS)	12.448 ± 0.64	95	39.06 (TS)	14.025 ± 0.67	95
19.53 (TS)	11.420 ± 0.75	87	19.53 (TS)	13.458 ± 0.00	92
9.77 (TS)	12.345 ± 0.34	95	9.77 (TS)	13.290 ± 0.15	90
4.88 (TS)	11.882 ± 0.22	91	4.88 (TS)	14.180 ± 0.03	107

Abbreviations: CPA, Cyclophosphamide (Positive control); MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide)

[a] Mitotic index = Number of metaphase cells/total number of cells screened × 100.

Table 2. Mitotic index observed in experimental phase II with exposure of whole blood culture to short term treatment (4 ± 1 hour) in presence of metabolic activation system (10% v/v)

Dose Levels (µg/mL)	Mitotic index	% Mitotic index
0.0 (VC)	13.052 ± 1.01	NA
60 (CPA)	10.325 ± 0.73	79
2500 (TS)	12.864 ± 0.43	99
1500 (TS)	11.596 ± 0.45	89
1000 (TS)	11.97 ± 0.77	92
750 (TS)	11.511 ± 0.15	88
500 (TS)	11.692 ± 0.06	90

Abbreviations: CPA, Cyclophosphamide (Positive control); TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

Table 3. Mitotic index observed in experimental phase II with exposure of whole blood culture to continuous treatment (22 hours) in absence of metabolic activation system.

Dose Levels (µg/mL)	Mitotic index[a]	% Mitotic index
0.0 (VC)	13.052 ± 1.01	NA
2 (MMC)	10.325 ± 0.73	79
2500 (TS)	12.864 ± 0.43	99
1500 (TS)	11.596 ± 0.45	89
1000 (TS)	11.97 ± 0.77	92
750 (TS)	11.511 ± 0.15	88
500 (TS)	11.692 ± 0.06	90

Abbreviations: MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

[a] Mitotic index = Number of metaphase cells/total number of cells screened × 100.

considerable increase in the number of structural chromosome aberrations when compared to vehicle control groups (Tables 3-5).

Therefore, confirmatory chromosome aberration assay clearly indicate that the Test Sample, in the absence of S9 metabolic activation system does not induce chromosome aberrations at the concentrations tested. However, the positive control group showed an increase in the frequency of aberrant cells and thus demonstrated sensitivity of the test system.

Assay with S9 activation–short exposure

The confirmatory chromosome aberration assay was conducted with about 4-hour exposures to the Test Sample, in the presence of activation system. The assay was carried out at five concentrations of the Test Sample (2500, 1500, 1000, 750 and 500 µg/mL).

The results of mitotic indices at all the five concentrations of the Test Sample, were found to be comparable to the vehicle control group. No cytotoxicity was observed at any of the dose levels of the Test Sample in the presence of metabolic activation. The results of chromosome aberrations including gap and chromosome aberration excluding gap at various dose concentrations (2500, 1500, 1000 µg/mL) in the presence of metabolic activation did not reveal any induction of chromosome aberrations when compared to

Table 4. Chromosome aberration analysis in experimental phase I with exposure of whole blood culture to short term treatment (4 ± 1 hour) in absence of metabolic activation system

Dose Levels (µg/mL)	% Aberrated cells (structural)		% Numerical aberration
	Including Gap	Excluding gap	
0.0 (VC)	2.00 ± 0.71	0.50 ± 0.71	0.20 ± 0.71
2 (MMC)	12.00 ± 0.41	12.00 ± 0.41	0.00 ± 0.00
2500 (TS)	1.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00
1250 (TS)	0.50 ± 0.41	0.50 ± 0.71	0.00 ± 0.00
625 (TS)	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00

Abbreviations: MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

Table 5. Chromosome aberration analysis in experimental phase II with exposure of whole blood culture to continuous treatment (22 hours) in absence of metabolic activation system

Dose Levels (µg/mL)	% Aberrated cells (structural)		% Numerical aberration
	Including Gap	Excluding gap	
0.0 (VC)	1.50 ± 0.71	1.50 ± 0.71	0.00 ± 0.00
2 (MMC)	13.00 ± 1.41	12.00 ± 0.00	0.00 ± 0.00
2500 (TS)	1.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00
1250 (TS)	0.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00
625 (TS)	0.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00

Abbreviations: MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

Table 6. Chromosome aberration analysis in experimental phase I with exposure of whole blood culture to short term treatment (4±1 hour) in presence of metabolic activation system (10% v/v)

Dose Levels (µg/mL)	% Aberrated cells (structural)		% Numerical aberration
	Including Gap	Excluding gap	
0.0 (VC)	1.50 ± 0.71	0.50 ± 0.71	0.20 ± 0.71
2 (MMC)	12.5 ± 0.71	12.00 ± 1.41	0.00 ± 0.00
2500 (TS)	1.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00
1250 (TS)	1.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00
625 (TS)	0.50 ± 0.71	0.50 ± 0.71	0.00 ±0.00

Abbreviations: MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

Table 7. Chromosome aberration analysis in experimental phase II with exposure of whole blood culture to short term treatment (4±1 hour) in presence of metabolic activation system

Dose Levels (µg/mL)	% Aberrated cells (structural)		% Numerical aberration
	Including Gap	Excluding gap	
0.0 (VC)	1.00 ± 0.41	0.50 ± 0.71	0.20 ± 0.71
2 (MMC)	11.50 ± 0.71	9.50 ± 0.71	0.00 ± 0.00
2500 (TS)	1.50 ± 0.71	0.50 ± 0.71	0.00 ± 0.00
1250 (TS)	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
625 (TS)	1.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00

Abbreviations: MMC, Mitomycin C (Positive control), TS, Test Sample, VC, vehicle control (Dimethyl sulfoxide).

the vehicle control groups.

In the presence of metabolic activation (1%v/v), the positive control (60 µg/mL Cyclophosphamide) showed a significant increase in the number of structural chromosome aberrations when compared to vehicle control groups (Tables 1, 2, 6, and 7).

Therefore, it is clear from the results of the confirmatory chromosome aberration assay that Test Sample, in the presence of S9 metabolic activation system does not induce chromosome aberrations at the concentrations tested. However, the positive control group (Cyclophosphamide) showed an increase in frequency of aberrant cells and thus demonstrated sensitivity of the test system (Figures 1-8).

Discussion

For chromosomal aberration studies, mammalian cells were cultured in vitro, exposed to a test substance, harvested, and the occurrence of chromosome aberrations was measured. Human peripheral blood lymphocytes do not normally divide but they are stimulated to divide in culture by exposure to Phytohemagglutinin (PHA) (17). At predetermined intervals after exposure to the Test Sample, the lymphocytes were treated with metaphase arresting substance, Colcemid. Later the cells were collected, stained, and investigated for the presence of chromosomal aberrations.

Several mutagenic agents did not directly interact with the DNA but did so after being converted to active intermediates by liver enzymes (18). Due to limited capacity of human lymphocytes to metabolize the test sample an external metabolic activation system (rat liver S9 homogenate)

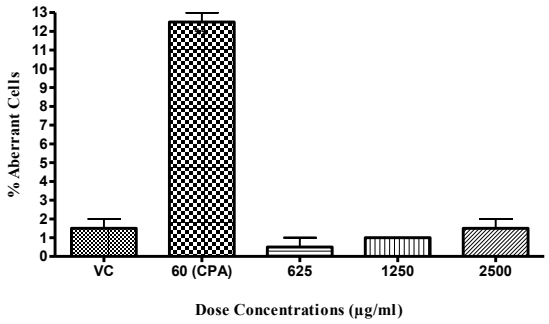

Figure 1. Experimental phase 1 percent aberrant cells observed with metabolic activation (including gap) at various concentrations of Test Sample.

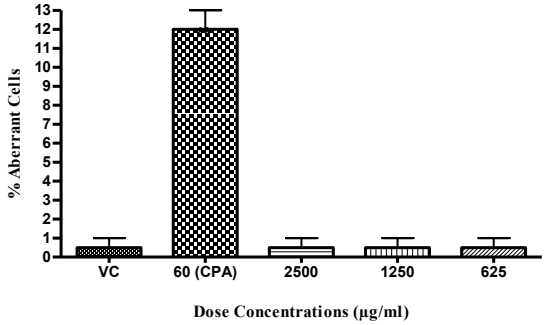

Figure 2. Experimental phase 1 percent aberrant cells observed with metabolic activation (excluding gap) at various concentrations of Test Sample.

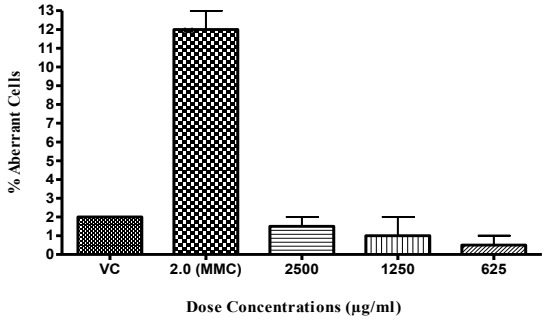

Figure 3. Experimental phase 1 percent aberrant cells observed without metabolic activation (including gap) at various concentrations of Test Sample

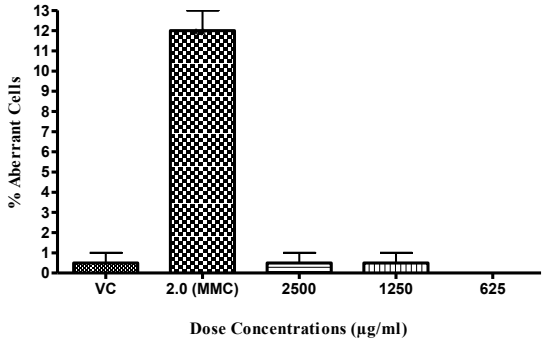

Figure 4. Experimental phase 1 percent aberrant cells observed without metabolic activation (excluding gap) at various concentrations of Test Sample

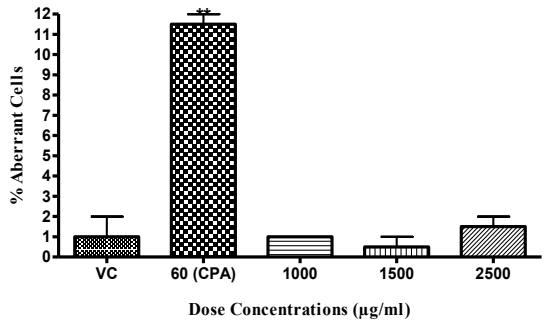

Figure 5. Experimental phase II percent aberrant cells observed with metabolic activation (including gap) at various concentrations of Test Sample

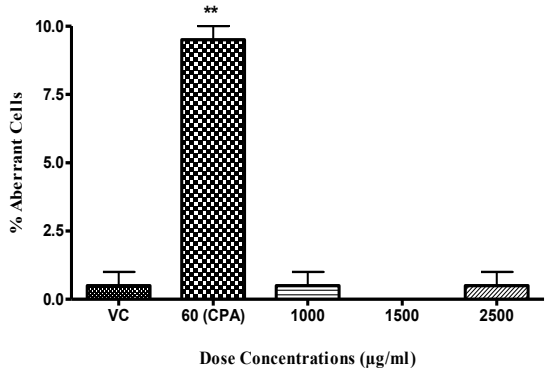

Figure 6. Experimental phase II percent aberrant cells observed with metabolic activation (excluding gap) at various concentrations of Test Sample

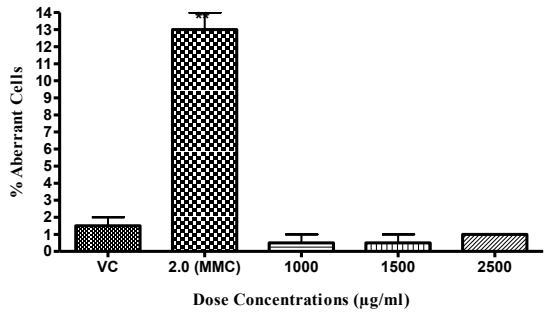

Figure 7. Experimental phase II percent aberrant cells observed without metabolic activation (including gap) at various concentrations of Test Sample.

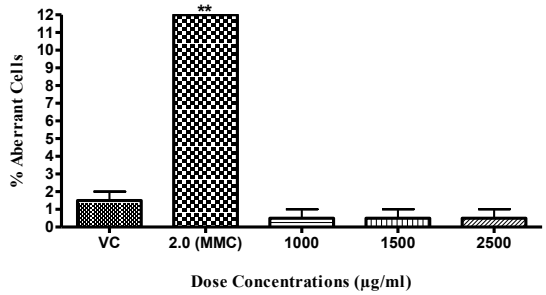

Figure 8. Experimental phase II percent aberrant cells observed without metabolic activation (excluding gap) at various concentrations of Test Sample

was incorporated to further improve the conversion as well as the ability of the assay to identify the clastogenic metabolic intermediates (19,20).

Numerical aberrations (a change in the number of chromosomes from the normal number of 46 for human lymphocytes) are not determined by this study plan.

Conclusion

From the results of this study, it is concluded that the hydro-alcoholic extract of *Picrorhiza kurroa* rhizome does not induce chromosome aberration up to 2500 µg/mL in final culture concentration both in presence (1% v/v) and absence of metabolic activation. Therefore, *Picrorhiza kurroa* rhizome extract can be used as a safe Ayurvedic medicine since it manifest its healing effects without causing genotoxicity. Nevertheless, more scientific data on in vivo activities in human is required to clarify the use of *Picrorhiza kurroa* as a therapeutic medicine.

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Authors' contributions

ABK conceived of the study, participated in its design and coordination and helped to draft the manuscript. HKM participated in the designing experimental protocol and performed the statistical analysis. VKS and NS participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

The authors have been entirely regarded ethical issues such as plagiarisms, data assembly, double publication or submission.

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